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REVIEW

Applying microfluidic technology to interpret the tumor immune microenvironment and cancer immunotherapy

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ABSTRACT In the past decade, cancer immunotherapy has emerged as a transformative treatment modality for diverse malignancies. Although impressive clinical efficacy has been demonstrated in some cancer patients, most patients respond poorly to immunotherapies. The complicated architecture and cellular composition of the tumor immune microenvironment (TIME) have substantial roles in the clinical outcomes of immunotherapies. Therefore, employing optimal *in vitro* models recapitulating the *in vivo* TIME characteristics is particularly important for interpreting the dynamic complexity of the TIME, evaluating drug efficacy, and developing novel immunotherapeutics. In recent years, microfluidic technology has been shown to be a valuable tool for mimicking dynamic crosstalk among the TIME *in vitro* through the manipulation of microscale fluids in an integrated device. Cellular behaviors, function and signal transduction, and tumor–immune interactions can be monitored in real time and analyzed in microfluidic chips by combining visualization technologies. Numerous recent studies have shown how to design and fabricate microfluidic chips for reproducing the complex three-dimensional architecture and dynamic changes in the TIME. This review comprehensively examines the application of innovative microfluidic technology in the field of cancer immunology research, focusing on interpreting dynamic crosstalk inside the TIME from bulk-cell to single-cell analyses, evaluating the efficacy of novel immunotherapies and preparing immunotherapeutic agents, and analyzes current limitations. This work aimed to propose a translational roadmap for leveraging microfluidics in elucidating mechanisms, biomarker discovery, high-throughput drug screening, and personalized immunotherapy development.

KEYWORDS Microfluidic chip; tumor immune microenvironment; immunotherapy; cell–cell interactions; cancer immunology

Introduction

Cancer is a global threat to human health[1]. In addition to traditional treatments, including surgery, radiotherapy, chemotherapy, and targeted therapy, immunotherapy ranks among the top 10 scientific breakthroughs according to *Science* in 2013[2] and has increasingly become a treatment option for some cancer patients[3,4]. Immune checkpoint blockade (ICB) therapy and cellular immunotherapy are promising cancer treatments that prolong the survival of cancer patients by activating or adaptively enhancing the immune system to attack tumor cells. Although impressive therapeutic outcomes have been achieved in multiple cancer types[5-7], most patients, especially patients with solid tumors, exhibit poor or partial responses to immunotherapies[8-11]. Therefore, exploring the mechanisms underlying primary or acquired drug resistance to immunotherapy and developing effective methods to evaluate the efficacy of novel immunotherapeutics are particularly important.

The tumor microenvironment (TME) is believed to co-evolve with tumor cells and modulate nearly every aspect of tumor progression. Complicated crosstalk among TME cells (fibroblasts, endothelial cells, and immune cells) and soluble molecules (cytokines and growth factors) have irreplaceable roles in regulating tumor immune escape and the antitumor efficacy of immunotherapies[12-14]. This co-evolution increases the enormous demand for applicable models to reflect the crosstalk observed in the TME, especially the tumor immune microenvironment (TIME). Traditional *in vivo* animal models and *in vitro* two-dimensional (2D) or 3D cell culture are usually used as experimental systems. Animal models can reflect the immune microenvironment *in vivo* when using immune competent mice and syngeneic cancer cell lines, spontaneous or induced tumorigenesis mice, humanized immune system mice, or patient-derived xenograft (PDX) mice. However, animal experiments are time-consuming, costly, ethically questionable, and cannot completely mimic human pathology.

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Among 2D static cell culture systems, the *in vitro* Transwell model is the most widely used cell–cell or cell–microenvironment crosstalk model because of model simplicity and good reproducibility. Nevertheless, the interaction between immune and cancer cells in a Transwell model is partly mediated by gravity[15], which differs from the dynamic interaction *in vivo*. Furthermore, Transwell models cannot reflect fluid characteristics and mimic the temporal and spatial dynamics encountered by immune cells targeting cancer cells[15], which is a key feature for evaluating the efficacy of immunotherapy. These advantages and disadvantages of animal and Transwell models are summarized in **Table 1**.

In the past decade, the application of microfluidic chips in the field of tumor immunology research has received increasing attention. Microfluidic technology involves the manipulation of microscale fluids in the microscale space. The microscale is compatible with many inherent microstructure scales of *in vivo* systems. Microfluidic cell culture systems allow the perfusion of culture medium throughout cell culture during *in vitro* studies and offer a more *in vivo*-like physical microenvironment. Thus, biological processes, such as temporal and spatial dynamic interactions between immune and cancer cells, can be effectively simulated. More importantly, microfluidics can reproduce cell confinement, which is a parameter of cell movement imposed on the interstitial space of the tissue and a key requirement for studying the behavior of moving cells, such as immune and cancer cells[27,28]. Notably, this feature is completely absent in Transwell models. Moreover, microfluidic chips can integrate different cell populations into different regions to reconstruct the human TME *in vitro*. Therefore, microfluidic chips may bridge the gap between Transwell and animal models and are capable of reproducing the complex 3D architecture of tissues and organs (**Table 1**) to better investigate the mechanism and function of the TIME and develop novel immunotherapeutics[29].

Hence, this review aimed to summarize the progress involving the application of innovative microfluidic technology or strategies for mimicking crosstalk inside the TME, evaluating the efficacy of immunotherapy and preparing immunotherapeutic agents (**Figure 1**).

Mimicking crosstalk inside the TME

Cell–cell crosstalk at the bulk cell level

Body tissues are highly organized and consist of a variety of cells with functions orchestrated by different signals in response to external or internal stimuli or stress. Cancer cells

Table 1 Comparison of tumor immune microenvironment models

	Advantages	Disadvantages
Animal model	1. Reflects the TIME *in vivo*; 2. Capable of modeling heterogeneity of *in vivo* responses and resistance; 3. Avoids the risks of conducting experiments on humans; 4. Models diseases with long incubation period, long course and low incidence.	1. Time-consuming; 2. Expensive; 3. Low throughput screening; 4. Results from animal models are not directly related to human response[16]; 5. Cannot fully summarize human pathology; 6. Raises ethical issues.
Transwell model	1. Mimics cell–cell or cell-microenvironment crosstalk in 2D static cell culture system; 2. Simple and reproducible; 3. Cheap.	1. Inability to recapitulate temporal and spatial dynamics interaction; 2. Cannot reflect fluid characteristics; 3. Morphology, physiology, gene expression and drug sensitivity differ from cells *in vivo*[17-19].
Microfluidic model	1. Capable of mimicking TIME[20]; 2. Analyzes spatial and temporal dynamic cell-cell interactions[15,21,22]; 3. Real-time imaging observation; 4. Suitable for 3D cancer cell growth; 5. Functional screening of drug combinations[23]; 6. Ability to provide drug gradients; 7. Liquid fluid can provide nutrient or small molecule and remove wastes; 8. Less reagents; 9. Multiple steps can be integrated in a single device[24]; 10. Eliminates gravity-mediated cell-cell interactions.	1. Inability to recapitulate biological *in vivo* interactions within the entire animal; 2. Difficult to maintain long-term culture (months); 3. Limitations when used with hydrophobic drugs[25]; 4. Size limited to small tumor tissues; 5. Rarely successfully commercialized for clinical use[26].

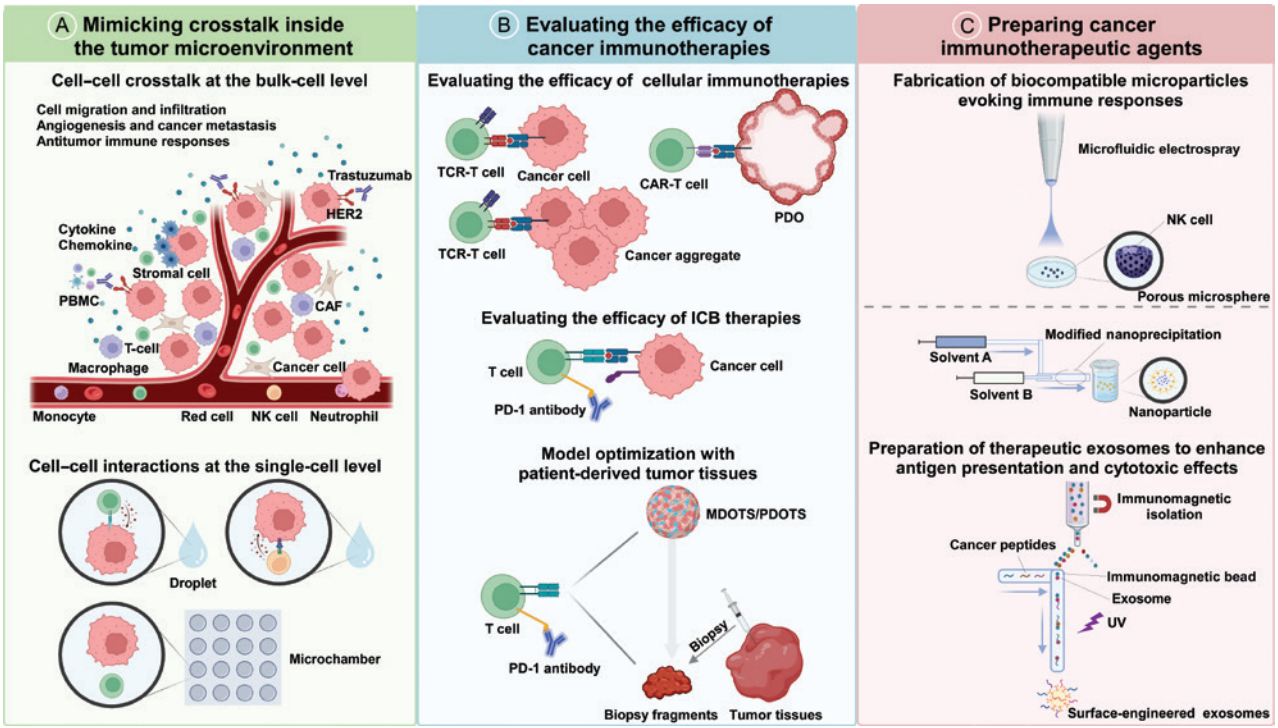


Figure 1 Overview of the application of microfluidic technology in mimicking crosstalk inside the TME, evaluating the efficacy of immunotherapies and preparing immunotherapeutic agents. (A) Mimicking cancer-immune crosstalk inside the TME during cell migration and infiltration, angiogenesis and cancer metastasis, and antitumor immune responses, spanning bulk cell (the upper panel) to single cell (the lower panel) analyses of TME dynamics. (B) Evaluating the efficacy of cellular immunotherapies (TCR-T and CAR-T cells) and ICB therapies (PD-1 antibody) with different cancer models, including cancer cell, cancer aggregate, patient-derived cancer cell organoid (PDO), murine-derived or patient-derived organotypic tumor spheroids (MDOTS/PDOTS), and tumor biopsy fragments. (C) Preparing various cancer immunotherapeutic agents, including porous microspheres, biocompatible nanoparticles, and therapeutic surface-engineered exosomes, by consolidating multiple complex procedures into integrated devices capable of simultaneous cell culture, capture, isolation, and analysis.

typically hijack some physiologic signals to recruit different types of cells, thus forming cancer tissues. Therefore, mimicking cell–cell crosstalk in the TIME at the bulk cell level in temporal and spatial dimensions is the basic strategy for designing a microfluidic model.

Microfluidic models generally consist of two-to-four connected channels or chambers. Distinct cell types can be seeded into extracellular matrix (ECM) components that contain hydrogels in separated but transparent channels or chambers. Culture medium with or without cells can flow through the channels toward the outlets to provide nutrients, remove waste, and simulate *in vivo* fluid characteristics and cell movement *via* the perfusion inlets (**Figure 2A**). These settings enable researchers to explore specific cell–cell crosstalk dynamics by mimicking *in vivo* microenvironments.

Cell migration and infiltration

Cell migration is the basis of multiple pivotal physiologic and pathologic processes, such as tissue formation, cancer metastasis, and immune infiltration and is dependent on cell–cell or cell–microenvironment communication. Lee et al.[30] fabricated a microfluidic device containing four connected channels, introduced macrophages and cancer cells into two inner adjacent gel channels, and perfused the culture medium through two flanking channels given that macrophage migration in the TME is important because of an ability to support tumor cell invasion through the ECM during metastasis[47,48] [**Figure 2B(I)**]. Lee et al.[30] used this device to investigate the factors that impact macrophage migration from the TME to tumor loci by calculating and controlling the velocity of interstitial flow to a range within tumor tissues *in vivo*. The results revealed that TME interstitial flow and the expression of chemokines [interleukin 8 (IL-8) and C-C motif chemokine ligand 2 (CCL2)] derived from cancer cells enhanced macrophage migration. Scott et al.[49] reported that cancer cell-derived colony stimulating factor-1 (CSF-1) signaling regulates macrophage migration and infiltration using a similar microfluidic device (**Figure 3A**). Bai et al.[31] explored the effects of macrophages on the dispersion of cancer aggregates, which can initiate cancer

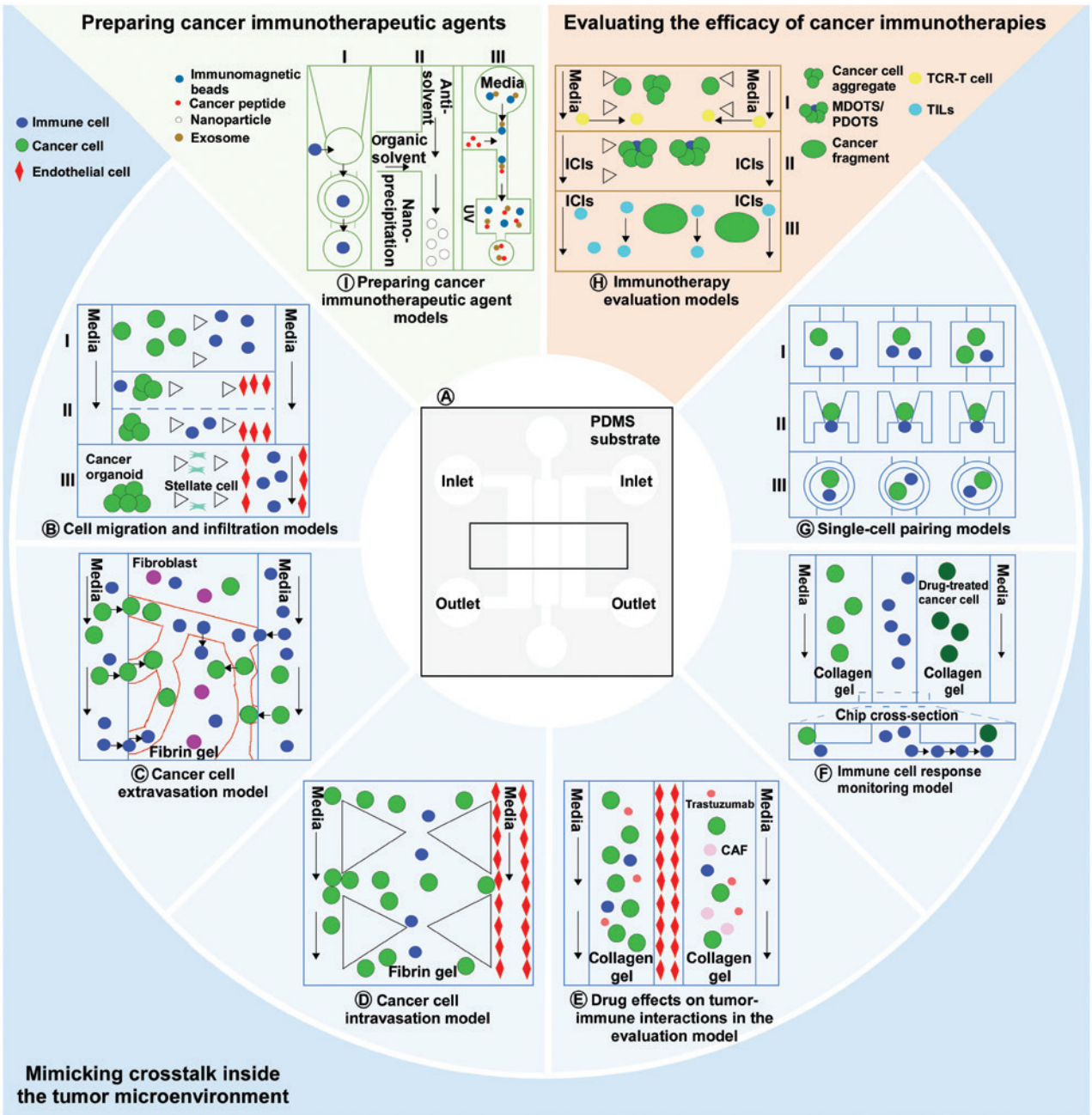


Figure 2 Schematic views of the application of microfluidic technology in tumor immunology research. (A) Typical microfluidic chip diagram. Cancer, immune, or stromal cells can be separately seeded in the central microchannel filled with ECM gel or collagen and an additional endothelial monolayer in the adjacent microchannel. Culture medium is added to the chips through the perfusion inlets. (B)–(I) Enlarged cross-sections of (A) showing the channel details of different microfluidic chips. PDMS, polydimethylsiloxane. (B) Cell migration and infiltration models. I. Introducing macrophages and cancer cells into two inner adjacent gel channels to determine the factors impacting the dynamic migration directedness and speed of macrophages toward tumor loci. II. Different subtypes of macrophages are co-cultured (upper panel) or separately cultured (lower panel) with cancer cell aggregates to determine the role on cancer aggregate dispersion. III. Endothelial cells, PSCs, and PDAC organoids were seeded into different channels to simulate the spatial structure of the tumor stroma. Stromal cells form a physical barrier that shields cancer organoids from migrating immune cells and affects the distribution of immune cells. Modified from the studies of Lee et al.[30], Bai et al.[31], and Geyer et al.[32], respectively. (C) Cancer cell extravasation model. Endothelial cells and fibroblasts (pink) are seeded with 3D fibrin gel in the middle channel. Five days later, endothelial cells form a microvascular network (red line) with lumens opening to the

flanking microfluidic channels, allowing the perfusion of cancer and immune cells. The immune cell subset and factors disrupting the endothelial barrier and promoting cancer cell extravasation are examined with this model. Modified from the study of Chen et al.[33]. The arrows represent the direction of the medium and cell flow. (D) Cancer cell intravasation model. The microfluidic chip consists of an endothelial (right), a cancer cell (left), and a 3D ECM channel (middle) for investigating the effects of immune cells (blue) on cancer cell (green) intravasation. Cytokines secreted by immune cells increase endothelial permeability and regulate cancer cell intravasation. Modified from the study of Zervantonakis et al.[34]. The arrows represent the direction of the medium flow. (E) Drug effects on tumor–immune interactions in the evaluation model. The complex tumor microenvironment is mimicked by co-culturing cancer, immune, and endothelial cells with (the second channel from the right) or without (the second channel from the left) the cancer-associated fibroblasts (CAFs) in a microfluidic chip to evaluate the effects of trastuzumab, a well-known HER2-targeted drug, on the interaction between cancer and immune cells. Modified from the study of Nguyen et al.[35]. The arrows represent the direction of the medium flow. (F) Immune cell response monitoring model. Recreating parallel 3D immune–cancer space for monitoring immune cell [interferon-α-conditioned dendritic cells (IFN-DCs)] migration toward drug-treated (the second channel from the right) or untreated (the second channel from the left) cancer cells and the interaction. Modified from the study of Parlato et al.[36]. The lower panel is enlarged cross section of the above dashed box to show the migration of immune cells toward the drug-treated cancer cells. The arrow represents the direction of medium and cell flow. (G) Single-cell pairing models. I. Controlling the initial cell concentrations and the ratio between immune and cancer cells to real-time image the dynamic interactions on a microfluidic microwell array device at the single-cell level. II. Cell-trapping device for capturing one cancer cell and one immune cell from opposite directions depending on cell size to detect the ability of single immune cell to kill a single cancer cell. III. Single cancer and immune cells are co-encapsulated in a droplet stably docked in a microarray on a microfluidic chip to assess how ICIs influence the cancer-immune interaction. Modified from the studies of Tu et al.[37], Zhang et al.[38], Antona et al.[39] and Agnihotri et al.[40], respectively. (H) Immunotherapy evaluation models. I. By incorporating HCC cells as dispersed individual cells or as cell aggregates in a 3D collagen gel region and tumor-specific TCR-T cells are perfused into adjacent channels. TCR-T-cell function is assessed under different oxygen concentrations and inflammatory cytokine treatments in a microfluidic chip. II/III. The efficacy of ICIs or TILs is evaluated in microfluidic chips integrating MDPTS/PDOTS (II) or tumor fragments (III). Modified from the studies of Pavesi et al.,[15] Aref et al.[41], Jenkins et al.[42], and Doty et al.[43] The arrow represents the direction of the medium and ICIs. TCR-T: T cell receptor engineered T cell, ICIs: immune checkpoint inhibitors, TILs: tumor-infiltrating lymphocytes, MDPTS/PDOTS: murine-derived or patient-derived organotypic tumor spheroids. (I) Preparing cancer immunotherapeutic agent models. I. Fabrication of porous microspheres encapsulating immune cells on microfluidic chips through an electrospray process under the electric field for tumor immunotherapy. II. Synthesis of nanoparticles with desirable sizes by modified nanoprecipitation on microfluidic chips. The inner fluid, an organic solvent (10% $CHCl_3$ + 90% THF) rapidly mixed with the outer fluid, an anti-solvent (a mixture of water and MeOH), resulting in the formation of nano-precipitations through the self-assembly process. III. Isolation of engineered immunogenic exosomes from on-chip cultured cells for cancer immunotherapy. The immunomagnetic bead-bound exosomes are mixed with cancer peptides, then exposed to UV light to be released *via* photoreaction. Modified from the studies of Wu et al.[44], Wang et al.[45], and Zhao et al.[46], respectively.

metastasis. Different subtypes of macrophages and cancer cell aggregates were injected into the same channel for direct contact culture or two adjacent channels for separate culture in a 3D collagen matrix [**Figure 2B(II)**]. The results demonstrated that direct contact between M2a macrophages and the aggregates is necessary for the release of cancer cells from the aggregates (**Figure 3A**). This microfluidic model enables the dynamic visualization of cancer cell aggregate dispersion and the interaction between cancer cells and macrophages. In addition, the model facilitates real-time monitoring and precise measurement of cell-to-cell distances.

The migration and infiltration of other immune cells in the TME, in addition to macrophages, have important roles in cancer progression. A 3D TIME was recreated *in vitro* using microfluidic technology to model dynamic neutrophil migration through a microfluidic channel on a porous membrane toward tumor spheroids within hydrogel microwells[50]. Neutrophil extracellular traps (NETs) formed in the extracellular collagen matrix predominantly mediate collective tumor invasion, suggesting that NET–stromal interactions represent potential targets for cancer therapy (**Figure 3A**). Monocytes constitute another key cell population in the TME. Monocytes can attenuate or support cancer progression based on the phenotype[51]. When T cells were added to the outside of a bilayer hydrogel embedding monocytes with tumor spheroids or dispersed cancer cells, monocytes and the hypoxic environment were found to increase T-cell infiltration into tumor loci by monitoring T-cell migration through the vascular layer and into the cancer cell/monocyte layer[52] (**Figure 3B**).

The dense tumor stroma acts as a barrier for immune cell infiltration into the tumor core, potentially presenting a challenge for cancer immunotherapy. Pancreatic stellate cells (PSCs) are the major stromal cells in pancreatic ductal adenocarcinoma (PDAC). The effects of PSCs on immune infiltration were demonstrated using a microfluidic-based PDAC model comprised of 40 chips with 3 microfluidic channels each. Endothelial cells, PSCs, and PDAC organoids were seeded into different channels to simulate the spatial structure of the tumor stroma [**Figure 2B(III)**]. Stromal

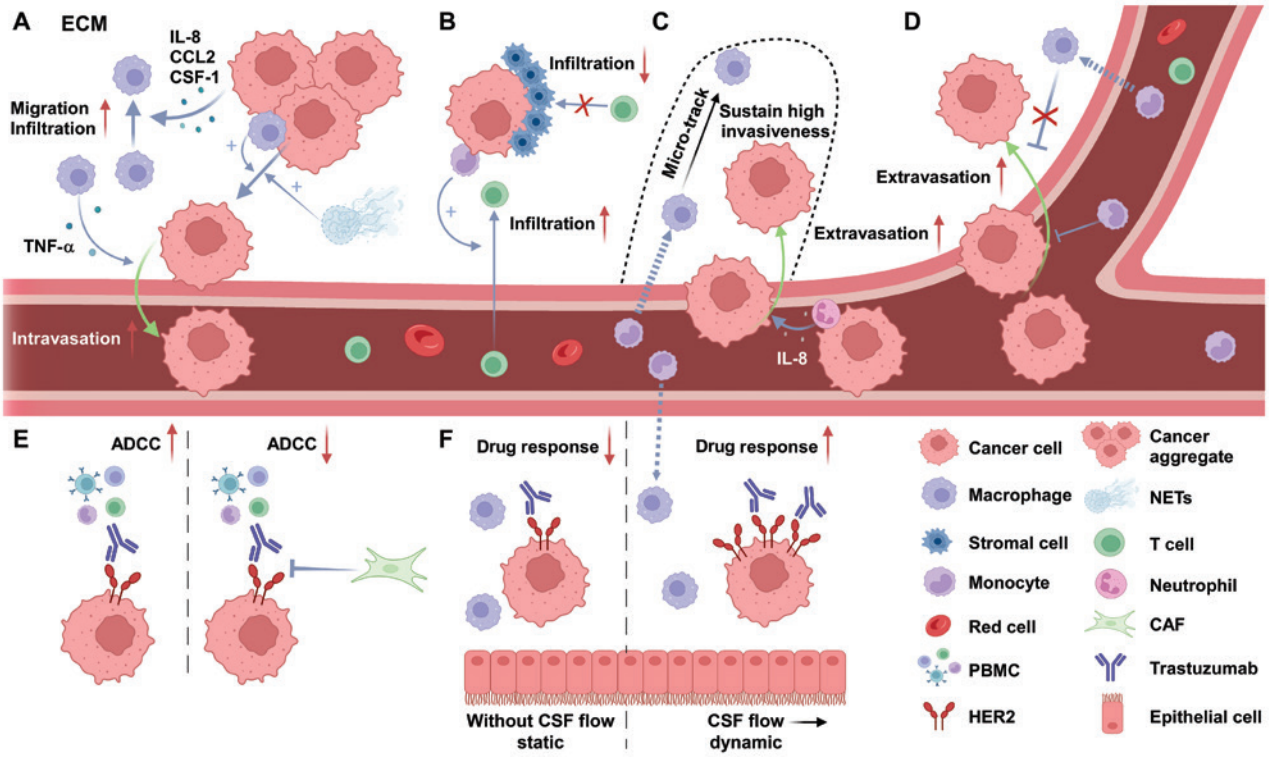


Figure 3 Overview of tumor–immune crosstalk inside the tumor microenvironment identified using microfluidic technology at the bulk-cell level in this review. (A) IL-8, CCL2, and CSF-1 derived from cancer cells enhance macrophage migration. M2a macrophages promote cancer aggregate dispersal when in contact with the cancer aggregates. NETs formed in the extracellular collagen matrix predominantly mediate collective tumor invasion. TNF-α secreted by macrophages increases endothelial permeability and regulates cancer cell intravasation. ECM, extracellular matrix; IL-8, interleukin 8; CCL2, C-C motif chemokine ligand 2; CSF-1, colony stimulating factor-1; TNF-α, tumor necrosis factor alpha; NETs, neutrophil extracellular traps. (B) Monocytes could increase T-cell infiltration into tumor loci, while stromal cells form a physical barrier that protects cancer cells from immune cell infiltration and recognition. (C) Inflamed neutrophils interact with cancer cells in blood vessels and secrete IL-8 to disrupt the endothelial barrier and promote cancer cell extravasation. Macrophages remold ECM and generate micro-tracks to allow extravasated cancer cells to sustain high invasiveness. (D) Monocytes can directly reduce cancer cell extravasation in a non-contact dependent manner in blood vessels. (E) Trastuzumab prolongs the interaction time between cancer cells and PBMC and induces an ADCC immune response, while CAFs antagonize this effect. PBMC, peripheral blood mononuclear cell; ADCC, antibody-dependent cell-mediated cytotoxicity; CAF, cancer-associated fibroblast. (F) *In vivo*-like CSF flow is recreated using dynamic fluid flow and applied to the microfluidic platform to recapitulate the choroid plexus dynamic microenvironment. HER2 expression on cancer cells is significantly higher in the dynamic condition compared to the static condition. Macrophages enhance the cytotoxic effects of anti-HER2 therapy. CSF, cerebrospinal fluid; HER2, human epidermal growth factor receptor 2.

cells were found to form a physical barrier that influences immune cell infiltration and shield cancer cells from recognition by migrating immune cells using this model (**Figure 3B**)[32]. This device represents a potentially effective tool for determining the mechanism underlying immune cell migration and infiltration and evaluating new cancer therapeutic approaches.

Angiogenesis and cancer metastasis

Angiogenesis is one of the hallmarks of cancer and shapes the TME by establishing new vascular networks. The tumor-associated vessels transport nutrients for tumor growth and provide a possible path for cancer metastasis, immune trafficking, or drug delivery. Cancer cells invade blood vessels, then transfer to remote organs through the blood circulation to form metastases, which are the leading cause of cancer-related deaths. This process is affected by various factors, such as immune cells and other soluble factors, that cannot be precisely quantified or easily manipulated in current animal or Transwell models.

Recent progress in microfluidic technology has enabled the development of *in vitro* assays that facilitate the study of vascular metastasis by mimicking the TME. Researchers can observe how extra- and intra-vasation of cancer cells are influenced by

immune cells *in vitro* by recreating the human vasculature on a microfluidic chip. In brief, human umbilical vein endothelial cells (HUVECs) suspended in fibrin gels are injected into the middle regions of the chip. The HUVECs become elongated, form vacuoles, and connect with neighboring cells to form lumens after 4–5 d (**Figure 2C**). Cancer and immune cells suspended in the medium can be perfused into microvascular networks by applying a hydrostatic pressure drop across the HUVEC gel region[33]. Chen et al.[53] reported that heterotypic aggregates are formed by inflamed neutrophils interacting with cancer cells in blood vessels because of mechanical trapping and neutrophil–endothelial adhesion using this type of microfluidic chip. The spatial localization of neutrophils and the secretion of IL-8 leads to disruption of the endothelial barrier and promotes cancer cell extravasation (**Figure 3C**). This process was further confirmed by Crippa et al.[54] using breast cancer cells. Moreover, Kim et al.[55] reported that macrophages generate micro-tracks to allow extravasated cancer cells to sustain high invasiveness regardless of the intrinsic metastatic potential in the presence of a microfluidic TME (**Figure 3C**), while Boussommier-Calleja et al.[56] reported that monocytes reduce cancer cell extravasation in a contact-independent manner. Monocytes lose the suppressive effects on extravasation and behave like macrophages after transmigrating through the vasculature[56] (**Figure 3D**). Recently, Lee et al.[57] developed a microfluidic device to determine the extravasation rates of cancer cells in the premetastatic niche. This device is regarded as a therapeutic screening platform for cancer extravasation.

In addition to extravasation, cancer cell intravasation is a rate-limiting step in the metastatic cascade that regulates the number of circulating tumor cells and thus presents a high risk for the formation of secondary tumors[58]. However, owing to the lack of physiologically relevant *in vitro* models, the mechanism underlying intravasation remains poorly understood. Zervantonakis et al.[34] used an *in vitro* 3D microfluidic model of the tumor–vascular interface involving triple coculture of endothelial cells, macrophages and cancer cells on a microfluidic chip to determine the effect of immune cells on cancer cell intravasation (**Figure 2D**). Tumor necrosis factor alpha (TNF-α) secreted by macrophages was found to increase endothelial permeability and regulate cancer cell intravasation. These results demonstrated the essential role of immune cells in cancer vascular metastasis. Microfluidic technology provides a valuable model for investigating the delicate mechanisms of tumor metastasis *via* blood vessels and developing effective therapeutics to slow cancer progression (**Figure 3A**).

Antitumor immune responses

Microfluidic chips enable real-time tracking of immune cell functions against cancer cells and evaluation of the ability to kill cancer cells. Nguyen et al.[35] tracked cell–cell interactions by reconstituting the human epidermal growth factor receptor 2 $(HER2)^+$ breast cancer microenvironment with four types of cell populations on a microfluidic chip (**Figure 2E**). The results demonstrated that trastuzumab, a well-known HER2-targeted drug, significantly prolonged the interaction time between cancer and immune cells and induced an antibody-dependent cell-mediated cytotoxicity (ADCC) immune response, one of the mechanisms underlying the *in vivo* antitumor efficacy of trastuzumab, while cancer-associated fibroblasts (CAFs) antagonized the effects of trastuzumab[35] (**Figure 3E**). Lim et al.[59] established a human brain choroid plexus (ChP)-on-a-chip to recapitulate key choroid plexus features, such as capillaries (an ECM barrier), epithelial layers, and pulsatile cerebrospinal fluid flow. The results showed that the cytotoxic effect on metastatic breast cancer cells in the ChP was considerably increased with this chip when trastuzumab and macrophages were applied under dynamic conditions compared to static conditions[59] (**Figure 3F**). Interferon-α-conditioned dendritic cells (IFN-DCs) were observed to migrate toward and phagocytize drug-treated cancer cells (a combination of romidepsin and IFN-α2b) by combining a microfluidic chip with advanced microscopy and a revised cell-tracking analysis algorithm rather than untreated cancer cells (**Figure 2F**)[36]. Using a similar method researchers tracked the migration-inducing effects between cancer and immune cells with specific gene knockouts or genetic polymorphisms to confirm the role of these genes in cancer-immune interactions within a mimicked TME[60,61]. More recently, researchers have been able to observe the trafficking of chimeric antigen receptor (CAR) T cells through blood vessels and penetration into gels containing cancer cells in a more physiologically relevant manner using a vascularized microfluidic chip. This approach may accelerate preclinical research on cellular immunotherapy for solid tumors[62].

The above findings indicate that microfluidic chips can better simulate the dynamic interactions between immune and tumor cells in the TME at the bulk cell level and facilitate an understanding of the mechanisms underlying vital pathologic processes, such as immune infiltration, cancer metastasis, and immune cell function, in response to drug treatment.

Cell–cell interactions at the single cell level

The heterogeneity of immune cells has attracted increasing attention in recent years. Complicated TME signals can modulate the differentiation, clonal expansion, and functional status of tumor-infiltrating immune cells, thus giving rise to diverse immune cell subsets with different characteristics and functions. For example, quite different killing abilities were reported among natural killer (NK) cells. Specifically, 49% of NK cells could not kill cancer cells, whereas some individual NK cells could kill as many as seven target cells[63]. Our previous study also revealed that the TME can educate infiltrating

$CD8^+$ T cells and upregulate the expression of some molecules to subdivide the functional subgroups of tumor-infiltrating lymphocytes (TILs) in the TME[64]. Therefore, deciphering the heterogeneity of immune cells in the TME could provide more precise information for understanding the mechanisms underlying tumor immune escape and the efficacy of tumor immunotherapy.

Conventional immune cell analysis technologies can only offer information about bulk immune cells at an average level, thereby masking the function and status of individual immune cells. Microfluidic chip technology provides the opportunity for high-throughput observation and analysis of immune cell heterogeneity at the single-cell level. An individual immune cell can be tracked for a long time on a microfluidic chip. Therefore, the entire contact history of each immune cell with cancer cells and the killing ability can be recorded and evaluated.

Several microfluidic chips have been designed to isolate single cells from cell populations with different methods. The first method is to control the initial cell concentrations and the ratio between immune cells and cancer cells on a microfluidic well array device with 6400 microchambers connected by flow channels, enabling massively parallel analysis of immune cell–cancer cell interactions at the single cell level [**Figure 2G(I)**][37].

The second method is based on the size difference between immune and cancer cells. Single NK and cancer cells could be captured from two opposite directions on a cell-trapping device in a microfluidic chip[38]. The trapped NK and cancer cells can directly interact, allowing the detection of the ability of single NK cells to kill cancer cells with or without pharmaceutical treatment [**Figure 2G(II)**].

The third method is based on an aqueous-in-oil droplet technology. NK cells were co-encapsulated with cancer cells in a microfluidic droplet array platform and evaluated the cytotoxic activity[39,40] [**Figure 2G(III)**]. The generated droplets were stably docked in an integrated microarray, enabling researchers to track individual cells and cell conjugates over several hours and analyze cell–cell interactions. Machine learning algorithms could be used to effectively measure NK cell dynamics and functional heterogeneity at the single cell resolution to better evaluate the dynamics of individual effector-target cell pairs and target death[65]. Moreover, Sullivan et al.[66] utilized a droplet microfluidic platform to assess how immune checkpoint inhibitors (ICIs) influence the interaction between cancer and $CD8^+$ T cells at the single cell level. Droplet microfluidic array platforms offer several advantages compared to other methods: (1) there is no need to design fixed cell trap devices according to cell size; (2) cells maintain high mobility within the droplets; and (3) cells are unaffected by adjacent cells because of paracrine effects. The respective advantages and disadvantages of the above three single cell pairing strategies in microfluidic models are summarized in **Table 2**.

Overall, single cell level studies with microfluidic chips are important for identifying hidden details in cell populations and revealing the heterogeneity of immune cells in the TME, including functional status, killing potential, and dynamic changes.

Evaluating the efficacy of cancer immunotherapies

In recent years, cancer immunotherapies, including cellular immunotherapies and ICB therapies, have shown remarkable efficacy in some cancer patients, whereas most patients, especially patients with solid tumors, have achieved a poor or no response. The level of programmed death ligand 1 (PD-L1) expression and tumor mutational burden (TMB) have been used as biomarkers for ICB therapies. However, the values of these biomarkers for predicting clinical efficacy have been limited according to a meta-analysis. Thus, whether immunotherapy should be used has become a dilemma, especially for patients who are non-responders. Inappropriate treatments may cause non-responders to absorb the costs of ineffective drugs and endure the side effects. Therefore, there is a significant demand for the development of novel immunotherapeutics and patient selection methods to enable precise cancer treatments.

Microfluidic chips are able to control the concentration of drugs or environmental factors. For example, Ayuso et al.[67] developed a microfluidic platform to establish gradients of oxygen, growth factors, or pH to evaluate the influence of TME stress on the antitumor function of immune cells. Therefore, microfluidic models may become valuable tools for predicting the efficacy of new immunotherapeutics and customizing personalized treatments.

Evaluating the efficacy of cellular immunotherapies

Cellular immunotherapies involve the adoptive transfer of isolated or engineered immune cells, such as TILs, NK cells, T-cell receptor-engineered T (TCR-T) cells, and CAR-T cells, to patients for cancer cell recognition and elimination. Pavesi et al.[15] designed a microfluidic chip incorporating human hepatocellular carcinoma (HCC) cells as dispersed individual cells or cell aggregates in a 3D collagen gel region to mimic an *in vivo* immunosuppressive microenvironment for selecting effective T-cell immunotherapies and tumor-specific TCR-T cells were perfused into adjacent channels. The migration and cytotoxic activity of various TCR-T cell populations could be dynamically imaged by regulating the culture conditions [e.g.,

Table 2 Comparison of single-cell pairing strategies in microfluidic models

	Advantages	Disadvantages
Well-based	1. Single or multiple cells pairing; 2. Ability to monitor cellular behaviors; 3. High throughput; 4. Real-time monitoring and end point analysis.	1. Unable to replicate dynamic cellular microenvironment at the single cell level; 2. Unable to accurately control the number of cells (dictated by Poisson statistics and initial cell concentration).
Trap-based	1. Efficient single cell pairing; 2. Ability to monitor cellular behaviors, from the point of cellular interaction and contact; 3. High throughput; 4. Real-time monitoring and end point analysis.	1. Stimulation of cells in bulk but not target; 2. Unable to avoid the paracrine effects on cellular behaviors due to incomplete spatial segregation of the trapped cells.
Droplet-based	1. Eliminates the need for size-specific cell trapping configurations; 2. Noise-free cellular microenvironment due to avoiding the paracrine effects of adjacent cells; 3. Single or multiple cell pairing; 4. Small volume of sample and reagent; 5. Sensitive due to low reaction volume; 6. High throughput; 7. Real-time monitoring and end point analysis.	1. Unable to replicate dynamic cellular microenvironment at the single cell level; 2. Random encapsulation.

oxygen levels and cytokine concentrations in the medium; **Figure 2H(I)**]. Lee et al.[68] evaluated the cytotoxic activity of various TCR-T-cell populations on HCC cell aggregates in the presence of monocytes seeded into the 3D hydrogel region of a microfluidic chip using a similar design.

Maulana et al.[69] established an *in vitro* tumor-on-a-chip model in which tumor aggregates or patient-derived breast cancer organoids (PDOs) were positioned in the hydrogel region adjacent to a vascularized microchannel to better recapitulate the TME encountered by CAR-T cells in solid tumors *in vivo*, thereby mimicking a perfused TME. Researchers monitored the recruitment and infiltration of CAR-T cells into solid tumors and dynamic cytokine release over 1 week using this model, thus providing a platform for evaluating the safety and efficacy of CAR-T cell therapies for solid tumors in a personalized manner[69]. Moreover, a more complex biomimetic organotypic microfluidic chip for real-time spatiotemporal monitoring of CAR-T cell extravasation, leukemia cell recognition, immune activation, cytotoxicity, and killing function has recently been reported. This chip was designed based on the tissue architecture of bone marrow and established an *ex vivo*, vascularized, and immunocompetent human leukemia bone marrow niche through precise structural design and the use of multiple types of human cells[70].

Park et al.[71] designed an injection-molded plastic array culture device composed of 12 rail-based microstructures embedded in microwells to evaluate the cytotoxic effects of NK cells on HeLa cells in a 3D microenvironment to improve the experimental throughput. In addition, Song et al.[72] incorporated clusters of colorectal cancer (CRC) cells into a tumor vasculature model to evaluate primary NK cell-mediated cytotoxicity against cancer cells using an injection-molded microfluidic platform. This microfluidic platform comprised of 28 wells in a standardized format further improved the experimental throughput for screening novel cellular immunotherapeutics[72]. These delicate microfluidic chips have promising applications for personalized treatment or high-throughput identification of effective cellular immunotherapies against cancers in preclinical and clinical settings.

Evaluating the efficacy of ICB therapies

Recent studies have elucidated the effects of ICB therapies on the clonal responses of TIL subsets[73], highlighting the critical relationship between drug efficacy and dynamic ICB drug–host immune interactions. Microfluidic chip technology has been used to simulate the immune microenvironment *in vitro* for screening and efficacy evaluation and facilitate ICB drug development.

Al-Samadi et al.[74] reported the first *in vitro* humanized microfluidic chip for assessing the efficacy of immunotherapeutic drugs in 2019, such as anti-PD-L1 antibodies and indoleamine 2,3-dioxygenase 1 (IDO 1) inhibitors, against head and neck squamous cell carcinoma (HNSCC) patient samples. Primary cancer cells were separated from the tumor tissues of HNSCC patients and seeded into a microfluidic chip together with immune cells and blood serum collected from the patients. Immune cell infiltration and cancer cell proliferation rates of samples from individual patients can be monitored in

response to the tested immunotherapeutic drugs[74]. Ao et al.[75] developed a high-throughput microfluidic platform for real-time imaging of immune-cell infiltration and target cancer cells killing to better understand immune-cell behavior within a 3D microenvironment. To this end, Ao et al.[75] trained a deep learning algorithm on clinical datasets and integrated the deep learning algorithm with the microfluidic platform, which created a powerful tool for identifying drugs that enhance T cell infiltration and immunotherapy efficacy.

TME heterogeneity has been recognized as a key factor influencing the clinical efficacy of ICB drugs. Cui et al.[76] integrated patient-derived tumor cells, human primary $CD8^+$ T cells, and human macrophages into a 3D brain-mimicking ECM with restructured brain microvessels to optimize anti-programmed death-1 (PD-1) antibody efficacy against different glioblastoma subtypes with heterogeneous TME characteristics. The results demonstrated that a CSF-1 receptor inhibitor enhances the efficacy of anti-PD-1 antibodies by regulating immune cell function using this glioblastoma-on-a-chip microfluidic system, providing a strategy for screening cancer subtype-specific drug combinations.

Model optimization with patient-derived tumor tissues

Microfluidic chip models have undergone continuous optimization to better recapitulate the *in vivo* TME. Early-generation microfluidic platforms typically use established cancer cell lines in dispersed formats. These models offer experimental accessibility and ease of manipulation but fail to capture critical *in vivo* microenvironmental features[29]. Tumor spheroids formed by 3D culture of cancer cell lines have been used to evaluate the efficacy of small molecules[77] and oligonucleotides as a substitute for animal models[78]. This 3D multicellular model demonstrates distinct cellular behaviors compared to dispersed cell cultures. Importantly, murine- or patient-derived organotypic tumor spheroids (MDOTS/PDOTS) exhibit significant advantages for evaluating the response to ICIs in contrast to conventional 2D- or 3D-cultured cancer cell lines. These organotypic models better recapitulate the local environment by preserving autologous lymphoid and myeloid cell populations[41] and have been used to determine responses or drug resistance to ICI treatment [**Figure 2H(II)**][41,42].

However, the enzymatic digestion process and relatively small size (approximately 40–100 μm) of these tumor spheroids result in a modified architecture that may not completely capture the complexity and heterogeneity of primary tumor tissues[79]. To enhance physiologic relevance, an increasing number of studies have focused on adapting freshly harvested tumor biopsy samples to ensure that the *in vitro* system faithfully reflects TME complexity and heterogeneity[80]. Moore et al.[81] and Doty et al.[43] prepared unprocessed tumor biopsy fragments with a needle attached to a syringe by directly coring tumor tissues freshly removed from mice or patients and obtained relatively large (approximately 150–300 μm) tumor fragments. These tumor fragments were cultured and monitored for at least 5 d and the cytotoxic ability of autologous TILs was analyzed using a microfluidic model [**Figure 2H(III)**].

The *ex vivo* culture of patient-derived organoid models, including circulating tumor cells and biopsy specimens, generally takes several weeks or even months. This process is time-consuming and results in the gradual loss of natural tumor immunity[82]. The use of a microfluidic chip could effectively increase the success rate of PDO culture and accelerate PDO growth. A microfluidic chip can maintain the viability of tumor biopsy samples for > 5 d and retain the native tumor immune microenvironment through the transport of oxygen and nutrients[83]. Zou et al.[83] designed a multilayer microfluidic chip for co-culturing PDOs with autologous peripheral blood mononuclear cells (PBMCs) and allogenic bone marrow mesenchymal stem cells to increase the success rate and growth rate of HCC organoid cultures and simulate the HCC TME. This microfluidic chip not only saved time and cost but also showed more precise prediction potential in assessing the response of patients with HCC to ICIs. Haque et al.[84] established a complex organotypic TME with prolonged cellular function and longevity by incorporating desmoplastic stroma and immune cells. This chip recapitulates the patient TME and thus could be used to individually evaluate the sensitivity to immunotherapeutic drugs, such as ICIs, prior to clinical application[84].

The advantages and disadvantages of patient-derived tumor tissue models in microfluidic chips are summarized in **Table 3**. The development of microfluidic technology will accelerate the identification of novel prognostic biomarkers and candidate drugs and improve the efficacy of personalized immunotherapy.

Preparing cancer immunotherapeutic agents

Cancer immunotherapy involves clinical applications of therapeutic agents designed to enhance immune responses against malignant cells. Microfluidic platforms can consolidate multiple complex procedures into integrated devices capable of simultaneous cell culture, capture, isolation, and analysis[24,85,86]. These systems offer significantly improved processing speed and operational convenience and reduced reagent consumption compared to conventional preparation methods. Various immunotherapeutic agents have been successfully fabricated using microfluidic technologies to effectively activate the immune system and eliminate cancer cells.

Table 3 Comparison of patient-derived tumor tissue models in microfluidic chips

	Advantages	Disadvantages
Organoid platform	1. 3D culture; 2. Able to be cultured *in vitro* for a long time; 3. High throughput; 4. Able to be genetically engineering and modified.	1. Lack of native stromal and immune cells; 2. Time-consuming; 3. Expensive; 4. Unable to exhibit the characteristics of whole organs.
MDOTS/PDOTS platform	1. Retains relevant tumor infiltrating lymphocyte and myeloid cell populations; 2. Provides sufficient quantity and quality of samples.	1. Unable to recapitulate T-cell priming or recruitment of naive immune cells to the tumor microenvironment; 2. Short-term *ex vivo* culture; 3. Size limit (approximately 40–100 μm); 4. Unable to fully recapitulate the complexity and heterogeneity of the tumor tissues due to enzymic digestion processes.
Tumor biopsy platform	1. Closely representative of the *in vivo* tumor microenvironment; 2. Easy to obtain samples; 3. High throughput.	1. Short-term *ex vivo* culture; 2. Size limit (approximately 150–300 μm); 3. Quantity and quality limit.

Fabrication of biocompatible microparticles evoking immune responses

Microfluidic technology enables the precise manipulation of multiple fluids within different microchannels and has been extensively utilized to fabricate multifunctional microparticles that can serve as delivery vehicles for small-molecule drugs, biomacromolecules, and even cellular therapeutics. The tunable microarchitecture of these microparticles creates an excellent environment compared to bulk delivery systems, preventing the encapsulated agents from contacting and interacting with the surroundings.

Recent studies have demonstrated the good biocompatibility, easy modification, and low immunogenicity of biofilm nanosystems (e.g., micellar nanocomplexes), which have significantly promoted the development of tumor immunotherapy by providing ideas for nanomedicine design and clinical translation[87]. For example, Wu et al.[44] developed a scaled-up method to generate porous microspheres that encapsulate NK cells *via* microfluidic electrospray for *in situ* tumor immunotherapy [**Figure 2I(I)**]. NK cells in the porous microspheres were protected from the complex external environment, thereby achieving significantly improved proliferation ability. With continuous proliferation, NK cells germinated from the surface of the porous microspheres, migrated into the tissues, then destroyed the tumor cells.

Moreover, photothermal conversion material-based photothermal therapy (PTT) has attracted increasing attention because of an ability to directly eliminate cancer cells and elicit antitumor immune responses[88,89]. Drugs/dyes can be encapsulated into nanoparticles through nanoprecipitation using a microfluidic system[90]. Microfluidic technology more effectively forms uniformly distributed nanoparticles with adjustable and larger particle sizes compared to conventional nanoparticle preparation methods, which may contribute to higher efficiency of photothermal conversion and cancer cell ablation. These findings indicate that microfluidic technology can be used to prepare biocompatible conjugated polymer nanoparticles as PPT agents [**Figure 2I(II)**] and evaluate immune responses *in vivo* and *in vitro* after PTT treatment[45].

Preparation of therapeutic exosomes to enhance antigen presentation and cytotoxic effects

Extracellular vesicles (EVs) can prime immune responses by presenting tumor antigens to immune cells. Immunogenic exosomes with an intrinsic payload of major histocompatibility complex (MHC) class I and II molecules and other co-stimulatory molecules can mediate immune responses, providing opportunities for the development of novel cancer vaccines and delivery systems for immunotherapy. Zhao et al.[46] introduced a 3D-printing-based microfluidic cell culture platform for integrating cell culture, exosome immunomagnetic isolation, exosome antigenic modification, and the photorelease of surface-engineered exosomes [**Figure 2I(III)**]. Engineered exosomes effectively enhanced antigen presentation and T-cell activation by decorating the exosome surface with melanoma peptides. Moreover, some studies have demonstrated the cancer therapeutic potential of NK cell-derived exosomes and developed a highly sensitive method to collect NK cell-derived exosomes on a chip with high purity and cytotoxic activity against cancer cells[91].

Therefore, microfluidic chips may serve as valuable tools for the preparation and development of personalized cancer immunotherapeutic agents.

Discussion

In this review, we summarize the recent advances in the application of microfluidic technology for understanding the TIME and cancer immunotherapy. Microfluidic technology can overcome many shortcomings of conventional 2D or 3D *in vitro* cell culture models by recapitulating the complex TIME to reveal the spatiotemporal dynamic interactions among immune, stromal, and cancer cells. The application of microfluidic technology provides promising tools for mimicking crosstalk inside the TME, evaluating the efficacy of cancer immunotherapies, and preparing immunotherapeutic agents. Therefore, microfluidic chips have been considered potential preclinical models for testing and predicting the efficacy of immunotherapies.

A key point in constructing an effective microfluidic chip is the type of cell or tissue to choose and how to culture the cell or tissue. Researchers have used different types of cells or tissues to recreate a TME based on different study aims. Endothelial cells are generally used to construct perfusable vascular structures. Established cancer cell lines, primarily isolated cancer cells, spheroids, and organoids, can be cultured as sparse or aggregated or mixed with stromal or immune cells in the presence of ECM components. Although traditional monolayer cell cultures have been widely used for decades, the lack of tissue architecture and complexity prevents accurate identification of *in vivo* biological processes. Organoid technology has revolutionized *in vitro* culture tools by creating 3D models to recapitulate the cellular heterogeneity, architecture, and functionality of native tissues[92]. Organoids preserve the complex tissue architecture and cellular diversity of cancers, enabling more accurate predictions of tumor progression and drug responses. Integration with microfluidic platforms will further enhance the ability to model tumor-environment interactions in real-time[93]. However, the absence of stromal and immune cells, as well as the extensive time required to produce a sufficient quantity of organoids, hinders the application in the study of tumor immune interactions.

Tumor biopsy fragments from syringe needles or freshly resected tumor tissues, such as MDOTS and PDOTS, are preferred because the fragments retain some characteristics of the TME in short-term *ex vivo* culture. However, the MDOTS/PDOTS platform is only capable of evaluating the interactions between tumor and tumor-infiltrating immune cells rather than recapitulating the priming or recruitment of naive immune cells into the TME. Tumor biopsy fragments from fine-needle aspirates (FNAs) and core-needle biopsies often yield far fewer cells compared to surgical biopsies and the viability of needle biopsies is frequently poorer. In addition, the use of tumor tissues is limited by the size of the samples. The tumor biopsy fragments ranged from 150–300 μm in diameter[43], whereas the MDOTS/PDOTS platforms ranged from 40–100 μm in diameter[41,42]. Although a larger sample size could better maintain the *in vivo* TME and heterogeneity, significant challenges remain in terms of survival rate and viability maintenance time. Previous studies of hypoxia in multicellular tumor spheroids have demonstrated the following: (1) spheroids < 200 μm in diameter show no hypoxia signatures; (2) spheroids develop distinct hypoxic cores when grown to 200–300 μm in diameter; and (3) spheroids > 500 μm in diameter exhibit central necrosis[42]. Furthermore, the influence of device dimensions, biophysical parameters (including interstitial flow and hypoxia), and metabolic conditions on tumor–immune interactions, especially cytokine signaling dynamics, remains poorly characterized and warrants systematic investigation. Future critical research directions should focus on extending the lifetime of *ex vivo* cultures, conducting clinical validation studies, and developing medium-to-high throughput system configurations.

Polydimethylsiloxane (PDMS) is the predominant material for molding microfluidic devices, offering the advantages of high optical transparency, tunable mechanical properties, chemical inertness and non-toxicity, biocompatibility, gas permeability, low autofluorescence, and facile surface modification *via* plasma treatment, ultraviolet (UV) irradiation, and silanization. However, some disadvantages of PDMS limit industrial applications, such as costly and laborious fabrication processes, poor scalability, high variability, unstable surface chemical properties, and easy adsorption of small molecules[94]. Therefore, alternative materials with enhanced surface stability, improved biocompatibility, maintained optical transparency, and simplified device fabrication processes for microfluidic applications are urgently needed.

Thermoplastic microfluidic platforms have attracted significant interest because of excellent material properties and lower production costs compared to PDMS. Several cost-effective thermoplastic polymers have been adopted for microfluidic device fabrication, including polymethyl methacrylate (PMMA), cyclic olefin polymer (COP), cyclic olefin copolymer (COC), polystyrene (PS), polycarbonate (PC), polyethylene glycol (PEG), and polyester terephthalate (PETE). These materials offer demonstrated biocompatibility, appropriate gas permeability, and excellent optical properties[95-98], providing a tremendous opportunity for mass production of microfluidic devices at lower costs.

In recent years, 3D printing, as an additive manufacturing technology, has received great attention in microfluidic manufacturing. Three-dimensional printing has several advantages, including a lack of need for cleanrooms, a low cost of consumables and equipment, high accessibility, fast

production, easy editing and reprinting of designs, multi-material compatibility, and multiphase printing capacity[99-101]. Therefore, 3D printing is expected to revolutionize microfluidic technology *via* faster and less expensive device prototyping and by providing opportunities for mass manufacture and commercialization.

Advances in higher throughput and highly parallelized microfluidic systems generate data at a sheer volume that surpasses conventional processing capabilities. Artificial intelligence (AI) has emerged as a pivotal tool in transforming this data deluge into actionable knowledge, enabling systematical discovery and enhanced interpretation of complex biological phenomena[102]. Importantly, AI offers a powerful means to overcome the inefficiencies and uncertainties of traditional drug discovery, while minimizing human intervention and bias[103]. The integration of AI with microfluidic chips yields a synergistic outcome, enabling rapid real-time analysis, the processing of large datasets with high accuracy and sensitivity, and the generation of reliable results. Chiang et al.[104] integrated a high-throughput microfluidic platform with AI-based algorithms to provide a cost-effective and non-destructive method for real-time assessment of tumor spheroid viability. Similarly, Sarkar et al.[65] and Ao et al.[75] developed AI-driven microfluidic systems to analyze the dynamics of individual effector-target cell pair conjugation and to identify drugs that enhance T cell infiltration and cancer immunotherapy efficacy, respectively. Despite this promise, this approach faces challenges, such as data quality vulnerability, high computational resource demands, and the need for expert result interpretation. We anticipate that ongoing technological progress will resolve these limitations in the foreseeable future.

Overall, given the dynamic and complex nature of the TME, it is difficult for a single *in vitro* or *ex vitro* model to fully replicate all aspects of the *in vivo* TME. Nevertheless, microfluidic chips have been proven to be valuable tools for cancer immunology research because microfluidic chips enable precise control over key parameters, such as cellular confinement, that better simulate TME characteristics. Future studies should address some critical translational challenges, including systematic validation against *in vivo* tumor models to verify device functionality and the correlation of microfluidic-derived data with clinical tumor specimens. Although preliminary investigations have begun to examine how device parameters (e.g., device height and gel region width) influence cytokine profiles, a comprehensive understanding remains to be achieved.

In this review, we summarized the application of microfluidic technology to mimic complicated crosstalk among TME cells, predicting the efficacy of immunotherapeutics and preparing immunotherapeutic agents. The development of microfluidic technology will accelerate the identification of novel prognostic biomarkers and the screening of candidate drugs, which may drive clinical and translational efforts to promote personalized immunotherapy and ultimately improve the prognosis of cancer patients.

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Conflict of interest statement

No potential conflicts of interest are disclosed.

Author contributions

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