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Review Article

In vitro co-culture models for studying organoids-macrophages interaction: the golden technology of cancer immunotherapy

Jinming Liu[1*], Biao Zhang[1*], Yuying Cui[2], Huiyi Song[2], Dong Shang[1,3]

[1]Department of General Surgery, Clinical Laboratory of Integrative Medicine, The First Affiliated Hospital of Dalian Medical University, Dalian, Liaoning, PR China; [2]Laboratory of Integrative Medicine, The First Affiliated Hospital of Dalian Medical University, Dalian, Liaoning, PR China; [3]Institute (College) of Integrative Medicine, Dalian Medical University, Dalian, Liaoning, PR China. [*]Equal contributors.

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Abstract: Macrophages, as the largest immune cell group in tumour tissues, play a crucial role in influencing various malignant behaviours of tumour cells and tumour immune evasion. As the research on macrophages and cancer immunotherapy develops, the importance of appropriate research models becomes increasingly evident. The development of organoids has bridged the gap between traditional two-dimensional (2D) cultures and animal experiments. Recent studies have demonstrated that organoids exhibit similar physiological characteristics to the source tissue and closely resemble the in vivo genome and molecular markers of the source tissue or organ. However, organoids still lack an immune component. Developing a co-culture model of organoids and macrophages is crucial for studying the interaction and mechanisms between tumour cells and macrophages. This paper presents an overview of the establishment of co-culture models, the current research status of organoid macrophage interactions, and the current status of immunotherapy. In addition, the application prospects and shortcomings of the model are explained. Ultimately, it is hoped that the co-culture model will offer a preclinical testing platform for maximising a precise cancer immunotherapy strategy.

Keywords: Organoids, macrophages, co-culture, interaction, immunotherapy

Introduction

During the past decade, three-dimensional (3D) organoid technology has sprung up and become more and more popular among researchers. The term "organoid" describes stem cells that grow in a defined 3D environment in vitro to form mini-clusters of cells that self-organise and differentiate into functional cell types, recapitulating the structure and function of an organ in vivo [1]. Organoid is a new model for research and therapy in the field of cancer, which will accelerate the individualised treatment of cancer [2]. Currently, researchers have successfully created a wide range of tumour organoids by enhancing various culture conditions, including those for colon [3], prostate [4], gastric [5], breast [6], pancreatic [7], endometrial/ovarian/cervical [8-11], urothelial [12], and renal [13] cancers.

These impressive 3D constructs offer a promising and near-physiological model for studying human cancers. They have the potential for diverse applications in cancer research and provide an intuitive, reliable, efficient, and ethically sound system for studying tumour biology and treatment-related research in vitro [14]. However, simple organoids often do not possess immune system components and are insufficient for simulating the in vivo microenvironment. It is, therefore, necessary to reconstruct the tissue microenvironment artificially. One strategy for overcoming the challenges of simulating the physiological environment in a two-dimensional (2D) culture and the inability to evaluate single components for in vivo experiments is reconstructing the tissue microenvironment [15, 16]. Due to the significance of reconstructing the microenvironment for investigating normal tissue function and disease pro-

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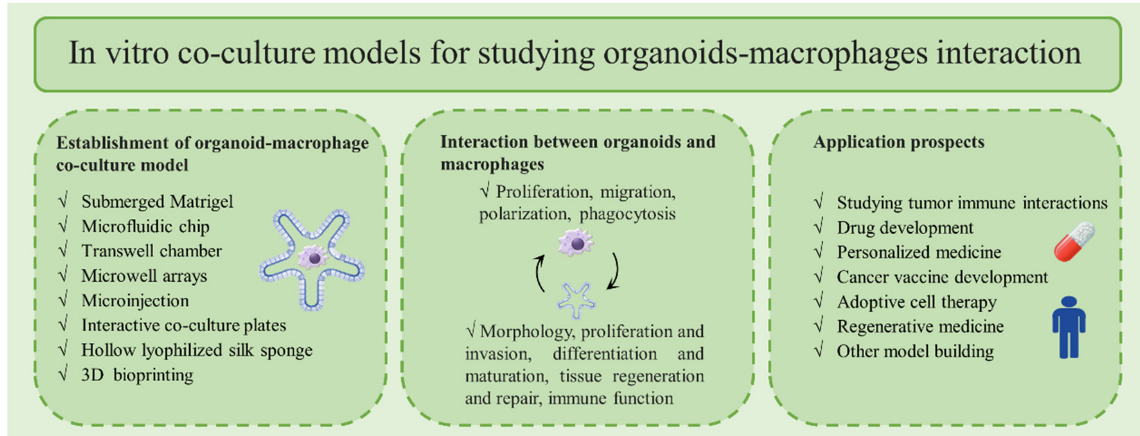

Figure 1. Graphical abstract. We first introduced the construction method of the co-culture models, then discussed the interaction between organoids and macrophages, and finally outlined the application prospects.

gression, there is a pressing need for a suitable co-culture system to establish physiologically relevant models [17]. As a result, more studies are being dedicated to co-culturing organoids and immune cells.

Within the vast and intricate immune system of the human body, macrophages serve as a critical type of immune cell that can be found in various tissues. They are the first line of defence in the human immune system, and can neutralise pathogens while alerting other immune cells to potential threats. Such a response enables the initiation and coordination of a comprehensive immune response [18]. Significantly, current immune checkpoint inhibitors target T cells but are still ineffective against immunosuppressive myeloid cells, which may account for some patients' subpar treatment responses. More recently, studies are considering whether to target other immune cells in immunotherapy. Recently, identifying and targeting phagocytic checkpoints of macrophages in cancer to subvert immunosuppression is emerging as a new generation of cancer immunotherapy [19, 20]. Additionally, compared with other immune cells, macrophages have the advantages of easy access and in vitro culture, leading to numerous significant advancements in the co-culture of tumour cells and macrophages [21-23]. Notably, macrophages can mediate tumour metastasis through blood and lymph. Compared with in vivo experiments, using in vitro co-culture models to study tumour invasion is more direct and convenient [24, 25]. Presently, there are three primary purposes for constructing an organoid-macrophage co-culture model. The first is to promote the formation of organoids through the interaction between histiocytes and macrophages. The second is using cancer organoids for cancer treatment. Finally, cytokines are secreted during co-culture to regulate the Matrigel of organoids [26].

This review comprehensively presents the methods of constructing the co-culture model of organoids and macrophages, mainly focusing on the interaction between organoids and macrophages. Essentially: (1) Organoids can regulate the proliferation, migration, polarisation, and phagocytosis of macrophages under co-culture conditions, and (2) Macrophages can regulate the morphology, proliferation, differentiation and maturation, tissue regeneration and repair functions, and immune functions of epithelial organoids. The co-culture model aims to simulate the intricate diversity and physical structure of the tissue microenvironment to the fullest extent possible. It can not only be used in studies of tissue interactions, but also has potential for applications in drug development, cancer immunotherapy, personalised treatment, and regenerative medicine (**Figure 1**).

Establishment of organoid-macrophage co-culture model

Several co-culture models of organoids and macrophages are presented in **Figure 2**, with a description of their benefits and drawbacks

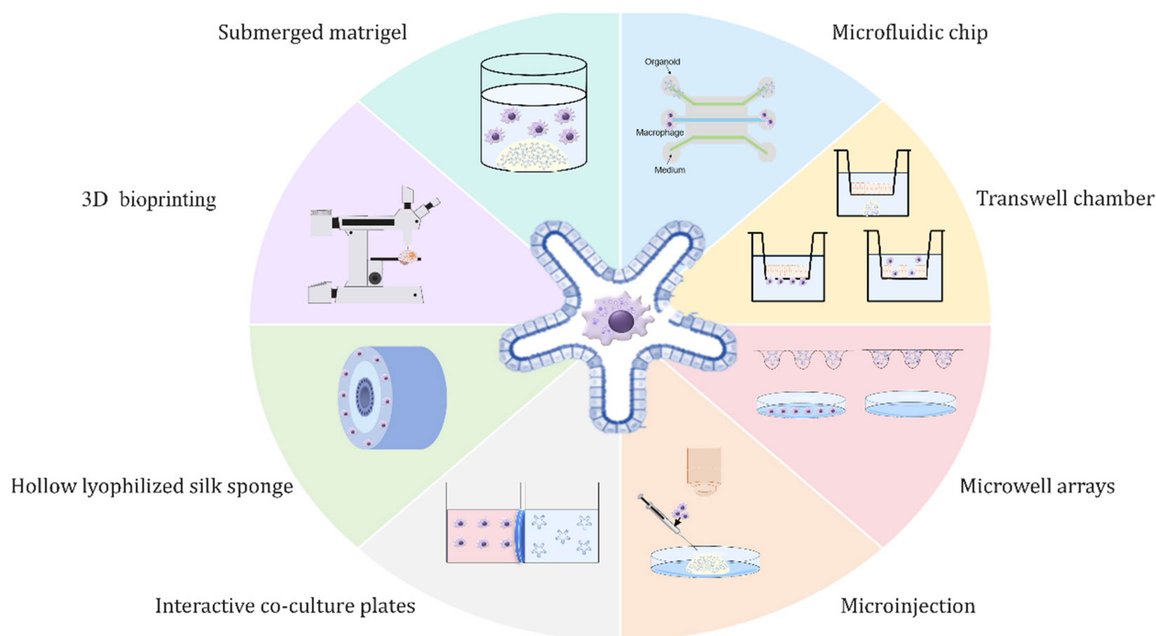

Figure 2. Eight models of macrophages and organoid co-culture. These include submerged Matrigel, microfluidic chip, transwell chamber, microwell arrays, microinjection, interactive co-culture plates, hollow lyophilised silk sponge, and 3D bioprinting.

presented in **Table 1**. These models not only better simulate the immune microenvironment in vivo, but can also be applied in various research studies under different conditions. Co-culture models suitable for high-throughput screening of particular diseases are anticipated to develop into potent instruments for precision immunotherapy.

Submerged matrigel culture

Submerged Matrigel is currently the most widely used and convenient technique for the co-culture of organoids and macrophages. Presently, the technology has been applied to the retinal, liver, lung, kidney, intestine, breast, and other tissue organoids [27-29]. The shape of Matrigel is primarily hemispherical, but when combined with the Transwell chamber, it can also be tiled on the chamber or orifice plate. It is commonly found in intestinal organoids and lung organoids [30]. This method can not only mix monocytes with tumour cells in Matrigel to form organoids but also add macrophages to the culture medium of organoids. For example, Xu et al. incubated and polarised macrophages in alginate cryogels, and then added breast cancer cells to construct organoids [31]. More often, macrophages or macrophage cell lines, either derived from mouse bone marrow or patient monocytes, are cultured in a normal cell culture medium, the organoids are immersed in Matrigel, and then the macrophages are inoculated into the organoid culture medium [32, 33]. In the study of co-culture of irradiated organoids from mammary glands and macrophages, the infiltration of macrophages in the organoids was discovered, paving the way for further research on organoid-macrophage interactions [33].

The advantages of this model are that the source is easy to obtain, the operation is relatively simple, and the expansion of cultivation is easily realised [34, 35]. In addition, this model has good versatility, allowing multiple types of organoids to be co-cultured with cells, and is a commonly used basic model [36]. However, compared with other models, organoids and cells cannot be easily separated, which is not convenient for observation and subsequent detection. Nor can high-throughput drug screening be achieved. In addition, compared with hollow lyophilised silk sponges, hemispherical organoids also have the risk of core necrosis. Macrophages cannot be passaged multiple times with organoids, so the culture time is shorter than that of microfluidic chips.

Table 1. Advantages, disadvantages, and applications of co-culture models

Co-culture models	Type	Advantages	Disadvantages	Comparisons	Application prospect
① Submerged matrigel culture	Retina, liver, lung, kidney, intestine, breast.	Easy to obtain; simple operation; easy amplification; good versatility.	It is not easy to separate and observe continuously; unable to conduct high-throughput drug screening.	① is the most commonly used model and has low technical requirements. Compared with ② ③ ④ ⑥ ⑦, it is not easy to separate. Compared with ② ④, high-throughput drug screening cannot be achieved, and macrophage culture time is shorter. Compared with ⑦, there is a risk of organoid core necrosis.	Study tumour-immune interaction, drug development, predict response to ICI treatment, regenerative medicine, and other model building.
② Microfluidic chip	Brain, heart, blood vessels, stomach, intestine, liver, and pancreas.	Easy separation, large-scale cultivation, precise control, real-time monitoring; reagent saving; open lumen, andextend culture time.	High technical requirements, high cost, long development time, lack of organisational compatibility and unable to stably cultivate microbial colonies.	Like ④ ⑤ ⑧, ② can achieve precise control. Compared with ④, ② can not only achieve high-throughput drug screening but also simulate in vivo drug delivery.	High throughput drug screening, and high throughput drug screening, and regenerative medicine.
③ Transwell chamber	Intestine, stomach, breast, and lung.	Economical, easy to obtain, customisable pore size, easy to operate; rapid turnover and moderate throughput, and easy separation.	Low repeatability and long processing time.	③ is the second most common model. Like ① ⑥, it is economical and easy to obtain. Like ④ ⑥ ⑦, it is easy to separate. The culture time and reproducibility were worse than ② ④ ⑤.	Study the response of organoids to the apical stimuli and paracrine communication, study epithelial cells and macrophages separately or collectively, understand how macrophages affect organoids through exogenic signalling molecules, cancer vaccine development, and other model building.
④ Microwell arrays based on polymer film	Intestine.	Make organoids more uniform, and variable in size, easy amplification, and can be combined with other models; can perform additional operations.	High technical requirements and high cost.	The best high-throughput effect characterises ④. Like ⑤, it can be monitored and imaged in real-time. Compared with①, ④ and ⑧ can make organoids more uniformly and variable in size. ④ can be combined with ① ② ③ ⑤.	Analyse juxtacrine and paracrine cell signalling concurrently, high-throughput detection, cell monitoring; automated imaging, and drug susceptibility testing; personalised therapies, study tumour-immune interaction, and cancer vaccine development.
⑤ Micro-injection	Lung and intestine.	Rapidity, high-throughput, precise measurement, inject cells into organoids accurately and reproducibly, and extend culture time.	Structural damage, limited injection of cells, not easy to separate, high levels of expertise, lengthy practice periods, and expensive equipment.	Compared with ① ⑥, it expands the contact surface, but has equipment and technical limitations and is not easy to separate. Because ⑤ can cause damage to organoids and has many limitations, it is not as developed as other models.	Drug testing; predicted response to therapies, and microinjection of bacteria and viruses.
⑥ Interactive co-culture plates	Stomach and adipose tissue.	Without cross-contamination, easy to use, affordable, large-scale cultivable, easy separation.	A particular kind of culture plate, membrane or biofouling.	⑥ is very similar to ③, but ⑥ requires a specific culture plate, and there may be a pressure difference on both sides of the membrane. Compared with ② ④, the drug high-throughput screening of ⑥ was less effective. It can be sterilised by laser.	Study tumour-immune interaction, study migration and polarisation, makes cytokine determination easier, cancer vaccine development, and bacteria-related research.
⑦ Hollow lyophilised silk sponge	Intestine.	Aerobic; replaceable, can add immune cells, simulate the stiffness of soft tissue, and raw material is economical and widely sourced.	The complex preparation process, high technical requirements, and high cost.	⑦ is characterised by its ability to replenish immune cells. Like ⑥, ⑦ is easy to separate and observe migration. Like ⑧, ⑦ can well imitate the stiffness of soft tissue, but it is technically challenging.	Regenerative medicine, drug delivery, tissue engineering, and observe the migration of macrophages to the epithelial layer.
⑧ 3D bioprinting	Brain and pancreatic islet.	High-resolution microstructure, can combined with microfluidic chips.	Slow procession, hardly suitable for high-throughput detection and poor adaptability of bioink.	⑧ Can best simulate the microenvironment in vivo. ⑧ can be combined with ② and ⑥. However, compared with ② ④ ⑥, ⑧ is not suitable for high-throughput screening.	Help identify tumour locations, more accurately mimic in vivo conditions, and Make studying interactions easier; adoptive cell therapy; regenerative medicine.

Microfluidic chip

The microfluidic organoid chip is an emerging 3D cell culture device capable of developing organoids to a diameter of millimetres [37]. It effectively controls fluid flow and nutrient supply through multiple interconnected pores while also monitoring oxygen levels and metabolite concentrations. This enables the Care Team to closely simulate the physiological environment. Currently, the chip is being utilised in various organoids such as the brain, heart, blood vessels, gastrointestinal tract, liver, and pancreas [38]. For instance, macrophages derived from human peripheral blood monocytes or mouse macrophage lines (RAW264.7) and tumour spheroids derived from mice or patients are integrated into the microfluidic chip platform. The platform was developed from the original PDMS 2-lane microfluidic chip to the membrane-free 3-lane OrganoPlate platform for high-throughput screening of compounds [39, 40]. Xin Cui et al. used a PDMS 2-lane platform to implant glioblastoma (GBM) spheroids and macrophages into hydrogels containing 3D artificial vascular organs to simulate the GBM microenvironment in vivo. Two parallel channels are encapsulated in a collagen-I hydrogel. Endothelial cells are seeded into the lumen of one channel to generate 3D monolayer vascular organoid, while adjacent channels are seeded with glioma cells or used as a repository of cytokines. Macrophages are suspended in the hydrogel, and information exchange is completed through the hydrogel [39]. This platform is capable of real-time observation of tumour-immune-vascular interactions, adjustable cell-cell or cell-matrix interactions, as well as inflammation in the GBM microenvironment. Claudia Beaurivage et al. used the membrane-free 3-lane OrganoPlate chip, which consists of three channels. The upper channel was inoculated with human intestinal organs (HIO), the middle channel was inoculated with extracellular matrix (ECM) gel, and macrophages were directly embedded in ECM gel. The bottom is the medium channel. By forming a meniscus, obstacle-free cultivation is achieved [40].

In short, this platform is versatile, can meet the requirements of multiple conditions, and is easy to separate between organoids and cells [41]. In addition, large-scale culture, precise control, real-time monitoring, and reagent saving can be achieved [42, 43]. Compared with ordinary Matrigel tests, it also forms an open lumen, which compensates for the difficulty in exposing drugs to the lumen due to the spherical nature of the organoids and avoids necrosis of the organoid core [44, 45]. Therefore, the culture time is extended to a certain extent [46]. It is worth noting that compared with the microporous array, it can not only perform high-throughput drug screening but also apply drug delivery mode to simulate in vivo drug delivery [47]. The model is of great help for drug testing and predicting response to therapies and regenerative medicine [48]. However, further improvements are still needed to integrate multiple tissue types on microfluidic platforms to more perfectly simulate the in vivo environment and study in vivo dynamics [42, 48]. Stable cultivation of microbial colonies has also not yet been achieved [49]. For most researchers, the platform has high technical requirements, high cost, and requires a long development time.

Transwell chamber co-culture

A polycarbonate membrane between the upper and lower layers of the culture medium separates the Transwell chamber. Due to the permeability of the polycarbonate membrane, the components in the upper and lower layers of the culture medium can be interconnected [50]. The use of the Transwell chamber for the co-culture of macrophages and organoids can be divided into two models: 2D and 3D.

2D cultures can be achieved in intestinal and gastric organoids. For example, 3D intestinal organoids are fragmented or ground and subsequently seeded onto the upper chamber of Transwell. Once fragmented, intestinal organoids grow and form polarised confluent monolayers. Macrophages adhere to the other side of the permeant membrane or face the epithelial cell basement membrane to achieve co-culture, allowing the immune cells (such as macrophages) to be added not only at the top of the intestinal epithelium but also at the basal side. It is convenient to study the response of organoids to, for example, apical stimuli or paracrine communication [51, 52]. Similar to this, trypsin was used to break down gastric organoids into monolayer structures that were then seeded on one side. Macrophages derived from bone marrow were seeded onto the

opposing side. This makes it possible to study epithelial cells and macrophages separately or collectively [53]. Compared with 3D culture, however, 2D culture lost the original 3D structure of the organ.

The 3D culture was realised in breast organoids. Bone marrow-derived macrophages were induced to polarise into the M2 subtype and then seeded on the upper layer of the chamber. Breast organoids were seeded on the bottom of the container and indirectly co-cultured through the culture medium. This model is suitable for studying how macrophages affect organoids through exogenic signalling molecules [54]. Xu et al. cultured lung organoids and alveolar macrophages in Transwell in 2D and 3D, respectively. Compared with 3D culture, 2D culture reduced the blockage of Matrigel, allowing more contact between macrophages and lung organoids, and better results were obtained [30].

The Transwell model is economical and easy to obtain and is the most common model besides Matrigel. The pore size of the model can be customised and is relatively easy to operate. In the co-culture of intestinal organoids and immune cells, rapid turnover and moderate throughput can be achieved. Easy separation is its largest advantage over other models. Nevertheless, the repeatability is low, and the processing time is lengthy [52, 55]. For large-scale culture, it can be combined with microwell arrays [56]. In addition, like the microporous array, it can dynamically measure the material between two chambers to study the kinetics. However, compared with a microfluidic chip, it has the disadvantage of not allowing nutrients to flow. It also enables dynamic measurements of substances between two chambers to study dynamics but does not enable the flow of nutrients [57].

Direct and indirect co-culture of microwell arrays based on polymer film

Kakni P et al. studied the co-culture of intestinal organoids and macrophages using a polymer membrane-based microporous array, which is divided into direct co-culture and indirect co-culture. To create the microwell arrays, Kakni P et al. employed microthermoforming techniques, utilising 50 μm thin polycarbonate films. These arrays were specifically designed to match the dimensions of a 24-well plate in advance [58].

The initial step in direct co-culture involved the macrophage seeding into microwells, followed by centrifugation to expedite cell seeding. Subsequently, organoid fragments were seeded into the microwells. By utilising this microwell array, it becomes feasible to analyse juxtacrine and paracrine cell signalling [59] concurrently.

In an indirect co-culture experiment, identical microwells were utilised. Initially, the 24-well plate was employed to seed the macrophage cell line RAW264.7 at the lower part. Subsequently, the microwell arrays were placed, and organoid fragments were seeded into each microwell. An O-ring was positioned beneath the microwell array to prevent contact between the microwell array and macrophages. In this manner, information exchange between organoids and macrophages is accomplished through paracrine signals [59]. Additionally, Ozawa F et al. deposited fibroblasts in alginate microwell arrays using electrodeposition, followed by seeding embryonic stem cells or liver cancer cells to form spheroids [60]. It may be applied to the co-culture of macrophages and organoids in the future.

Compared with the traditional Matrigel model, the microwell array model can make organoids more uniform, variable in size, and more easily expanded for culture [58]. Compared with Transwell, it has the advantages of a larger scale and higher throughput. Again, there are cost and technical limitations. However, it is worth noting that its advantage is that it can be combined with other systems, such as microfluidics and can also perform additional operations such as microinjection [61, 62]. In addition, the platform enables high-throughput detection, cell monitoring, and automated imaging, and facilitates personalised therapies [59, 63]. For example, the development of drug sensitivity testing has been hampered by the long time to establish patient-derived organoids (PDOs). The microwell array can predict the response of patients to anticancer therapy within a week [64]. In addition, unique indirect co-culture also facilitates the isolation of organoids and cells [59].

Microinjection

In this model, macrophages are directly injected into organoid matrigel with microneedles

under the microscope, or monocytes are induced into macrophages after injection, which has been applied in lung organoids and intestinal organoids, respectively. At present, in the existing methods of constructing lung organoids with Matrigel, the basal layer cells are wrapped outside. Functional cells in the polarised growth state of the lumen are not easy to contact with external stimuli and often require microinjection. In the study of Vazquez-Armendariz AI et al., they injected up to 50 cells under the microscope into the central regions of bronchoalveolar lung organoids (BALOs) on day 14 of culture with the prepared single-cell suspension at a rate of 6 cells/min. After quantifying the alveolar macrophage numbers throughout time, it was discovered that > 80% of the initial alveolar macrophage could be found on Day 10 after injection with 87% viability. These macrophages survived effectively for at least 14 days [65], which was confirmed in another study [66]. Furthermore, human intestinal organoids were seeded with monocytes generated from induced pluripotent stem cells, which were subsequently stimulated to develop into macrophages by M-CSF [67].

Microinjection has the advantages of rapidity, high throughput, and precise measurement, and it can accurately and reproducibly inject cells into organoids [68-70]. Compared with ordinary Matrigel culture, it is a true direct contact co-culture that increases the contact surface area between macrophages and organoids and facilitates more intuitive monitoring of the morphological changes of both over time under a microscope [67]. In addition, it also has the effect of prolonging the culture time. However, organoids may sustain structural damage as a result of microinjection. Only a limited number of cells can be injected each time, and it is limited by the size and differentiation of organoids, which is not conducive to the isolation of macrophages [71]. They are not widely used due to several issues, including requiring high levels of expertise, lengthy practice periods, and expensive equipment [70]. At present, the application of microinjection in organoids mainly focuses on the injection of bacteria and viruses, with research on immune cells such as macrophages still developing [72].

Interactive co-culture plates

Utilising sterile and disposable interactive co-culture plates with two distinct wells connected by an O-ring and a filtration device, the two cell populations were able to share growth media and secreted substances without coming into contact, eliminating cross-contamination. Gastric organoids and human type 2 innate immune cells (ILC2) cells were combined and seeded in the same matrigel on one side, and the complete organoid growth medium was given. Mouse bone marrow-derived macrophages were seeded on the other side and given macrophage basal medium [73]. Additionally, Park SB et al. used a self-made interactive co-culture plate combined with 3D printing technology. One end of the co-culture plate used 3D printing technology to add alginate Matrigel containing human adipose-derived mesenchymal stem cells (ADMSCs), and the other end macrophages to achieve the purpose of simulating type 2 diabetes caused by insulin resistance in vitro. The authors also used this model to evaluate the therapeutic effect of acarbose, metformin, and rosiglitazone on insulin resistance [74].

This model is easy to use, affordable, large-scale cultivable, and facilitates cell isolation. This model can be used to study migration and polarisation, and makes cytokine determination easier. It can also be used for drug screening, but the effect is not as good as microfluidic chips and microwell arrays. The bottom of the co-culture plate used by Park SB et al. also has holes to facilitate laser electron beam sterilisation. However, a particular kind of culture plate is needed, while membrane biofouling is a recognised problem. Due to the pressure difference on both sides of the membrane, cells may be adsorbed on the membrane, which can be solved by improving the material of the membrane [75].

Hollow lyophilised silk sponge

The cylindrical scaffold shape was employed in this model because previous intestinal models demonstrated that the intestinal epithelium structured in a tubular pattern caused low oxygen tension in the lumen, allowing anaerobic bacteria to proliferate [76-78]. The silk fibrin extracted from silkworm cocoon is treated as a scaffold material, which is divided into inner and outer layers, and can be removed by sliding. Cell suspensions of colonoids were seeded on the film surface on the inner silk scaffold, and monocyte-derived macrophages

were seeded throughout the porous outer silk scaffold. The establishment of this model takes three weeks. High Wnt medium needs to be used in the first two weeks, to allow colonic epithelial cells to aggregate into a single layer of epithelium and fail to differentiate. Next, the differentiation medium was used. Then, macrophages were divided into two groups and added to the model. The first group was added after the first week of culture, and the second group replaced the first group after the second week. The two steps were required due to the metabolic activity of monocyte-derived macrophages in high Wnt or differentiation medium decreased by up to 50% on day 7 [79], resulting in a replaceable outer layer of macrophages. Finally, macrophages migrate into the epithelial layer under inflammation, affecting epithelial morphology and cytokines.

A critical feature of the double cylindrical scaffold is that it can replace the outer layer containing macrophages without destroying the inner scaffold layer containing epithelium, which is easy to separate, and observe the migration of macrophages to the epithelial layer. Bone marrow-derived monocytes from peripheral blood constantly supplement intestinal macrophages in vivo. At the same time, co-culture of other immune cells and organoids typically approaches the terminal time point and is unable to supplement the immune cell population [80]. This technology can add immune cells to make up for this shortcoming. Additionally porous, its unique material also has the characteristic of multiple pores, which better simulate the stiffness of soft tissue [79]. This model can also be applied in other domains like regenerative medicine, drug delivery, and tissue engineering [81]. Its raw material, silk protein, is economical, widely sourced, and has good biocompatibility. However, the preparation process is complicated and there is no mature commercial model to achieve large-scale application [82]. There are also limitations regarding organoid types. It is only applicable to single-layer epithelial organoids such as intestines and skin [83].

3D bioprinting

A significant advancement in model-building technology is 3D bioprinting. It has achieved co-culture with macrophages in the brain and islet organoids. Marcel Alexander Heinrich et al. constructed a co-culture model of brain glioblastoma organoids and macrophages. The 3D bioprinting model requires bioink, which is obtained using 3% w/v gelatin metacryloyl and 4% w/v gelatin after condition optimisation. Macrophages (RAW264.7) were first combined with bioink at 37°C, then moved to a syringe and left motionless for 15 minutes, to create a gel to print. The brain model was printed using a specially designed bioprinter, but a cavity was left in the designated location. Next, glioblastoma cells (GL261) were added to the bioink and printed in the reserved position. This macrophage-filled brain organoid model can help identify tumour locations, more accurately mimic in vivo conditions, and make studying interactions easier [84]. Zhu et al. used gelatin methacryloy, decellularised extracellular matrix, and platelet-rich plasma as bioink, together with macrophages, to construct islet organoids using 3D printing. The results showed that the bioink induced macrophage M2 polarisation, attenuated inflammation, and significantly induced angiogenesis [85].

3D printing technology can produce high-resolution microstructure, which can be used to reproduce TME. The above model makes it easy to separate organoids from macrophages. In recent studies, 3D printing was combined with microfluidic chips and interactive co-culture plates, taking into account the pre-existing ECM and native tumour cellular heterogeneity, to achieve bottom-up reproduction of tumours in vivo [86]. However, compared with microfluidic chips and microwell arrays, the currently available 3D bioprinters still make the process slow and hardly suitable for high-throughput detection. Additionally, bioink used in different models needs to meet different viscosity, stiffness, and gel dynamics. The development of bioink and supporting devices for large-scale applications will help to improve the manufacturing speed and scalability [87, 88].

Interaction between organoids and macrophages under co-culture conditions

Previously, this review has outlined the benefits and drawbacks of the recognised co-culture models of organoids and macrophages. In general, however, they match the corresponding models based on the research purposes of

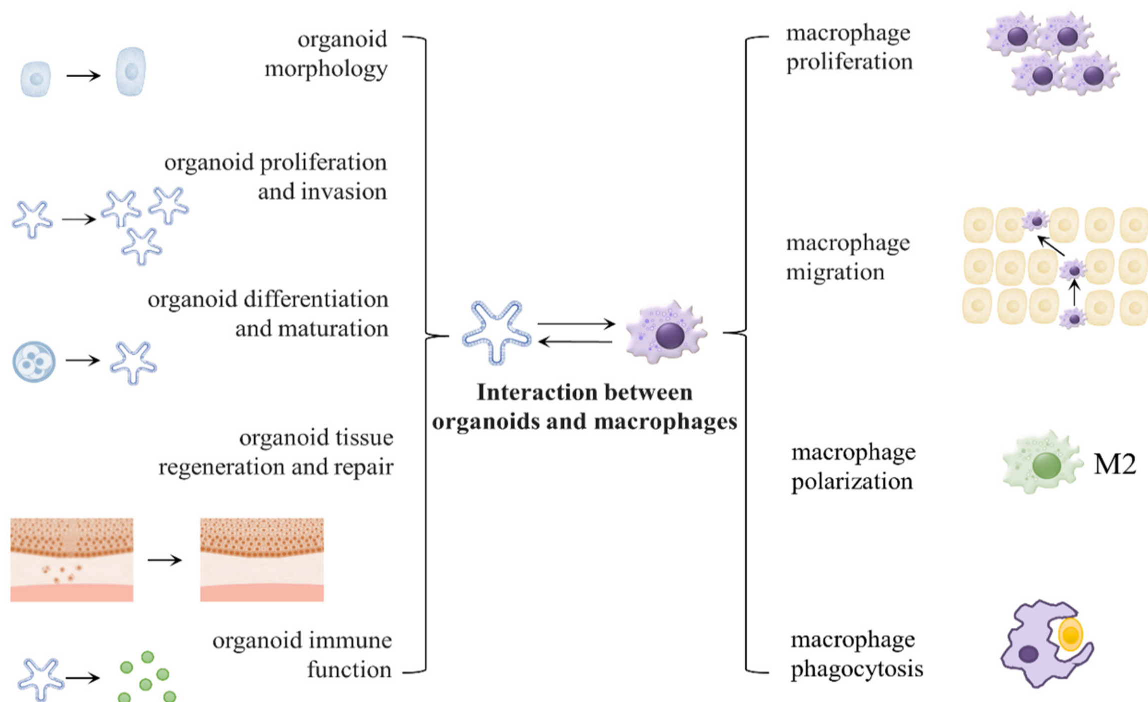

Figure 3. Interaction between organoids and macrophages under co-culture conditions.

their respective experiments. Based on this, researchers obtained some results on the interaction between organoids and macrophages. Below is presented an overview of the co-culture model's interactions between organoids and macrophages. Generally, organoids can affect the proliferation, migration, M2 polarisation, and phagocytosis of macrophages. As for organoids, macrophages can affect the morphology, proliferation (and invasion), differentiation and maturation, tissue regeneration and repair function, and immune function of organoids (**Figure 3**).

Effect of organoids on macrophages

Organoids affect macrophage proliferation: The survival, proliferation, and differentiation of macrophages are significantly regulated by Macrophage Colony Stimulating Factor (M-CSF) [89]. M-CSF can promote monocyte survival, monocyte-to-macrophage transformation and macrophage proliferation [90, 91]. Adenosine (ADO) and prostaglandin E2 (PGE2) activate a similar transcriptional pathway in M-CSF-induced differentiated macrophages that involves the upregulation of growth factors and the downregulation of inflammatory mediators [92]. In Kuen j et al., the increase of M-CSF was detected in the co-culture supernatant, which played an essential role in the survival, proliferation and differentiation of macrophages [93].

Organoids affect macrophage migration: Macrophages are recruited to the site of damage or inflammation, to get rid of the initial inflammatory signals and ultimately encourage wound healing and tissue repair. Intestinal epithelial cells play a crucial role in regulating the intestinal immune system. Intestinal organoids release cytokines under inflammatory stimuli. The release of pro-inflammatory cytokines leads to increased migration of macrophages towards the inflamed epithelium [79]. Gastrin can stimulate gastric parietal cells to secrete sonic hedgehog (Shh) signals to induce macrophages to migrate to gastric organoids, which is associated with the significant increase of Gli target genes PTCH1, CCR2 protein in macrophages during co-culture. In addition, Shh signalling can also induce macrophage chemotaxis through an SMO-dependent pathway mediated by Akt signalling [73]. In the study of Hacker BC et al., the mammary gland was irradiated in vitro before organoid formation as the irradiated organoid group, and the normal organoid was used as the control, which were

co-cultured with macrophages, respectively. Live cell images showed that macrophages colocalised with organoids after 24 h, indicating that macrophages migrated [33, 94]. It is worth noting that the tightness of organoids may be one of the influencing factors. Interestingly, the tighter the connection between pancreatic cancer cells and the spheroids formed by fibroblasts, the easier it is to attract the migration of macrophages [93].

Organoids affect M2 phenotype macrophage polarisation and phagocytosis: Macrophage polarisation refers to the capacity of macrophages to change their phenotype under the influence of the microenvironment, thereby exhibiting diverse functions. There are two commonly observed phenotypes of macrophages: the classically activated or inflammatory macrophage (M1 phenotype) and the alternatively activated or healing macrophage (M2 phenotype). However, M2 phenotype macrophages can inhibit immune responses, support the development of tumour tissues, and act as a cell reservoir of various pathogens [95, 96]. In addition, M2 phenotype macrophages have complex roles beyond inflammation, such as organ morphogenesis, tissue renewal, and endocrine signalling [97, 98]. It appears that macrophages are more prone to polarisation towards the M2 subtype when co-cultured with organoids.

In the co-culture of organoids and macrophages, M2 phenotype macrophage markers like CD206, CD14, CD163, and arginase-1 were expressed. The pro-inflammatory factors IL-8 and IFN-γ were significantly reduced, and phagocytosis was enhanced. It indicates that the immune microenvironment induces the adhesion changes and phenotypic changes of macrophages, leading to the elongation and enlargement of macrophages, becoming anti-inflammatory tumour-promoting M2 phenotype macrophages [32, 51, 93]. In addition, the production of anti-inflammatory factors can also confirm this. In the study by Chakrabarti j et al., gastrin-driven activation of the Shh signalling pathway resulted in the expression of IL-33 in gastric organoids and the subsequent release of IL-13, which induced the polarisation of M2 macrophages [99]. In another study, brain glioblastoma organoids were used to study angiogenesis, and it was found that organoids promoted immunosuppression and polarised macrophages to the M2 subtype with pro-angiogenic activity, thus more closely simulating the pathological situation in vivo. Activated M2 phenotype macrophages secrete increased anti-inflammatory cytokine TGF-β1 and IL-10 to promote capillary epithelial cell proliferation and angiogenic sprouting in organoids [39]. Zhu et al. 3D printed islet organoids using specific bioink and also found macrophage M2 polarisation. CD206 (M2 marker) was highly expressed, while iNOS (M1 marker) was low expressed, helping to reduce immune rejection and promote angiogenesis [85].

Effect of macrophages on organoids

Macrophages affect organoid morphology: Co-culturing has been shown to affect the morphology of intestinal organoids in recent investigations substantially. For instance, it would result in a highly significant increase in the height of intestinal epithelial cells, revealing the potential role of macrophages in encouraging intestinal epithelial maturation and thickening the physical barrier [51, 79]. In one study conducted by Kakni P et al., intestinal organoids gradually lost their crypt-villus structure and became spherical structures after co-culture. This situation is significant with increasing macrophage numbers because macrophages release more pro-inflammatory cytokine TNF-α. Through the internalisation of occludin protein in the cytoplasm, the loss of zonula occludens-1, and the phosphorylation of myosin light chain, TNF-α is linked to the breakdown of the integrity of the epithelial barrier [100-102].

Macrophages promote the proliferation and invasion of tumour organoids: As mentioned earlier, M2 phenotype macrophages, usually considered tumour-associated macrophages (TAMs), promote the proliferation and invasion of tumour cells [103]. Enhanced collagen hydrolase activity, increased levels of MMP-2 and MMP-9, disrupted basement membranes, and enhanced tumour cell invasion were detected in human skin squamous cell carcinoma organoids co-cultured with macrophages [99]. According to Denardo DG et al.'s study, CD4+T cells produced IL-4, activated TAMs, elevated macrophage epidermal growth factor (EGF) mRNA expression, and promoted tumour invasion and metastasis that was EGFR dependent

[104]. In Xu et al., a direct breast cancer cell (BCC) - TAM co-culture organoid model was developed. The results show that the expression of cancer stem cell markers (ALDH1A1, SOX2, and ALCAM) and major ECM components (such as collagen, fibronectin, and integrin) increased in the co-culture group. The Transwell migration assay also showed that the number of migrated cells in the co-culture group was higher. These outcomes indicate that co-culture significantly enhanced the invasive phenotype of breast cancer organoids, including enhanced stemness, migration, and ECM remodelling [31].

Macrophages affect the differentiation and maturation of organoid tissues: In co-culture, macrophages can promote stem cell differentiation and promote the differentiation and maturation of organoids. For example, co-culture differentiated stem cells from apical papilla (SCAPs) to a cap-like apical papilla organoid, which was reflected in the elevated expression of SCAP differentiation markers such as dentin sialophosphoprotein (DSPP) and dentin matrix acidic phosphoprotein (DMP-1) [105, 106]. In another study, co-culture drove airway epithelial cell differentiation and promoted lung organoid maturation, which was reflected in a significant increase in the total percentage of terminally differentiated epithelial cells (alveolar epithelial cells (AEC I) and ciliated cells), along with a decrease in cell proliferation and stress signals [65]. Mammary stem cells (MaSCs) are kept active, and mammary organoids continue to develop when macrophage-produced TNF-α intracellular PI3K/CDK1/Cyclin B1 signalling in mammary cells [54].

Macrophages affect organoids' tissue regeneration and repair function: M2 phenotype macrophages can reduce inflammation while promoting tissue regeneration and wound healing. The M2 macrophage markers Arg1 and Ym1 detected in the co-culture of lymph node organoids and macrophages are engaged in reducing inflammation and promoting tissue regeneration [32, 107, 108]. Additionally, M2 phenotype macrophages express a group of matrix metalloproteinases, MMP, which are zinc-containing endopeptidases involved in wound repair [109]. MMPs are essential for tissue remodelling through extracellular matrix degradation and pro-angiogenic cytokine activation [110-113]. Significantly, a high expression level of MMP-9 was also detected in the co-culture medium [32].

In addition, macrophages can also take effect through other signalling pathways. Glioblastoma perivascular macrophage-epithelial cell interactions via alpha(v)beta(3) integrin and Src-PI3K-YAP signalling pathway regulate pro-angiogenic activity in vitro. These two signalling pathways can be promoted by cell-cell or cell-matrix interactions [39]. In the co-culture of gastric organoids and macrophages, under histamine stimulation, the expression of CD44 variant isoform 9 (CD44V9) and IL-33 were significantly increased. CD44V9 has been shown to induce spasmolytic polypeptide/TFF2 expressing metaplasia (SPEM). The SPEM markers TFF2 and gsii, marker genes wfdc2, olfm4 and CFTR were also upregulated, which played an important role in gastric epithelial regeneration [73, 114].

Macrophages affect the immune function of organoids: Under inflammatory conditions, co-culture promotes the immune function of organoids. When organoids are exposed to inflammatory cytokines (exogenously added or secreted by macrophages, such as TNF-α), they exhibit innate immune response activity and release a variety of pro- and anti-inflammatory cytokines that trigger an immune response [115]. Compared with macrophage culture alone, the secretion levels of these cytokines were lower, suggesting that intestinal organoids have some immune regulatory function, which seems to be capable of regulating the immune response of macrophages. Additionally, direct co-culture produced higher levels of cytokines than indirect co-culture, indicating that the contact of intestinal organoids with macrophages seems to have a significant impact on the interaction [59]. In the co-culture of tumour organoids and M2 phenotype macrophages, the proliferation and function of T cells were inhibited, which represented the decline of immune function. The activation of CD4+ and CD8+ T cells was impaired, which was reflected in the significantly decreased expression of activation markers CD25, CD69, and 4-1BB. Finally, the expression of immune checkpoint molecules such as PD-1 and CTLA-4 was also significantly downregulated [93].

The application prospects of the organoid-macrophage co-culture model in immunotherapy

The co-culture platform of immune cells with tumour organoids has the potential to enhance personalised research in immunotherapy, revitalising the field of cancer immunotherapy. Currently, immunotherapy for tumours primarily consists of therapies such as immune checkpoint inhibitors (ICIs), adoptive T-cell therapy, tumour vaccines, and oncolytic virus therapy [116]. Studies have shown that ICIs can interfere with the tumour's suppression of macrophage phagocytosis ("do not eat me" signal) and can also activate macrophage phagocytosis ("eat me" signal) [117].

Blockade therapy targeting immune checkpoints is dependent on the correlation between tumour cells and the respective ligands of immune cells. For instance, CD47, a transmembrane protein present in tumour cells, interacts with SIRPα ligands on phagocytes, resulting in resistance to phagocytosis and evasion of immune responses by cancer cells [118]. In a study conducted by Dooling et al., tumour organoids containing macrophages and cancer cells were utilised to elicit tumour-specific IgG antibodies by interrupting the macrophage checkpoint CD47-SIRPα and utilising monoclonal antibodies. This approach led to a decrease in tumour metastasis and an enhancement in overall survival rates [119]. Song et al. demonstrated that the ubiquitin ligase UBR5 is crucial for the development of spheroids related to ovarian cancer (OC) and the infiltration of macrophages. UBR5 stimulates the recruitment and activation of macrophages by utilising essential chemokines and cytokines, utilising β-catenin-mediated p53 signalling to encourage organoid formation. Disrupting UBR5 activity can improve the efficacy of cisplatin treatment. Co-treatment with either anti-PD-1 mAb or adoptive T cell therapy notably boosts the present immunotherapeutic response against OC [120]. Xu and colleagues established bladder cancer organoid and xenograft models that were treated with either anti-PD-1 antibody or control IgG2a. Analysis at the single-cell level revealed that a higher percentage of immune cells, particularly macrophages, infiltrated the group that responded to immunotherapy compared to the group that did not respond [121]. Interestingly, both human and murine tumour-associated macrophages express PD-1, and the anti-PD-1 monoclonal antibody can accumulate in macrophages through FCγ receptors [122, 123]. Once cancer vaccines enter the body, macrophages, as antigen-presenting cells, also promote the activation of anti-tumour immune responses. With the success of dendritic cell vaccines, targeting other immune cells, such as macrophages, is expected to address current limitations such as tumour type and safety [124]. In addition, co-culture models have shown promise for oncolytic virus therapy. The oncolytic cowpox virus (VACV) replicates and specifically kills tumour cells. VACV was designed to express GM-CSF, which induces monocyte-to-macrophage polarisation [125]. It is hoped that more oncolytic viruses will be used in co-culture models in the future. Therefore, there is significant importance in focusing on organoid-macrophage co-culture for the advancement of new immunotherapy strategies targeting macrophages.

Recently, Ferreira N et al. constructed a pancreatic cancer organoid-peripheral blood mononuclear cell (PBMC) co-culture model using the Matrigel. They used the artificial intelligence OrganoIDNet algorithm to analyse the results of live cell imaging, which can accurately detect the response of pancreatic cancer organoids to standard chemotherapy (gemcitabine) over time. This method can also be used for organoid-immune cell co-culture and is expected to evaluate the immunotherapeutic effect of individual PDOs in real time [126]. Stüve P et al. analysed organoids and immune cell co-cultures using machine learning-enabled image cytometry, which can accurately detect organoids in co-cultures. These auxiliary technologies have substantially facilitated the development of co-culture models [127].

Limitations and application prospects

The application of co-culture strategies makes the organoid model closer to the natural state in vivo. However, it is crucial to acknowledge that no model, including the organoid model, can fully replicate the complexity and functionality of the real in vivo environment. Inherent limitations and intrinsic defects are inevitable. The lack of multiple cellular components in the microenvironment in vivo is a significant chal-

lenge in current organoid models. Furthermore, numerous obstacles restrict the clinical application of organoids.

Firstly, establishing and maintaining organoids can be expensive [128]. Secondly, microfluidic chip systems require highly challenging finishing technologies. Not all research laboratories have access to microfabrication facilities and related expertise at any time. Therefore, it is still necessary to develop commercial services that provide customised design and application-specific equipment so that more investigators can enter the field of 3D organoid modelling [129]. Thirdly, the survival time of immune cells co-cultured with organoids still needs to be further extended to simulate chronic inflammation. Finally, vascularising organoids continues to be a difficult task. Although the co-culture system can promote the vascularisation of organoids, it only endows the vascular characteristics of organoids and cannot achieve the functional perfusion of blood vessels [130].

The application of these models was also discussed, which can improve the effect of personalised cancer immunotherapy and help understand the tumour microenvironment [131-133]. For example: (1) Study tumour-immune interactions to detect the specific role of immune cells such as macrophages in tumour progression and remission. (2) Drug development (drug screening tests specific compound libraries). (3) Personalised medicine (e.g., screening the efficacy of immune checkpoint inhibition ICIs to determine the most effective treatments for specific patients) [134]. (4) Study the impact of macrophage polarisation on ICI treatment for potential cancer vaccine development [135]. (5) Study human chimeric antigen receptor macrophages for cancer immunotherapy and supplement adoptive cell therapy [136]. (6) Regenerative medicine (organoid transplantation into the body). (7) Help the establishment of co-culture models of other immune cells and organoids.

Conclusion

The organoid-macrophage co-culture model better simulates the physiological environment in vivo. It can not only improve our understanding of tumour-immune interactions, but more significantly, it can be used as a tool to evaluate patient-specific responses before immunotherapy, helping to personalise treatment. If existing limitations are addressed in the future, they may facilitate the development of cancer immunotherapies that target macrophages and rapidly enter the clinic in combination with other immunotherapies.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Huiyi Song, Laboratory of Integrative Medicine, The First Affiliated Hospital of Dalian Medical University, 222 Zhongshan Road, Dalian 116023, Liaoning, PR China. E-mail: huiyisongmail@163.com; Dong Shang, Department of General Surgery, Clinical Laboratory of Integrative Medicine, The First Affiliated Hospital of Dalian Medical University, 222 Zhongshan Road, Dalian 116023, Liaoning, PR China. E-mail: shangdong@dmu.edu.cn

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