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Immunotherapy discovery on tumor organoid-on-a-chip platforms that recapitulate the tumor microenvironment

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ABSTRACT

Cancer immunotherapy has achieved remarkable success over the past decade by modulating patients' own immune systems and unleashing pre-existing immunity. However, only a minority of cancer patients across different cancer types are able to benefit from immunotherapy treatment; moreover, among those small portions of patients with response, intrinsic and acquired resistance remains a persistent challenge. Because the tumor microenvironment (TME) is well recognized to play a critical role in tumor initiation, progression, metastasis, and the suppression of the immune system and responses to immunotherapy, understanding the interactions between the TME and the immune system is a pivotal step in developing novel and efficient cancer immunotherapies. With unique features such as low reagent consumption, dynamic and precise fluid control, versatile structures and function designs, and 3D cell co-culture, microfluidic tumor organoid-on-a-chip platforms that recapitulate key factors of the TME and the immune contexture have emerged as innovative reliable tools to investigate how tumors regulate their TME to counteract antitumor immunity and the mechanism of tumor resistance to immunotherapy. In this comprehensive review, we focus on recent advances in tumor organoid-on-a-chip platforms for studying the interaction between the TME and the immune system. We first review different factors of the TME that recent microfluidic *in vitro* systems reproduce to generate advanced tools to imitate the crosstalk between the TME and the immune system. Then, we discuss their applications in the assessment of different immunotherapies' efficacy using tumor organoid-on-a-chip platforms. Finally, we present an overview and the outlook of engineered microfluidic platforms in investigating the interactions between cancer and immune systems, and the adoption of patient-on-a-chip models in clinical applications toward personalized immunotherapy.

Contents

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1. Introduction

In recent years, immunotherapy has become a powerful and evident approach capable of re-educating the immune system to fight cancer, and has been rapidly developed into one of the leading options for cancer treatment [1]. Unlike conventional anti-cancer therapies, immunotherapy is a biological therapy designed to modulate the functions of a patient's own immune system to recognize and attack the cancer cells, based on monoclonal antibodies or cells produced in human body or engineered *ex vivo*, such as chimeric antigen receptor (CAR) T-cells, which can produce durable remission even in advanced tumors with relatively fewer side effects [2]. Compared with conventional anti-cancer therapies which exhibit low selectivity between normal and cancer cells, immunotherapy can target specific cancer cells and prevent recurrence due to the memory of the immune system [3]. There are various types of immunotherapy such as adoptive cell therapy which includes the injection of functional antitumor immune cells engineered and expanded *ex vivo*, immune checkpoint inhibitors (ICIs) which target the negative regulators of the immune system, cytokines and immune modulators, cancer vaccines, and oncolytic viruses [4,5].

During the development of these different types of immunotherapy, the tumor microenvironment (TME) that comprises cancer cells, immune populations, stromal cells, and soluble molecules such as cytokines and growth factors, plays a significant role [6]. The TME affects multiple aspects of the tissue such as metabolism, vascularization, and the immune system, which have been shown to be critical to tumor development [7,8]. Due to the special structure and properties, a tumor can produce local biochemical differences, such as oxygen gradients and hypoxia inside tumors, pH, and growth factors, to shape tumor features [9]. A high level of hypoxia in the tumor core, along with relevant soluble mediators severely hinders the transportation of immune cells to the tumor, causing resident immune cells to be polarized to change phenotypes and become tumor-promoting [10,11]. As a result, effective immune cells are difficult to gain access to the tumor mass, while immunosuppressive cells, including M2-tumor associated macrophages (M2-TAM), regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSCs) accumulate in the tumor site [12]. The TME affects various aspects of tissue homeostasis, leading to relapses in most aggressive cancer treated by conventional chemotherapy, radiotherapy, or surgical resection [13]. Therefore, restoring and strengthening the antitumor immunity has become a promising direction to develop future cancer therapies [14]. New efficacious immunotherapeutic strategies have revolutionized the therapeutic treatment of some types of metastatic cancers which were previously considered incurable with unexpected clinical benefits [15].

While these advancements of immunotherapy have proved the potential of modulating the immune system to treat tumors, most of current immunotherapeutic approaches have so far been efficient only in certain tumor types and minorities of patients. Meanwhile, the combination of immunotherapy with traditional drugs is being investigated to maximize antitumor efficacy to more patients and cancer types [16]. Extending the application of immunotherapy as a more widely accepted treatment approach for multiple cancer types requires a better understanding of the interactions between tumor cells, immune cells, cytokines, and inhibitory factors within the TME [17]. In this light, although *in vivo* models remain a gold standard, microfluidic platforms, which converge micro-physiological tumor models and microfabrication techniques, have been extensively investigated and employed to provide patient-specific information on cancer-immune interactions and predict individual responses to immunotherapy. Microfluidic platforms are devices with various micrometric structures, consisting of channels and chambers filled with tiny amounts of fluids or 3D matrix that can support the co-culture of cells or tissues, which enable the study of physiopathological systems in a highly controllable manner [18–25]. Through precise control of fluid flows and volumes, culture medium and matrix, surface properties, and stimuli in microfluidic devices, TME features including hypoxia, acidic pH, vascular dysfunction, nutritional starvation, and immunosuppressive mechanisms can be well recapitulated in microfluidic platforms [26,27]. Accordingly, microfluidic platforms recreating the TME, also known as "tumor-on-a-chip", enable a systematic understanding of the individual and synergistic effects of various components in tumor progression [15,28]. They can regulate key parameters such as shear stress, and are increasingly being utilized to simulate 3D TME structures through immune-cancer cell co-cultures, thus enhancing the similarity to the TME *in vivo* and immunotherapy outcomes [13,15,17,29]. Various structures of microfluidic platforms have been designed and fabricated to establish tumor models *in vitro* and discover effective anti-cancer therapies [30,31].

This review focuses on recent advances achieved by using microfluidic platforms for immunotherapy investigations (Fig. 1). We first comprehensively review the different factors of the TME that must be taken into account in microfluidic-based strategies to generate advanced tools for the investigation of cancer-immune crosstalk. Then, we highlight the latest advances in the evaluation of different immunotherapies' efficacy using microfluidic platforms through engineering customized microfluidic designs for the faithful reproduction of the TME. Finally, we pre-

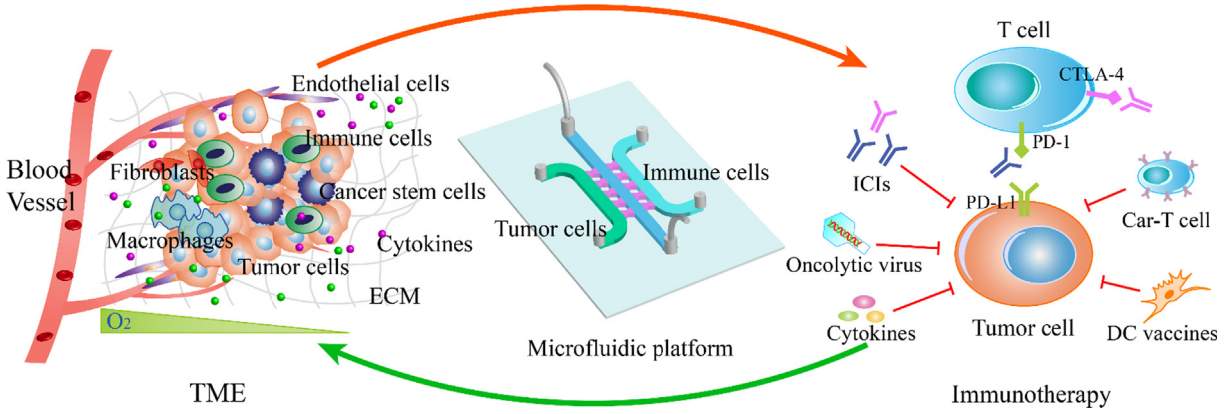

Fig. 1. Engineered tumor-on-a-chip platforms for investigation of cancer immunotherapy by recapitulating the TME.

sent an overview and outlook of engineering microfluidic platforms in investigating interactions between cancer and immune systems, and the adoption of patient-on-a-chip models in clinical settings towards personalized immunotherapy.

2. Modeling tumor microenvironments on microfluidic platforms for immunotherapy

The TME consists of multiple cell types (immune cells, endothelial cells (ECs), fibroblasts, mesenchymal stroma/stem cells (MSCs), etc.) and extracellular components (extracellular matrix, cytokines, growth factors, hormones, etc.) that surround tumor cells and are nourished by a vascular network [32]. The TME has attracted more and more attention for its significant influence on tumor initiation, progression, and metastasis, as well as effects on tumor therapeutic responses and efficacy [33]. Developing more *in vivo* similar TME models can not only provide a reliable method to predict sensitivity to cancer therapeutics and evaluate therapy efficacy, but also promote our understanding of the tumor-TME interactions, and advance the investigation of personalized or precision functional medicine [34].

Microfluidic devices are powerful for modeling the TME, as multiple factors in TME can be controlled separately and precisely in microfluidic platforms [35–38]. They can be easily customized to comprise multiple chambers and channels with different structures according to different experimental requirements, which enables precise spatial control of cell distribution and fluid flow rates, thus facilitating the research of many important biochemical and biomechanical processes [39–41]. These channels can also be used to construct physical or chemical gradients in a microfluidic device which are more precise and sustained than in conventional *in vitro* systems [42,43]. As to the tumor immune microenvironment modeling, microfluidic devices are capable to establish well-defined gradients to simulate the recruitment of immune cells from the blood flow through inflammatory chemokine/cytokine gradients [44]. In addition, these microfluidic systems allow the imitation of perfusion of immune cells in vascularized networks, which may be the key to replicating the recruitment of immune cells to the tumor site [45–47]. Furthermore, multiple different features can be combined within one device. Combined methods can control a wide range of TME features, including cellular compositions and ratios, flow rates, soluble factors, oxygen levels, matrix structure, matrix stiffness, and mechanical properties, allowing a better and systematic understanding of the cancer-immune crosstalk [48]. Herein, we introduce different features of the tumor immune microenvironment reconstructed on microfluidic platforms, including 3D cell culture, extracellular matrix, cell co-culture, vasculature, chemokine gradient, hypoxia, and biophysical forces.

2.1. 3D cell culture

2D culture systems have been utilized by researchers for a long time to investigate tumor biology and tumor therapies due to their low cost and ease of use. Microfluidic devices have been used for 2D cell cultures. For 2D culture, different types of cells are cultured in microchannels as monolayers to study cell physiological activity and responses to drugs. However, there are some drawbacks of 2D culture such as the lack of cell–cell interactions and specific cell-extracellular environment crosstalk, which are essential to physiological functions to include cell proliferation, differentiation, migration and responses to stimuli [49]. Compared to 2D culture, 3D culture is increasingly exploited as a tumor model to recapitulate the complex and heterogeneous TME [9,50]. Multiple 3D culture systems have been developed for *in vitro* tumor modeling, including hydrogel culturing cells, cell spheroids, organoids, 3D bioprinting, etc.

Microfluidic 3D models are becoming more and more popular to mimic the physiological architecture of tissues and organs by the utilization of extracellular matrices, such as collagen or biocompatible polymers, to realize cell culture in a 3D environment [50,51]. As an example, an easily customizable 3D model was developed by Pavesi *et al.*, in which the TME conditions were regulated and the functions of different T cell preparations was tested [52]. Human cancer hepatocytes were seeded in a 3D collagen region of the microfluidic device as dispersed cells or cell aggregates (Fig. 2A). Tumor-specific T cell receptors engineered human T cells (TCR–T cells) were then added in neighboring channels to analyze their ability to migrate and kill the tumor cells with varying oxygen levels and inflammatory cytokines. The results show that only in the 3D model, the influence on TCR–T cells function by oxygen levels and the inflammatory environment could be detected. Another form of 3D cell culture is tumor spheroids, which can be produced by 3D aggregations of cells [53]. For instance, Su *et al.* developed a facile and high-throughput microfluidic 3D cell culture system with spheroid-in-gel culture arrays, using hydrogel confinement via the geometrical limitation of channel heights and capillary burst valve effects [54]. By hanging a drop culture on a patterned polydimethylsiloxane (PDMS) substrate, breast cancer (MCF-7) spheroids were formed, which were then directly integrated on another chip in separate hydrogel beads at corresponding positions, avoiding extra manual cell spheroid handling and transferring steps.

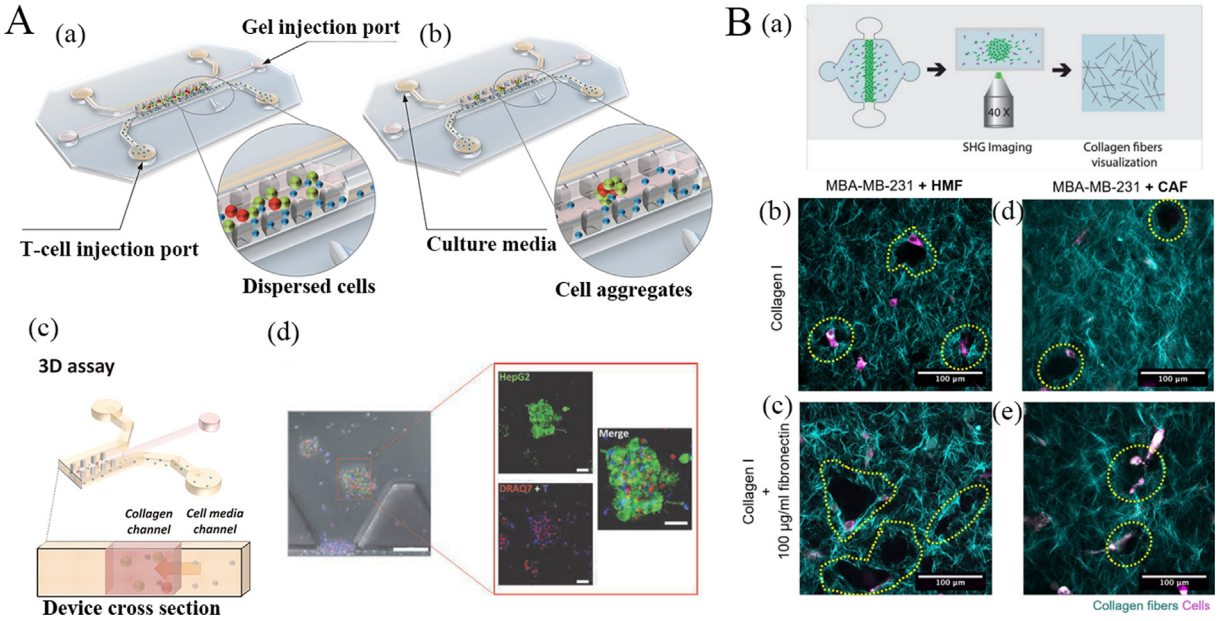

Fig. 2. 3D cell culture of cells in ECM in microfluidic devices. (A) A 3D microfluidic device for investigation of TCR-engineered T cell function. (a) 3D image of the device with dispersed cells. (b) 3D image of the devices with cell aggregates. (c) The directional chemotaxis in a 3D microfluidic device of engineered T cells. (d) HepG2 cell aggregates in collagen gel in the device. Reproduced from Ref. [52] with permission of JCI. (B) The effect of 3D heterogeneous ECMs on the migration of MDA-MB-231 cells. (a) Second harmonic generation (SHG) imaging for the matrix visualization. (b–e) Gaps were formed in the matrix due to collagen degradation and remodeling. MDA-MB-231 cells co-cultured with human mammary fibroblasts (HMFs) in the collagen matrix (b) compared with fibronectin-rich matrix (c). MDA-MB-231 cells co-cultured with cancer-associated fibroblasts (CAFs) in the collagen matrix (d) compared with fibronectin-rich matrix (e). Reproduced from Ref. [55] with permission of MDPI.

2.2. Extracellular matrix (ECM)

ECM is secreted by cells for biophysical, biochemical and structural support of cells, and is composed of the non-cellular component of tissues [56,57]. The tumor stroma is composed of a variant ECM compared with normal tissues, which is produced by all cell types within the TME, resulting in an intricate physical network that not only plays an important role in tumor cells' invasion and metastasis, but also affects the response to therapeutic treatment [58,59]. The ECM consists of various components, to name a few, collagen, fibronectin, laminin, proteoglycans, etc. The composition and organization of the ECM, cell–cell interactions, and the tumor cell architecture constitute a physical barrier to the delivery of tumor-infiltrating immune cells [60,61]. Special ECM within TME has fundamental influence on cell signaling, angiogenesis, and tissue biomechanics [62–64]. A human tumor-derived matrix "Myogel/fibrin" was exploited by Al-Samadi *et al.* to culture a tongue cancer cell line (HSC-3) and immune cells from three healthy donors in a 3D microfluidic chip for immunotherapy efficacy testing [65]. The tumor-based matrix "Myogel" was originated from leiomyoma tissue and suitable for 3D *in vitro* assays of human cancer, offering an ideal TME for the cultured cancer cells due to its neoplastic origin. Recently, an interesting microfluidic model was developed by Lugo-Cintrón *et al.* to study the effect of 3D heterogeneous ECMs on the migration ability of MDA-MB-231 cells, which is a highly invasive human breast cancer cell line [55]. More cancer cell migration was induced by a fibronectin-rich matrix with co-cultured normal breast fibroblasts. An increase of metalloproteinases (MMPs) secretion was observed in cancer-stromal co-cultures to increase migration distance (Fig. 2B). This model imitates the *in vivo* TME for tumor cells invasion, enabling the analysis of tumor cell migration in a 'physiologically' relevant environment. In addition, some interesting work was carried out by Sabhachandani *et al.* to develop an integrated droplet microfluidic platform for high-throughput production of immunogenic diffuse large B cell lymphoma (DLBCL) spheroids [66]. A novel hydrogel, combined of 1% alginate and 0.15% puramatrix, was used as ECM for 3D cell culture. Cancer cells, fibroblasts, and lymphocytes were co-cultured into spheroids in this combined hydrogel, which was found to promote cell adhesion and aggregation. Immunomodulatory drug lenalidomide was applied to cell spheroids in this platform, allowing for dynamic analysis of cell proliferation and therapeutic efficacy, and a direct anti-proliferative effect was observed on activated B-cell-like DLBCL spheroids compared with untreated spheroids.

2.3. Cell-cell interactions

In the TME, there are various cell types including tumor cells, endothelial cells, fibroblasts, immune cells, mesenchymal stroma/stem cells (MSCs) [67]. The interactions between different cells are crucial to tumor development [68,69]. Herein, we review some important studies based on microfluidic platforms to investigate tumor-immune cell and tumor-stromal cell interactions within the tumor immune microenvironment (TIME) [70–72], such as immune cells, mesenchymal stromal cells, fibroblasts, and endothelial cells.

2.3.1. Immune cells

Both innate immune cells (dendritic cells, macrophages, neutrophils, natural killer cells, etc.) and adaptive immune cells (T and B lymphocytes) impact tumor cells by direct contact or through chemokine and cytokine signaling in the TME, which influence the behaviors of a tumor and its response to therapeutic treatments [73]. Within the altered TME, immune cells can vary their activation status and localization, and thus either support or suppress therapeutic efficacy. For instance, in the TME, tumor-infiltrating immune cells are re-educated to be tumor supportive through immune silencing and promote tumor progression [74,75].

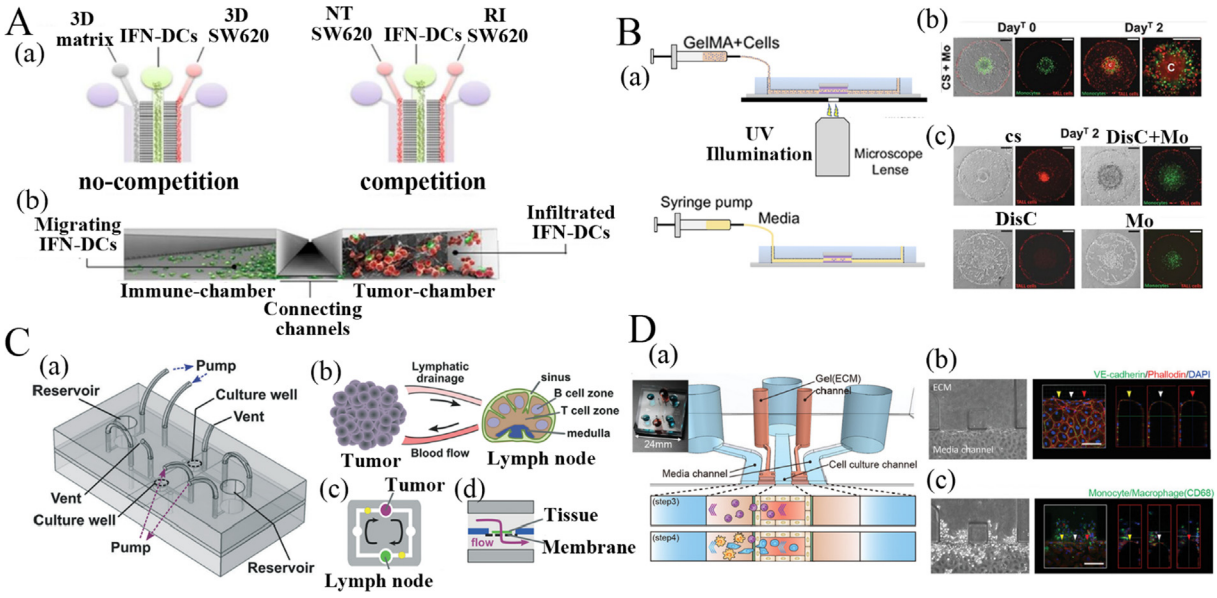

Fig. 3. Cell interactions between tumor cells and immune cells. (A) Design of the 3D microfluidic device to analyze DC-cancer cells interaction. (a) Schematic representation of two experimental designs: no-competition and competition settings. (b) A section view of the microfluidic device showing the migrating of DCs toward tumor cells. Reproduced from Ref. [76] with the permission of Springer Nature. (B) (a) Photopatterning of cell-laden GelMA hydrogels within the microfluidic device. (b) Day T0 panel, merged and fluorescence images of T cells adhered onto the periphery of a bilayer GelMA hydrogel containing a cancer spheroid, monocytes, and endothelial cells (CS + Mo) immediately after introducing T cells. The Day T2 panel is a fluorescence image of the cell-laden construct 2 days after infiltration. Monocytes and T cells were fluorescently labeled by green and red dyes, respectively. (c) Brightfield and fluorescence images of the bilayer hydrogels with cancer spheroids, dispersed cancer cells, and monocytes (DisC + Mo), DisC, and monocytes (Mo) within the interior of the hydrogel at day T2. Adapted from Ref. [78] with permission of American Association for Cancer Research. (C) A microfluidic chip for modeling tumor-lymph node interactions. (a) 3D schematic of the microfluidic device with connecting tubes. (b) tumors and lymph nodes crosstalk *in vivo*. (c and d) Top view (c) and side view (d) of the dual-slice chip. Adapted from Ref. [79] with permission of The Royal Society of Chemistry. (D) (a) Photograph and schematic of the microfluidic device showing migration of cancer cells and monocytes into the hydrogel. (b) The endothelial network is formed in the 3D model. (c) Invasion of monocytes in the 3D model. Reproduced from Ref. [80] with the permission of Wiley.

2.3.1.1. Dendritic cells (DCs). DCs play a key role in the antitumor response of the adaptive immune system. They have the specific role to initiate interactions between the immune system and cancer cells by recognizing cancer cells, collecting tumor antigens (Ags), and presenting to naive T cells. Then, activated T cells transfer to the tumor site to interact with cancer cells. Studies have shown that interferon-α (IFN-α) can increase DCs' phagocytic function and the ability to induce Ag-specific T-cell response. For example, a novel microfluidic platform developed by Parlato *et al.* recreated the interaction between cancer and immune systems with specific 3D environmental properties, for analyzing human DCs' behaviors toward tumor cells [76]. Combining the microfluidic platform with advanced microscopy and a cell tracking analysis system, it was made possible to evaluate the guided motion of IFN-DCs towards drug-treated cancer cells and the phagocytosis activity (Fig. 3A), allowing the understanding of IFN-DC-cancer cell interactions, which was largely guided by the CXCR4/CCL12 axis. Additionally, a lymph node-on-a-chip flow device designed to investigate the T cell-DC interaction was realized by Rosa *et al.*, enabling the real-time analysis of dynamic interactions between adherent dendritic cells and flowing lymphocytes [77]. The attachment and detachment of CD8 + cytotoxic T cells (antigen-specific and unspecific) and CD4 + T helper cells to DCs (activated or non-activated) were studied at different flow shear stresses. This system proved the ability to selectively induce adhesion of antigen-specific T cells via serial contacts and showed that antigen-specific attachment and detachment occurred at different shear stresses. Shear stress of 0.1–1 Dyn cm^{-2} was suitable for cell-based affinity isolation of antigen-specific T cells by unknown antigens.

2.3.1.2. Lymphocytes. Preclinical testing of different types of T cells that are engineered for targeting tumor cells was also realized on microfluidic platforms. For instance, to test T-cell recruitment, Aung *et al.* developed a perfusable multi-cellular tumor-on-a-chip platform including cancer cells, monocytes, and endothelial cells, which were spatially confined within a gelatin hydrogel by 3D photopatterning in a controlled manner [78]. An endothelial layer around the constructs was created by the migration of endothelial cells towards a chemokine gradient (Fig. 3B). The effect of monocytes and cancer cells' interaction on T-cell recruitment was examined, and results showed that spheroid cultures recruited more T-cells compared to dispersed cancer cells, and the co-culture of monocytes with cancer cells increased T-cell recruitment. Another microfluidic platform was reported by Moore *et al.*, termed EVIDENT (Ex Vivo Immuno-oncology Dynamic Environment for Tumor biopsies), which accommodated up to 12 individual tumor biopsy fragments interacting with flowing tumor-infiltrating lymphocytes (TILs) in a dynamic microenvironment [81]. The system promises real-time high-resolution imaging of the interaction between tumor fragments and TILs to assess TIL-mediated tumor killing and tumor responses to ICI treatments.

Single-cell analysis can reveal more cellular details hidden in bulk analysis [38,82–88]. Single-cell level research for high-throughput parallel analysis of immune cells' heterogeneity through cell pairs with tumor cells was reported by Tu *et al.*, using a microfluidic microwell array [89]. By precise control of the numbers and ratios of tumor cells and T cells, this system can analyze the dynamics of CD8 + T cell and leukemia cell interactions inside 6,400 microwells at the same time. Compared with bulk investigations, single-cell level experiments revealed heterogeneity in T cell killing capability and time-dependent killing dynamics by studying the interactions between T-cell and tumor cell pairs.

Instead of T cells, Shim *et al.* exploited slices of a murine lymph node to co-culture with tumors or normal tissues on a microfluidic chip with recirculating media [79]. A small volume of media could

continuously circulate in this multi-compartment microfluidic chip through two tissue samples to realize organ-level cross-talk by secreted factors (Fig. 3C). When co-cultured with tumor slices, lymph node slices were more immunosuppressed than the slices co-cultured with normal tissues, indicating that the device was able to model some important features of tumor-immune interactions.

2.3.1.3. Monocytes. Monocytes are one of the important components of TME, which can modulate T cell activities. They can transfer their phenotype from immunocompetent to immunosuppressive and become tumor-associated macrophages (TAMs) under the influence of TME-specific signals [90,91]. TAMs play an important role in tumor progression and metastasis, accounting for up to 50% of the tumor mass. TAMs are 'plastic' and can polarize into two major subtypes: the normally activated pro-inflammatory M1 macrophages which promote T helper (Th) 1 responses and have anti-tumor activity, and the alternatively activated M2 macrophages which can promote tissue repair and Th2 responses [92]. Bai *et al.* developed a microfluidic system to integrate human monocyte-derived macrophages and tumor cell aggregates in a 3D hydrogel scaffold, with a co-cultured endothelial monolayer to construct an *in vitro* TME [93]. The influence of M0, M1, M2a, M2b and M2c macrophages in human lung adenocarcinoma (A549) aggregate dispersion was studied to represent epithelial- mesenchymal transition (EMT). M2a macrophages were observed to infiltrate and release tumor cells from cell aggregates, indicating that ICAM-1 and integrin β2 interactions could induce tumor dissemination by contact. Another microfluidic co-culture chip was employed by Zhao *et al.* to investigate the effect of lactate shuttling on the phenotype polarization and distribution of macrophages and transitional cell carcinoma of the bladder (TCCB) cells [94]. TCCB cells were confirmed to reprogram macrophages into M2 phenotype. Lactate was found to inhibit M1 polarization and induce M2 polarization of macrophages, while the inhibition of macrophage-cancer cell lactate flux significantly suppressed this process. To investigate the pre-metastatic niche of tumors and the contributions of recruited cells, Kim *et al.* developed a microfluidic system that integrated endothelial cells and ECM scaffolds on the chip (Fig. 3D) [80]. Collagen type 1 hydrogel was used to create suitable conditions for cell proliferation and invasion and an EC monolayer was constructed on a locally produced thin basement membrane as a barrier to extravasating monocytes and cancer cells. Monocyte-derived matrix metalloproteinase 9 was found to destruct endothelial tight junctions to facilitate tumor cell extravasation. The invasiveness of tumor cells was significantly increased through moving within characteristic "microtracks" generated by macrophages.

2.3.2. Mesenchymal stromal cells (MSCs)

MSCs tend to migrate to inflammation tissues, especially cancer inflammatory sites, by cytokines and chemokines signalling and can alter the functions and phenotype of immune cells [95]. Normal MSCs can regulate inflammatory responses to initiate the repair process, while tumor site-resident stromal cells (TSR-MSCs) can change the inflammatory status of a tumor into immunosilent conditions and promote metastasis via EMT. Zhao et al. developed a set of microfluidic devices with microwell array to generate tumor and stromal cell spheroid pairs to investigate the tumor-stromal metastatic process *in vitro* [96]. By convenient pipetting and centrifugation, 240 addressable tumor-stroma cell pairings could be produced. Cell spheroids pairing could be precisely controlled in the micropits at different heights with one-to-one interacting correspondence. By quantitative profiling of the dynamic tumor-fibroblast enveloping process and confocal time-lapse imaging, it was found that tumor spheroid possesses higher invasiveness and mobility to wrap the stromal spheroid. By using different extracellular factors such as the prometastatic factor vascular endothelial growth factor A (VEGFA), this metastasis phenotype could be inhibited or promoted. Lee *et al.* exploited a co-culture model based on microchannel plate that integrated tumor spheroids with pancreatic stellate cells (PSCs) in a 3D collagen matrix to simulate the TME *in vivo* by reproducing EMT and chemo-resistance [97]. Human pancreatic cancer cell line PANC-1 cells and PSCs were co-cultured in type I collagen, where PANC-1 cells could form 3D tumor spheroids and the spheroids number increased under co-cultured conditions with PSCs (Fig. 4A). In co-cultured cell spheroids, the expression of EMT-related biomarkers

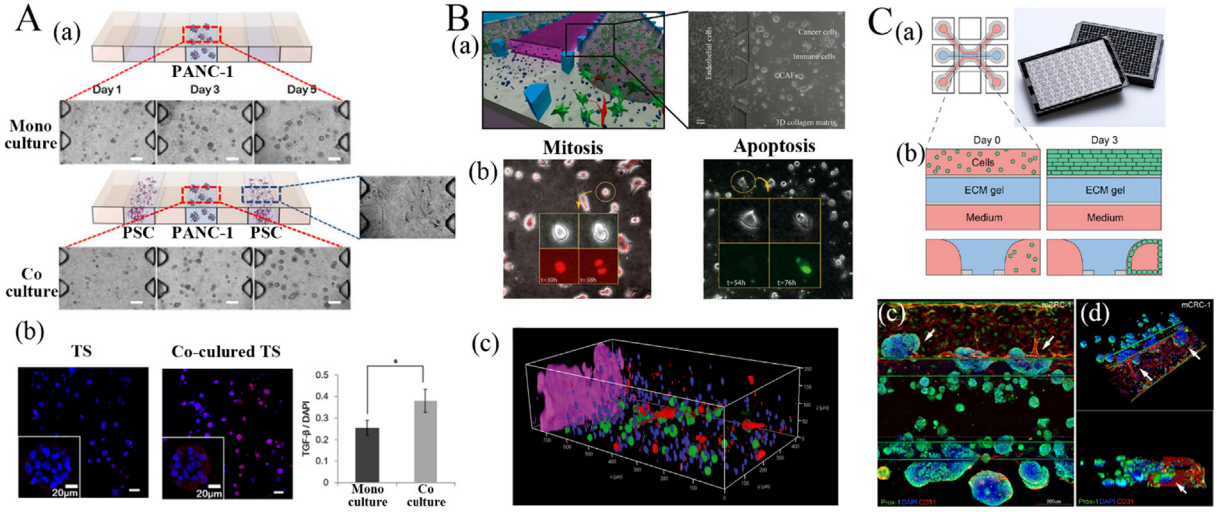

Fig. 4. The influence of MSCs, CAFs and TECs on tumor-immune interactions. (A) Influence of PSCs co-culture on PANC-1 spheroids growth. (a) PANC-1 spheroids formed in a collagen-filled microchannel plate when cultured alone or with PSCs. (b) Expression of cytokines in PANC-1 tumor spheroids under co-culture conditions. Adapted from Ref. [97] with permission of BMC. (B) Reconstitution of a tumor ecosystem with cancer cells, immune cells, endothelial cells, and fibroblasts. (a) Schematic of the microfluidic chip and photograph of the microchannel. (b) Mitosis and apoptotic tumor cells. (c) A confocal image showing the 3D positioning of BT474 cancer cells (green), Hs578T CAF cells (red), PBMC immune cells (blue), and human umbilical vascular endothelial cells (HUVECs, violet). Adapted from Ref. [98] with permission of Elsevier. (C) Lymphatic vessel/tumor co-culture in a 3D microfluidic model. (a, b) Schematics of the 3-lane microchip. (b) imLECs formed a monolayer tube on the top perfusion channel. (c) Interactions between the colon cancer organoids and the imLEC vessel. (d) 3D image of colorectal cancer (CRC) organoids invading a LEC vessel. Reproduced from Ref. [99] with permission of The Royal Society of Chemistry.

such as TGF-β and vimentin was increased compared to tumor cell spheroids. Removal of TSR-MSC and inhibition of MSCs migration can be a possible method to enhance antitumor responses and boost anti-metastatic pathways. On the other hand, MSCs' tendency and ability to migrate to the tumor site could be an advantage to transfer antitumor reagent carriers to release immune-stimulatory factors into the tumor tissue to modulate the TME.

2.3.3. Cancer-associated fibroblasts (CAFs)

CAFs play a vital role in modulating the immune contexture, and have been shown to provide critical signals that support tumor progression and generate an immunosuppressive environment by secreting various soluble factors such as TGF-β [100]. CAFs constitute an important source of cytokines and growth factors such as VEGF to significantly influence the immune landscape, and CXCL12 to inhibit cytotoxic T lymphocyte (CTL) infiltration into the TME, which together promote tumor progression, angiogenesis, and therapeutic resistance. Nguyen *et al.* achieved 3D co-cultures of multiple types of cells (including cancer cells, immune cells, endothelial cells and fibroblasts) in a microfluidic device to reproduce an ex vivo human tumor ecosystem ($HER2^+$ breast cancer) (Fig. 4B) [98]. Trastuzumab (Herceptin) is a targeted antibody to interact against the HER2 receptor, and was used to validate the complex dynamics of this tumor-on-chip. CAFs were found to antagonize the effects of trastuzumab, which can promote long cancer-immune interactions and lead to anti-tumor immune responses. Jeong *et al.* presented another microfluidic system-based tumor tissue culture model that integrated CAFs and 3D tumor spheroids within a hydrogel matrix [101]. Under co-culture conditions the fibroblasts were activated by increased α-SMA expression. Activated fibroblasts could enhance the growth of human colorectal carcinoma HT-29 cells into 3D tumor spheroids and improve the resistance of tumor cells against anti-tumor drug paclitaxel.

2.3.4. Tumor-associated endothelial cells

Tumor-associated endothelial cells (TECs) constitute an essential part of the dysfunctional vasculature in the TME, and play an important role in shaping the immune environment. TECs are different from normal endothelial cells in various aspects including proliferation, migration, and responses to growth factors and therapeutic drugs. Tumor cells can induce the immunosuppressive activities of TECs to influence anti-tumor immunity and therapeutic responses. Zervantonakis *et al.* developed a microfluidic device to study the permeability of spatially confined endothelial cells. Results showed that the secretion of tumor necrosis factor alpha (TNF-α) by macrophages led to higher intravasation rates and endothelial barrier impairment [102]. Additionally, a microfluidic lymphatic vessel model was reported by Frenkel *et al.* to investigate lymphangiogenesis and the interaction with colon cancer organoids (Fig. 4C) [99]. Immortalized lymphatic endothelial cells (imLECs) were injected into a microfluidic chip with free-standing ECM and formed a perfusable vessel-like structure. A stable co-culture model was established by introducing mouse colon tumor organoids to the lymphatic vessel for the investigation of lymphangiogenesis and tumor cell metastasis. Han *et al.* established *in vivo* like inflammatory models in a microfluidic device for quantitatively measuring the 3D transendothelial migration of neutrophils during the inflammatory process [103]. This platform incorporated ECM, stabilized concentration gradients of multiple inflammatory molecules, and the co-culture of neutrophils and endothelial cells, enabling direct observation of the reconstituted inflammation. The effects of human interleukin-8 (IL-8) and N-formyl-methionyl-leucyl-phenylalanine (fMLP) on *in vivo*-like transendothelial migration of neutrophils were quantitatively detected.

2.4. Vascularized tumor-on-a-chip models

The vascular system of tumors is highly dysfunctional and abnormal, which exhibits chaotic blood flow and impaired leukocyte extravasation [104,105]. The structurally and functionally aberrant tumor vasculature contributes to protumorigenic and immunosuppressive TMEs by maintaining the tumor's permissive environment characterized by hypoxia, acidosis, high interstitial pressure, and physical barriers to T cells' infiltration [106]. Blood endothelial cells of tumor vessels can actively suppress the recruitment, adhesion, and activity of T cells, while lymphatic vasculature in tumor tissues remodels to facilitate tumor cells' metastatic and immunosuppression [107,108]. The involvement of the vasculature in the design of tumor-on-a-chip was a key advance for recapitulating the TME complexity [109–113]. With advancements in microfluidic techniques, the chip-based 3D microvascular models provide a reproducible and precise platform to investigate the tumor microvasculature *in vitro*, achieving greater biological relevance for TIME modeling [114–119].

To study the influence of inflamed neutrophils on hematogenous dissemination, Chen *et al.* employed a multiplexed microfluidic device to model the human microvasculature and realized physiologically similar transport of circulating cells [120]. In the artificial microvascular, tumor cells and LPS-stimulated neutrophils (PMNs) formed heterotypic cell aggregates, which were arrested by neutrophil–endothelial adhesions and mechanical trapping. PMNs were chemotactically limited by tumor-derived CXCL-1 and self-secreted IL-8, which resulted in significant neutrophil sequestration and increased tumor extravasation. The recent work by Haan *et al.* employed a high-throughput, artificial membrane-free 3D endothelium-on-a-chip system to investigate transendothelial migration of T cells under different flow conditions [121]. Primary human T cells could adhere to endothelial vessels when perfused in the microvessel. Under the influence of CXCL12 gradients/melanoma cells and TNFα-mediated vascular inflammation, T cells could be induced to undergo *trans*-endothelial migration (Fig. 5A). To investigate the endothelial immune barrier, Kim *et al.* developed a microfluidic tumor vasculature model with endothelial cells expressing Fas ligand (FasL), which could bind to the receptor (Fas, CD95) and induce cell apoptosis to maintain immune homeostasis (Fig. 5B) [122]. Tumor cells and the TME promoted vascular endothelial cells to express FasL on the cell membrane and formed an immune barrier to kill anti-tumor cytotoxic T cells, which could be enhanced under a hypoxic microenvironment. Furthermore, the effects of monocytes on tumor cell extravasation were investigated by Boussommier-Calleja *et al.* in a 3D vascularized microfluidic system [123]. They demonstrated monocytes' differentiation into macrophages as they migrated from the intravascular compartment to the extravascular microenvironment in this model, which replicated physiological differences between different monocyte subsets. Normal monocytes could directly decrease tumor cell extravasation by non-contact dependent signaling. In contrast, when monocytes trans-migrated through the vasculature and became macrophage-like, there was little effect on tumor cell extravasation.

2.5. Chemokine gradients

Gradients of soluble molecules modulate cell behavior and signaling in various multicellular systems [124]. Chemokine-induced chemotaxis is a key factor to influence cell behavior during tumor progression and immune responses [125,126]. Gradients of chemokines, cytokines and growth factors play an important role in the TIME, and influence on switches of stromal cell states, as well as the migration of T cells. Microfluidic devices can realize biochemical gradients by diffusion or convection [127,128]. Gradi-

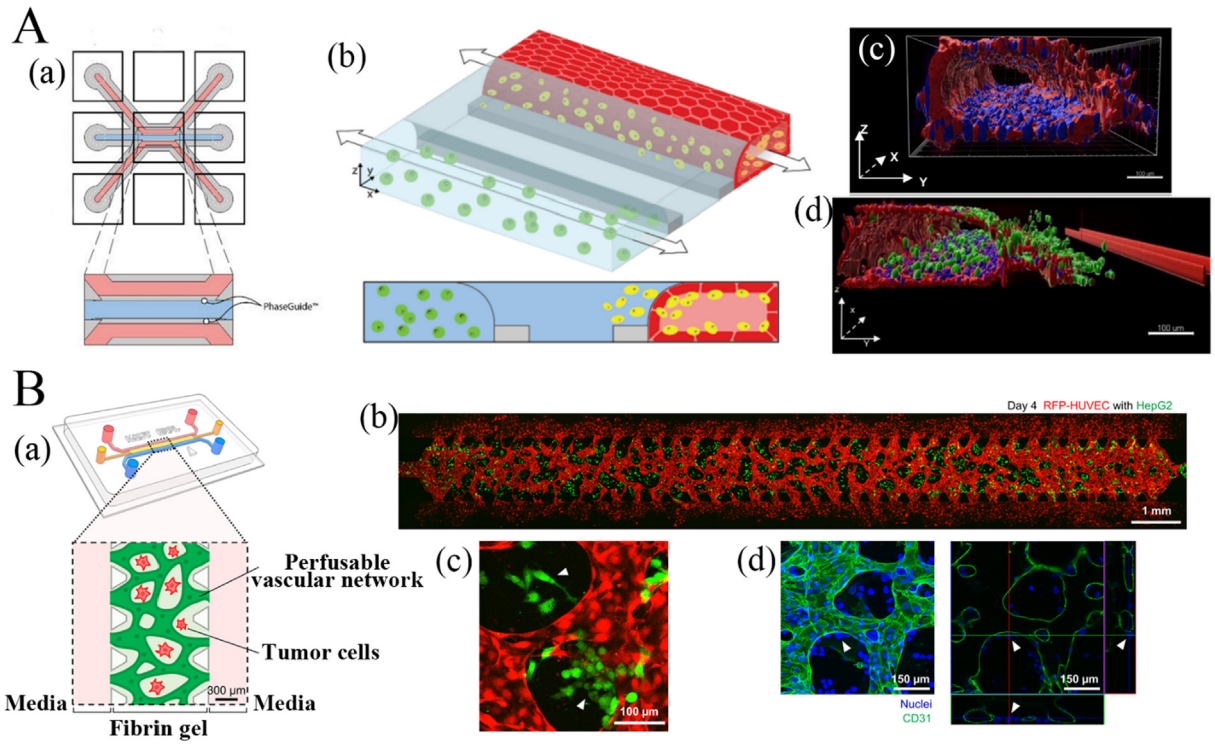

Fig. 5. Tumor-on-a-chip models recapitulating vasculature systems. (A) A 3D endothelium-on-a-chip system to study transendothelial migration of T cells. (a) Schematic of the microfluidic chip. (b) T cells (yellow) migrated through the HMEC-1 vessel towards A375 tumor cells (green). (c) 3D image of the tubular structure. (d) 3D image showing the migration of T cells across the endothelial vessel. Adapted from Ref. [121] with permission of MDPI. (B) On-chip micro-vascular network (MVN) involving tumor cells in a 3D fibrin gel. (a) A schematic of the microfluidic device and 3D MVN. (b) RFP-HUVECs (red) formed the MVN with HepG2 cells (green) inside the microchannel. (c) An enlarged image of the MVN (red) and HepG2 cells. (d) When HepG2 tumor cells were attached to the MVN, CD31 (green) remained on the MVN without degradation. Adapted from Ref. [122] with permission of American Chemical Society.

ents can be established by allowing controlled diffusion of chemokines across microfluidic chambers [129]. By mixing different concentrations of biochemical factors, convection-based devices can also produce gradients [130]. A recent application was demonstrated by Grigolato *et al.* to establish a microfluidic device for fully automated, quantitative assessment of neutrophil chemotaxis, which allowed the precise determination of CXCL2 and CXCL8 concentrations to induce mouse and human neutrophil chemotaxis [131]. Specific modulators of neutrophil chemotaxis were examined, showing that IL-4 receptor signaling neutrophils inhibited their migration towards CXCL2 and CXCL8. Another microfluidic device was developed by Sonmez *et al.*, which realized different chemokine gradients in flow-free chambers and controlled surface ECM, to investigate chemotaxis either at the single-cell level or at the population level (Fig. 6A) [132]. The microfluidic chip consisted of two PDMS layers that sandwiched a 0.4 μm polycarbonate (PC) membrane filter to separate gradient channels and the cell chamber. By the diffusion of soluble factors from two side flow channels into the middle flow-free microchamber, concentration gradients could be generated. The combination of a controlled fibronectin surface concentration and a CXCL12 chemokine gradient was applied to study the chemokinetic response and chemotaxis of Jurkat cells for modeling of T lymphocyte motility. When surface fibronectin was increased in a dose-dependent manner, the chemotactic response of Jurkat cells to CXCL12 gradient decreased. To track cell migration at single cell level, Frick *et al.* exploited an automated microfluidic system with precisely controlled diffusion-based chemokine gradients. Cell positioning could be easily realized in 3D microenvironments [133]. Gradients of the chemokine CCL19 were applied to dendritic cells to quantitatively assess key migration characteristics.

2.6. Hypoxia

The normal oxygen level in healthy tissue and organs is about 3–6%; when the oxygen content is lower than 3%, it's described as hypoxia. Hypoxia is a key feature of a tumor, which can influence cancer responses to therapies and facilitate immune evasion [134]. In tumors, the hypoxic conditions can include EMT of cancer cells and abnormal growth of the vascular system in angiogenesis, leading to tumor metastasis [135]. Complex signaling pathways activated by hypoxia such as hypoxia-inducible factors (HIF) and VEGF have been investigated by numerous studies [136]. However, the direct responses of tumor cells to hypoxia were difficult to measure by conventional methods [137]. Tumor-on-a-chip models can offer an efficient method to study hypoxia *in vitro* [138–141]. For example, Zheng *et al.* developed a multi-organ microfluidic system with controlled oxygen concentrations to study hypoxia influence on lung cancer-liver metastasis and to screen drugs [142]. Lung cancer A549 cells were used to establish a lung cancer-liver model at the organ level under hypoxic or normoxic conditions (Fig. 6B). In this system, the expression of EMT transcription factors (Snail 1 and Snail 2) was elevated by the HIF-1 α pathway to promote cancer metastasis. The EMT transcription factor and HIF-1 α levels were found to be positively correlated with the levels of tumor metastasis damage factors such as gamma-glutamyl transpeptidase (γ-GT), alkaline phosphatase (ALP), and alpha-fetoprotein (AFP) from liver cells. To investigate CAR-T cell-mediated cytotoxicity and how solid tumors escape immunosurveillance under hypoxia conditions, Ando *et al.* designed a microfluidic system that recapitulated a 3D tumor model with oxygen gradients and fluid flow around the cancer cells for CAR-T cell delivery [137]. A tumor model was constructed by human ovarian

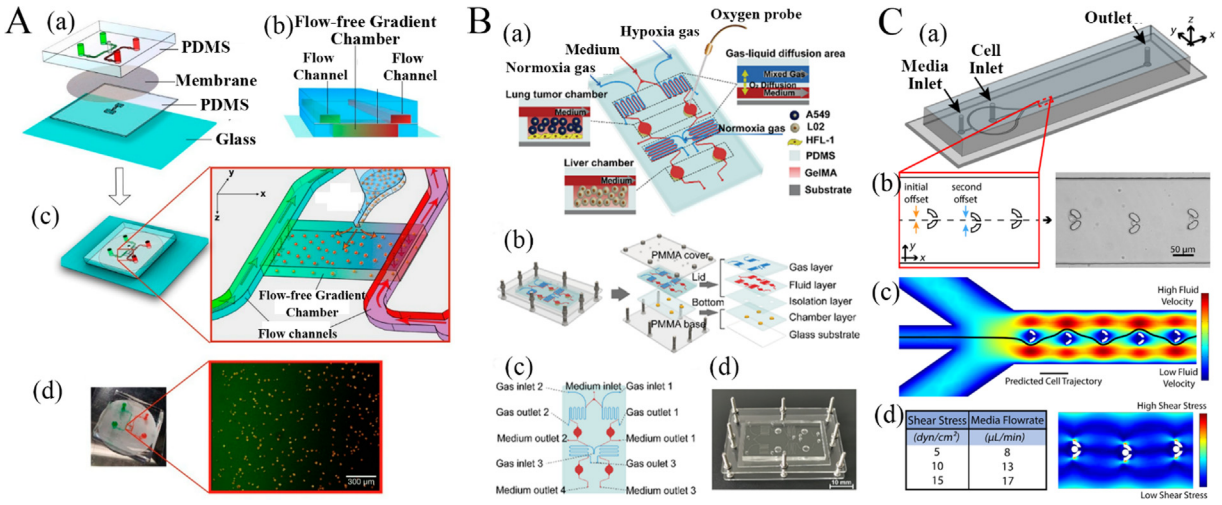

Fig. 6. Realization of chemokine gradient, hypoxia and sheer stress in microfluidic devices. (A) A microfluidic device with flow-free gradient chambers to study the chemotaxis of Jurkat cells. (a) Components of the microfluidic device. (b) Schematic of the flow-free gradient chamber. (c) Generation of a gradient across the cells in the chamber. (d) Photograph of the actual microfluidic device and fluorescent image of Jurkat cells. Reproduced from Ref. [132] with permission of MDPI. (B) An oxygen-concentration-controllable microfluidic device for analysis of the hypoxia-induced lung cancer cells metastasis. (a) Schematic of each area of the device. (b) The multilayer structure of the device. (c) The functions of each hole in the device. (d) Image of the microfluidic device. Reproduced from Ref. [142] with permission of American Chemical Society. (C) A microfluidic device to study the influence of FSS on single circulating tumor cell (CTC). (a) Schematic of the microfluidic device. (b) Structure of the traps to capture single cells. (c) Multiphysics simulations of the velocity profile of the device by COMSOL. (d) FSS magnitudes calculation by media flow rates. Reproduced from Ref. [148] with permission of AIP.

cancer cells with an oxygen gradient generated by cellular metabolism in a 3D micro-patterned hydrogel. Fig. 7C(a) shows the mechanism of the production of oxygen gradients for immunotherapy. CAR-T cell cytotoxicity and infiltration ability could be tested in the heterogeneous oxygen microenvironment of this hypoxia device. The influence of dual gradients of matrix stiffness and oxygen was investigated by Wang *et al.* by creating an easy-to-operate and highly integrated microfluidic device, capable of producing more stable, linear, continuous, and diffusive hydrogel stiffness gradients over a well-defined oxygen gradient [143]. Tirapazamine (TPZ), a hypoxia-sensitive anti-cancer drug, was applied to A549 cells under a spatially hydrogel stiffness gradient and a perpendicular oxygen gradient. Results showed hypoxia-induced cytotoxicity of TPZ and matrix stiffness-dependent cell drug resistance.

2.7. Biophysical forces

Biophysical forces play a diverse role in tumors such as the transportation of the nutrients through blood vessels and the changes in mechanical properties in a tumor matrix [144]. Mechanical force can alter cell behaviors and induce tumor metastasis by changing cytoskeleton structures and actomyosin-mediated contractility, and triggering mechanoreceptors of cells [145,146]. The rapid tumor growth can also produce mechanical compression to induce hypoxia in tumor core and reduce fluid perfusion rates. Biophysical forces, such as compression, tension, shear stress, and ECM stiffness, can be easily reproduced on tumor-on-a-chip platforms via dynamic flows, stretchable substrates, and the application of mechanical force directly [146,147]. For instance, a microfluidic system was employed by Landwehr *et al.* to characterize the biophysical response of single breast cancer cells in a circulatory system during the metastasis process [148]. MCF7 and MDA-MB-231 cells were exposed to different fluid shear stress (FSS) to study the single-cell response of multiple breast cancer types. Increased duration and magnitudes of FSS induced greater deformability of both MDA-MB-231 and MCF7 cells (Fig. 6C). To imitate cardiac tissue contractions, Lind *et al.* developed a convenient approach to fabricate a new class of instrumented cardiac micro-physiological devices with multimaterial 3D printing [149]. Based on piezo-resistive, high-conductance, and biocompatible soft materials, six functional inks were designed to allow the integration of soft strain gauge sensors within the micro-architectures, leading to the self-assembly of physio-mimetic laminar cardiac tissues. Although a lot of studies have been performed to investigate the influence of biophysical forces on tumor progression, few research studies focused on the influence of biophysical forces on tumor-immune interactions, which could be a new direction for immunotherapy research on microfluidic platforms.

3. Immunotherapy evaluation on tumor organoid-on-a-chip platforms

Although cancer immunotherapy has achieved great success and advancements in recent years, only a small number of patients have long-term responses due to intrinsic and acquired resistance. As increasing immunotherapies are being developed, and combinational therapies have been proved effective for many types of cancer compared with monotherapy [150], there is a huge demand to develop platforms that can accurately test the efficacy of these therapies in preclinical and clinical experiments [151]. A variety of microfluidic systems have been used to evaluate immunotherapeutic responses to immunotherapy alone or in combination with other therapies. Here, we discuss the application of these novel models to test the efficacy of immunotherapies, and highlight their applications regarding how tumor organoid-on-a-chip models can be utilized to clarify the underlying mechanisms, including adoptive cell therapy, immune checkpoint inhibitors, cancer vaccines, oncolytic virus therapy, cytokine therapy, high-throughput immunotherapy assays and personalized therapy. Representative microfluidic platforms for immunotherapy are summarized in Table 1.

3.1. Adoptive cell transfer (ACT)

In ACT therapy, immune cells from either a donor (allogeneic cells) or a patient (autologous cells) are firstly isolated, subsequently modified and expanded *ex vivo*, and then injected back

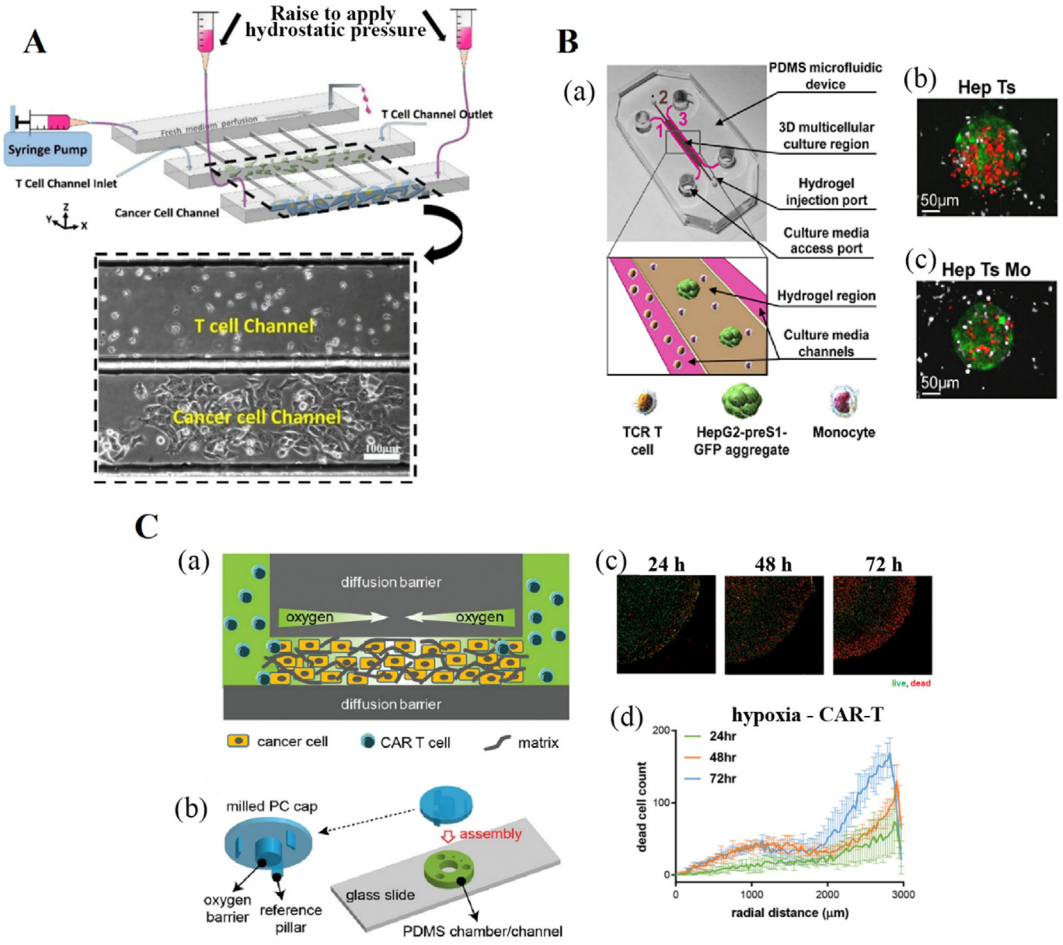

Fig. 7. Microfluidic platform for immune T cell-mediated cytotoxicity. (A) Schematic and photograph of the microfluidic device simulating physical barriers to evaluate the anticancer response of CTLs against tumor interstitium; CTLs and cancer cells were cultured in the middle and bottom channels, respectively, which were named as T-cell and cancer-cell channels for clarity. Reproduced from Ref. [154] with the permission of Springer Nature. (B) Photograph and schematic of the 3D multicellular TME microfluidic model for the investigation of the impact of monocytes on TCR-T cells cytotoxicity against tumor cell aggregates (a), and the confocal images of the GFP-labeled target HBV-HCC cell aggregates (Hep) treated using TCR-T cells (Ts) with (b) and without (c) monocyte (Mo), in which the presence of dead target cells was DRAQ7$^+$ (in red), TCR-T cells were labeled with Cell tracker violet dye (in white), while monocytes were unlabeled. Reproduced from Ref. [158] with the permission of Frontiers. (C) Illustration of the principle of microfluidic device operation (a) and schematics of the assembly of the microfluidic device (b) recapitulating an oxygen gradient and matrix microenvironment for T cell infiltration. (c) Live/dead staining of the 3D tumor models incubated under hypoxia with CAR-T cells for 24, 48, and 72 hr. (d) The radially quantified numbers of dead cells from CAR-T cell-treated conditions for the hypoxic microdevice conditions. Adapted from Ref. [160] with the permission of Wiley.

to the patient [152,153]. The main ACT products are tumor-infiltrating T cells, TCR-transduced T cells, CAR-T cells, NK cells, and CAR-NK cells. One of the most common studies of microfluidic-based immunotherapy is adoptive cell transfer therapy.

The organ-on-a-chip approach is broadly utilized to investigate the T cells' activity for cancer immunotherapy. Chen *et al.*, studied the characteristics of migration and anticancer performance of tumor antigen-specific CTL that targets hepatic cancer cells by antigen-specific and allogeneic recognition using a microfluidic-based platform [154]. The microfluidic device consisting of three 50 μm-high main channels interconnected via 5 μm-high slit channels was used to simulate physical barriers in the tumor interstitium, in which slit channels mimicked the narrow interstitial paths constrained by the fibrous capsule (Fig. 7A). To simulate increased interstitial fluid pressure (IFP), medium-containing syringes were connected to the cancer cell channel and raised to apply hydrostatic pressure to the center of the tumor. The results, however, showed antigen-specificity of CTLs against the tumor cells did not significantly impact the infiltration rate of CTLs into the cancer cells but it affected the cytotoxicity of the CTLs. The presence of the increased IFP in the tumor center led to the promotion of CTLs recruitment to tumor peripheries but restricted the success of infiltration. In addition, the achieved results proved the importance of incorporating the physical obstacles of the tumor interstitium in the development of CTLs-based tumor immunotherapy. Poggi and his co-workers [155] fabricated a double-channel microfluidic chip to investigate the capability of zoledronic acid (ZA)-spherical polymeric nanoparticles(SPN)-activated T cells to extravasate and reach CRC cells. The device enabled the investigation of the dynamics of T cell migration through a vascular bed and across an endothelial monolayer to reach tumor cells. The findings indicated that ZA-SPNs-expanded T cells could overcome the vascular obstacle and the ECM and migrate into tumor cells. Moreover, Briones *et al.*, fabricated a microfluidic platform that consisted of hydrodynamic traps and a pneumatic valving system to detect single-cell Granzyme B (GrB) activity in cells for prediction of the efficacy of cancer immunotherapy [156]. A fluorometric single-cell enzymatic activity assay was used to profile GrB levels in the blood through T cell tumoricidal activity. It was found that GrB expression can be measured to monitor specific T cells' anti-tumor activity. The challenge for this proposed method is the

Table 1
Summary of recent representative microfluidic platforms for cancer immunotherapy.

Author	Technique	Cell	Microfluidic chip design	Immunotherapy/ Application
Pavesi *et al.*, 2017 (Ref. [52])	3D cell co-culture	HepG2 cells, TCR-T cells	Three chambers separated by two rows of micro-pillars for cell co-culture and migration	ACT. Evaluation of TCR-T cells against solid tumors
Al-Samadi *et al.*, 2021 (Ref. [65])	3D cell co-culture, human tumour-derived matrix "Myogel/fibrin", personalized testing	HSC-3 cells, HNSCC cells, PBMCs	Three chambers connected by a set of microchannels to allow molecular communication and cell migration	ICI. Personalized immunotherapeutics for head and neck cancer patients
Sabhachandani *et al.*, 2019 (Ref. [66])	3D cell spheroids, ECM hydrogel combined of alginate and puramatrix	SUDHL-10 cells, HS-5 cells, PBMCs	T-junction droplet generation part and cell spheroids docking array	Evaluation of immunomodulatory drug lenalidomide
Parlato *et al.*, 2017 (Ref. [76])	3D cell co-culture	SW620 CRC cells, DCs	Three chambers connected by a set of microchannels to allow molecular communication and cell migration	Evaluation of immunotherapy efficacy by tracking DCs migration toward cancer cells
Aung *et al.*, 2017 (Ref. [78])	3D cell co-culture	MCF7 cells, MDA-MB-231 cells, THP-1 cells, HUVEC cells	Two concentric annulus with cells encapsulated in hydrogel	ACT. Assessment of T-cell recruitment with breast cancer-immune model
Moore *et al.*, 2018 (Ref. [81])	Flow control and pressure control system	TIL cells, MC38 cells	An array of micropillars to trap tumor cells, with medium and TIL flow around	ICI. Evaluation of TIL-mediated cytotoxicity and tumor response to ICI
Shim *et al.*, 2019 (Ref. [79])	Continuous recirculating flow	Lymph node and tumor slices	A microfluidic device with recirculating flow to transfer signaling molecules between tissue slices	Modeling tumor-lymph node interaction
Tu *et al.*, 2020 (Ref. [89])	Single-cell level cell co-culture	C1498 cells, T cells	Microfluidic microwell array	Investigation of cancer -immune interactions at the single cell level
Kim *et al.*, 2019 (Ref. [80])	3D cell co-culture	MDA-MB-231 cells, THP-1 cells, ECs	Three chambers connected by an array of microchannels to permit molecular communication and cell migration	Macrophages remodeling of endothelium-interstitial matrix to form a pre-metastatic niche
Nguyen *et al.*, 2018 [98] (Ref.)	3D cell co-culture	BT474 cells, Hs578T cells, PBMCs, HUVECs	Three chambers separated by two rows of micro-pillars for cell co-culture and migration	Dissecting effects of anti-cancer drugs and CAFs by on-chip immunocompetent tumor microenvironments
Chen *et al.*, 2018 (Ref. [120])	Microvasculature	Neutrophils, HUVECs, A375-MA2 cells	Eight independent hydrogel regions where microvascular networks were connected by branching channels.	Inflamed neutrophils sequestered at tumor cells via chemotactic confinement promote tumor cell extravasation
Haan *et al.*, 2021 (Ref. [121])	3D cell co-culture, microvasculature	HMEC-1 cells, T cells, A375 cells	Three chambers separated by two barriers for cell co-culture and migration	Transendothelial migration of T cells
Kim *et al.*, 2021 (Ref. [122])	3D cell co-culture. microvasculature	HepG2 cells, HUVECs, Jurkat cells	Three chambers separated by two rows of micro-pillars for cell co-culture and migration	Microfluidic tumor vasculature model to recapitulate an endothelial immune barrier
Boussommier-Calleja *et al.*, 2018 (Ref. [123])	3D cell co-culture, microvasculature	HUVECs, MDA-MB-231 cells, MDA-MB-435 cells, human monocytes	Five channels enclosing 3 rectangular compartments	The effects of monocytes on tumor cell extravasation
Sonmez *et al.*, 2020 (Ref. [132])	3D cell culture, flow-free gradient	Jurkat cells	Two parallel flow channels and a cell culture chamber separated by a membrane filter	Chemotactic responses of Jurkat cells
Ando *et al.*, 2019 (Ref. [160])	3D cell co-culture, oxygen gradient	CAR-T cells, SKOV3 cells	A PDMS fluidic component with a milled PC cap	ACT. Evaluation of CAR-T efficacy in a hypoxic tumor model
Chen *et al.*, 2020 (Ref. [154])	Interstitial fluid pressure	CTL, BNL cells, HEPA1-6 cells	Three chambers connected by an array of microchannels to permit molecular communication and cell migration	ACT. Evaluation of CTL mediated anticancer response against tumor interstitium simulating physical barriers
Lee *et al.*, 2018 (Ref. [158])	3D cell spheroids	TCR-T cells, HepG2 cells, monocytes	Three chambers separated by two barriers for cell co-culture and migration	ACT, ICI. Characterizing the role of monocytes in T cell cancer immunotherapy
Ayuso *et al.*, 2019 (Ref. [163])	3D cell spheroids, concentration gradient	NK-92 cells, MCF-7 cells, HUVECs	A cell culture chamber with two flanking lateral lumens	ACT. Evaluating natural killer cell cytotoxicity
Ayuso *et al.*, 2021 (Ref. [164])	3D cell co-culture, concentration gradient	NK-92 cells, MCF-7 cells, HUVECs	A cell culture chamber with a lumen located at one end of the microchamber	ACT. Evaluation of the role of tumor environmental stress on NK cell exhaustion

(*continued on next page*)

Table 1 (*continued*)

Author	Technique	Cell	Microfluidic chip design	Immunotherapy/ Application
Ke *et al.*, 2017 (Ref. [165])	optoelectronic tweezers	L929 cells, NK-92MI cells, K562 cells	four-leaf-clover-shaped (FLCS) microwells	ACT. NK cells and cancer cells interactions
Jiang *et al.*, 2021 (Ref. [183])	3D cell spheroids, high-throughput	MDA-MB-231 cells, Jurkat cells	Microwell arrays for cell culture and complimentary micropillar arrays for cytokine monitoring.	ICI. Modeling ICI and tumor interactions
Cui *et al.*, 2020 (Ref. [184])	3D cell co-culture	Glioblastoma cells, human macrophages, hBMVECs	A peripheral channel designated for patterning 3D brain microvessels, an intermediate tumor stromal region (middle ring), and a core medium region	ICI. GBM-on-a-Chip to optimize anti-PD-1 immunotherapy
Doty *et al.*, 2020 (Ref. [185])	High-throughput	NSCLC cells, MC38 cells, CT26 cells, B16F10 cells, TIL cells	An array of pillars to trap tumors in the center of the channel while allowing for medium and TILs flow around	ICI. Modeling ICI efficacy
Zhao *et al.*, 2019 (Ref. [198])	3D-printing molded microfluidic device	THP-1 cell, JAWSII cells	Cylindrical cell culture chamber and S-shaped channel for exosomes engineering	Cancer vaccine. Engineered exosomes for cytolysis activation
Quagliarini *et al.*, 2021 (Ref. [200])	LNP-pDNA complex formulation	HEK-293 cells, human primary keratinocytes, CaSki cells	Staggered herringbone micromixer (SHM)	Cancer vaccine. Microfluidic formulation and transfection of DNA-loaded LNPs
Lee *et al.*, 2020 (Ref. [206])	3D multicellular tumoroids	A549 cells, MRC-5 cells, HUVECs	Two microphysiological systems connected by block-to-block linkage	Oncolytic viruses.
Lucarini *et al.*, 2017 (Ref. [208])	3D cell spheroids	A375 cells, SK-MEL-28 cells, SC cells, WM793 cells	Three chambers separated by two barriers for cell co-culture and migration	Cytokine therapy. Evaluation of the synergistic effect of the demethylating drug DAC and IFN-I
Wang *et al.*, 2021 (Ref. [225])	High-throughput droplet microfluidics	K562-Her2 cells, PBMCs, SK-BR-3 cells, T cells, Jurkat cells	Cross-junction for droplets generation	Screening for antibodies with special functionalities
Aref *et al.*, 2018 (Ref. [233])	3D cell spheroids	Patient-derived organotypic tumor spheroids, T cells	Three chambers separated by two barriers for cell culture in a central channel	ICI. Personalized therapy

retrieval of the identified lymphocyte cells in the patient peripheral blood mononuclear cells (PBMC) with GrB overexpression. More research can be done to find out the parameters involving GrB overexpression and the mechanism of its cytotoxicity.

In addition to endogenous tumor-infiltrating lymphocytes T cells, genetically engineered T cells such as highly specific TCR-T cells and CAR-T cells have been utilized in ACT therapy. By inserting exogeneous TCR-T cells into cancer cells, T cells specificity can be precisely redirected to selected tumor antigens [157]. TCR-T cells immunotherapeutic activities have been recently studied using microfluidic lab-on-a-chip technology. Lee *et al.* used a 3D intrahepatic TME microfluidic device (Fig. 7B(a)) to test the antitumor activity of TCR-T cells engineered to identify hepatitis B antigen-expressing hepatocellular carcinoma cells (HBV-HCC) in the presence of immunosuppressive monocytes and programmed cell death protein-1 (PD-1)/programmed death-ligand 1 (PD-L1) axis [158]. It was shown that a decrease in the TCR-T cells cytotoxic activity in the presence of monocytes (Fig. 7B(b-c)) and the full phenomenon reversion via anti-PD-L1/PD-1 antibodies. In addition, it was seen that in the presence of monocytes, only retrovirally transduced TCR-T cell cytotoxic activity was suppressed to cancer cells by PD-L1/PD-1, while the cytotoxic activity of mRNA electroporated TCR-T cell was not affected.

Besides TCR-T cells, CAR-T cells as another type of exogenous T cells are genetically engineered to produce a specific receptor to an antigen on the surface of cancer cells [15]. These cells can remain active for up to a decade after injection and has the potential of becoming a one-time therapy [159]. Tumor organoid-on-a-chip models have been utilized to investigate CAR-T cells' immunotherapeutic activities. Ando *et al.* established a microfluidic platform recapitulating a 3D tumor model integrated to an oxygen gradient and fluidic channels surrounding the tumor to investigate CAR-T cell activity under a hypoxic gradient (Fig. 7C) [160]. CAR-T cells were delivered through microfluidic channels that surrounded the tumor cells, and spatiotemporal examination of CAR-T cell infiltration and cytotoxic activity within the hydrogel was achieved. The results showed that at 24 hr, CAR-T cells cytotoxicity increased against cancer cells at the periphery of the hypoxic tumors and cancer cells increased their PD-L1 expression in hypoxic conditions. In addition, to investigate the long-term functionality of CAR-T cells in the TME, a co-culture study was performed at 48 and 72 hr in the 3D tumor models. As shown in Fig. 7C(c), extending incubation time from 24 hr to 48 hr and 72 hr enhanced CAR-T cells cytotoxicity in hypoxic samples. The dead cells count was averaged within concentric circles with a radial distance of 500 μm at the inner, intermediate, and outer regions corresponding to oxygen levels of 0.2–0.3%, 1.5–4%, and 6–17%, respectively. As shown in Fig. 7C(d), the number of dead cancer cells significantly increased at the edge under hypoxia after 48 and 72 hr.

In comparison to T cells, NK cells show superior safety in several clinical settings and their risk of on-target/off-tumor toxicity to normal tissues is relatively low [161]. NK cells' immunotherapeutic activities have been also recently investigated with the tumor organoid-on-a-chip technology. Wu *et al.* produced porous alginate microspheres on a large scale via microfluidic electrospray and

encapsulated them with NK cells for effective tumor immunotherapy by protecting NK cells from the modulation of TME and from the rejection by the host's immune system after injection of NK cells [162]. Encapsulated NK cells in microspheres showed higher cytotoxicity *in vivo* compared with free NK cells due to the budding of NK cells from the outer surface of the microspheres, leading to direct interactions with surrounding tumor tissues. The results also showed that in their early stage, the NK cell-loaded microspheres could kill cancer cells by secreting perforin and granzymes. Ayuso *et al.* developed a multi-chamber microdevice including a 3D breast cancer spheroid in a 3D ECM and two flanking lumens lined with endothelial cells to study NK cell cytotoxicity [163], as shown in Fig. 8A(a-b). The results of the NK cells cytotoxicity test against the MCF7 spheroid (Fig. 8A(c)) demonstrated that NK cells induced MCF7 cells mortality in a dose–response manner. At a lower NK cell ratio, cytotoxic activity was not homogenous across the spheroid; however, the induced cytotoxicity of NK cells was observed throughout the entire spheroid at a higher ratio (Fig. 8A(d)). The same group used an *in vitro* microfluidic platform to study NK exhaustion due to tumor environmental stresses reproduced on the chip [164]. The microfluidic platform design allowed to mimic nutrient, pH, proliferation, and necrosis gradients across 3D tumor cells. The results showed NK cells initially exhibited cytotoxicity and destroyed a high percentage of cancer cells. But the suppressive environment created by the tumor on the chip gradually reduced NK cell cytotoxicity, causing NK cell exhaustion. Moreover,

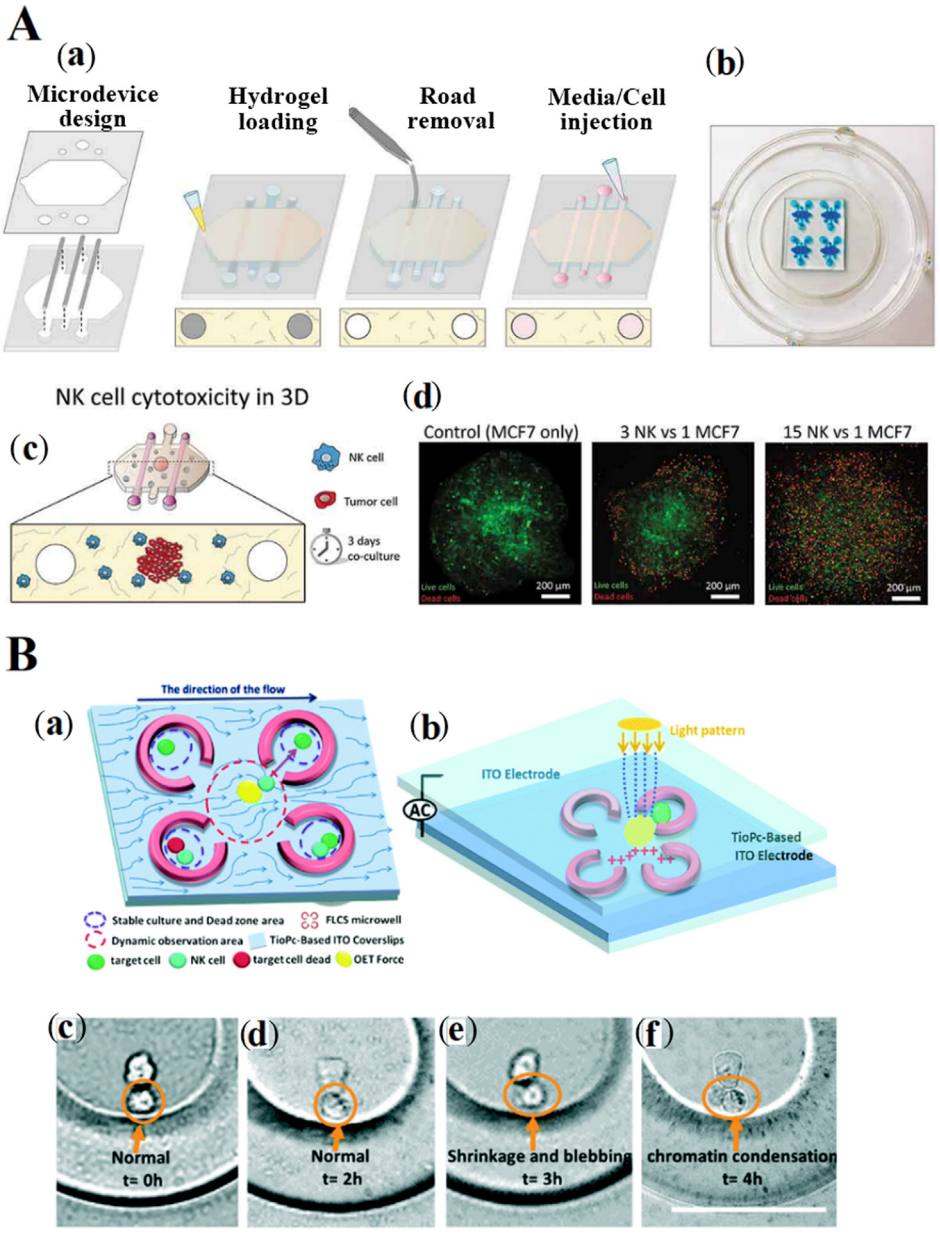

Fig. 8. Microfluidic platform for evaluation of NK cells immunotherapy efficacy. (A) The microdevice to study NK cell's cytotoxicity against MCF7 cells. (a) Schematic of the microfluidic chip fabrication and application process. (b) Image of a microfluidic device array. (c) Schematic of the NK cell cytotoxicity in 3D collagen hydrogels. (d) Confocal images of the NK cell cytotoxicity test at different NK cell ratios. MCF7 cancer cells were labeled with cell tracker green and viability was evaluated after 3 days in culture. Reproduced from Ref. [163] with permission of Taylor & Francis Group. (B) Illustrations of the TiOPc-based microfluidic device for cancer immunotherapy. (a) The diagram of the two steps of microfluidic device operation: cell manipulation to direct cell–cell contact and cell–cell interaction; (b) Schematic diagram of the TiOPc-based FLCS microfluidic device; (c-f) A normal morphology of the target cell after t = 0 hr (c), t = 2 hr (d), t = 3 hr (e, Shrinkage and blebbing of the target cell), and t = 4 hr (f, Chromatin condensation of the target cell). Reproduced from Ref. [165] with permission of The Royal Society of Chemistry.

NK cell exhaustion lasted for 7 days after removing NK cells from the microdevice. However, the analysis was limited to 7 days; thus, additional studies involving long-term analysis are needed to ascertain if these stress-induced alterations are permanent. In addition, Ke *et al.,* fabricated a TME Lab-on-a-Chip model, equipped with titanium oxide phthalocyanine (TiOPc)-based optoelectronic tweezers (OET) for allowing direct cell–cell contacts to evaluate NK cells and tumor cells interactions (Fig. 8B) [165]. A slow flow velocity zone in the center was created that provided an appropriate environment for OET force to manipulate single cells (Fig. 8B (a)). The OET non-contact force was produced using the light-induced non-uniform electrical field and single cells were delivered into the four-leaf-clover-shaped (FLCS) microwells (Fig. 8B (b)). After contacting the NK cells with the tumor cells in the microfluidic device, the target cancer cells showed shrinkage and blebbing in 3 hr and then dying in 4 hr (Fig. 8B(c-f)). In comparison to conventional analysis, the microenvironment lab-on-a-chip model exhibited greater NK cell activities because the continuous fluid flow and shear stress were avoided in the specific device sections where secreted proteins were washed out and interactions between cells and NK cell cytotoxicity were affected.

3.2. Immune checkpoint inhibitors (ICIs)

Malignant tumor cells promote an immunosuppressive state that favors immune evasion and tumor growth. To overcome these immunosuppressive conditions, ICIs play a significant role in blocking the impacts of selected inhibitory pathways [166,167]. In 2011, with the approval of ipilimumab from the Food and Drug Administration (FDA), ICIs or immune checkpoint blockade (ICB) were considered as therapeutic reagents and provided a breakthrough in cancer management [168,169]. These inhibitors not only introduce a new mechanism to treat cancer, but also allow for durable responses with a less toxic side effect [170]. Compared to traditional cytotoxic chemotherapies, ICIs play the role of strengthening the host immune system to fight cancer, via a set of inhibitory and stimulatory pathways that directly affect the function of immune cells [171,172].

The expression of ICIs is variously and closely modulated in every stage of immune cell activities [173–176]. For example, co-stimulating CD28 is fundamentally expressed in T cells, but a negative regulator cytotoxic T-lymphocyte-associated Protein 4 (CTLA-4) is promptly conveyed to the surface of the cell after T cell activation, for the purpose of downrating the immune response [177]. Therefore, inhibiting CTLA-4 can promote an enhanced activation and priming of antigen-specific T cells, resulting in proliferation in the draining lymph nodes [168]. In contrast, PD-1, which is highly expressed from T cells after constant exposure to antigen stimuli, interacts with ligand PD-L1 expressed on numerous cancer cells as well as tumor-infiltrating immune cells that contribute to immune escape [178]. Inhibiting PD-1/PD-L1 will eliminate suppression and reestablish pre-existed T cells' function, mainly at the site of the tumor. The purpose of ICIs treatment modality is to restore immune function from the unregulated immune environment through blocking the crucial regulator in the immune system [171]. Up to now, seven ICIs have been approved by the FDA, including one inhibitor for CTLA-4, three inhibitors targeting PD-1, and three inhibitors for PD-L1 [179,180].

Microfluidic techniques facilitate the investigations in cancer-immune interactions and allow high-efficiency evaluations of ICIs treatment responses [164,181,182]. The perceptions achieved from the repetition of the TME, such as understanding improvements of the immune system in cancer suppression, have encouraged new designs in targeted and effective cancer therapy. Since patient responses to ICIs are highly variable, it's important to promptly evaluate ICIs efficacy. Jiang *et al.* developed an array of miniaturized bioreactors, termed immunotherapeutic high throughput observation chamber (iHOC), for testing the effect of anti- PD-1 antibodies through monitoring immune interactions between cancer spheroid (MDA-MB-231, PD-L1 +) and T cell (Jurkat) [183]. A complementary micropillar array was coated with capture antibodies towards interleukin-2 (IL-2) (Fig. 9A(a)). T cells inhibition from tumor spheroids and reactivation by ICIs were observed by detecting IL-2 secretion and tumor infiltration. The tumor immune interactions were depicted from the iHOC by measuring IL-2 concentrations using a micropillar array where antibodies were coated on the surface. As shown in Fig. 9A(b), the number of infiltrating Jurkat T cells was significantly increased after treatment with the anti-PD-1 antibody. A higher penetrating distance in the direction of the spheroids center indicated an improvement of T cell infiltration and survival in the TME model after the ICI treatment. To explore the efficacy of the PD-1 checkpoint immunotherapy in glioblastoma (GBM), Cui *et al.* exploited a patient-specific "GBM-on-a-Chip" microfluidic platform to analyze the heterogeneity of immunosuppression in TME. The study also optimized immunotherapy efficiency of anti-PD-1 at different subtypes of GBM (Fig. 9B) [184]. It was identified that the molecular difference of the GBM subtypes showed diverse epigenetic as well as immune signatures, which would induce distinct immunosuppression mechanisms. Results revealed that, compared with proneural GBM, the mesenchymal GBM niche allured a tiny amount of allogeneic CD154 + CD8 + T-cells but a huge amount of CD163 + TAMs, and expressed higher immune checkpoints targeting PD-1/PD-L1 and TGF-β1, IL-10, and CSF-1 cytokines. After introducing a CSF-1R inhibitor BLZ945 to remove CD163 + M2-TAMs, the PD-1 inhibitor nivolumab exhibited enhanced efficacy to promote the functionality of CD154 + CD8 + on T-cell and GBM apoptosis.

To compare ICI therapies in two different targets, namely, anti-CTLA4 and anti-PD-1, in three syngeneic mouse tumor models, Doty *et al.* designed a cyclin olefin copolymer (COC) based microfluidic device with an array of pillars to trap tumors in the center of the channel while allowing for medium and TILs to flow around and over tumor fragments [185]. Unmodified tumor fragments were cultured in this device with viability over 7 days or more by dynamic perfusion. Through real-time imaging and analysis of high-resolution confocal, mechanisms of TIL migration, infiltration, and lymphocyte-mediated killing within tumor fragments were readily accessible, demonstrating excellent correlations between *in vitro* tumor biopsy and *in vivo* syngeneic mouse model responses of the checkpoint inhibitors. The same group also applied a microfluidic platform to study lung tumor biopsies responses and evaluations of resident TILs to PD-1 antibodies for the treatment of non-small-cell lung carcinoma (NSCLC) [186]. The device facilitated with a real-time monitoring system was fabricated via a commercialized printable resin (Pro3dure GR-10), providing a transparent, noncytotoxic, physiologically relevant, high-resolution microfluidic system. The device sustained tissue fragments biopsy under dynamic perfusion for 72 h, and established a personalized tumor model. This method of modeling and analyzing tumor responses from confocal microscopy provides an improved prediction of immunotherapy efficacy for individuals. Additionally, Lu *et al.* reported an integrated microfluidic system for exosome isolation and detection (EXID) to analyze the abundance of the exosomal PD-Ll protein marker by using immune magnetic capture beads and anti-PD-L1 fluorescence probing [187]. Exosome isolation, biomarker labeling, and quantification were incorporated in a single microfluidic chip, which significantly reduced the total analysis time to < 2 h. Seven categories of cell lines including cancer cell lines and control samples were profiled using the EXID system, and the observed noticeable variations in PD-L1 abundance among cancer cell lines highlighted the need

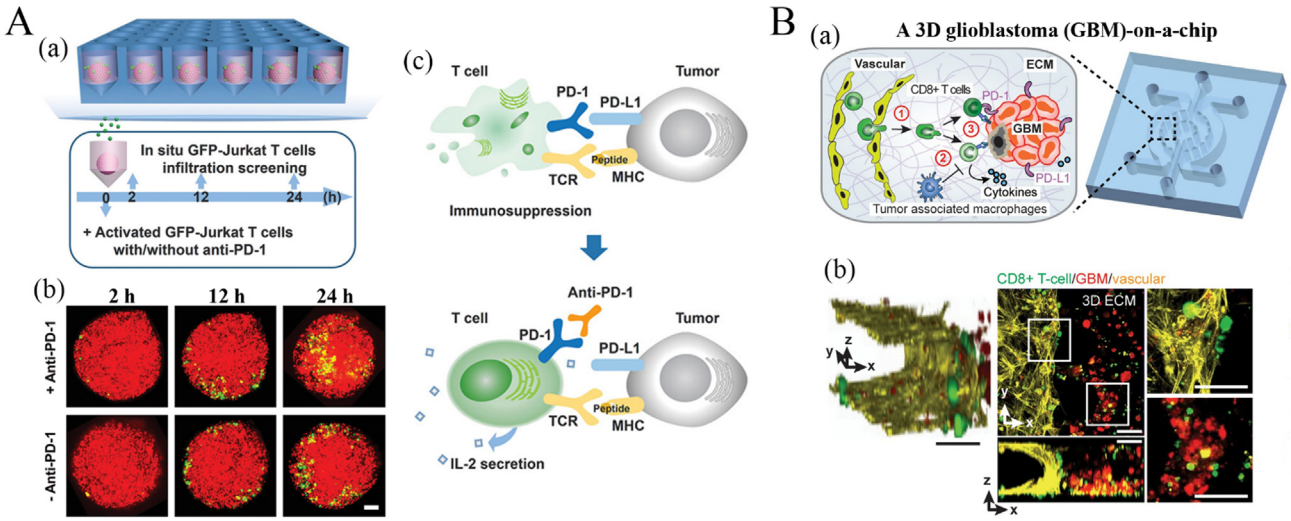

Fig. 9. Microfluidic systems for evaluation of ICIs functions and efficacy. (A) (a) Scheme of the high-throughput chambers for T cells infiltration assays. (b) Confocal fluorescence images of the tumor spheroids infiltrated by Jurkat T cells. (c) Function of anti-PD-1 antibody to influence the interaction between tumor cells and T cells. Adapted from Ref. [183] with the permission of Wiley. (B) GBM-a-on-Chip system to investigate PD-1 immunotherapy. (a) Scheme of the microfluidic device. (b) Confocal immunofluorescence image with a 3D brain microvessel (yellow) with CD8 + T-cells (green) and GBM tumor cells (red). Adapted from Ref. [184] with permission of eLife.

for personalized treatments, providing guidance to immunotherapy strategies for different types of tumors.

3.3. Cancer vaccines

Different types of cancer therapeutic vaccines have been tested including viral, bacterial, and yeast vectors, immunogenic peptides, immune cells, and dead cancer cells, and used to present Ags to effector T cells and improve immune system activation against tumor cells [188,189]. Cancer vaccines stimulate dendritic cell activity and lead to greater T-cell responses [190]. The combination of cancer vaccines with ICIs has shown promising results. Provenge is the first cancer vaccine approved by the FDA that relies on activating autologous dendritic cells ex vivo and reinfusing them to prime the endogenous T cells to elicit antitumor responses [29,191,192]. The broad application of cancer vaccines is limited because of several challenges. The low immunogenicity of the vaccine and the negative regulatory mechanisms orchestrated by the tumor-inducing immune tolerance are usually the main reasons for the poor efficacy of cancer vaccines [193,194].

Microfluidic tumor organoid-on-a-chip platforms have been applied to predict clinical responses after cancer vaccination and thereby to optimize immunization strategy [195]. For example, Lu *et al.*, devised a cell electrofusion device integrated with titanium electrodes on a glass wafer that paired and fused homogeneous and heterogeneous cells, and the device was employed for the production of dendritic cells-tumor fusion vaccines to elicit anti-tumor immunity [196]. Hydrodynamic trapping in combination with positive dielectrophoretic force (pDEP) was utilized to achieve cell fusion. The microfluidic device contained 960 pairs of trapping channels which showed a pairing efficiency of 68% with a fusion efficiency of 64%. The fused cells could be easily extracted from the chip, which made it distinguishable from other designs. The experimental procedure was simple, efficient, and repeatable. The chip had the potential to improve the current cell fusion techniques and defeat key barriers to build and develop an automated, large, and efficient dendritic cell-tumor fusion vaccine therapy.

Extracellular exosomes can also prime immune system activities by presenting parent cell signaling proteins or tumor antigens to immune cells [197]. A streamlined microfluidic cell culture platform was developed by Zhao *et al.* integrated with harvesting, antigenic modification, and photo-release of surface engineered exosomes, enabling the production of MHC peptide surface engineered exosomes for cytolysis activation [198]. By modifying melanoma tumor peptides on the exosome surface, the ability of exosomes in antigen presentation and T cell activation was enhanced to significantly increase antigen-specific CD8 + T cell proliferation (Fig. 10A). Compared with native exosomes, it was observed that gp-100 engineered exosomes significantly increased the cellular uptake from dendritic monocytes by almost two folds, (Fig. 10A(c)). The proliferative response of CD8 + T cells was induced by gp-100 exosomes when cultured with LPS-activated JAWS cells (an immature dendritic cell line derived from a C57BL/6 mouse) with ~30% proliferation rate at the 100: 1 ratio of exosomes: dendritic cells.

To isolate MHC-I restricted tumor-specific peptides for reliable tumor antigen characterization, Feola *et al.* exploited a microfluidic chip (PeptiCHIP) to determine and characterize tumor-specific ligands on clinically relevant human samples [199]. They studied the ability to immobilize a pan-HLA antibody on solid surfaces using well-characterized streptavidin–biotin chemistry, toward the implementation of a microfluidic through-flow system, highlighting the potential to exploit microfluidics approaches in personalized immunopeptidome analysis from individual tumor biopsies to design of tailored cancer vaccines. Lipid nanoparticles (LNPs) have also emerged as a new gene therapy method in cancer immunotherapy and DNA vaccination. Quagliarini *et al.* realized the microfluidic formulation and transfection of DNA-loaded LNPs to HEK-293 cells, human primary keratinocytes, and CaSki cells [200]. DCs that act as antigen-presenting cells (APCs) and mediate between innate and adoptive immunity have an essential function in cancer vaccination [78,201]. García-Salum *et al.* reported that autologous DCs loaded with an allogeneic heat shock (HS)-conditioned melanoma cell-derived lysate, induced T-cell-mediated immune responses in stage IV melanoma patients [202].

3.4. Oncolytic viruses

Oncolytic viruses (OVs) are another type of immunotherapy that use the innate capability of a virus to destroy tumor cells with the potential to initiate an antitumor response. OVs target tumor cells inside and kill them while sparing healthy cells [203]. Several

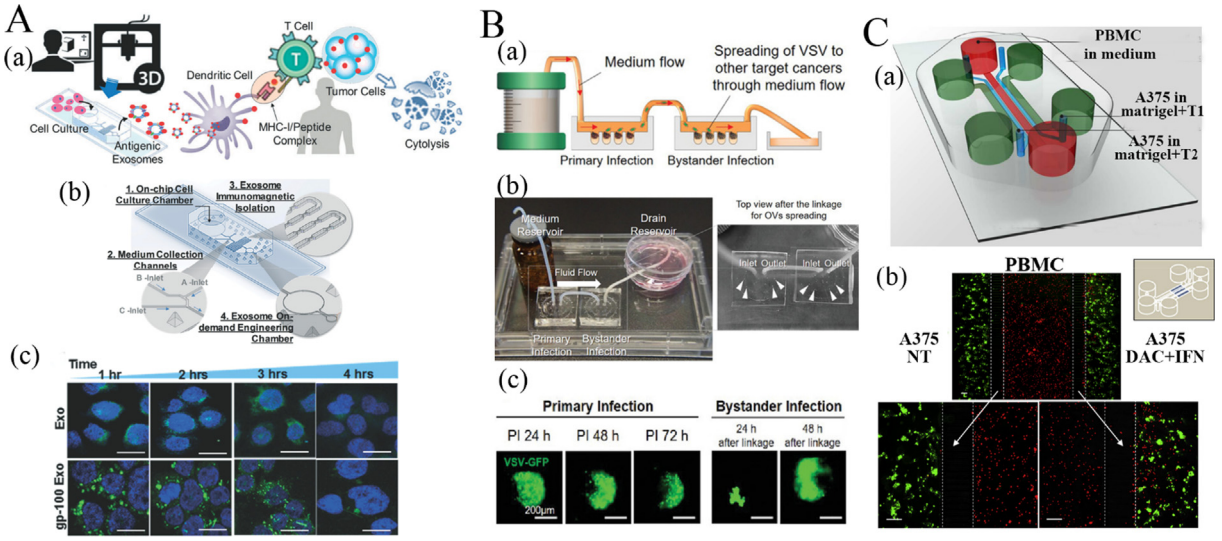

Fig. 10. Cancer vaccines, oncolytic viruses and cytokine therapy tested on microfluidic platforms. (A) Engineering of exosomes on a microfluidic platform for cancer immunotherapy. (a) Illustration of the 3D-printing molded PDMS microfluidic culture device for streamlined engineering of antigenic exosomes employed in activating anti-tumor responses. (b) Schematic of the microfluidic culture chip for engineering immunogenic exosomes directly from on-chip cultured cells in real-time. (c) Confocal microscopy images of the DC uptake of tumor-targeting antigenic (TTA) peptide gp-100 surface engineered exosomes, compared with non-engineered native exosomes. Adapted from Ref. [198] with permission of The Royal Society of Chemistry. (B) Microfluidic platform-based MPS to study both oncolytic infection and bystander infection into targeted tumor cells by spreading OVs. (a) Design of the 3D *in vitro* MPS with *in vivo* simulating microenvironments; (b) Photograph of the 3D *in vitro* microfluidic platform-based MPS for the spread of VSV; (c) PI time-dependent changing of VSV-GFP expression intensity in the primary infection and the bystander infection. Adapted from Ref. [206] with the permission of Wiley. (C) (a) Scheme of a microfluidic system to investigate IFN-I and DAC influence on murine and human melanoma cells. (b) PBMCs migration toward DAC/IFN-I–treated human melanoma cells in the microfluidic device. Adapted from Ref. [208] with permission of Elsevier.

clinical trials proved the safety of OVs derived from different families of viruses [29,204,205]. Unfortunately, the application of OVs for cancer immunotherapy is extremely limited to most metastatic cancers because of TME, acquired specific immunity against the virus, and complete viral clearance, causing repeated therapy to become impossible [205]. Lab-on-a-chip technology can be used to address these challenges to improve the OVs immunotherapeutic efficacy.

Tumor organoid-on-a-chip models recently appeared as privileged tools to study oncolytic viruses as cancer immunotherapeutic agents. Lee *et al.*, introduced a 3D *in vitro* microfluidic chip-based microphysiological system (MPS) allowing real-time monitoring of oncolytic infection and spread of OVs [206]. For assessment of the spread and bystander infection of OVs via fluid flow, bystander infection was realized by block-to-block linkage of the primary infected MPS with uninfected 3D multicellular tumoroids (MCTs) integrated with MPS (Fig. 10B(a-b)). To identify the oncolytic virotherapy effect due to the spread of OVs, replicable VSV-GFP (vesicular stomatitis virus-green fluorescence protein) was used to determine the location of the infection in 3D MCTs that were formed by fluorescence tracker-labeled cells. GFP expression in 3D MCTs within connected MPS was tracked to monitor the spread of VSV-GFP through fluid flow. The post-infection (PI) time-dependent decrease in VSV-GFP expression intensity in the primary infection and the enhancement of VSV-GFP fluorescence intensity in the bystander infection verified the spread of the replicable VSV-GFP (Fig. 10B(c)). The same group investigated the anti-tumor responses of oVSV (oncolytic VSV) through the application of the developed 3D *in vitro* tumor organoid-on-a-chip model based-MPS [207]. The 3D multicellular tumor spheroids (MCTSs) in the MPS were infected with oVSV-GFP (oVSV expressing GFP). In comparison to the 3D MCTS, the 3D MCTS-integrated MPS was an improved *in vitro* cancer model because it simultaneously consisted of 3D MCTSs and a microdevice. Results demonstrated that oVSV antitumoral characteristics could be easily monitored in the 3D MCTS-integrated MPS and the antitumoral activity of oVSV differed from that in a 2D system.

3.5. Cytokine therapy

Cytokines are soluble proteins that play the role of mediating cell-to-cell communication, providing growth, differentiation, and signals of inflammatory or anti-inflammatory to different cells [209]. They are important immunomodulatory agents that form responses from the immune system and, on the other hand, as well participate in the immune suppression response [6,210]. Through controlling the cytokine, endogenous protection could be reconstructed or even improved. Thus, cytokine immunotherapy holds great potential for cancer treatment. Cytokines can take effect during every phase of cancer-immune interactions, such as antigen priming improvements, enhancement of recruiting effector immune cells into the tumor immune microenvironment (TIME) or increasing their cytotoxicity [211,212]. Cytokines affect tumor growth through anti-proliferation, pro-apoptotic activities, mediately stimulating effector cells' cytotoxicity, such as interferon-alpha (IFN-α) and interleukin-2 (IL-2), even if the efficacy was only modest [15,213]. Recent interest in the anti-tumor properties of cytokines has induced expanding clinical trials of cytokine-based drugs, as well as their combination with other immunomodulatory drugs [214].

Microfluidic tumor organoid-on-a-chip that can model more sophisticated and clinically relevant TME and precisely control the flow of molecules and cells, can efficiently introduce functional interactions between cytokines, immune cells, and tumor cells from patients. For instance, a Matrigel-based microfluidic device (Fig. 10C(a)) was fabricated by Lucarini *et al.* to study synergistic effects of the demethylating drug decitabine (DAC) and interferon- I (IFN-I) [208]. The combined utilization of DAC and IFN-I significantly inhibited the growth of human melanoma cells *in vitro* and murine *in vivo*. After DAC/IFN-I treatment, cell growth of melanoma cells was reduced, apoptosis was augmented, and migration was diminished, with increased recruitment of immune cells toward the tumor (Fig. 10C). The results showed even a low dose of DAC plus IFN-I resulted in various antitumor effects on human metastatic melanoma cells and murine. In a microfluidic

device, untreated A375 cells (NT, left channel) and A375 plus DAC/IFN (right channel) showed significant variations through preferentially migrating to DAC/IFN-I–treated human melanoma cells (Fig. 10C(b)). The combined treatment stimulated significant inhibition of tumor cell growth after 4 days, which resulted partially in G2/M−S−phase cell cycle arrest and partially in the induction of apoptosis.

Additionally, T-cell engaging bispecific antibodies (TCBs) are a novel class of cancer immunotherapeutic agents, which can bind to tumor cell surface antigens and CD3 T-cell receptors, for improving the clinical efficacy and safety. Kerns *et al.* reported two organs-on-chip devices to evaluate the safety of TCBs targeting tumor antigens [215]. Lung-Chip and Intestine-Chip both could predict and illustrate target-dependent TCB liabilities depending on sensitivity tests to the target expression and antibody affinity. The Lung-Chip was composed of a top microfluidic channel where adult human alveolar cells was seeded and cultured through an air–liquid interface and a flexible membrane. With such a porous membrane, the top epithelial channel was separated with seeded primary lung microvascular cells from a bottom vascular channel. The Alveolus Lung-Chip demonstrated lung toxicities observed in cynomolgus mediated by Folate Receptor 1-TCB that can lead to close proximity between CD3 expressing cytotoxic T-cells and FOLR1 expressing tumor cells. The Intestine-Chip model obtained the liabilities of TCB targeting human-specific antigen, presented TCB affinity, and demonstrated differential and toxicity outcomes depending on target expressions between different intestinal regions. In another example, Businaro *et al.* devised a microfluidic model to investigate the interactions between cancer and the immune system, as well as how interferon regulatory factor 8 (IRF-8) gene expression regulates the immune and melanoma cells crosstalk [216]. IRF-8 knockout mice were highly permissive to the growth of B16 melanoma as immune cells failed to exert immunosurveillance.

4. High-throughput immunotherapy assays

Cancer therapeutic screening assay is a key method for the evaluation of therapeutic agents (such as drug or immune components) efficiency and toxicity, providing valuable information to early-phase clinical trials [21,217–220]. It is imperative for ex vivo models that can recapitulate the tumor-stromal complexity, meanwhile providing reproducibility and simplicity to high-throughput systems [221–223].

The microfluidic technique is a promising method where primary high-throughput anticancer therapeutic agents can be screened, meanwhile approaching immune-infiltration and other immune studies. [181,224]. For instance, a highly efficient droplet-based microfluidic platform for next-generation cancer immunotherapy was developed by Wang *et al.*, including a lentivirus transduction system capable of screening numerous antibodies to identify desired potentials [225]. The droplet microfluidic technology allowed simultaneous analysis and screening with unparalleled throughput of numerous individual antibody-secreting cells for their antibody affinity and secretion rate at the single-cell level (Fig. 11A). From a combinatorial antibody library, rare active anti-Her2 × anti-CD3 bispecific antibodies

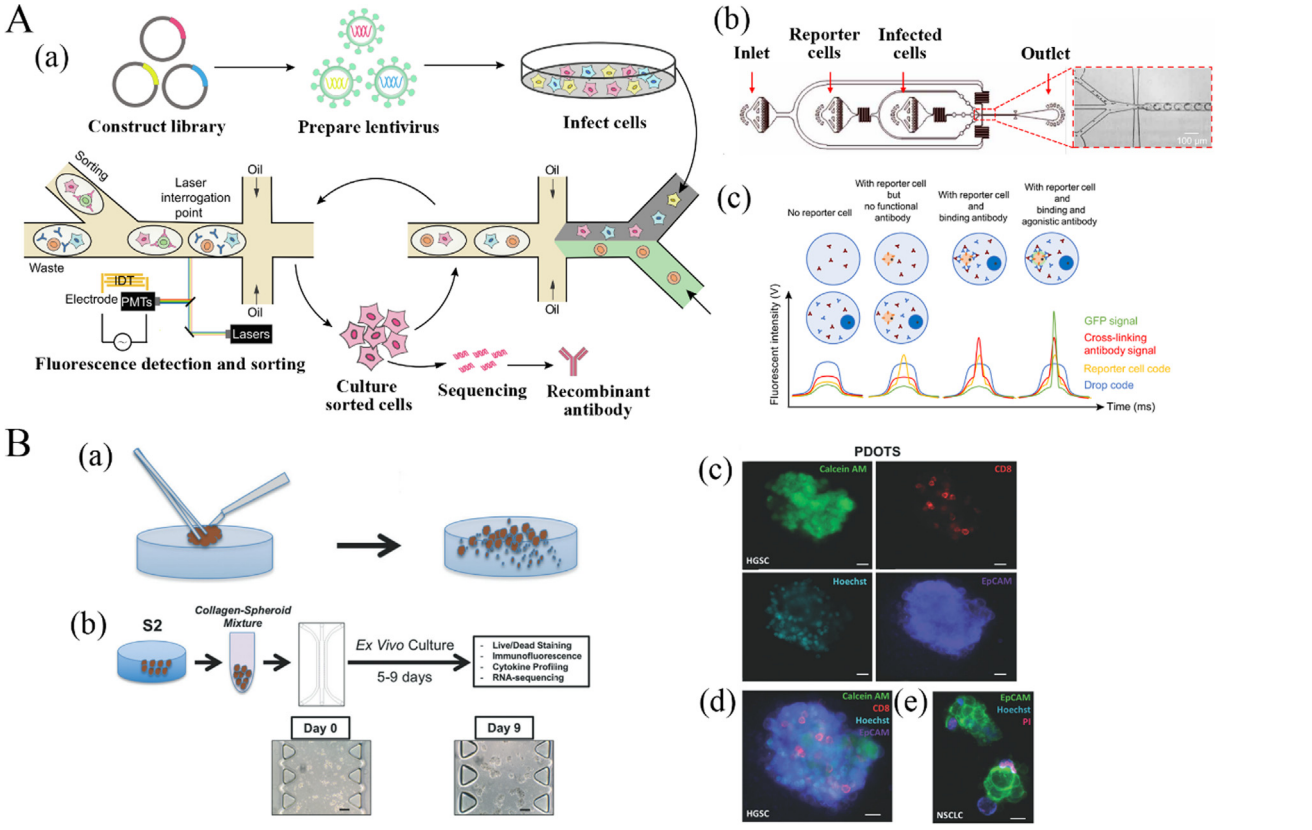

Fig. 11. High-throughput immunotherapy assay and Personalized immunotherapy test. (A) A high-throughput droplet-based microfluidic system for functional antibody screening for cancer immunotherapy. (a) Schematic of the antibody screening process with a droplet microfluidic system. (b) The droplet chip was used to produce droplets to co-encapsulate antibody-secreting cells with reporter cells. (c) Screening CD40 agonist antibody from a monoclonal antibody library. Adapted from Ref. [225] with permission of American Association for the Advancement of Science. (B) Patient-derived organotypic tumor spheroids cultured in thee microfluidic device to study ICB. (a) A tumor sample was dissociated physically and enzymatically into cell spheroids and dispersed cells. (b) Cell spheroids were injected into the microfluidic device for further culture. (c and d) Immunofluorescence (IF) staining of high-grade serous carcinoma (HGSC) PDOTS indicating alive cells (green), tumor cells (purple), CD8 T cells (red), and all nucleated cells (blue). (e) IF image of non-small cell lung carcinoma (NSCLC) PDOTS indicating all nucleated cells (blue), tumor cells (green) and dead cells (red). Adapted from Ref. [233] with permission of The Royal Society of Chemistry.

and effective costimulatory receptor CD40 agonist antibodies were successfully discovered through this platform. Another application of high-throughput microfluidic immunotherapeutic was reported by Park *et al.*, who designed a 3D cytotoxicity assay to evaluate the cytotoxicity of lymphocytes [226]. Polystyrene platforms were fabricated by mold injection to produce plastic culture substrates and hydrophilic rail-based microstructures. The multi-well device provided high-throughput potential by introducing multiple assays to be simultaneously achieved in a single platform. The results demonstrated that 3D ECM significantly decreased NK cells migration and approaching HeLa cancer cells, producing lower cytotoxicity. With the enhancement of a 3D matrix, the NK cells effectively destroyed cancer cells after instantaneous contact with cancer cells. Furthermore, a microfluidic device with a radial channel design was developed by Wu *et al.*, which allowed for simultaneous chemotaxis tests of various kinds of cells with various gradient conditions [127]. To increase the throughput in a single device, eight separated units of gradient generation networks were integrated. Based on the radial octameric design of the microfluidic chip, eight simultaneous chemotaxis assays were allowed to introduce eight different conditions (such as concentrations, chemoattractants, and time points), which were impossible for conventional single unit chemotaxis systems. Two slower-migrating human breast cancer cell lines (MDA-MB-231 and MCF-7) and fast-migrating human neutrophils were introduced to evaluate cell immigration and the versatility of the system.

5. Personalized immunotherapy

Personalized oncology targets the unique features of a patient's tumor to improve therapy efficacy, while cancer immunotherapy aims to activate and regulate the patient's immune system to attack the tumor [227,228]. The combination of these two strategies results in a new direction as personalized immunotherapy. Patient-specific microfluidic models are relatively more simple and modest compared with existing complex models using cancer cell lines [228,229]. It's a big challenge to perform preclinical immunotherapy testing in microfluidic devices using patient-derived cells [230–232]. Targeting this challenge, Aref *et al.* evaluated murine- and patient-derived organotypic tumor spheroids (MDOTS/PDOTS) to screen for the response of patient tumors to ICB therapy, with RNA-sequencing to discover changes in the TIME (Fig. 11B) [233]. MDOTS/PDOTS containing tumors, immune, and stromal cells were cultured in collagen hydrogel, in which PD-1 blockade could interact with autologous immune cells. This system could not only recapitulate tumor sensitivity and resistance to ICB, but also have the capability to test synergistic effect of PD-1 blockade and other factors such as inhibitors of CDK4/6 and TBK1, which enhanced the effect of PD-1 blockade. Molecular analysis with RNA-seq also demonstrated relative expansion of M0 macrophages and CD8 + T cells in PDOTS treated with dual ICB (α PD-1 + α CTLA-4) compared to single ICB or blank control. In another recent report, Al-Samadi *et al.* developed a 3D microfluidic chip to test individual responses to immunotherapy, with isolated patients' cancer cells, immune cells and serum, and loaded with PD-L1 antibody and IDO 1 inhibitor [65]. For the first time, it was observed that IDO 1 inhibitor, instead of PD-L1 antibody, induced immune cells to migrate towards cancer cells both in HSC-3 and in two head and neck squamous cell carcinoma (HNSCC) patient samples. Efficacy of IDO 1 inhibitor and PD-L1 antibody was tested with two patient samples for tumor cell proliferation and was found to be patient dependent. Results showed that PD-L1 antibody was the most effective drug for the first patient, while IDO 1 inhibitor was the most effective for the second patient. This personalized *in vitro* microfluidic assay could be used to predict the efficacy of different immunotherapeutic drugs for individual patients.

6. Conclusion and outlook

Following the unexpected success in the treatment of multiple types of cancers which was previously difficult to treat such as melanoma, immunotherapy has advanced substantially over the past decade and become an indispensable therapeutic method for many types of cancers [234]. Despite the great advancement of immunotherapy, only about 20–30% of total cancer patients with different cancer types are able to benefit from immunotherapy, and even among these patients with effective responses, intrinsic and acquired resistance exists and remains a significant challenge. Thus, a deeper understanding of the immune system, tumor, TME, and the cancer–immune interactions is essential to further investigations. More sophisticated preclinical models are required to perform relevant functional applications, such as efficient/effective evaluation of immunotherapy combinations and dissecting the immune contexture in the TME. Microfluidic-based tumor-on-a-chip models integrating patient-derived tumor cells and physiological features of the TME have emerged as innovative promising tools to overcome the drawbacks of other *in vitro* models and animal models [235].

As presented in this review, tumor organoid-on-a-chip platforms have been widely used to recapitulate the TME of various tumor types, and to decipher the influence of different factors on the TME. In Table 1, we summarize the technique, cell types, microfluidic device design and applications of recent microfluidic platforms pertaining to cancer immunotherapy. The TME, including different phenotypes of immune cells, dysfunctional vasculature, cytokines, hypoxia, and other factors, plays a crucial role in cancer immunotherapy. Understanding the interactions between these different factors could be the first step to develop a novel and effective cancer immunotherapy. With rapid development in tissue engineering and biomaterials, microfluidic platforms not only permit 3D cell co-culture, precise flow control, perfusion of vascularized structures to mimic *in vivo* tumor microenvironments, but also facilitate real-time imaging analysis. Modeling the TME with tumor-on-a-chip technologies can realize long-time and high-resolution monitoring of cancer–immune cell interactions, which is critical to understanding how tumors modulate the TME to act against anti-cancer immunity at both cell and tissue levels [26]. Microfluidic platforms utilizing patient-derived tumor cells or tissues have been shown to be a reliable and effective tool for the evaluation of various immunotherapies, implying great potential for personalized medicine and overcoming drug resistance [86]. In addition to the tumor-on-a-chip technologies, the integration of multiple analysis approaches can allow on-chip multi-parameter analysis of immune responses [15]. These systems will greatly deepen our understanding of tumor's immune evasion, and they are becoming an essential and versatile tool for cancer modeling and tumor–immune interaction investigation, thus offering predictive, diagnostic and therapeutic values to promote cancer immunotherapy [223,236–239].

Although various microfluidic systems have been utilized to model TME-immune interactions, and demonstrated credible results in immunotherapy, some limitations still exist. For example, the extent to which current tumor-on-a-chip models approximate *in vivo* biological processes remains unclear. Thus, the application of multi-model validation is imperative, and key biological results obtained from 3D microfluidic models need to be evaluated by clinical results. In addition, retrieving cells from microfluidic devices for further biochemical analysis and cancer treatment is complicated and not straightforward. The low cell

number in microfluidic devices can also be a limitation when more cells are required for normal biochemical assays or if secreted molecules need to be quantified. Furthermore, PDMS is found to adsorb small hydrophobic molecules, which can influence the accuracy of drug screening assays. New advances in biomaterials may provide an alternative to address this problem [35,240–242].

It is expected that over the next few years, more and more tumor organoid-on-a-chip platforms will be developed using patient-derived samples to study how individual patients will respond or resist immunotherapies toward clinical applications and the pharmaceutical industry [228]. Miniaturization of microfluidic systems is compatible with limited input samples such as patient-derived biopsies, allowing for parallel tests with either tissue fragments or digested dispersed cells, on a much larger scale than current macro-size models. With throughput microfluidic assays using biopsy samples, much more information can be obtained to offer a clinically meaningful time-frame for personalized therapy, and to provide a feasible path to integrate human tissue-based tests into preclinical studies. Additionally, the combination of nanomaterials and nanotechnology with microfluidic platforms can further enhance detection sensitivity and cancer therapy such as by combining nanomaterial-mediated drug delivery and control, targeted drug delivery, and phototherapy with immunotherapy [21,238,243–248]. With greater physiological relevance, these platforms have the potential to become the gold standard for preclinical screening of multiple therapies without the risk of adverse side effects in human experiments. Taken together, by combining the immunological features with TME, tumor organoid-on-a-chip platforms will be able to predict patient responses to different therapies and provide personalized therapies to cancer patients based on synergistic combinations of immunotherapy and other therapies, progressing towards clinical usage for precision medicine and routine R&D practice in the pharmaceutical industry.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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