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Novel research model for in vitro immunotherapy: co-culturing tumor organoids with peripheral blood mononuclear cells

Peng Li[1,2], Minli Huang[1,2], Yifan Ma[3], Yongbin Zhang[1*] and Changhong Shi[2*]

Abstract
Tumor organoids have emerged as powerful tools for in *vitro* cancer research due to their ability to retain the structural and genetic characteristics of tumors. Nevertheless, the absence of a complete tumor microenvironment (TME) limits the broader application of organoid models in immunological studies. Given the critical role of immune cells in tumor initiation and progression, the co-culture model of organoids and peripheral blood mononuclear cells (PBMCs) may provide an effective platform for simulating the interactions between immune and tumor cells in vitro. This model stands as a robust instrument for dissecting the TME, elucidating the molecular interactions, and exploring the therapeutic applications of chimeric antigen receptor (CAR)-engineered lymphocytes, as well as other cancer treatment modalities. This review systematically evaluates the advantages and disadvantages of the co-culture model, identifies its technical bottlenecks, and proposes corresponding optimization strategies. By summarizing the latest research advancements in this co-culture model, our goal is to provide valuable insights for further model optimization and clinical application, thereby promoting immunological research and bridging the gap between experimental outcomes and clinical practice.

Keywords Organoids, Peripheral blood mononuclear cells, Co-culture, Immunotherapy, Tumor

Introduction

Cancer remains a major worldwide health problem, even though substantial progress in developing treatments has been made in the past few decades [1]. Solid tumors are considered as a "complex organ" that comprise malignant cells and several other non-malignant cell types such as the stromal tissue [2]. Stromal tissue is the supportive and connective tissue of the host comprising many different cell types, including fibroblasts, resident epithelial cells, pericytes, myofibroblasts, vascular and lymphovascular endothelial cells, and infiltrating cells that form the tumor microenvironment (TME). In the TME, interactions among tumor cells, non-malignant cells, and

*Correspondence:
Yongbin Zhang
yongbinzhang@gzucm.edu.cn
Changhong Shi
changhong@fmmu.edu.cn
[1]Animal Laboratory Center, Guangzhou University of Chinese Medicine, Guangzhou 510405, China
[2]Division of Cancer Biology, Laboratory Animal Center, The Fourth Military Medical University, Xi'an 710032, Shaanxi, China
[3]Gansu University of traditional Chinese medicine, Lanzhou 730030, Gansu, China

extracellular components create tumor tissue heterogeneity, complicating treatment [3, 4].

Although immunotherapy has made significant progress in the treatment of certain cancers, such as melanoma and non-small cell lung cancer [5], the complex nature of the TME has exacerbated the difficulties associated with immunotherapy. Therefore, there is an urgent need for new models to evaluate the effect of immunotherapy. The common cancer cell line model, namely long-term two-dimensional (2D) culture, can lead to cell polarization at the base and top of tumor cells, altering the immune checkpoint molecule expression [6]. This model cannot demonstrate the interaction between tumor and immune cells. The humanized tumor model can simulate human immune environment by simultaneous transplanting human immune cells and human tumor cells/tissue into immunodeficient mice [7]. Through this approach, the effectiveness of immunotherapy strategies can be evaluated and the interaction between tumors and the human immune system can be studied. Nevertheless, this model integrates human immune cells with mouse innate immune cells, but it is constrained by lengthy modeling times, high expenses, and an inability to support high-throughput drug screening [8]. Organoid models preserve the heterogeneity and three-dimensional (3D) structure of patient tumor cells, providing a molecular and physical basis for cell interactions; however, these models cannot reproduce the complex TME. Therefore, co-culturing patient tumor-derived organoid (PDO) and the non-malignant cells in TME around the epithelium can simulate the complex TME in vitro [6], which provides an essential tool to guide precise clinical treatment.

Given the crucial role of immune cells in tumor occurrence and development, tumor organoid and peripheral blood mononuclear cells (PBMCs) co-culture model has been established to simulate the interaction between immune and tumor cells (Fig. 1). PBMCs consist of the mononuclear cells fraction, isolated from peripheral

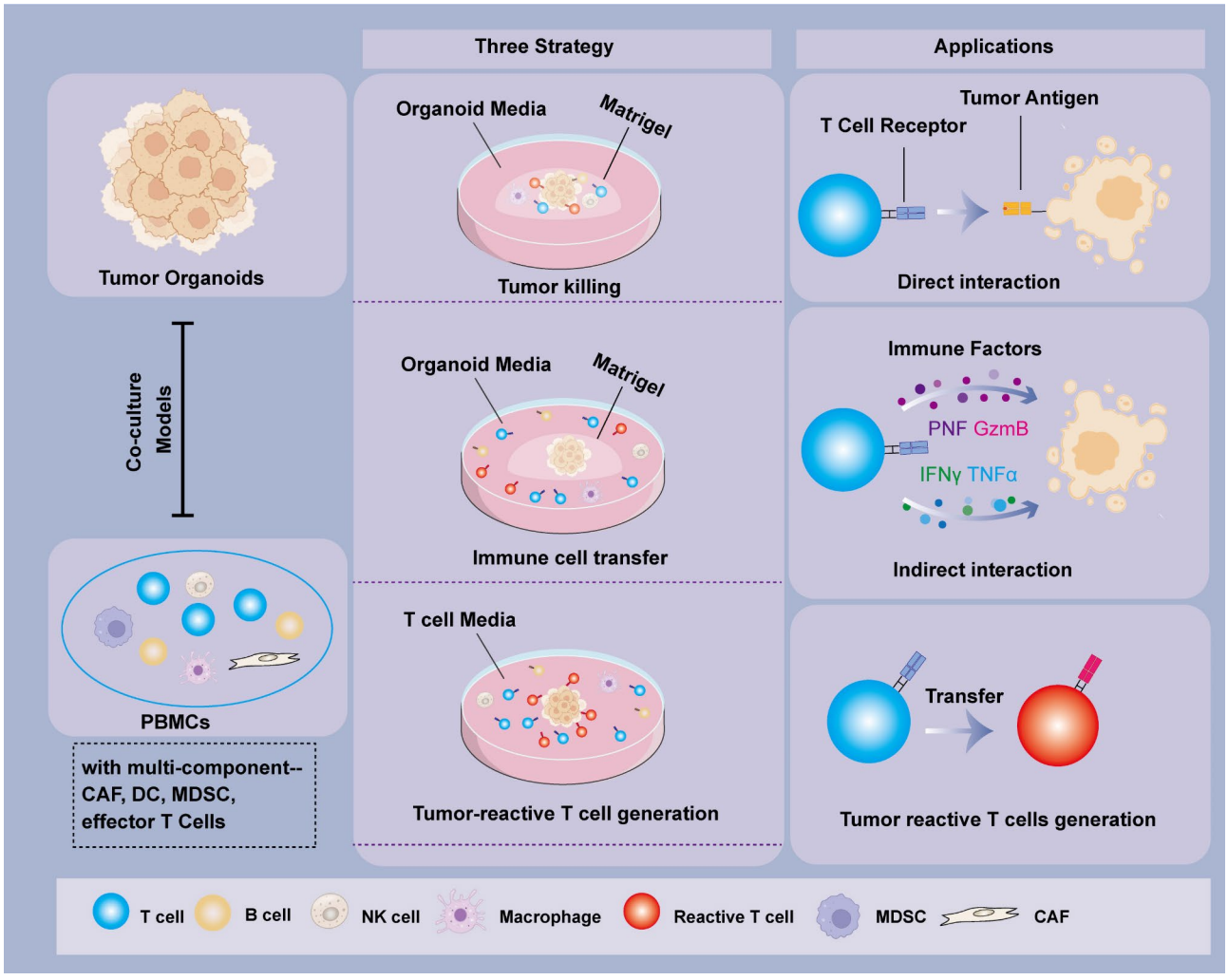

Fig. 1 Basic characteristics of organoid and peripheral blood mononuclear (PBMC) co-culture model

blood, mainly including lymphocytes (T cells, B cells, and NK cells), monocytes, dendritic cells, and additional rarer hematological populations [9]. Compared to tumor-infiltrating lymphocytes (TILs) extracted from tumor tissues, which target tumor-specific antigens, PBMCs contain a wider array of immune cells and provide a more straightforward, quicker method for collection, isolation, and expansion [10]. This co-culture system simulates the interaction between tumor and immune cells to a certain extent, reveals the immune response mechanism in tumor and personalized precision medicine, and exhibits great potential in immunotherapy research. However, co-culturing PBMCs with organoids is complex and has several technical challenges that must be overcome. This review focused on the organoid and PBMC co-culture system, primarily introducing the occurrence and development of co-culturing organoids with immune cells, the technical aspects of modeling, and the potential applications.

Basic characteristics of the organoid co-culture model

In general, fresh tumor tissues were digested into small cell clusters or single cells and then cultured in Matrigel to establish tumor organoid [11]. This method simulates the extracellular environment of collagen-rich basement membranes in human tissues, facilitating cells grow in 3D mode, and preserving cell-cell interactions. Growth factors, such as Noggin, R-spondin-1, and Wnt3a, are often added to the culture medium to promote organoids proliferation. The components of the culture medium can penetrate the Matrigel and provide nutrients to the organoids. Many tumor organoids from different epithelial tumors have been established through surgical resection and tumor biopsies [10, 12].

PBMCs are obtained from autologous or allogeneic peripheral blood samples using the Ficoll-Paque method. Then, PBMCs and organoids are co-cultured to rapidly and effectively simulate the immune microenvironment in vivo. Depending on the method and purpose of the experiment, co-culture models can be performed at least three ways : (i) PBMCs and organoids are co-cultured in Matrigel to study the interaction between PBMCs and tumor cells [13]. PBMCs and organoids are co-cultured in Matrigel to establish a 3D environment that closely emulates the TME. This innovative approach facilitates a more accurate examination of the intricate interactions between PBMCs and tumor cells. The incorporation of Matrigel permits the infiltration of immune cells, including T cells and NK cells, into the extracellular matrix. This enables direct engagement with tumor cells, thereby promoting more precise immune responses, including cytotoxic activity and cytokine secretion. Moreover, the 3D structure afforded by Matrigel enhances the migration and invasion of tumor cells, providing a more authentic replication of in vivo conditions. This co-culture system is especially advantageous for investigating immune cell activation, immune evasion mechanisms, and the comprehensive dynamics of immune-tumor interactions. It offers insights that may remain elusive in conventional 2D cultures [10]. (ii) PBMCs are added to the exterior of the Matrigel, while organoids are placed within the Matrigel. PBMCs are placed on the surface of the Matrigel, with organoids positioned within its matrix. This co-culture model allows for a more detailed exploration of the indirect interactions between PBMCs and tumor cells, such as signaling via soluble factors and chemokine- guided cell migration. Furthermore, it offers a means to assess the infiltration capabilities of PBMCs within a three-dimensional matrix, particularly their capacity to navigate toward and infiltrate organoids. By mimicking the immune cell infiltration process within the tumor microenvironment, this system provides valuable insights into how immune cells navigate the extracellular matrix (ECM) and interact with tumor cells. It also serves as a robust platform for examining the migration and functionality of therapeutic immune cells in vivo [14]. (iii) PBMCs and organoids are directly co-cultured in the presence of T-cell medium, which supports the generation of tumor-reactive T-cells (Fig. 1). The direct co-culture model, devoid of Matrigel, facilitates direct interaction between PBMCs and organoids in T-cell medium, eliminating physical barriers and rapidly inducing immune responses. Under these conditions, T cells can directly recognize and attack tumor cells, triggering cytotoxic mechanisms. This model is particularly adept at studying T-cell activation, including changes in surface markers, cytokine secretion, and cytotoxic responses against tumor cells. The absence of Matrigel allows immune cells to more efficiently identify and target tumor cells within the organoids, leading to a faster generation of tumor-reactive T cells. This setup offers a streamlined and effective platform for investigating the expansion of tumor-specific T cells, T-cell-mediated tumor cell apoptosis, and the direct interactions between immune cells and tumor cells, making it especially useful for rapidly assessing T-cell function and efficacy [15, 16].

Regardless of the methodology, co-culturing organoids with PBMCs allows researcher to explore tumor immunology and novel immunotherapy targets [17, 18]. This model sustains organoid structure and growth, revealing immune cell viability, their interactions with organoid and the quantifiable patient-specific cytotoxic effects (Table 1).

The primary advantages of organoid co-culture model

Currently, at least four strategies have been developed to study the interactions between organoids and

Table 1 Summary of Co culture system of Tumor Organoid with PBMC

Cell Type	Co-cultures	Autologous/ Allogeneic	Ratio	Time	Additional condition	Conditions of Cultivation	Modeling for	Reference
NSCLC/dMMR(CRC)	PBMC	Autologous	20:1/5:1	2Week/3Day	IL-2 IFNγ	T cell medium	Generation of tumor-Reactive T cells and organoid killing assays	16
PC	PBMC, CAFs	Autologous	1:1	72 H	Anti-human CD3/CD28 dynabeads	Matrigel domes	Development of 3D organotypic microenvironment models	28
PDAC	PBMC, CTL, DC, MDSC	Autologous	N	72 H	N	Matrigel	Test the efficacy of combinatorial therapies and targeted therapies, based on modulating the tumor microenvironment	29
PC	PBMC, NK	Autologous	1:1	7–14 Day	IL-2	75% factor-reduced Matrigel	Understand how PDAC tumor cells might act to directly influence NK phenotype	38
PC	PBMC	Autologous	1:1/1:10	7Day/72H	N	Human T cell medium	Identify and expand tumor-targeting cytotoxic T cells and Organoid killing assays	15
PDAC	PBMC	Autologous (Mmouse)	1:20	10Day	anti-CD28	T cell medium	establish a potential individualized ex vivo model system to support T cell-based therapies.	40
GC	PBMC, CTL, MDSCs	Autologous	N	48 H	N	Matrigel	Identify the mechanisms by which HER2 regulates the expression of PD-L1 in gastric cancer	30
CCA	PBMC/T	Allogeneic	1:20	7Day	Anti-human CD3/CD28 dynabeads and IL-2	10%BME	Modelling immune cytotoxicity for cholangiocarcinoma	14

Abbreviations: CRC, Colorectal cancer; dMMR, Deficient Mismatch Repair; PC, Pancreatic cancer; PDAC, Pancreatic Ductal Adenocarcinoma; GC, Gastric Cancer; CCA, Cholangiocarcinoma

immune cells (Table 2). In the air-liquid interface (ALI) model, tumor biopsies are directly cultured at the air-liquid interface to form cell suspensions containing more types of cells derived from TME (including endogenous immune cells and other non-epithelial cell types) [19]. The microfluidic 3D cultures utilize microfluidic technology to construct 3D cell culture models on a chip, precisely controlling the flow and composition of fluids through microchannels to simulate the microenvironment for cell growth [20]. Organoid chips build upon this by integrating specific cell types to achieve multicellular co-cultures [21]. 3D bioprinting fabricates complex 3D tissue structures via layer-by-layer printing of cell-containing "bio-inks" [22]. Although these methods each have their advantages in simulating the human physiological environment, they also display limitations such as operational complexity and high costs. In contrast, PDO and PBMC co-culture systems based on matrices or suspensions offer higher operability and repeatability, serving as effective tools for studying the interactions between immune cells and tumors.

In terms of functionality, PDO models preserve the histology, gene mutations, and complex epithelial lineages of the tumor tissue. Therefore, Cytotoxic T Lymphocytes (CTL) produced by co-culturing patient-derived autologous lymphocytes with self-organoids are considered tumor-related host reactions [23]. This co-culture system can effectively generate patient-specific $CD8^+$ T- and $CD4^+$ T-cells, and induce IFN-γ secretion, and cause the immune cells from different patients to exhibit varying degrees of tumor-killing effects [15, 16, 24]. These findings demonstrate the potential of this system to reveal individual differences in immune function, providing new insights into the interaction between immune and tumor cells, expanding the application prospects of personalized immunotherapy.

Co-culturing tumor organoids with effector T-cells

Effector T-cells can be produced by a co-culture system of organoid and PBMCs. Compared to PBMCs, effector T cells have a better ability to recognize and kill tumor cells. The clinical value of organoid and effector T-cells

Table 2 Construction strategies of tumor organoid culture systems modeling the tumor immune microenvironment

Method	Definition	Advantages	Disadvantages
Submerged Matrigel culture	Suspend cells in collagen-rich Matrigel matrix, then culture beneath the medium.	Easy to enrich and expand tumor organoids; easy to operate.	Lacks native immune and stromal components; requires additional supplementation.
Microfluidic 3D culture	Uses microfluidics on chips to build 3D cell models, precisely controlling fluid flow and composition through microchannels to simulate the microenvironment for cell growth	Allows simulation of blood flow; studies immune cell migration; suitable for high-throughput screening.	Requires additional immune cells, subject to size limitations; needs specialized equipment.
Organ-on-a-chip	An advanced application of microfluidic 3D culture, integrating specific cell types on a microchip to simulate key functions and microenvironments of human organs	Multi-cell type interactions more closely mimic in vivo conditions; suitable for studying long-term immune responses.	Requires additional immune cells, subject to size limitations; needs specialized equipment.
ALI culture	Place tissue or organoids on a fixed scaffold, with one side exposed to air and the other side in contact with nutrient-rich culture medium.	Preserves diverse immune and fibroblast cells; suitable for studying air-exposure-related immune responses; simulate pathogen invasion and immune cell reactions.	Creates uniformly sized organoids; limited to native tumor-infiltrating immune cells; not suitable for all cell and tissue types; immune cells and fibroblasts in organoids decline over 1–2-month period.
3D Bioprinting	Uses a special printer to layer-by-layer print "bio-ink" containing cells, constructing complex three-dimensional tissue and organ structures.	Precisely controls cell placement and structure, allowing creation of models with multiple types of immune cells, customizing simulation of specific immune microenvironments, fully automated high-throughput.	Significant technical challenges; high cost and time investment.

co-culture model increase in the immune-oncology field considering the inherent diversity of human leukocyte antigen (HLA) and T-cell receptor (TCR) genes [25] (Fig. 1). For example, co-culturing tumor organoids and autologous PBMCs can enrich tumor-reactive T-cells with mismatch repair-deficient (dMMR) colorectal and non-small-cell lung cancer (NSCLC) cells [16]. These effector T-cells can be used to assess the efficiency of killing autologous tumor organoids. More importantly, these effector T cells and TILs have more than 50% overlap with TCR gene [16], indicating that this co-culture system exhibits a phenotype similar to the tumor immune interaction in vivo [12, 13].

TILs therapy is an adoptive cell therapy that involves isolating TILs from a patient's own tumor tissue, expanding and activating them in vitro, and then reinfusing them back into the patient to directly treat tumors [23, 26]. However, there are certain limitations associated with TILs therapy, such as insufficient quantity or activity of TILs, and individual differences, unpredictability and differences in treatment response. Therefore, screening for tumor-reactive T-cells with good tumor-killing activity through co-culture may provide a feasible alternative or predictive method for TILs therapy (Fig. 1).

In conclusion, the organoid and PBMC co-culture model can produce tumor-responsive T cells in *vitro*, which can be used to screen targets for adoptive cell therapy and enrich the source of adoptive cells, which is conducive to the development of adoptive cell therapy [17].

Co-culturing tumor organoids with multiple components and PBMCs

Multicomponent co-culture models, which progressively add other non-malignant cells to the base of tumor organoids and PBMCs, have become an important direction by closely approximating the TME [27–29] (Fig. 1). For example, cancer-associated fibroblasts (CAFs) are a major component of the tumor stroma. By constructing a multi-components co-culture model that includes pancreatic cancer organoids, CAFs, and PBMCs to facilitate the study of immune cell infiltration in vitro. It was found that the degree of immune cell infiltration in the co-culture environment is related to both tumor cells and CAFs [28]. This indicates that, even in an in vitro setting, stromal and tumor cells can engage in effective interactions. This finding underscores the capability of the co-culture model to simulate the dynamic interactions between tumor stroma and tumor cells, highlighting its utility in exploring immune responses within a controlled environment. Another study found that co-culturing pancreatic cancer organoids with CTL with immunocheckpoint inhibitors yielded comparable results [29]. Then, in order to verify whether immunosuppressive cells can exert their immunosuppressive effects in an in *vitro* model, myeloid-derived suppressor cells (MDSCs) were added to construct a multi-component co-culture system. Using this co-culture system, researchers found that MDSCs effectively inhibit the proliferation and function of PBMCs; however, when a MDSC inhibitor exists, the inhibition effects is relieved.

Establishing a multi-component PBMC co-culture model is important for studying the function of non-malignant cells and replicating the TME [18, 30]. This multi-component co-culture model not only proves that immunosuppressed cells can survive and function in *vitro*, but also demonstrates its feasibility and effectiveness. In the future, the development of multi-component co-culture models will more realistically reflect the immune microenvironment and reveal the roles and mechanisms of non- malignant cells in the immune process.

Factors affecting the co-culture system

Sources and structures of tumor organoids

Long-term organoid culture models have been established from primary cancer biopsy samples [26, 27, 31]. These organoid models exhibit different success rates [32–51] (Supplemental Table 1). Tumors with high malignancy have a higher success rate, whereas those with low malignancy display a lower success rate [31, 34, 52]. Additionally, different pathological classifications are essential influencing factors. For example, the efficiency of establishing continuously propagated organoid lines from pancreatic neuroendocrine neoplasm samples was only 10%; however, that of pancreatic ductal adenocarcinoma (PDAC) samples reached 80% [31]. This affects the efficiency of constructing organoid models and limits the application scenarios of the co-culture models. Therefore, more comprehensive cultivation conditions should be explored to improve the success rate of different types of organoids, making co-culture models more widely applicable to various cancers.

Moreover, different types of organoid display different 3D structures and are primarily divided into solid, vacuolar, and mixed [31]. Compared with solid and mixed structures, vacuolar structures need a shorter digestion time, display a larger diameter, and are more susceptible to infiltration by immune cells. To better evaluate the therapeutic effect and reduce the impact of spatial structure differences, more evaluation indicators need to be considered, such as organoid diameter, the structural damage degree, apoptotic cell count, and cytokine secretion levels. Finally, due to the 3D structure, cell counting can be extremely challenging. Therefore, it is necessary to digest organoids into single cells to obtain accurate cell counts prior to co-culture.

Sources and quality of PBMCs

The PBMC sources can be divided into autologous and allogeneic sources. Several studies have demonstrated that autologous PBMCs can effectively simulate the in vitro immune response of individuals in co-cultures of pancreatic cancer and colorectal cancer [16]. However, autologous PBMC amplification and acquisition are challenging, especially in patients undergoing radiotherapy and chemotherapy. A potential solution is to obtain T-cells from different healthy individuals and test their responses to immune drugs to determine the immunogenicity of individuals.

Allogeneic PBMCs are easily obtained and can be conveniently expanded to sufficient numbers to study the sensitivity of tumor cells to immunotherapy at different time points [6]. However, the mismatch of HLA between immune and tumor cells challenges the quality of immunotherapy evaluation. HLA mismatch can lead to the inhibition of CD8+cell activation, delays the release of tumor killer factors, and limit T-cell recognition and killing function against tumor cells, resulting in a limited immune response [53, 54].

Some scholars currently believe that using next-generation sequencing technology to match allogeneic PBMCs with tumors may be a solution [55]. Research has found that tumor organoids effectively retain most of the HLA alleles and maintain patient-specific HLA neo-antigen features. By comparing the alleles of HLA-A/- B/- C alleles on organoids with PBMCs in healthy individuals, they found that half of the allele matches were significantly attacked by T-cells [55], confirming that tumor organoids serve as killing targets and providing insights into the effectiveness and feasibility of immunotherapy.

Meng Q et al. [15] found that incubation of engineered T-cells with autologous pancreatic cancer organoids induces IFN-γ secretion within 24 h and increases by 48 h. However, incubation with allogenic organoids does not induce detectable IFN-γ secretion during the first 24 h, but IFN-γ secretion can be detected by 48 h. The delayed response to allogenic organoids was likely due to an HLA-mismatch-mediated response. This indicates that in the co-culture system, allogeneic immune cells can still recognize tumor cells and exert killing effects through other pathways. Overall, in the co-culture system of organoids and allogeneic PBMCs, the matching of MHC-1 is an important challenge that cannot be ignored and significantly affects the quality of experimental evaluation.

In addition, the quality evaluation of PBMCs also includes the absolute number and proportion of effector cells [14, 15]. $CD4^+$ T-cells kill tumor cells through a FasL-mediated mechanism, whereas $CD8^+$ T-cells kill tumor cells through the direct and indirect secretion of IFN-γ and granzyme B [56](Fig. 1). Therefore, the proportion and number of effector cells in PBMCs, particularly $CD4^+$ and $CD8^+$ T-cells, are the key factors affecting the tumor killing effect.

Proliferation and activity of PBMCs

The number of effector T-cells may be insufficient under natural conditions, leading to an insufficient killing effect

on tumors [14]. Therefore, it is necessary to stimulate the immune cells to expand or express specific tumor antigens in vitro to enhance the cytotoxic effect of the immune cells. Three methods are applied to activate PBMCs (Fig. 1): (i) Activation of T-cells by exogenous compound or cytokines. For example, phorbol myristate acetate (PMA), ionomycin, and IL-2 can effectively stimulate T-cell proliferation, activation, and increase the living cell counts of $CD4^+$, $CD8^+$, and $CD56^+$ natural killer cells in a co-culture or PBMC culture alone [14, 57, 58]; (ii) Simulated antigen presentation. Antibodies and synthetic small-molecule agonists are usually used, which simulate the interaction between antigen presenting cells and T-cells [14]. For example, by binding anti-CD3 and anti-CD28 antibodies with immunomagnetic beads, the magnetic beads can provide primary and co-stimulatory signals for T-cell activation and expansion [14]. CD3 is composed of multiple subunits that bind to T-cell receptors (TCRs) and transmit signals to activate T-cells [59]. Also, CD28 is a co-stimulatory molecule that binds to B7 molecules on antigen presenting cells, providing a second signal to activate T-cells. The activation of CD28 can promote the proliferation, survival, and functional enhancement of T cells, while also contributing to the formation of immune memory. (iii) Co-culture to generate specific T-cells. When co-culturing autologous PBMCs with tumor organoids, PBMCs interact with tumor cells, changing the immune cell number and type in PBMCs, even TCR genes, thus producing effector T-cells with patient tumor specificity [15, 16]. These types of T-cells express patient-specific antigens and are relatively easy to generate in sufficient quantities.

The effector (E) to target (T) cell ratio

The effector (PBMC)-to-target (Tumor organoids) cells ratio is also an unavoidable issue, related to co-culture time, conditions, cell types, and experimental purposes. Generally, the number of PBMCs needed is calculated with the formula: PBMCs needed = (V × 22,000) * n, where V denotes the volume of the dome and n represents the sample size [13]. However, studies employ different ratios depending on their specific objectives. For evaluating cell toxicity, a ratio of at least 1:1 is recommended to maximize the observation of the killing effect, thereby enabling swift and precise assessments of PBMC toxicity. For research focusing on cell interactions or signaling pathways, a ratio lower than 1:1 is preferable, as it more accurately mimics natural cellular interactions, thus revealing the subtleties of cell communication. For instance, in a study where pancreatic cancer organoids were co-cultured with autologous PBMCs to produce tumor-reactive T cells, a 1:1 effector-to-target ratio was used for co-culturing in T-cell medium for two weeks [15]. Subsequently, tumor-reactive T-cells and organoids were co-cultured at a 1:1 ratio in Matrigel for 20 h to assess the T-cells' killing effect, and a 1:10 ratio was used for 72-hour co-cultures to evaluate cytokine release levels into the supernatant [15]. In another experiment, co-culturing gallbladder cancer with allogeneic PBMCs, the authors employed an effector-to-target ratio of 20–30:1 to amplify the attack on target cells [14].

In experiments where effector cells, such as CD4+and CD8+T cells, are isolated from PBMCs, their enhanced infiltration capability allows for a reduced ratio in killing experiments. In an analysis of the tumor-extrinsic toxicity of T cells in donor-matched intestinal cancer organoids, a 1:2.5 effector-to-target ratio was utilized, focusing solely on the primary effectors (CD4+and CD8+T cells) [13]. These ratio adjustments demonstrate the flexibility in experimental design, aiming to replicate specific biological scenarios according to research goals, thereby improving the accuracy of the outcomes.

Matrigel concentration

The extracellular matrix (ECM), the main components of Matrigel, primarily derived from non-human sources, contains tumor-related factors that can modulate the immune system and reduce anti-tumor responses, potentially causing unwanted immune reactions [60]. The ECM's physical properties, such as its stiffness and pore size, can affect immune cell migration, direction, and invasiveness. A dense ECM can nearly block immune cell penetration into tumor organoids [16]. In certain co-culture setups, organoids are separated from the ECM using enzymes and then cultured with immune cells in suspension or on a Matrigel-free surface [61]. However, without ECM, organoids lose their 3D structure and wall-adherent growth, which can limit proliferation and alter cellular behavior [16]. Thus, fine-tuning the ECM concentration is essential for optimal organoid culture conditions.

A study delving into the co-culture of cholangiocarcinoma (CCA) organoids with PBMCs examined the efficacy of diverse Matrigel concentrations [14]. The investigators discovered that a 10% Matrigel suspension adeptly maintains the organoids' 3D structure, offering a stable scaffold without altering their shape or size. In a subsequent study validating the cytotoxic effects of CAR-T cells on bladder and kidney cancer organoids, the organoids were typically embedded in a 50% matrigel, followed by the addition of X-VIVO 15 medium containing CAR-T cells around the Matrigel dome for co-culture. This concentration of Matrigel effectively replicated the interaction between immune cells and tumor cells, establishing a reliable experimental framework for the further assessment of CAR-T cells' therapeutic potential [62, 63]. In another experiment, researchers directly co-cultured pancreatic cancer organoids with PBMCs

in T cell medium to stimulate tumor-reactive T cells. The main advantage is simplification of the experimental, which avoids non-specific immune responses from Matrigel or other 3D scaffolds and enhances immune cells' direct exposure to tumor antigens, leading to more efficient tumor-reactive T cell generation. This approach is especially advantageous for high-throughput screening or rapid production of tumor-reactive T cells. It's important to note that the type of tumor cells influences the suitable Matrigel concentration. For instance, gastric cancer organoids can break down Matrigel, requiring an increased concentration or more frequent medium changes [64]. In summary, the Matrigel concentration should be adjusted flexibly according to the specific requirements of each experiment.

Due to the limitations inherent in matrigel for experimental use, such as high cost, unclear origin, ethical concerns, and batch-to-batch variability, there is a growing trend towards exploring alternative matrices, such as GelMA and other hydrogels, which offer distinct advantages and are gaining traction in research settings [65]. Synthetic hydrogels, produced in various ways, are expected to replace natural Matrigel due to their higher purity and controllability. This enhances standardization and quantification, reducing non-immune-specific interference in co-culture experiments. In a prior study, intestinal organoids derived from patients were co-cultured with PBMCs and embedded in a 100% 3D hydrogel—a blend of collagen I and matrigel—for culture. These constructs were then utilized for the histological basis of multiplex immunofluorescence imaging [13]. Such a robust ECM effectively reproduces the mechanical properties of intestinal tissue and replicates key in vitro immune processes, including bystander signaling, immune cell migration, and immune cell infiltration.

Therefore, researchers are encouraged to perform gradient experiments to identify optimal concentration or explore Matrigel substitutes. The aim is to keep organoids suspended in liquid, avoiding wall adhesion, so that a thin film allows immune cells to penetrate and interact with tumor organoids.

Medium components

The exact medium components are customized for specific tumor tissues [66, 67]. To better maintain the similarity between the organoid and original tumor phenotype, more cytokines are added into the organoid culture media to ensure that tumor cells maintain a 3D structure in vitro and express the characteristics of the original tumor cells [8]. For example, WNT3A and R-spondin activate the Wnt signaling pathway, whereas Noggin and A-8301 are involved in tumor growth factor-beta inhibition. Epidermal growth factor (EGF) participates in activating the EGF signaling pathway. Forskolin is an adenylate activator, and XMU-MP-1 is a protein kinase MST1/2-inhibitor that regulates the Hippo pathway [31]. These medium components are essential for tumor organoid growth.

These cytokines can ensure better survival and proliferation of organoids in vitro; however, they can also affect PBMCs. Among them, forskolin and niacinamide inhibit T-cells, whereas IL-2 can promote T-cell proliferation [14]. Adding exogenous growth factors and small molecules during organoid growth may lead to unnecessary cloning selection, and medium components can interact with the tested drugs, resulting in an ambiguous conclusion. Therefore, a culture medium with fewer growth factors should be used to minimize clonal screening and avoid inaccurate drug treatment results. Furthermore, some co-culture systems only select a T-cells medium [14–16]. This method can produce tumor-reactive T-cells with organoid specificity; however, this condition cannot support the survival and proliferation of tumor cells. Therefore, further exploration is needed to determine more suitable culture medium components for co-culture to support tumor and immune cell survival and function.

Application of the co-culture systems

Exploring the immune interaction and immune escape

Immune cells usually can recognize and kill tumor cells, inhibit the development of tumor-initiating cells, and participate in immune surveillance to eliminate abnormal cells, preventing tumor formation. In the tumor microenvironment, immune cells also regulate tumor growth and metastasis by releasing inflammatory factors and cytokines. However, tumor cells can evade the immune system's attacks, such as by weakening antigen presentation capabilities to promote growth. Tumor-initiating cells typically "hijack" the signal pathways that regulate adult stem cell function through the acquisition of mutations that overcome normal cell cycle control mechanisms [68–70]. This involves the interactions between multiple immune cells and tumor cells, where the recognition and killing capabilities of immune cells and the escape mechanisms of tumor cells can impact the formation of tumor-initiating cells, forming a complex network of immune responses. Thus, it is very important to understand the interaction mechanism between immune cells and tumor cells using organoid with PBMC co-culture model (Table 3). Tumor-targeting cytotoxic T-cells from patients with pancreatic cancer have been identified and produced by co-culturing PBMCs with autologous tumor-derived organoids. These T-cells expressed tissue-resident memory T-cells (TRMs) or $CD103^+$ TRMs [18]. The interaction between $CD103^+$ TRMs and E-cadherin expressed in tumor epithelial cells activates T-cells in human solid tumors. Therefore, in the co-culture model, the appearance of the TRM phenotype suggests that

Table 3 Application of organoid and PBMC co-culture model

Application Fields	Challenges	Future Directions	Reference
Exploring immune interaction and immune escape	Lack of blood vessels, suitable 3D scaffolds, and other components hinder accurate immune microenvironment simulation; Growth factors in Matrigel may activate immune cells, causing nonspecific immune responses.	Multi-component co-culture to simulate the tumor microenvironment; Optimize Matrixgel composition and concentration, and introduce new technologies (e.g., microfluidics, dynamic culture systems, or 3D printing) as alternatives to Matrixgel.	15,65–68,76
Evaluating immunotherapy (e.g., ICIs, CAR-T therapy)	Individual differences in immune therapy response; Difficulty in long-term assessment of immune response.	Develop personalized immunotherapy; Combine humanized immune models for multi-angle in vitro and in vivo analysis.	30 ,79
Developing immunotherapy targets and adoptive cell therapies	Technical bottlenecks in high-throughput screening; Tumor organoid models and standardization issues.	Screen immunomodulatory drugs or targeted molecules as alternatives to adoptive cell therapy; Standardize outcome evaluation.	15

PBMCs were activated in vitro and produced a phenotype similar to that of a solid tumor.

Both cancer cells and tumor-infiltrating immune cells depend on glucose, and impaired immune cell metabolism contributes to cancer cells evading immune surveillance. Research utilizing this co-culture model to explore the intracellular program regulation differences in nutrient acquisition among different immune cell subpopulations and tumor cells will be important [71]. This selective nutrient distribution mechanism provides new perspectives for cancer treatment strategy development and can also be employed to track tumor progression. Incorporating co-culture models to deeply explore immune-tumor cell interactions.

Immune escape is the most critical processes in tumor development, inducing and recruiting immunosuppressive cells (such as T-regulatory cells, bone marrow-derived suppressor cells, and tumor-associated macrophages), and increasing the expression of various immunosuppressive molecules (such as PD-1 and PD-L1) [72]. Co-culturing organoids with PBMCs can provide a foundation for in-depth research on immune escape (Fig. 2). Researchers previously developed a mouse pancreatic cancer organoid/CTL/MDSC co-culture model to explore whether MDSCs can inhibit the effect of immune checkpoint inhibitors on the killing effect of PDAC tumors [73]. First, pancreatic cancer organoids were co-cultured with monocytes, dendritic cells, and CTLs, and subsequently treated with an anti-PD-1 inhibitor. The results revealed a significant organoid-killing effect; however, this killing effect was inhibited when MDSCs were added. Adding tyrosine kinase inhibitors, such as cabozantinib and sunitinib (MDSC inhibitors), successfully blocked the immunosuppressive function of MDSCs on CTL proliferation, which restored the immuno-killing effect in the co-culture system. Furthermore, autologous PBMCs were co-cultured with tumor organoids, and PD-1, PD-L1, TIM3, TIGIT, LAG3, and natural killer group 2 member A (NKG2A) receptors were blocked using inhibitory antibodies or soluble proteins to clarify the function of checkpoint protein expression [15]. Blocking NKG2A significantly increased IFN-γ production compared with blocking other ligands, implying that NKG2A regulates cytotoxic T-cell activity. These results demonstrate that a co-culture platform can be applied to identify checkpoint inhibition strategies to block immune escape and improve immunotherapy effectiveness.

Therefore, an immunosuppressed microenvironment was reproduced successfully in an organoid-PBMC co-culture model. This model can be used to reveal the immune escape process and the relative mechanism in vitro.

Evaluation of immunotherapy

More immune checkpoint inhibitors, such as CTLA-4 and PD-1/PD-L1, have been shown to exhibit clinical benefits in patients with advanced cancers, including melanoma, NSCLC, and dMMR colorectal cancer (CRC) [73, 74]. Despite these encouraging results, many patients do not respond to the immunotherapies that are currently available. Low immunogenic antigens, defective antigen presentation, and the expression of alternative immune checkpoint molecules may explain treatment failure [75]. The accuracy of drug evaluation in vitro, especially immunotherapy drug evaluation, depends on optimizing cell culture models (Table 3). The high failure rate of preclinical compounds in clinical trials demonstrates the limitations of cell line models. Therefore, co-culturing PBMCs with tumor organoids may be a valuable tool for the in vitro evaluation of immunotherapy.

Autologous gastric cancer organoids and immune cell co-cultures are an appropriate model in vitro to study the effects of anti-HER2-targeted therapy combined with anti-PD1 [29].

Rastuzumab inhibits HER2 dimerization with other isoforms and promotes endocytosis, triggering cell-mediated immunity. The combination of Rastuzumab and anti-PD-1 therapies has shown synergistic antitumor effects. Parallel cultures of tumor organoids and T-cells, with or without drugs, could create a simple system for

discovering new therapeutic targets and drugs, as well as potential candidates for immunotherapy. In another study, combining the Hedgehog inhibitor GANT61 with chemotherapy drugs led to decreased cancer cell proliferation and increased cell death in PDOs co-cultured with PBMCs [76]. Most importantly, this result is consistent with that in mice, strongly suggesting the effectiveness of this co-culture model.

More and more studies have demonstrated that the organoids-PBMC co-culture model can be widely used to evaluate the sensitivity of immune-targeting drug. By further comparing the results of the co-culture model and the immune humanized model, the effectiveness of this model in precision therapy is further improved.

Developing immunotherapy targets and adoptive cell therapies

In addition to analyzing the interaction between tumor and immune cells, small molecule compounds and antibodies are increasingly being used to enhance the sensitivity of tumor cells to T-cell interactions (Fig. 2). PBMCs-pancreatic cancer organoids co-culturing was used for high-throughput immunotherapy evaluation, and the expression levels of 17 immune checkpoint receptors was monitored [15]. Relative inhibitory antibodies were subsequently used to block immune checkpoint receptors. Blocking NKG2A induced the highest level of IFN-γ production, indicating that targeting NKG2A represents an effective strategy for PDAC treatment. These experiments demonstrated the ability of the co-culture platform for high-throughput, which may identify checkpoint inhibition strategies for effective immunotherapy.

Adoptive cell therapy has become an important field in immunotherapy. Co-culturing autologous PBMCs with PDAC organoids offers an unbiased approach to isolate tumor-reactive T-cells and assess individual patients' tumor cells' sensitivity to T-cell-mediated attacks (Fig. 2). Meng et al. [15] compared the TCR genes identified in tumor-reactive T-cells with TILs to understand the differences between TCR genes selected in T-cells derived from a co-culture platform and those naturally enriched in tumor tissue. A 50% overlap among the top 10 TCRs was observed between tumor-reactive T-cells and TILs, indicating that the interaction between autologous PBMCs and organoids in the co-culture system is similar to that in vivo. Thereafter, the α and β chain sequences of the top five TCRs in tumor-reactive T-cells were determined to generate a chimeric TCR gene. $CD8^+$ T-cells, in the PBMCs derived from a healthy donor, were engineered to express this chimeric TCR gene. Incubating these engineered T-cells with autologous organoids induced IFN-γ secretion within 24 h, increasing significantly by 48 h. These observations demonstrated that the ability of the TCR genes identified in organoid-reactive T-cells was sufficient to confer the tumor-targeting ability to engineer human T-cells. Patient-specific T-cells can be generated from a co-culture model of organoids and PBMCs, which provides novel cell sources and targets for TCR-T therapy [15].

These results demonstrate that the use of this co-culture platform enables high-throughput screening of target and the development of effective immunotherapy drugs. The characterization of immune cells within the co-culture system can also contribute to the development of adoptive cell therapies.

Prospects of the co-culture system

Culturing tumor organoids with PBMCs can replicate cancer-immunity cycles, encompassing the priming or activation of effector T-cells, their migration or infiltration into tumor tissues, and their recognition or killing of cancer cells [30, 77]. This model has successfully simulated the interaction between tumors and immune cells to a certain extent, better representing the heterogeneity of patients' tumors. Furthermore, the model has successfully been used to reveal the immune escape mechanism, evaluate immunotherapy effectiveness in vitro, and develop immune-related drugs and adoptive cell therapies. In the future, efforts should focus on developing multi-component co-culturing systems, a more standardized outcome evaluation with culture conditions that simulate the interactions among various microenvironment components in vivo and ensure the repeatability and credibility of the experimental results. Moreover, combining co-culture and immune humanization models facilitate multiple perspectives analysis in vitro and in vivo into reality.

Nevertheless, many challenges still exist in the co-culture modeling process, limiting its wide application. The ability of immune cells to infiltrate remains a critical challenge, particularly in the context of tumor immune responses. In vivo, the ECM and other physical barriers within the TME are pivotal to the infiltration and migration of immune cells. These cells must navigate through these structures to reach the tumor core effectively and exert their effector functions [3]. However, in vitro models, especially those utilizing Matrigel or similar 3D co-culture systems, often fail to accurately mimic this complex infiltration process. Consequently, further experimental exploration is needed to determine the optimal types and concentrations of Matrigel, while also considering the integration of microfluidic technology, dynamic culture systems, or 3D printing techniques. Such approaches would help maintain the structural integrity of the co-culture model and ensure efficient immune cell infiltration into tumor cell clusters, enabling more precise assessments of tumor immune evasion

mechanisms and the efficacy of immunotherapies. Matrigel, derived from mouse tumor cells, contains various proteins and growth factors that may activate immune cells, leading to non-specific immune responses to its components [16]. This potential for non-specific immune activation by Matrigel is a significant concern in co-culture models. To address this, using highly purified matrix materials, synthetic hydrogels, or polymer scaffolds could offer effective alternatives. These strategies can reduce the presence of unnecessary growth factors and proteins in the matrix, thereby minimizing non-specific immune cell activation. Furthermore, conducting rigorous control experiments can assist in distinguishing the true immune response to tumor cells from the non-specific reactions induced by Matrigel.

Moreover, compared with the immune infiltrating cells in tumor tissues, the T-cell numbers and types, as well as the TCR genes expressed in PBMCs, are quite different [15]. Multi-component co-culture systems may act as a solution because they provide an optical immune environment for different T-cell [27]. Moreover, the absence of a functional vascular system leads to inadequate nutrient and oxygen supply, resulting in the formation of a necrotic core, particularly in the centers of larger organoids [78]. Microfluidic devices could be used as an alternative by placing tumor cells in a specific area on the chip and delivering nutrients, oxygen, and drugs to the cells through liquid flow to simulate the interaction and biological reaction between organs [79]. In addition, during in vivo development, blood vessels provide essential signaling cues to guide and instruct cell migration and differentiation [80, 81]. Various methods have been implemented to enhance oxygen delivery to organoids, aiming to overcome limitations and decrease oxidative stress. Introducing vascular networks is expected to boost oxygen and nutrient supply to the organoid core. Additionally, a variety of criteria, such as bright field and immunofluorescence imaging, and ELISA for cytokine release, are used to evaluate co-culture models, assessing immune cell activity and infiltration. Considering the different experimental objectives, it's essential to standardize the criteria and evaluation indicators for co- culture modeling to ensure the universal applicability and repeatability of experimental outcomes.

Conclusion

In summary, cancer treatment methods should be diversified and personalized due to the heterogeneity and genetic instability of cancer cells. Organoids retain the histological characteristics, cellular diversity, genetic heterogeneity, and mutation diversity of the originating tumor tissue. Since organoid-PMBC co-culture model better simulate complex TME, provides insights into interactions between immune and tumor cells. Thus, this co-cultivating system may be an effective tool for developing cancer immunotherapy strategies and improving personalized medicine.

Author contributions

SC, LP, ZY and HM were responsible for the literature search and writing, reviewing, and editing the manuscript. LP and MY contributed to the creation of the graphical illustrations. All authors reviewed the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics and Consent to participate

Not applicable.

Informed consent

Not applicable.

Competing interests

The authors declare no competing interests.

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