Review Article

Application of Immunocompetent Microphysiological Systems in Drug Development: Current Perspective and Recommendations

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Abstract

Immune responses are heavily involved in the regulation and pathogenesis of human diseases, including infectious diseases, inflammatory and autoimmune conditions, cancer, neurological disorders, and cardiometabolic syndromes. The immune system is considered a double-edged sword serving as a powerful host defense mechanism against infection and cancerous cells and causing detrimental tissue damage when the immune response is exaggerated or uncontrollable. One of the challenges in studying the efficacy and toxicity of drugs that target or modulate the immune system is the lack of suitable preclinical human models that are predictive of human response. Recent advancements in human microphysiological systems (MPS) have provided a promising in vitro platform to evaluate the response of immune organs ex vivo, to investigate the interaction of immune cells with non-lymphoid tissue cells, and to reduce the reliance on animals in preclinical studies. The development, regulation, trafficking, and responses of immune cells have been extensively studied in preclinical animal models and clinically, providing a wealth of knowledge by which to evaluate new in vitro models. Therefore, the application of immunocompetent MPS in drug discovery and development should first verify that the immune response in an MPS model recapitulates the complexity of the human immune physiology. This manuscript reviews biological functions of immune organ systems and tissue-resident immune cells and discusses contexts-of-use for commonly used immunocompetent and immune organ MPS models. Current perspective and recommendations are provided to guide the continued development of immune organ and immunocompetent MPS models and their application in drug discovery and development.

1 Introduction

The unprecedented progress in science and technology over the past decade has contributed to a marked increase in the use of microphysiological systems (MPS), including organs-on-chips, resulting in extended application of MPS across multiple stages of the drug discovery and development process. The use of animal studies in drug development has undoubtedly contributed to the success of marketed drugs (Monticello et al., 2017). In parallel, however, the increased use of MPS models can be at least partly explained by the observed discordance between animal study results and human clinical trial outcomes as well as the need to...
reduce, refine, and replace (3Rs) animal usage in drug research and development (Leenaars et al., 2019). While many different MPS platforms are used in both pharmacology and safety studies (Baran et al., 2022), a requirement for all MPS models is their potential to recapitulate key physiology of parental organs, enabling the assessment of specific biological functions without the need to conduct in vivo studies. Therefore, a thorough and complete evaluation of human MPS models will likely contribute to their increased adoption, resulting in a significant reduction in animal use, positively impacting the 3Rs, and may better predict the efficacy and safety of new drugs in humans (Peterson et al., 2020).

The immune system is a unique organ system because it comprises localized organs, circulating leukocytes, and tissue resident cells that exhibit highly interactive potential with other cell types. In recent years, there has been a growing interest in developing and applying various innate immune MPS models and incorporating immune cells into tissue chips, primarily due to the rising prominence of immunotherapies and the challenges of predicting immunotoxicity in disease-free preclinical models (Polini et al., 2019; Atkins et al., 2020; Denayer et al., 2014).

1.1 Immune system

The immune system consists of a series of organs, tissues, cell types, and secreted factors that facilitate the differentiation, activation, and migration of immune cells throughout the body (Kindt et al., 2007). Many diseases develop when the immune system becomes dysregulated due to predisposing genetic factors and/or after exposure to pathogens or xenobiotics, leading to conditions such as allergy, autoimmunity, chronic inflammation, and cancer. The immune system is a key component of many disease processes, and yet immune components are often missing or are insufficiently incorporated into in vitro models and are subject to species differences in animal models used in drug development.

The innate immune system is the body’s first line of defense against invading pathogens. It consists of anatomical barriers such as the skin, mucous membranes, sweat, tears, and saliva as well as innate protein factors including the complement system, such as the skin, mucous membranes, sweat, tears, and saliva as well as innate protein factors including the complement system, chemokines, and cytokines. Innate leukocytes lack antigen specificity; rather, the cells recognize conserved pathogenic structures (e.g., bacterial lipopolysaccharide) and can reside within tissues and respond to signals of tissue damage and inflammation. Several types of innate leukocytes develop from myeloid or lymphoid progenitor cells. These innate cells consist of natural killer (NK) cells, innate lymphoid cells, mast cells, basophils, eosinophils, monocytes, macrophages, neutrophils, and dendritic cells (DCs), which can perform antigen non-specific functions including phagocytosis, antigen presentation, production of cytokines and chemokines, and cell killing.

In contrast, adaptive immunity is acquired after an encounter with a specific antigen and consists of antibody and cell-mediated (B cell and T cell) components. A series of gene segments that encode antigen receptors on B cells and T cells undergoes genetic recombination events to produce a diverse repertoire of antigen specificities. B cells, which egress from the bone marrow, can undergo maturation and clonal expansion within the germinal centers of the spleen after antigen-specific recognition by their receptor and secrete antibodies to help facilitate antigen clearance. CD4+ and CD8+ T cells enter the periphery from the thymus and can become activated through binding of their T cell receptors (TCR) to peptides presented in the context of major histocompatibility complex (MHC) molecules on antigen presenting cells, which can occur in secondary lymphoid organs, including the lymph node. Once activated, T cells can perform effector functions including cytokine production and cytolytic activity.

While hematopoiesis occurs primarily in the bone marrow and thymus, immune cell trafficking within lymphoid organs and infiltration into non-lymphoid tissues is essential for mounting a protective immune response. In fact, leukocytes can traffic through the blood and lymphatic vessels to almost all organs and tissues, including lung or liver, where some take up residence, while others infiltrate in response to infection or inflammation and can become key drivers of immune-related pathologies.

1.2 Immune response in drug development

The immune system is a dynamic and highly regulated host defense system. An enhanced or suppressed immune system may promote different types of disease. Therapeutic interventions are developed to oppose dysregulated immune responses. When developing a drug to directly stimulate an immune response, proof-of-efficacy studies are often requested. In pharmacology studies, not only the amelioration of the disease should be demonstrated, but a corresponding immune analysis is included to confirm the intended immune activation. The augmentation of immune responses such as upregulation of T cell activation markers and cytokine production are often used as biomarkers in both preclinical and clinical studies. Vice versa, when suppressing the immune system with a drug, a relevant pharmacological model, if available, will be used to demonstrate the reduction of disease score and the dampening of targeted immune responses.

The evaluation of immunotoxicity of pharmaceuticals, biologics, and cell therapies is recommended in preclinical studies when immunomodulation is a potential liability. When enhancing immunity is an intentional therapeutic approach, adverse effects such as cytokine release syndrome, immune-related adverse events, and off-target, on-tumor activity are points to consider (Ramos-Casals et al., 2020; Kerns et al., 2021). This is represented by immune checkpoint inhibitors, anti-CD3 bispecific engagers, and recently emerging immune cell-based therapies. On the contrary, when dampening immune responses and inflammation is desired to treat patients, suppression of T cell, NK cell and antibody responses is often determined to evaluate immunotoxicity risks.

Similar tests can be applied when modulation of immune responses is not an intentional pharmacological strategy but when standard toxicology studies reveal causes for concern for non-oncology drugs (as in International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use [ICH] S8 immunotoxicology guidance (ICH, 2005)). Biotherapeutics have the potential to cause immunogenicity, which could lead to decreased exposure and reduced efficacy, while some small molecules may cause hypersensitivity. Therefore, suitable models of the human immune system are in high demand for pharmacology and toxicity studies.
2 Opportunities for immunocompetent MPS models in preclinical evaluation

2.1 In silico analysis: applications and limitations
Great progress has been made in the development of in silico (computational) tools for predicting immunogenicity risk for proteins (Groell et al., 2018) or allergic contact sensitizing potential (Ta et al., 2021). Computational models are also being explored for use in predicting the efficacy and toxicity of agents during drug development (Raies and Bajic, 2016). The challenge with computer-based models, however, is that they include assumptions based on a limited understanding of the complex molecular and intercellular dynamics within the immune system, which limit their usefulness.

Immune MPS have the potential to generate data that can feed into these models and bridge the gap between in silico tools and in vivo models to aid in the development of predictive in silico models, which could be extended to other potential applications such as human lymphoid organ pharmacokinetics, vaccine efficacy, or cellular therapy off-tumor activity.

2.2 In vitro assays: translatability and limitations
Various in vitro immune cell assays have been developed utilizing primary cells or cell lines from either human or preclinical species. The variety of available assay systems based on single cell suspensions is broad, including evaluation of pan-T cell responses, cytokine release assays, antigen-specific immune responses (both B and T cells), DC activation and antigen presentation, mast cell function, among many others. Similarly, the nature of the methods used to evaluate these endpoints is also broad, including assessment of cell proliferation, ELISA, immunophenotyping, etc. A description of these assays is beyond the scope of this manuscript, but the reader is referred to a couple of excellent recent reviews (Corsini and Roggen, 2017; Gersmolec et al., 2017). In vitro assays are often applied early in the development of drugs, are relatively high-throughput compared to in vivo studies, use small amounts of compound or biologic, and are consistent with the 3Rs. Specific mechanistic questions can be asked, and the concentrations at which effects on the immune system are observed can be compared to clinically efficacious concentrations or systemic exposures.

Despite these advantages, such in vitro assays may offer limited information and few in vitro tests have been adopted for routine testing of chemicals and pharmaceuticals. Perhaps more than any other organ system, immune cell responses are often the consequence of cell-to-cell interactions or inter-cellular crosstalk. Importantly, many immune cell responses are initiated in three-dimensional (3D) in vivo structures such as lymph nodes, spleen, thymus, liver, and bone marrow, and 2D culture may not be sufficient to provide the necessary tissue microenvironment for the resident immune cells. For example, there are currently no 2D models that can mimic the structure of germinal centers and produce a primary antigen-specific humoral response. Efforts have been made toward this goal (e.g., Collinge et al., 2010) for recall responses to flu antigen. Often in vitro systems use mature cell types, and the effect of compounds on hematopoietic development and thymic maturation may be bypassed in vitro. These limitations speak to the need for developing 3D systems that more accurately replicate in vivo systems.

2.3 In vivo models: translatability and limitations of animal studies and humanized mice
Various in vivo animal models are used during the development of pharmaceuticals to evaluate drug metabolism and pharmacokinetics, efficacy, and safety, as well as for the testing of environmental chemicals. These include rodents (both rats and mice), rabbits, dogs, nonhuman primates (NHP), and minipigs. While rodent models are most often used for immunotoxicity assessments of chemicals, the species selected depends upon its pharmacological relevance. Typically, two species are assessed for toxicity (ICH, 2013a,b), but for some biologic therapeutics, NHP may be the only relevant species, or there may be no relevant species at all. In these cases, regulatory filings are largely dependent on in vitro human assessments and, sometimes, off-target toxicity in one species.

Preclinical models cannot always predict human outcomes. With respect to development, the immune system of different species matures at different rates (Skaggs et al., 2019). While the basic components of the developed immune system are similar across species, they are not identical. From an immunotoxicity perspective, these often-subtle differences are perhaps best highlighted by the tragic “TeGenero Incident” where healthy human volunteers experienced severe cytokine release syndrome in response to administration of a superagonist CD28 antibody (Suntharalingam et al., 2006). Preclinical studies, including in vivo NHP studies, failed to predict this adverse event, and subsequent studies demonstrated that this was most likely due to differences between humans and NHP in CD28 expression on the surface of human CD4+ effector cells (Eastwood et al., 2010).

A number of preclinical in vivo immunotoxicity models are used to predict human outcomes but with varying degrees of accuracy. The T-dependent antibody response (TDAR) assesses antigen-specific humoral responses following immunization with a T-dependent antigen as a functional approach for measuring immune competence (Luster et al., 1992), especially when used in combination with other immune function tests. The TDAR is amongst the best characterized immunotoxicology tests, evaluated for multiple eliciting antigens (Lebrec et al., 2014), and was included in the ICH S8 immunotoxicology guidance (ICH, 2005) as a key functional assay to assess immunotoxicity. However, despite significant concordance between preclinical species and humans with respect to the impact of chemicals and drugs on the TDAR response, quantitative risk assessment remains challenging (Lebrec et al., 2014). Specifically, it is unknown what level of increase or decrease in an animal TDAR is linked with an adverse outcome or pharmacological change in humans.

Over the last several years, humanized mice have been investigated for evaluation of efficacy and safety of pharmaceuticals. Models involving reconstitution of various mouse strains with components of the human immune system, including peripheral blood mononuclear cells (PBMCs), CD34+ hematopoietic progenitor cells, or fetal bone, liver, and thymus, have been used to
model cytokine release and other immune-related adverse events (Allen et al., 2019; Yan et al., 2019; Weaver et al., 2019; Ye et al., 2020; Wang, M. et al., 2018). These models can be used to answer specific questions but are limited by several factors. For example, engraftment of all cell types is not equal and may not represent the distribution observed in humans as exemplified in PBMC reconstitution models, where T cells typically engraft to a greater extent than B cells. The development of graft versus host disease (GvHD) also poses a challenge, limiting the length of the studies, as well as leading to basal expression of some proinflammatory cytokines. Work is ongoing in these areas, for example using double knockouts of the mouse MHC class I and class II to reduce GvHD or expressing transgenic human cytokines, chemokines, and growth factors in mice to enhance engraftment of certain immune cell types. The most immediate future for such models in the pharmaceutical industry appears to be in the realm of efficacy models rather than safety applications.

2.4 Standards for characterization of immunocompetent MPS models

Standards are important for supporting the characterization, and thereby qualification and assessment, of models. They can facilitate development of these models through the application of standard operating procedures (Low et al., 2021), which allow comparisons between and within labs for a specified model, providing a measure of reliability and reproducibility (Allwardt et al., 2020; Marx et al., 2020). Through this, the biological performance of the model can be characterized for a specified context-of-use, thereby determining whether a model is “fit-for-purpose”. This can be done by establishing performance standards for both analytical validation and biological qualification (Marx et al., 2020; Low et al., 2021). Ultimately, this will increase confidence in MPS, accelerating end-user adoption (Allwardt et al., 2020; Marx et al., 2020) and facilitating regulatory acceptance. The application of standards in the development and characterization of immune system MPS is discussed in detail in the following sections.

3 Immune system MPS and context-of-use application

The development, maturation, and activation of immune cells takes place in a well-controlled environment in many organs including bone marrow, thymus, spleen, lymph nodes, and various tissue-associated tertiary lymphoid tissues. Interactions and crosstalk between immune cells and non-lymphoid tissue cells within immune organs are important to regulate the activity of the immune system. Studies with dissociated single cell suspensions in a 2D culture plate often cannot capture such complexity provided by cell-to-cell contact or numerous soluble factors to assess for effects on immune cell phenotypes and function. Various 3D immune organ culture systems have been in development to improve the recapitulation of physiological conditions (Tab. 1). In addition, there has been a growing interest in developing multiple organs-on-chip or human-on-a-chip via connecting chambers containing individual organs-on-chip to mimic in vivo tissue interactions (Abaci and Shuler, 2015; Esch et al., 2014).

3.1 Bone marrow

The bone marrow (BM) is the site where blood cells develop from hematopoietic stem cells (HSCs). HSCs reside in niches, with dividing and non-dividing HSCs mainly found in the endothelial/perisinusoidal niche, where they are in close contact with the blood vessels and mesenchymal stem cells (MSCs), which are an important source of factors required for HSC maintenance (Raic et al., 2019).

The BM is therefore an important organ both for drug targeting and off-target toxicity. Current drug studies rely on 2D in vitro and in vivo models for safety and efficacy. These various preclinical models have improved in the past years and significantly contribute to pharmacology and safety assessment, though with limitations in terms of human predictivity (Mahalingaiah et al., 2018). Traditional 2D cultures lack the complexity and longevity of the BM. The colony forming unit (CFU) assay to drive HSC to form lineage-specific colonies is time consuming, low-throughput, and only suitable for acute toxicity assessment (Olaharski et al., 2009; Yadav et al., 2016). While in vivo rodent models have been demonstrated to predict clinical responses (Olson et al., 2000), translatability of BM toxicity in vivo can be challenging as inter-species differences, such as the predominance of neutrophils in human peripheral blood and lymphocytes in the rat (Friberg et al., 2010), can lead to translational disconnects (Zhang, J. et al., 2019).

To predict human lineage-specific toxicity in the BM more accurately, more physiologically relevant models are required (for an example of a BM-on-a-chip, see Fig. 1). A number of 3D or MPS models of the BM have been developed in recent years. Most BM MPS have been developed from animal tissues to demonstrate their functional competency. For example, a 3D scaffold-based perfusion bioreactor system has been developed in which nucleated mouse BM cells were loaded onto porous, ceramic-based 3D scaffolds and cultured for 3 weeks under perfusion, following which HSCs and precursor cells were introduced in the culture medium and cultured for 1 week. This model demonstrated reconstruction of the BM stem cell niche and CFU in vitro and that spleen colonies could form in lethally irradiated mouse recipients. The authors identified applications for this model such as HSC expansion for transplantation, to generate an ectopic BM to provide a healthy environment when the niche is damaged, and to test new drugs for cancer and bone related disorders (Di Maggio et al., 2011). In another BM microfluidic device, BM was extracted from a mouse and cultured in a microfluidic chip for at least 2 weeks, maintaining blood cell production in vitro and demonstrating responses to radiation and countermeasure drugs (Torisawa et al., 2016). In one study (Sieber et al., 2018), researchers used human tissue-derived primary MSCs and primary hematopoietic stem and progenitor cells (HSPCs) co-cultured on a scaffold to mimic human BM. By measuring stem cell factor and fibronectin production along with the expression of relevant genes (e.g., nestin, osteopontin), it was demonstrated that the MSCs provide the niche for HSPC culture. The authors demonstrated the maintenance of HSPCs in their native state (maintained multi-lineage differentiation potential) for 4 weeks (analyzed by flow cytometry). The microfluidic device had a dedicated BM compartment and a second
## Tab. 1: Characterization and context-of-use for immune system MPS and immunocompetent MPS

| Organ                     | MPS                        | Cell components           | Phenotypic characterization | Functional capabilities                                      | Application (endpoints and test articles) | Limitations                                                                 | References                                                                 |
|---------------------------|----------------------------|---------------------------|----------------------------|-------------------------------------------------------------|-------------------------------------------|--------------------------------------------------------------------------------|----------------------------------------------------------------------------|
| **Immune system MPS**     |                            |                           |                            |                                                             |                                            |                                                                                 |                                                                            |
| Bone marrow               | Bone marrow-on-a-chip      | Stromal cells, erythroid, myeloid lineages | Cell surface markers, IHC | Cell surface marker expression, antigen presentation, cell activation | Safety and efficacy; cell toxicity and recovery post-treatment | No lymphoid cells; termination of chip to extract cells limiting the conditions that can be tested; cannot assess cellular egress | Di Maggio et al., 2011; Bruce et al., 2015; Houshmand et al., 2017; Sieber et al., 2018; Chou et al., 2020 |
| Spleen                    | Spleen-on-a-chip (functional unit of red pulp) | None                        | H&E                        | Detection of RBC deformities and filtration                | Mechanical device; not cell-based       |                                                                                  | Kang et al., 2014; Rigat-Bruagarolas et al., 2014                           |
| Lymph node                | Lymph node-on-a-chip, lymphatic-on-a-chip, artificial lymph node | Stromal cells, T and B cells, DCs, monocytes | Cellular markers, cytokines, immunoglobulins, formation of germinal centers, IHC | Cellular trafficking; antigen presentation; cell activation; cell differentiation, IgG production, autoimmunity | Evaluating effects of immune modulating agents such as checkpoint inhibitors, vaccines, and adoptive cell therapies | Models often incorporate PBMCs, which do not sufficiently recapitulate the cell populations in the LN; engineering challenges with recreating the complex tissue architecture of the LN | Birmingham et al., 2020; Giese et al., 2010; Goyal et al., 2021; Kraus et al., 2019; Mitra et al., 2013; Moura Rosa et al., 2016; Shanti et al., 2020; Wagat et al., 2021 |
| Thymus                    | Regenerative thymus organ culture, thymic epithelial cell (TEC) thymocyte co-culture | TEC, thymocytes             | FOXP1 expression, T cell markers | Thymic selection, T cell tolerance induction and maturation | Thymus transplantation in DiGeorge syndrome; T cell proliferation response | Low efficiency of TEC differentiation and T cell development | Anderson and Jenkinson, 2007, 2018; Bredenkamp et al., 2014; Okabe et al., 2015; Poznansky et al., 2000; Seet et al., 2017; Tajima et al., 2016; Vianello and Poznansky, 2007 |
| Mixed                     | FBR-on-a-chip              | HUVEC, monocytes/ THP-1 | Cellular markers, cytokines | Foreign-body response, cellular migration | Implant materials | Limited scope                                                                 | Sharifi et al., 2019                                                        |
| **Immunocompetent MPS**   |                            |                           |                            |                                                             |                                            |                                                                                 |                                                                            |
| Liver                     | Liver-on-chip, organoid, 3D liver spheroids, quad culture liver chip, 3D printed liver, etc. | Kupffer cells, infiltrating myeloid and lymphoid cells | Cell surface markers (activation vs. inhibition), immune cell infiltration, TEs, cytokines and chemokines, H&E | Signaling cascade, homeostasis, protein adduction, chemotaxis | Pharmacology, safety, and efficacy of the test articles | Donor-matching of immune cells to parenchymal cells; activation of immune cells prior to incorporation into liver MPS; co-culture of immune cells with parenchymal cells or inclusion with flowing media to mimic "infiltration" | Baudy et al., 2020; Ehrlich et al., 2019; Kanabekova et al., 2022; Deng et al., 2019 |
| Lung                      | Lung-on-a-chip (e.g., on-chip) | Endothelial cells, epithelial cells | Cell surface markers, H&E, IHC, Cytokine and chemokine production, cell | Pharmacology, toxicity, bacterial and Gas exchange, mimicking cell migration to | Charcoal absorption, bleeding time | Balogh Sivars et al., 2018; Benam et al.,                                      |                                                                 |
| Organ | MPS | Cell components | Phenotypic characterization | Functional capabilities | Application (endpoints and test articles) | Limitations | References |
|-------|-----|----------------|----------------------------|-------------------------|------------------------------------------|-------------|------------|
| Brain | Brain-on-a-chip, organoids | Microglia | Cell surface markers, electrophysiology, cytokines | Nerve impulses, cell crosstalk | Efficacy, safety, target validation | Limited to use of cell lines and iPSC differentiated cells that do not fully mature | Jacob et al., 2020; Jagadeesan et al., 2020; Park et al., 2018 |
| Vasculature | Vasculature-on-a-chip platforms | Perivascular macrophages, Müller glia (organ specific) | Adhesion molecule expression (ICAM, VCAM, selectin), ECM production, H&E | Immune cell recruitment, rolling adhesion, perfusion of more complex organ MPS | Leukocyte binding, leukostasis, vascular breakdown (leakage) | Not ideal for static culture (organoid) | Delannoy et al., 2022; Lu et al., 2022; Kim et al., 2017 |
| Thrombosis-on-a-chip, vessel-on-a-chip | Endothelial cells, human whole blood, occasionally vascular smooth muscle cells | Immunohistochemical demonstration of tight junction formation, expression of tissue markers (e.g., vWF, tissue factor, ICAM-1, VCAM-1, osteopontin, ADAMTS13, etc.) | Visualization of thrombi formation; quantification of platelet aggregation, fibrin deposition, etc. | Safety, mechanistic investigations, efficacy | Low throughput; need for whole blood donor | Barile et al., 2018; Costa et al., 2017; Dupuy et al., 2021; Jain et al., 2016, 2018; Mathur et al., 2019; Rajeeva Pandian et al., 2020; Tsai et al., 2012; Westin et al., 2013; Zhang, Y. S., et al., 2016, 2017 |
| Skin | Skin-on-a-chip | Keratinocytes, dermal epithelial cells, endothelial cells, fibroblasts, Langerhans cells, dendritic cells, T cells | Cell surface markers, H&E, IHC, RNA and protein profiling | Barrier integrity, cytokine and chemokine production, cell recruitment, endocytosis | Pharmacology, inflammation (sodium dodecyl sulfate), toxicity (doxorubicin, UV), bacterial adhesion | Mimicking cell migration from lymph nodes | Bock et al., 2018; Chau et al., 2013; Hardwick et al., 2020; Jean et al., 2009; Kandarova et al., 2006; Kwak et al., 2020; Lee et al., 2014; Pellevoisin et al., 2018; Ramadan and Ting, 2016; van den Bogaard et al., 2014; Wallstabe, J. et al., 2020 |
| Tumor | 3D immune cell infiltration and cytotoxicity, engineering complex 3D tumor immune microenvironments | T cells, NK cells, monocytes, regulator T cells (Tregs) | Cell proliferation, apoptosis, cytokine production, microscopy analysis and surface markers | Anti-tumor response, chemotaxis, cell recruitment | TME physiology, pharmacological efficacy, and safety of immunomodulatory agents | Incorporation of stroma, vasculature, and immune environment simultaneously | Ando et al., 2019; Ayuso et al., 2019; Bhattacharya et al., 2020; Candini et al., 2019; Datta et al., 2020; Schnalzer et al., 2019; Wallstabe, L. et al., 2019 |
3.2 Spleen

The spleen is the largest secondary lymphoid organ, capable of performing a variety of functions. It is connected to the circulatory system and plays a central role in preventing sepsis, removing viruses, bacteria, and other pathogens, and also removing older, damaged, senescing, or infected red blood cells (RBCs). The spleen is divided into two inter-mingled major regions, the red and white pulp, separated by the marginal zone. The red pulp, composed largely of blood-filled sinuses, is involved in the mechanical filtration of RBCs as well as removal of pathogens and cellular debris.

Attempts have been made to develop MPS models that recapitulate the function of the red pulp. Specifically, one study developed a blood cleansing microfluidic design (Kang et al., 2014), while

Another recent publication (Chou et al., 2020) described a human BM model comprised of primary human-derived BM mononuclear cells and BM stromal cells (from femoral head). Wright-Giemsa staining identified erythroid and neutrophil cells while flow cytometry indicated differentiation of CD34+ cells into neutrophil, megakaryocyte, and erythroid lineages with maintenance of CD34+ cells over 4 weeks. This model was able to recapitulate clinically relevant drug (5-FU and AZD2811) toxicity (flow cytometry was used to detect lineage-specific toxicity).

Great progress has been made in the development of BM chip models, which can be maintained for up to 4 weeks and enable the differentiation and maintenance of cells of the different blood lineages. The presence of these different cell types in a single model is important for determining drug toxicity to the bone marrow, and the presence of CD34+ cells in the model described by Chou et al. (2020) allow for post-treatment recovery to be assessed. Further studies are needed to determine the optimal media composition able to promote differentiation of different cell lineages, particularly for the lymphoid compartment. Advancement in cell sampling techniques to reduce the number of required chips in turn increases the number of conditions that can be tested, and development of disease models will further drive this field forward.

Fig. 1: Schematic demonstration of a bone marrow-on-a-chip model

(A) Schematic of bone and the cell types present in the bone marrow. (B) Schematic of the cross-sectional view of a human bone marrow-on-a-chip. The model is initiated with stem cells and stromal cells within a matrix, and over time the stem cells differentiate into myeloid and lymphoid progenitors. Created with BioRender.com.
another described a human spleen-on-a-chip (Rigat-Brugarolas et al., 2014), which essentially mimics some of the filtering functions of the spleen with accurate recognition of RBC types. This latter microengineered device recreates the hydrodynamic forces and physical properties of the red pulp to maintain filtering functions.

The white pulp consists of lymphocytes and is largely involved in developing adaptive immune responses. It is organized very similarly to the white pulp observed in lymph nodes, with specific T and B cell zones, and is where antigen-specific immune responses are initiated. Lymphoid follicles are rich in B cells, with the periarteriolar lymphoid sheaths rich in T cells. This structure of the spleen allows B cells, T cells, and DCs to converge for efficient B cell maturation and activation. Thus, a splenic model must allow evaluation of effects on lymphocyte activation, maturation, and robust and reproducible antigen-specific antibody responses. To date, the development of splenic MPS models has generally lagged behind that of other models for immune organ niches including the bone marrow and lymph node (discussed in a later section).

3.3 Thymus
The thymus is a primary lymphoid organ located in the upper front part of the chest. It is composed of two lobes with a central medulla and an outer cortex in each lobe. The maturation of T cells takes place inside the thymus and is highly dependent on the interaction with stromal cells.

Growing thymic components in 2D culture can only provide very limited support of thymopoiesis, while 3D culture of the stroma better supports the development of immature T cells (Ropke, 1997; Robinson and Owen, 1977). In fact, to study thymic T cell development, several complex in vitro culture systems have been established. Fetal thymus organ cultures (FTOC) are well-established in vitro systems to study thymic T cell development including positive and negative selection and T cell tolerance upon interaction with autologous MHCs and endogenous antigens (Anderson and Jenkinson, 2007a). Modified versions of FTOC, such as hanging drop cultures and reaggregate thymus organ cultures, have been used to analyze thymus colonization and thymic epithelial cell function, respectively (Anderson and Jenkinson, 2007b). However, the human FTOC are difficult to use due to the high variability between experiments and between donors, and the limited availability of primary thymic tissues. Additionally, the maximum length of culture is only one week. To improve the culture duration and assay reproducibility, tissue-engineered thymic organoids were developed with either thymic fragments or a delta-like canonical notch ligand 1 (DLL1)-transduced stromal cell line to successfully support the development of functional human T cells from hematopoietic precursor cells (Seet et al., 2017; Poznansky et al., 2000). To form the organoids, compaction reaggregation by centrifugation of stromal cells and precursor T cells as well as various 3D scaffolds were used by different groups. The 3D scaffolds have included synthetic material such as CellFoam matrix, a carbon network coated with tantalum, and natural extracellular matrix of the thymus composed of laminin, collagen, and fibronectin (Poznansky et al., 2000). Instead of using organoids, fibroblasts, a key cell type of thymic stroma, were successfully reprogrammed into thymic epithelial cells (TECs) via overexpression of transcription factor forkhead box N1 (FOXN1) (Bredenkamp et al., 2014). The FOXN1-induced TECs supported the development of both CD4+ and CD8+ T cells in vitro, while in vivo transplantation of these TECs developed a fully organized and functional thymus. Even though this work was conducted in laboratory animals, the in vivo reprogrammed TECs may shed light on an alternative approach for human thymus transplantation. Pluripotent stem cell-derived TECs in a 3D sphere culture have also been used to support T cell development in mice (Okabe et al., 2015).

The quality of the thymic culture is commonly characterized by the phenotypic and functional measurement of the T cells. The different developmental stages of T cells are determined by their cell surface antigens, and fully matured T cells should proliferate and express activation markers in response to antigen presenting cells. The diversity of TCR repertoire is usually measured by flow cytometric analysis of TCR Vb frequencies or by deep sequencing of both Vα and Vβ CDR3 regions. Despite the extensive effort, currently in vitro thymic culture still only recapitulates partial thymic function (Vianello and Poznansky, 2007). In addition, the differentiation of T cell subsets, especially the development of regulatory CD4+ T cells, has not been well characterized, suggesting a limitation of current in vitro models to support the maturation of different T cell subsets.

Recently, with the emergence of T cell therapy for cancer, reprogramming allogeneic T cells into autologous T cells is a new field to involve in vitro thymus MPS. Presumably, the hematopoietic precursor cells from allogeneic donors can develop central tolerance against recipient cells when encountering medullary TECs from the recipients (Kyewski and Klein, 2006). A more complex model such as a thymus-on-chip system with further improved T cell selection and maturation will be the next generation of in vitro thymic culture systems.

3.4 Lymph nodes
Microfluidic lymph node-on-a-chip (LNoC) cultures are being explored as a tool for evaluating the efficacy, potency, and immunotoxicity of immunomodulatory agents such as immune checkpoint inhibitors, vaccines, and adoptive cell therapies. In addition to evaluating effects on antigen-specific immune responses, LNoC could be multiplexed with other MPS to measure effects on intercellular interactions and trafficking of immune cells between the LN, other lymphoid tissues, and other organs such as the gut, skin, lung, and tumor. The dynamic and complex architecture and specialized cell types of the LN make it a challenging organ to engineer, thus groups have been initially working to recapitulate specific LN areas and/or functions (Morsink et al., 2020).

LN are secondary immune organs encapsulated in connective tissue with extensions (trabeculae) through which lymphatic fluid flows in carrying antigens and APCs viafferent vessels that pass through the subcapsular, cortical, and medullary sinuses before exiting through efferent vessels. High endothelial venules line the post-capillary venule in the deep cortex through which lymphocytes, including naïve T cells, travel to the deeper cortical regions. The outer cortex contains B cell follicles, which consist primarily of proliferating B cells and plasma cells located within germinal centers, while the inner cortex consists mainly of
T cell zones including follicular helper T cells with macrophages and DCs present throughout. Beyond the cortex is the paracortex area followed by the inner medulla area, which is populated with a variety of lymphocytes including B cells, plasma cells, and macrophages. Different stromal cell types including fibroblasts and reticular cells also populate the LN tissue to help facilitate normal lymphocyte function and trafficking. Ultimately, a biomimetic LN MPS should recreate the structural microenvironment of a native LN and contain the relevant cell populations organized in appropriate anatomical domains as well as mimic the flow pattern of lymphatic fluid through the LN (Shanti et al., 2020). Functionally, immune cells must be able to migrate in response to environmental cues (e.g., cytokines/chemokines) and for APCs to effectively present antigen to T cells and B cells to facilitate antigen-specific activation, proliferation, differentiation, and antibody class switching.

In 2010, Giese et al. reported on an artificial LN bioreactor system created to evaluate localized immune cell functions. The device was seeded with human PBMCs including TNF-α-matured DCs. Micro-organoids containing B cells, T cells, and DCs formed within 7 days of culture and promoted B cell maturation and plasma cell development. Introducing DCs treated with a hepatitis A vaccine or cytomegalovirus antigens induced cytokine production and antigen-specific IgM that could be modulated by exposure to dexamethasone. Kraus and colleagues (2019) used a similar system to evaluate the immunogenicity of protein aggregates. When exposed to heat, the anti-VEGF antibody bevacizumab was found to form protein aggregates and subsequently induce a Th1 and proinflammatory response when introduced to the culture system.

A LNoC device was used to examine DC chemotaxis and effects on T cell activation (Mitra et al., 2013). Human T cells isolated from PBMCs were seeded into the bottom chamber of the device while DCs derived from the MUTZ-3 cell line were seeded into the top chamber. The chambers were connected by channels perfused with media. When a CCL19 chemokine gradient was introduced, 35% of mature DCs showed migratory movement, with some demonstrating complete migration through the chemotaxis channel. Comparatively, 16% of mature DCs showed only slight movement in the absence of chemokine and <3% of immature DCs showed movement regardless of the presence of the chemokine gradient. The mature DCs were also shown to induce T cell activation in the system.

Moura Rosa et al. (2016) developed and an LNoC system to model the paracortical region of the LN to examine DC-T cell interactions under different physiological conditions. A monolayer of mouse DCs treated with bacterial lipopolysaccharide and loaded with ovalbumin (OVA) peptides was introduced into one chamber. OVA-specific T cells were introduced to a second chamber, and migration of T cells to the DC monolayer was measured and found to be independent of the flow direction. Under varying conditions to mimic the physiological shear stress of the LN T cell zone, the CD4+ T cells showed long stable interactions with the DCs whereas CD8+ T cell interactions were more transient. These interactions were also stronger for OVA-specific T cells compared with non-specific T cells. Under lower shear stress conditions, OVA-specific T cells steadily accumulated at DCs.

Researchers at the Wyss Institute also developed a LNoC model to recapitulate the structure and function of the B cell follicle areas of the LN (Goyal et al., 2021). The bottom channel of a microfluidic platform was filled with Matrigel and Type I collagen and populated with a high density of human blood-derived T cells and B cells at equal ratios while the top channel was continuously perfused with culture medium. The B and T cells self-assembled into 3D germinal center-like follicles within 3-4 days of culture under flow. B cells underwent class switching and differentiated into antibody-secreting plasma cells by 7 days of culture after exposure to IL-4 and anti-CD40 or Staphylococcus aureus bacterial antigens. Delivering DCs with influenza vaccine also induced the production of flu-specific IgG and cytokines consistent with serum responses to the vaccine in humans.

Birmingham et al. (2020) engineered a LN subcapsular sinus-on-a-chip to study LN metastasis and the effects of changes in wall shear stress that occur in an inflamed LN and remodeled microenvironment. Changes in the fluid flow microenvironment were shown to regulate E-selectin-enabled adhesion of human metastatic colon and pancreatic carcinoma and monocyte cell lines. Furthermore, co-perfusion of monocyte cell line THP-1 was shown to further increase metastatic cell adhesion. These results suggest that the biophysical effects of LN inflammation and remodeling can regulate mechanisms of metastatic LN invasion. Incorporation of additional immune cell components into this system could help increase our understanding of the interactions of metastatic cells within the LN to help identify more effective cancer treatment strategies.

Ideally, a LNoC model should allow for examination of effects on cellular trafficking, antigen presentation, cellular activation and differentiation, and antigen-specific antibody production that are human-relevant and highly reproducible. Over the past decade, great progress has been made in the development of LNoC systems but to date, none of the systems have been able to fully reproduce the complete tissue structure and the humoral response, suggesting further bioengineering work is needed. An additional challenge has been that the cells introduced into the LN MPS devices are often derived from PBMCs and/or immortalized cell lines and may not accurately recapitulate the leukocyte subpopulations present in the native LN environment. Cell suspensions prepared from lymphoid tissue sources such as tonsils, which, as organoid structures, have been demonstrated to support antigen-specific somatic hypermutation, affinity maturation, and class switching of human B cells (Wagar et al., 2021), are being evaluated for their potential to recapitulate a functional lymphoid microenvironment.

### 3.5 A foreign body response on-a-chip

A microfluidic platform has been developed to model the foreign body response (FBR) to implants (Sharifi et al., 2019). This system was used to measure monocyte-endothelium interactions, transendothelial migration, and activation against implant materials. The polydimethylsiloxane (PDMS)-based system includes a bottom chamber containing titanium microbeads (foreign body) surrounded by ring-channels containing gelatin methacryloyl (GelMA) hydrogel with monocyte chemoattractant protein-1 (MCP-1). Above
is a polyethylene terephthalate membrane topped with a monolayer of human umbilical vein endothelial cells (HUVECs) to model the endothelial barrier between the vascular lumen and tissue. THP-1 monocytes or primary human monocytes were perfused into the top chamber connected to a peristaltic pump to mimic vascular circulation. MCP-1 release from the extracellular matrix hydrogel induced transendothelial migration of circulating monocytes. THP-1 monocytic cells were shown to differentiate into a proinflammatory M1 phenotype in response to titanium microparticles. Proof-of-concept experiments using individual human donor-derived monocytes revealed inter-individual differences in the FBR to titanium microparticles, evidenced by differences in M1 & M2 phenotypes. The FBR-on-a-chip platform is proposed to be a personalized, high-throughput approach that could be used to identify suitable implant materials.

4 Immunocompetent organ MPS

4.1 Implementation of immune cells into non-immune MPS models

Immune cells are known to play a role in a number of autoimmune and inflammatory diseases. In recent years, however, unregulated immune cell responses have also been linked to diseases that were previously not considered to include an immune cell component. This highlights the need for the development of immunocompetent MPS representative of different immunocompetent tissues, such as liver, lung, gut, brain, skin, vasculature, and tumor. This will be discussed below in greater detail in the context of different tissues/organs (Tab. 1).

4.2 Liver

The liver is a complex organ of the human body, the cardinal site for metabolism, and, unlike the other organs, can regenerate. At the cellular level, the liver comprises mainly two cell types: the parenchymal and non-parenchymal cells (NPCs). The parenchymal cells, predominantly hepatocytes, are responsible for production of acute phase proteins and other plasma proteins, including albumin. The NPCs are composed of sinusoidal endothelial cells (LSECs), Kupffer cells (KC), hepatic stellate cells, and pit cells, mainly liver-specific NK cells. There is also a constant flow of infiltrating peripheral immune cells including monocytes, neutrophils, activated NK cells, helper and cytotoxic T cells in the liver, especially during organ injury, fibrosis, and autoimmune hepatitis (Ramadori and Saile, 2004). Importantly, recruitment of extra-hepatic immune cells is necessary for injury resolution and initiation of hepatic repair (Kubes and Jenne, 2018; Karlmark et al., 2008). Drug-induced liver injury (DILI), which is responsible for more than 50% of acute liver failure in the clinic, is a major cause for drug failure (Hussaini and Farrington, 2007).

Stimulatory Fc receptor expression on tissue resident LSECs and KCs is hypothesized to provide crosslinking and activation conditions for various monoclonal antibody drugs to induce liver toxicity. A recent study suggested that IL-27 released from KCs in response to T cell costimulatory receptor agonist antibodies stimulates T cells and other innate immune cells in the liver and leads to inflammatory responses in hepatic stellate cells and hepatocytes (Bartkowiak et al., 2018).

A pharmaceutical survey suggested a very low correlation between preclinical liver toxicity and clinical outcome, indicating a predictive value of liver toxicity in monkeys, dogs, and rats for human liver toxicity of 50%, 27%, and 33%, respectively (Liu et al., 2011). At present, various complex in vitro liver models are being developed, such as micropatterned models, hepatocyte-derived spheroids, 3D-bioprinted liver, stem cell-derived liver organoids, and liver-on-chip models to address this issue (Baudy et al., 2020; Ehrlich et al., 2019; Kanabekova et al., 2022; Deng et al., 2019). These in vitro liver models go beyond the 2D he-
patocyte sandwich culture model; however, they can be time-consum- ing to develop and optimize for late-stage drug translation. Therefore, it is extremely important to have well-defined context(s)-of-use for these advanced models.

The specific context-of-use and application will dictate the com- position of the liver MPS. For example, if a liver MPS model is to be used for identifying a mechanism of immune-mediated DILI, inclusion of KCs and/or other relevant infiltrating leukocytes will be necessary. Consideration should be given as to whether immune cells should be included in the co-culture with parenchymal and endothelial cells or if the endothelial and hepatocyte compartments should be separated and immune cells included in the flowing me- dia to better recapitulate immune cell infiltration into the liver. Immune cell pretreatment (e.g., activation) and donor-matching of immune cells with hepatic parenchymal cells should be considered when designing immunocompetent liver MPS. Overall, an ideal liver MPS model will include immune components to recapitu- late immune related clinical safety and efficacy for a large body of emerging immunomodulatory drugs (Fig. 2).

4.3 Lung
The lung, a complicated tissue to model (Ainslie et al., 2019), comprises an intricate network of immune cells that are critical for host defense but are also involved in the pathogenesis of al- lergic and autoimmune disorders. Investigations of allergic air- way inflammation have extensively characterized the role that resident immune cells in the lung play in the pathogenesis of these disorders (Lambrecht and Hammad, 2012a) and this has in part been recapitulated in MPS (Lambrecht and Hammad, 2012a; Benam et al., 2016). There are also other examples where lung MPS have been adapted to include other immune cells (Balogh Sivars et al., 2018; Yu et al., 2016; Chandorkar et al., 2017), some of which are described below. However, to date, neutro- phils, monocytes, and macrophages that extravasate into the lung and initiate signaling cascades in response to antigen, resulting in immune cell infiltration and inflammation (Lambrecht and Hammad, 2012a,b; Lambrecht et al., 1998; Nakano et al., 2012; Condon et al., 2011; Boda et al., 2018), are lacking in commer- cially available MPS. Furthermore, downstream activation of T cells, B-cell production of IgE, and basophil degranulation found in human disease have not been explored using complex in vitro lung models.

One of the reported lung MPS integrating components of the immune system is a small airway chip composed of primary hu- man airway epithelial cells and lung microvascular endothelial cells with a type 1 collagen-coated polyester membrane and an air-liquid interface that has shown cellular changes in response to IL-13 similar to other models for asthma (Manson et al., 2020; Benam et al., 2016). While not able to exhibit the full spectrum of pathologic events (Holgate, 2012), this MPS platform was also capable of demonstrating changes including poly (I:C)-medi- ated upregulation of E-selectin and VCAM-1 on endothelial cells and evidence of neutrophil recruitment when neutrophils were added to the fluidics channel (Benam et al., 2016). More recently, the human airway chip was demonstrated to model human lung response to respiratory viral infection, suggesting its use in eval- uating efficacy of anti-viral therapeutics (Si et al., 2021). When primary human neutrophils were perfused into the vascular chan- nel, the cells were recruited to the apical surface of H1N1 or H3N2 influenza virus-infected lung endothelium and then trans- migrated to the airway epithelium where the cells demonstrated functional activity against infected cells, increased cytokine production, and reduced viral titers. Introduction of SARS-CoV-2 pseudovirus into the air channel led to subsequent infection of lung-airway epithelial cells that was inhibited when neutralizing SARS-CoV-2 mAb or antiviral drugs were introduced.

There is promise for developing a lung chip to accommodate a greater number of immune cell types (e.g., macrophages, eosinophils, mast cells, monocytes, T cells, dendritic cells, and B cells), allowing for comparisons of discrete or multiple phases of airway disease, such as resident macrophage changes and pe- ripheral immune cell homing, maturation, activation, and result- ing changes to the tissue microenvironment. Airway MPS should allow for transmigration of immune cells between the epithelial and endothelial layers, thus it requires a membrane with suffi- cient pore size that still preserves the tight pseudostratified epi- thelium structure. Recent work has described the various resident and homing immune cell populations found in the human lung, which may be helpful for future immunocompetent lung MPS design (Travaglini et al., 2020; Yu et al., 2016).

4.4 Gut
The gastrointestinal (GI) tract is one of the most complex organs in the human body, both structurally and functionally. The small and large intestines together facilitate many pivotal functions in- cluding digestion, absorption, peristalsis, excretion, and host pro- tective mucosal immunity to various invading pathogens, toxins, and resident microbiota (Steinway et al., 2020). The intestinal epithelium is a physical barrier between the lumen and tissue and provides vital protection to the host from external pathogenic mi- croorganisms, allergens, and internal gut microbiota. Pathogenic infection, immune dysregulation, and epithelial disruption result in a disturbed homeostasis and are recognized to be associated with a variety of diseases including inflammatory bowel diseases (IBD), multiple sclerosis, and autoimmune hepatitis (Wei et al., 2020). Therefore, intestinal homeostasis is an absolute necessity (Peterson and Artis, 2014). Genetically, chemically, and immu- nologically manipulated mouse models have been developed to study gut physiology and disease pathology; however, they do not fully mimic human physiology (Wikswo, 2014). Therefore, there are numerous ongoing in vitro efforts to decouple drug-re- lated GI liabilities from efficacy to improve prediction of safety outcomes in the clinical setting over that of preclinical models.

Gut MPS are advanced in vitro models that aim to recapitu- late the in vivo gut environment using multiple cell types together with mechanical stimuli to mimic physiologically relevant func- tions. Various advanced MPS models, including organ-on-chip, organoids/spheroids, and 3D bioprinting, are under development to address such critical tasks. However, most of these systems lack physiological biomechanics, immune components, and con- trollable protocols for a longitudinal host-microbiome co-culture (Steinway et al., 2020). Being the largest immune organ in the
human body, studying only biological responses through gut MPS models would incompletely cover disease pathology, drug safety, and efficacy. To address these caveats, work on advanced gut MPS models is underway to emulate the human immune system in vitro. Possible incorporation of microbiome components (inflammatory vs regulatory) in addition to immune cells and intestinal epithelial cells (IECs) would be an ideal human MPS system that could be beneficial to study various parameters including immune cell crosstalk with epithelium, dysbiosis, homeostasis, inflammatory vs regulatory immunity.

For example, a gut-on-a-chip system (Xiang et al., 2020; Shanti et al., 2018; Ambrosini et al., 2020) facilitated investigation into immune responses at the luminal phase triggered by gut microbiota in comparison to harmful bacterial products. This model can be further leveraged by studying inflammatory and regulatory immune responses based on the specific requirements. Technology pioneered by Hans Clevers (Sato et al., 2011) has enabled the generation of gut organoids that incorporate multiple epithelial cell types. Co-culturing organoids with immune cell or immune cell-derived factors permits the study of the interplay between epithelium and immune cells (Bar-Ephraim et al., 2020). Elegant work with a biochip made of polystyrol was used to study immune responses induced by commensal and harmful bacteria. This MPS device included resident APCs to differentiate between normal physiology and the acute innate immune response activated by bacterial lipopolysaccharide (Maurer et al., 2019).

Efforts should be made to include additional GI immune resident cells, such as innate lymphoid cells (ILCs), known to be involved in GI homeostasis and a variety of diseases including ulcerative colitis, Crohn’s disease, and inflammatory fibrosis (Wang, S. et al., 2018; Buela et al., 2015). Addition of gut infiltrating immune cells such as monocytes, macrophages, DCs, T cells, B cells, and granulocytes can also be useful to evaluate drug-mediated safety and efficacy. An ideal GI MPS system would include autologous IECs, immune cells, gut microbiota, and other important GI components integrated with basolateral flow to the liver and apical access.

4.5 Brain
The central nervous system (CNS) includes the brain and the spinal cord, and for years was considered an immune-privileged tissue separated from the systemic immune system by the blood brain barrier (BBB). However, recent findings of neuroimmune interactions occurring under homeostatic and pathological conditions have redefined the concept of immune-privileged (Louveau et al., 2015).

Penetration through the BBB allows the infiltration of immune cells into the CNS and the development of diseases, with examples including multiple sclerosis (MS), Alzheimer’s disease, Parkinson’s disease and Huntington’s disease (Sweeney et al., 2018). In addition to infiltrating immune cells, microglia have been implicated in a number of neurodegenerative diseases including Alzheimer’s disease, Parkinson’s disease, and frontotemporal dementia (Bachiller et al., 2018). Microglia are brain tissue resident macrophages that play an important role in the maintenance of neuronal function under healthy conditions. During inflammation, microglia participate in the destruction of neuronal tissue.

The aforementioned diseases represent areas of unmet clinical need as developing drugs to treat neurological diseases have had limited success (Butlen-Ducuing et al., 2016). One reason is a lack of physiologically relevant in vitro human models besides limited in vivo animal models. This is changing with the advent of MPS platforms, including brain-on-a-chip, brain organoids, and bio-printed models, into which immune cells can be incorporated.

An example is a human microfluidic 3D model of Alzheimer’s disease, incorporating neuronal cells, astrocytes, and microglia (Park et al., 2018). In this model, neuronal cells were engineered to produce β-amyloid and tau protein aggregation, resulting in chemokine release and enabling the recruitment of microglia to be tracked. Activation of microglia resulted in neuronal cell death, while inhibition of their activation rescued the neuronal cells.

A loss of barrier function contributes to the development of MS. T cells play a significant role in the development and progression of MS, following the breakdown of the BBB (van Langaal et al., 2020). A model described by Jagadeesan et al. (2020) incorporating iPSC-derived endothelial cells, neuronal cells, and astrocytes paves the way to incorporate immune cells and study the effects of drugs in the context of a mixed population of cell types in a human BBB model system.

Jacob and coworkers demonstrated that a patient-derived glioblastoma organoid model recapitulates inter- and intratumoral heterogeneity, and the model could be applied for assessing the efficacy of CAR-T cell therapies (Jacob et al., 2020).

The development of immunocompetent brain models poses several challenges. The protocols for differentiation of human neuronal cells from iPSCs are long, complex, and labor intensive. For diseases that often do not manifest until people attain a mature age, recapitulation of an aged brain, let alone a diseased aged brain, remains a significant challenge. Taking Parkinson’s disease as an example, the hallmark features of pathology are a loss of dopaminergic neurons in the substantia nigra. The roles of different cell types involved in the initiation and progression of Parkinson’s disease are not fully understood, but there is increasing evidence that microglia are implicated. Reproducing the cardinal features of Parkinson’s disease is the ultimate goal; however, testing the model for therapeutic efficacy is limited as there are no drugs currently available to benchmark against.

4.6 Vasculature
Immunocompetent endothelial MPS should include immune cells that commonly circulate in human whole blood. The exact composition of the immune cells chosen may depend on the tissue or disease area of interest, e.g., peripheral vasculature vs lymphatic vessels. PBMCs isolated from whole blood are a source of primary human immune cells for use in such models. These cells can be co-cultured with endothelial cells to produce immunocompetent MPS. If a specific immune cell type or population is desired, isolation of those subsets from PBMCs can be performed and then recombined to achieve the right composition. Tissue digests can also be performed to isolate organ-specific immune cell populations.
A common interest when investigating endothelial-immune cell biology is rolling-adhesion and extravasation (McEver and Zhu, 2010). Rolling adhesion is an important aspect of the immune response when immune cells are recruited from the periphery to sites of inflammation via chemoattractant gradients. To accurately model this process in vitro, the MPS must include laminar flow and thus, shear stress. Endothelial cells in vivo experience shear stress as blood flows through the vessels. This shear stress is required for normal endothelial cell morphology and function (Levesque and Nerem, 1985), and optimal rolling adhesion of leukocytes (Lei et al., 1999). The flow rate and oscillation properties can be adjusted to model different regions of the vasculature. Examples could include capillaries and large arteries, which have differing flow rates, or arterial branch points, which experience disturbed laminar flow (Davies et al., 2013; Malek et al., 1999). Many chip-based MPS platforms include some version of fluidics-driven flow.

Potential applications of an endothelial MPS model might be in determining the activity of small molecules or biologics designed to limit immune cell activation, rolling adhesion, and extravasation, e.g., integrin inhibitors (Kim et al., 2017; Lu et al., 2022; Delannoy et al., 2022). This type of system could also be used to evaluate the effects of therapeutics on disturbed flow-induced endothelial lesions, which are vascular regions susceptible to atherosclerotic plaque formation, or for the study of vasculitis. There are also safety applications for endothelial MPS platforms which include examination of compounds that may exhibit vascular toxicity or cause leakage by perturbing intercellular junctional complex.

Thrombosis-on-a-chip is a type of MPS specifically modeling cardiovascular disease. Rudolf Virchow described three factors contributing to the formation of thrombi in 1856: 1) alterations in blood flow such as turbulence or stasis, 2) changes in blood constituents, and 3) damage to the vessel wall (Kumar et al., 2010; Ramot and Nyska, 2007). These original tenets, termed Virchow’s triad, have been repeatedly revisited as new data have come to light. These include further elucidating pathways to thrombi formation, stabilization, and potential for embolism with increased emphasis on mechanisms of endothelial cell damage, platelet biology, perturbations to the coagulation and fibrinolytic systems, and the roles of red and white blood cells and cytokines (Owens and Mackman, 2010; Ramot et al., 2013; Raskob et al., 2018; Twomey et al., 2018). Despite increased mechanistic knowledge of thrombosis dynamics, a need has remained for translational in vitro models, particularly for biological therapeutics, which has paved the way for the development of vessel-chip and thrombosis-on-a-chip devices capable of incorporating fluidics and real-time monitoring and visualization of hemodynamics (for a detailed review, refer to Hosseini et al., 2021).

The design and application of such models has expanded considerably in recent years with a focus on investigating drug-induced thrombosis, probing mechanisms of disease-mediated prothrombotic states and vascular inflammation, and testing effects of therapeutic interventions (Barrile et al., 2018; Jain et al., 2018; Mathur et al., 2019; Westein et al., 2013). Microfluidic devices of varying geometries have been reported, with some utilizing the geometry itself to recapitulate stenosis by progressively narrowing the luminal space and studying effects on platelet behavior (Costa et al., 2017; Westein et al., 2013). Most have employed a primarily linear trajectory in modelling vessel structure (Barrile et al., 2018; Costa et al., 2017; Jain et al., 2016, 2018; Mathur et al., 2019; Zhang, Y. S. et al., 2016); however, some developers have incorporated increasingly complex bifurcations and bends that more closely mimic the microvasculature of capillary beds (Tsai et al., 2012; Zhang, Y. S. et al., 2017). Rajeeva Pandian and colleagues fashioned a linear vasculature channel with invasions reminiscent of deep vein valves at each end, which enabled visualization of platelet aggregation and fibrin deposition at the valve cusps and in the vessel lumen (Rajeeva Pandian et al., 2020). The model was responsive to cytokines and antimicrobials and demonstrated the effects of the degree of stenosis on flow velocity and amount of fibrin deposition (Rajeeva Pandian et al., 2020). The majority of models reported have relied on what could be considered a simple but effective cellular design, specifically HUVECs or human microvascular endothelial cells to line the engineered vessel channels with addition of human whole blood to the vasculature lumen (Barrile et al., 2018; Costa et al., 2017; Jain et al., 2016; Tsai et al., 2012; Westein et al., 2013; Zhang, Y. S. et al., 2016). Recent investigations have incorporated human blood outgrowth endothelial cells as a patient- and disease-specific source of primary human cells, further expanding biological relevance and potential for disease modeling (Mathur et al., 2019). Jain and colleagues utilized a human lung alveolus-on-a-chip model consisting of primary alveolar epithelial cells and an endothelial cell-lined chamber (Jain et al., 2018). Though initially intended as an alveolus model, addition of human whole blood to the endothelial-lined vascular chamber provided for an organ-level assessment of hemodynamics and reproduced activation of thrombosis through perturbations of the epithelial layer (Jain et al., 2018).

Though advancements in thrombosis-on-a-chip and vessel-chip models are ongoing, there remains an opportunity to further expand modelling focus and/or probe model capabilities. Namely, Barrile et al. (2018) reported recapitulation of the thrombotic effects of a monoclonal antibody in a vessel-chip model that were not identified in preclinical studies. The model also allowed the researchers to investigate modifications of the antibody to arrive at one that did not elicit thrombogenic effects. While this study certainly exemplifies the potential power of such an in vitro system to probe the effects of a biological therapeutic in a preclinical setting, published studies of additional biologics would be helpful to determine the robustness and broader applicability of such models.

Few, if any studies have examined the predictive capabilities of thrombosis-on-a-chip or vessel-chip models to identify small molecules with thrombogenic potential in a robust manner. Lastly, thrombosis-on-a-chip and vessel-chip models rely on the use of human whole blood to provide a physiologically relevant source of platelets and blood components; however, downstream analyses have centralized their focus primarily on platelets. There is a paucity of analyses on the contributions of other blood components and cells to thrombogenesis in an MPS setting. Both platelets and neutrophil extracellular traps (NETs) have been identified as having a primary role in the early events of thrombogenesis in vivo in animals and humans (Martinod and Wagner, 2014; Savchenko et al.,
2014) yet data are limited. Only recently have there been reports on neutrophil interactions with the endothelium (Dupuy et al., 2021) and/or subsequent NET formation in the context of human MPS models. There is significant opportunity to increase knowledge and probe the capabilities of thrombosis-on-a-chip and vessel-chip models with respect to the full representation of immune cells in human whole blood samples. Lastly, low model throughput and the need for whole blood can often be time-consuming and a costly limiting factor to the utility of vessel-chip and thrombosis-on-a-chip models in the drug discovery setting. Knowledge of human whole blood donor disease state and/or pharmaceutical use can be critical to ensuring sound study design and proper interpretation of results. Within the setting of pandemics, such as COVID-19, human whole blood donor access and implications of disease history are much more prominent in the context of hemodynamic studies. Future improvements to address such issues would make these models more adaptable to a screening setting.

4.7 Skin
Skin models represent one of the most widely reported MPS models of those described (Hardwick et al., 2020) and thus may pose the best opportunities to study immune responses in an in vitro setting. Additionally, animal testing for chemical contact hypersensitivity potential is not permitted for cosmetic ingredients or products in Europe. Immunocompetent skin MPS ideally would build upon current models including epidermal and dermal layers while adding primary human DCs, T cells, and mast cells with a phenotype and function similar to that demonstrated in intact human skin, allowing in vitro drug testing directly on human cells (Matejuk, 2018; Morsink et al., 2020).

Characterization of individual immune cell types in skin MPS have been performed to answer specific questions. For example, addition of CD4+ T cells was useful to demonstrate T cell migration into the dermis and interaction with epithelial cells where the T cells were activated in a separate culture step prior to addition to the skin MPS (van den Bogaard et al., 2014). In a separate study, DCs were differentiated separately and added to an agarose-fibronectin gel layer to monitor migration and differentiation (Chau et al., 2013). In another skin model composed of human keratinocytes cultured on a polycarbonate filter at the air-liquid interface, Langerhans cell progenitors were introduced during tissue seeding (which then differentiate in the supra-basal epidermal layer) to evaluate skin irritation and dermal sensitivity (Lee et al., 2014; Kandárová et al., 2006; Pellevoisin et al., 2018).

Collagen skin models and vascularized skin models comprised of primary keratinocytes, fibroblasts and microvascular endothelial cells isolated from human biopsies were used to demonstrate inflammation-induced tissue damage with pre-stimulated auto- or allogeneic PBMCs (Wallstabe, J. et al., 2020). Bock and colleagues presented a reconstructed human full thickness skin model incorporating Langerhans-like cells derived from either the MUTZ-3 AML cell line or monocytes for skin sensitization investigations (Bock et al., 2018). Additional skin MPS have employed a mixture of cell lines and primary cells to construct the different cellular components (Ramadan and Ting, 2016; Kwak et al., 2020), but when selected, cell lines should be compared side-by-side to the primary cell counterpart under similar test conditions (or to humans) to demonstrate the relevant phenotype and function is represented in the MPS.

Together, more comprehensive skin MPS could be designed with both epidermal/dermal and resident immune cell populations to allow for allergen or toxin exposure to the epidermal cell layer with DC antigen capture, presentation, processing, and downstream stimulation of T cell recruitment via the fluidics channel. The utility of validated immunocompetent skin MPS could include the study of skin injection site reactions with peptide and protein-based pharmaceuticals and examination of test article effects via direct (topical) or indirect (systemic) exposure on resident or migratory immune cells with respect to their role in the pathogenesis of disease such as atopic dermatitis or psoriasis. Specifically, Jean and coworkers (2009) reported on a model for psoriasis using a bilayered skin substitute containing a bottom layer of fibroblasts and a top layer of keratinocytes isolated from healthy and patient cells which could be adapted into immunocompetent skin MPS.

4.8 Tumor
A plethora of preclinical and clinical trials are currently underway to investigate a rapidly evolving field of new oncology immunotherapies for both liquid and solid forms of tumors. Owing to the enormous clinical successes of immune-checkpoint blockades such as pembrolizumab (anti-PD-1) and ipilimumab (anti-CTLA-4) (Robert et al., 2015), the current strategies of immunotherapies focus on either augmenting the immune effector cells’ capacity to kill tumor cells or overcoming immune suppression induced by tumors or tumor microenvironment (TME) (Shimizu et al., 2018; Ishiguro et al., 2017). Of those modalities that aim at stimulating effector cells, a great deal of attention is still being paid to activate immune cells by either directly engaging signal 1 (e.g., CD3/TCR) or by modifying signal 2 co-stimulatory molecules (e.g., CD28, PD-1) to enhance cytolytic activity of effector cells (Goebeler and Bargou, 2020). For overcoming immune suppression, there are a variety of immunomodulatory drugs in development to modify inhibitory immune mediators expressed on various immune cells or non-immune cells in the TME (Bejarano et al., 2021).

Preclinical testing of these immunomodulatory compounds for potential efficacy and (immuno)toxicity is becoming a critical area of study due to the complex design of engineered antibodies and increasing number of potentially immunogenic drugs in the pipeline. Animal models are widely used for the evaluation of immune responses prior to commencing clinical trials. However, such models do not fully recapitulate the immune responses observed in humans. A variety of human MPS models have been developed to mimic the structural and functional complexity of the human tumor physiological system that is lacking in conventional 2D monolayer cell cultures. One of the major challenges in the development of these MPS platforms is incorporation of the human immune, stromal, and endothelial environment that truly recapitulates solid TME observed in vivo. Successful inclusion of these parameters justifies a very relevant TME that is immunosuppressive, hypoxic, and acidic in nature and filled with im-

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munoregulatory milieu that suppresses the anti-tumor activity of effector cells (Bhattacharya et al., 2020).

These advanced MPS are being engineered not only to recapitulate the physiology of human organs/tissues but also to mimic immune responses and a physiologically ideal TME (Datta et al., 2020). For instance, a customizable 3D model was recently developed that recapitulated the anti-tumor efficacy of TCR-engi-

5 Opportunities for immunocompetent MPS in drug development

5.1 Context-of-use

Before the advent of MPS, biopsies, discarded surgical tissue, or tissue from cadavers provided the best opportunity to study events in the context of multiple cell types and architecture. Ac-

5.2 Characterization/qualification recommendations

The perfect immunocompetent MPS model would recapitulate all aspects of a tissue. A-fit-for-purpose approach should start with first examining the needs and specific questions to be answered. An understanding of the tissue’s physiology, pathophysiology of the disease of interest, and the drug’s mechanism of action will help guide MPS design. The cellular composition must include benchmarking against primary healthy and diseased tissues. Given the structural and functional complexity of any tissue, the design of immunocompetent MPS can become quite intricate, not just in the cellular makeup but also in the source (e.g., autolo-

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The attrition rate of drugs in the clinic provides evidence that the historically used models may not be fit-for-purpose for those unsuccessful clinical candidates. A recent review examined failed antibody therapeutics between 2014 and 2019 and found late-stage failure of monoclonal antibodies could have been caused by inadequate understanding of human diseases, lack of prediction of anti-drug antibody, false biomarker identification, and poor translatability of preclinical results to clinical outcomes (Sun and Benet, 2020). For example, immunogenicity was cit-

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downstream of T cell extravasation are needed, then cytokine production or a longitudinal evaluation should be included.

When selecting immunocompetent MPS, it is important to keep the MPS minimally complex as needed to answer key investigational questions while mimicking the in vivo situation as far as possible. Characterization of the phenotype and function of both infiltrating immune cells and resident tissue cells should be performed and should be similar to that observed in human tissues under normal conditions and the pathologic condition of interest. Confocal fluorescence microscopy and flow cytometry are powerful tools to characterize both tissue cells and immune cells during MPS platform qualification. Cell-to-cell interactions, cytokine production, cell recruitment, and effects on tissue resident cells with and without stimulation should be validated with well-described positive and negative controls that act on a mechanism relevant to the test article or process under investigation.

5.3 Practical consideration for pharmaceutical use: challenges, gaps, and opportunities

While MPS models bring many advantages as discussed in previous sections, there still exist several challenges to their adoption, which will be outlined in the following section.

As complexity of these models increases, throughput decreases, making their use impractical for early stages of drug discovery where high or medium throughput screening assays are required (Low et al., 2021; Sung et al., 2019). Their complexity also means studies take longer to run than conventional cell culture, and this makes them technically challenging to set up and maintain, thus the technical robustness of the data is an important factor. Ideally, to recapitulate the organ’s physiological or pathophysiological properties, these models would mimic all relevant features, such as relevant scaffolds, cell-to-cell and cell-to-matrix interactions, oxygen, and nutrient gradients. However, typically, the models, though complex, do not meet all these criteria (Langhans, 2018). This may affect the physiological relevance of the model; however, it could also be considered that complete recapitulation of the in vivo scenario is not required for a representative model. Since the majority of MPS models only partially recapitulate in vivo tissue complexity and architecture, this may also limit applicability of traditional histopathological evaluation of tissue damage in MPS models (Sura et al., 2020), but should not preclude engagement of pathologists early in the MPS design, characterization, and validation phases. Assessment of biomarker released into media may also be hindered by low sample volume availability in organ-chip platforms and insufficient sensitivity of existing assays. Many MPS are also made from PDMS, which can bind small molecules, an undesirable feature. While this can be overcome, such as with structural modelling, alternative materials for fabrication would be preferable (Prantil-Baun et al., 2018).

PBMCs, comprised of a mixed population of lymphoid and myeloid lineage cells, have been widely used in drug discovery and academia owing to their relatively easy accessibility and minimum discomfort to donors when withdrawing the blood. PBMCs can be used as is or primary immune subpopulations can be isolated for incorporating into models. Depending on the purpose of the MPS being set up, PBMCs may or may not be fit-for-purpose. An assay to assess safety of a drug would require a model representative of healthy tissue and PBMCs from healthy volunteers. In contrast, assessment of efficacy requires a model representing a disease state and a mix of immune cells at different differentiation or activation stages. For example, Th17 cells are a rare population in peripheral blood of healthy donors but occur in elevated numbers in inflammatory diseases, including rheumatoid arthritis, IBD, and asthma (Tesmer et al., 2008). Taking peripheral blood from patients may provide more representative immune cell populations, but access to patient blood is limited. Another challenge is replicating the sophisticated architecture of immune tissues with immune cells from the periphery. This poses the question of how well PBMCs capture lymphoid organ activity. A comparison of LN mononuclear cells with PBMCs in patients with active Mycobacterium tuberculosis infection indicates differences in both activated T cells and regulatory T cells (Sahmoudi et al., 2018). Donor variability should be considered when determining the source of immune cells. Age, sex, diet, environmental and microbial exposure potentially contribute to the inter-donor variations (Liston et al., 2021). Pre-screening of donors based on immunophenotyping, or in vitro bioactivity assays might be needed to identify the best fitting donors for the study purpose. When there are no clear pre-screening criteria, repeating studies with immune cells from multiple donors will likely increase the chances of successful application of immunocompetent MPS.

When replacing traditional 2D cultures with increasingly sophisticated 3D models that use primary human cells to better represent human physiology, the throughput of the assays is reduced. For these to be adopted for routine use in drug discovery processes, key questions and the right level of complexity of the models should be chosen. For example, the increasing information around links of genetic variants to disease combined with advances in gene editing technologies means there is now the opportunity to run genome-wide screens (20,000+ genes in the human genome) for novel target identification. Taking as an example immune cell targets to treat IBD, the ideal scenario would be to have an MPS model where genes can be edited in the immune cell compartment and the consequences assessed on gut barrier function. For various organoids, the ideal is to incorporate an immune cell component replicating tissue inflammation and then implement the model at scale.

Looking into the future, MPS present opportunities for a more patient-centric approach to safety and efficacy testing. Through the use of patient-derived cells to create a “patient-on-a-chip” (Low et al., 2021), human models will be advantageous in assessing new therapeutic modalities, such as oligonucleotides or cell therapy, which are challenging to directly test in animal models (Low et al., 2021).

6 Impact on 3Rs

Despite wide use of animal models in drug discovery, challenges associated with translating data from animal models to humans are well documented (Mestas and Hughes, 2004). The
3Rs guiding principles refer to the replacement, reduction, and refinement of animal use in research and testing, first described in 1959 by Russell and Burch (Tannenbaum and Bennett, 2015). These principles encourage the identification of alternative methods to animal testing (replacement), employing methods that require the use of fewer animals (reduction), and the use of testing approaches that cause minimal distress to animals (refinement). The 3Rs have been widely embraced by the scientific community and incorporated into legislation governing animal use due to ethical concerns around animal welfare and limitations in translation to human biology. The 3Rs guiding principles are also recognized as an opportunity to promote the development of better scientific approaches and technologies (MacArthur Clark, 2018). In line with the expectations of the 3Rs, there is significant value in developing MPS to reduce animal usage by virtue of the ability to miniaturize studies in defined organ systems before committing to larger in vivo studies. To achieve this, the development of human MPS models has become the most prominent topic. While animal MPS have also been developed, their primary purpose is to serve as data comparators to results from in vivo studies.

Unlike most other tissue cells, immune cells circulate with blood flow and migrate into tissues, which makes it difficult to study tissue-associated immune responses in a static 2D cell culture. The function of the lymphoid organs and the biology of tissue-infiltrating leukocytes are generally studied in rodent disease models. The immunotoxicity of a drug candidate is often tested in animals as well. Human immunocompetent MPS offer a physiologically relevant culture system to allow a certain degree of immune cell trafficking; therefore, they have the potential to replace animal studies, reduce the usage of animals, or reduce extra procedures on animals in research and testing. For example, as stated in a previous section, the TDAR assay is used to evaluate immune alteration after drug treatment and may present an opportunity for the development of an improved, alternative method for evaluating effects on human immune function. There are ongoing research collaborations to develop human TDAR-on-chip assays that can demonstrate robust and reliable effects on functional antigen-specific immune responses. Replacement of the in vivo TDAR assay with a validated, immunocompetent MPS system could reduce animal usage or eliminate procedures of immunization and blood collection in toxicity studies. Multi-organ microfluidic chip systems with circulating immune cells have also been developed to mimic the in vivo immunosurveillance process, with a potential to replace humanized animal models (Sasserath et al., 2020). With further advancement of MPS, the use of animals and the procedures applied to animals to study human disease, drug efficacy, and safety can be significantly reduced.

7 Conclusion

For decades, immune systems have been studied in 2D cultures and in vivo animal studies with numerous breakthrough scientific discoveries. With the rapid advancement of microfluidic culture systems, developing sophisticated immune organ-on-chip systems will provide a significant opportunity to model pharmacology and immunotoxicity in humans. The selection of organs for which this is applied is largely driven by the needs of recapitulating the human physiological response for a particular context-of-use. Furthermore, immune components have been introduced into many tissue MPS platforms, acknowledging the contribution of immune cells in pharmacology and safety of therapeutic candidates. Though the regulatory acceptance of immune competent MPS is still under discussion, the overwhelming effort in the field surely provides strong scientific support.

Considering the immune system is a highly dynamic and replenishable system, many of the MPS models still do not fully contain all the required immune components, nor do they provide the conditions to allow lymphatic and hematological trafficking. Furthermore, a full spectrum of soluble factors including cytokines and chemokines is generally not provided in the immunocompetent MPS. Therefore, the application of MPS models should have a defined context-of-use and limitations of the system representing potential gaps between the system and in vivo physiology should be considered. Currently, a standardized approach to qualify various models has not been fully developed. The evaluation of the immune response in immunocompetent MPS is still largely dependent on concordance with previous findings in 2D culture and in vivo studies.

Taken together, the complex MPS models representing different organs together with immune cells serve as promising tools for understanding pharmacology or potential immunotoxicity and tissue damage following treatment with drug candidates. The comprehensive characterization, standardization, and validation of MPS will warrant studies that better mimic physiological conditions to ensure robust interpretation of the results.

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Conflict of interest
There are no conflicts to declare.

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