hiPSC-derived 3D cardiac microtissue models with integrated immune cells and vasculature

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Introduction

The heart and its microenvironment

The heart is one of the body's vital organs. Its rhythmic contraction continuously pumps blood through vessels around the whole body, transporting nutrient and oxygen to organs and tissues ensuring their health and function. The heart has four cavities (or chambers), two atria and two ventricles, one of each on left and right sides. These chambers control blood circulation: deoxygenated blood enters from the right atrium and is then transferred to the right ventricle from where it is pumped to the lungs for oxygenation. Oxygenated blood is next carried to the chambers on the left side via the pulmonary veins. Finally, oxygenated blood is circulated through the body via the aorta (HARVEY and LEAKE, 1929).

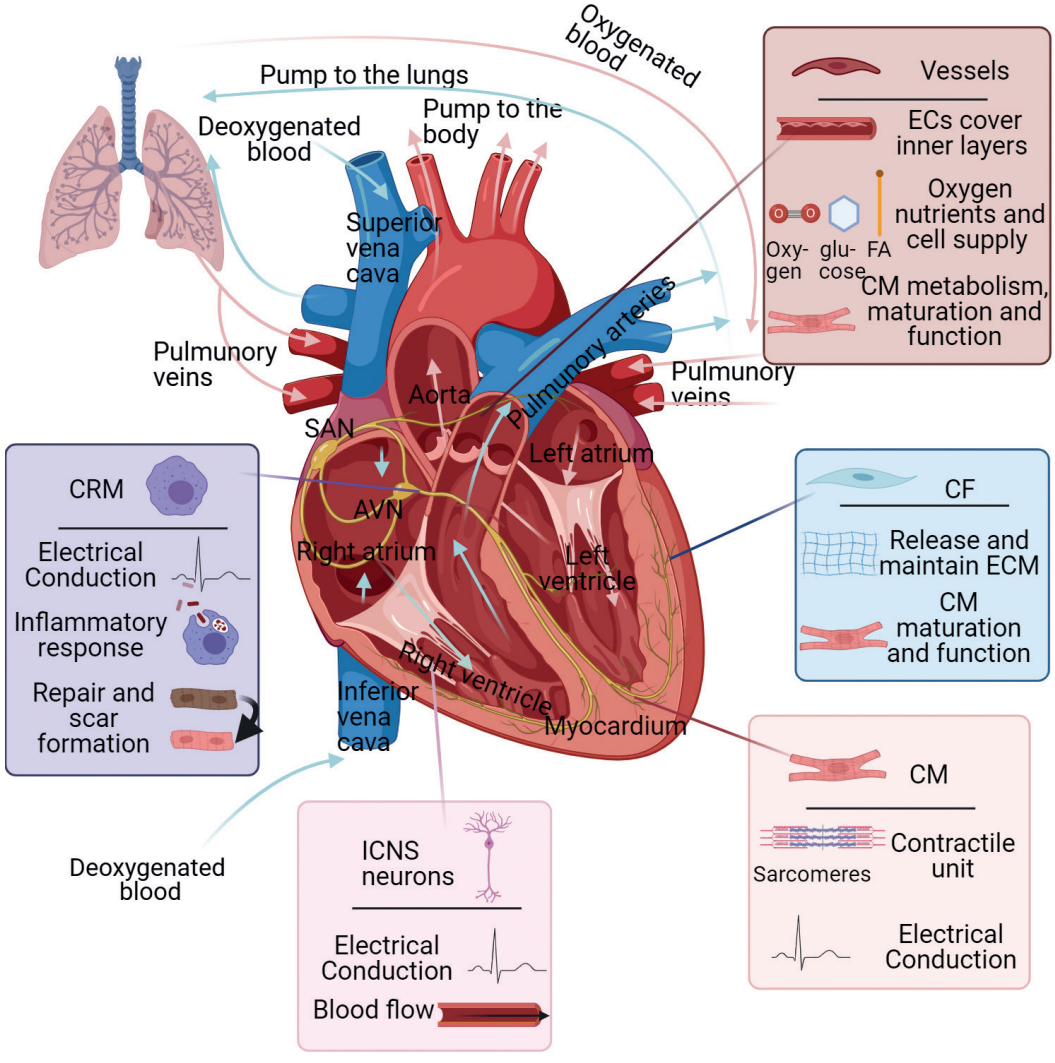

Figure 1: Schematic overview of the heart physiology and its cellular components. *SAN, Sinoatrial node; AVN, atrioventricular node; CRM, cardiac resident macrophages; ICNS, intrinsic cardiac nervous system; CM, cardiomyocytes; CF, cardiac fibroblasts; ECM, extracellular matrix; EC, endothelial cells.*

At the cellular level, the heart is a complex structure made up of multiple layers composed of multiple cell types (Figure 1).

The different cells in the heart can be distinguished in part by their expression of certain distinct proteins but also by their function, for example whether they contribute to contractility/electrical conduction, extracellular matrix (ECM) production, innervation and vascularization or whether they are part of the immune system. In order to function properly, well-regulated crosstalk between all of these cellular components as well as the heart cell microenvironment is essential (Reaume et al., 1995; Huang et al., 1998; Ming et al., 2009; Beny and Pacicca, 1994; Rook et al., 1992; Lo, 2000).

Cardiac contractility is critical as it circulates blood through the body via sequential contraction of each of the chambers (Moorman and Christoffels, 2003). Contractility is mainly regulated by cardiomyocytes (CMs) which contribute to the muscle wall of the myocardium. During embryonic development, the CM number and volume change significantly as they divide rapidly to form the complete heart at birth (de Boer et al., 2012). The fetal heart is composed of approximately 60% CMs. By contrast, in adult heart, CMs lose their proliferation capacity and contribute only 20-30% of the adult human heart by cell number, although form 80% of its total volume (Rubart and Field, 2006).
Specialized atrial and ventricular CMs are restricted to specific sites within each of the chambers. In addition to these CM types, there are also pacemaker cells and Purkinje fibers that are localized in the sinoatrial node (SAN). Together these form mechanical and electrical conduction system of the heart.

The ECM of the heart is involved in connecting cells and providing structural stability to the organ network and geometry (Novakovic et al., 2014; Rienks et al., 2014; Lockhart et al., 2011). It is mainly composed of interstitial matrix and basement membrane components: fibrillar and non-fibrillar collagens, fibronectin, hyaluronic acids, elastin and laminin (Rienks et al., 2014). ECM in the heart is mainly secreted and maintained by the cardiac fibroblasts (CFs) which form the largest population of non-myocyte cells. CFs also play an important role in regulating myocardium integrity in the case of cell death in the heart, for example after myocardial infarction (MI) (Dostal et al., 2015). After MI, CFs divide rapidly to replace the lost tissue and undergo a myofibroblast transition. This increases ECM secretion and the stiffness of the heart as a result of formation of the fibrotic scar tissue (Hall et al., 2021). Although these wound healing and repair functions of CFs are beneficial for proper heart function, excessive fibrosis can be detrimental, because the increased stiffness of the heart tissue makes contraction more work which can lead to heart failure and/or cardiac arrest (Lecomte et al., 1993).

The cardiac autonomic nervous system (ANS) contributes with a small population of cells to the heart. The ANS is a network of sympathetic and parasympathetic nervous systems that spreads from the brain to the heart and establishes the neuro-cardiac junction. At the organ level, there is also a rich intrinsic cardiac nervous system (ICNS) in the cardiac ganglia (Ardell and Armour, 2016). Sensory, motor and interconnecting neurons are integral parts

of the ICNS and they regulate the electrical and mechanical function of the heart (Zipes et al., 2017).

In this thesis, the main focus is on the vasculature and the immune components of the heart, how they contribute to its structure and function and how their interaction with the contractile cells of the heart can be modelled *in vitro*.

Vasculature

There are >90,000 km of blood vessels in the body, many in the heart. The heart is in fact among the most richly vascularized organs in the body. This is because the energy demand of CMs to beat 60 or more times a minute for many decades is enormous (Janssen et al., 2016). Vasculature in the heart forms a semi-permeable membrane through which it provides oxygen and selective nutrients to the surrounding cells in the organ. It is also involved in immune cell trafficking, for example in the case of inflammation. Vasculature is critical for supporting organ growth and function.

Constriction or complete closure of blood vessels of the heart caused by thrombosis (blood clots) or atherosclerosis leads to MI due to lack of oxygen which causes rapid death and permanent loss of CMs. It is therefore important to understand the interaction between different cell types involved in the structure and function of the vascular network of cardiac muscle.

On a cellular level, vasculature is formed by endothelial cells (ECs) and mural cells. ECs line the inner layer of the tubular (or lumenized) vessels where they control the vessel permeability, vasomotor tone, immune cell trafficking and angiogenesis (Aird, 2007). There are multiple EC compartments in the heart such as endocardium, capillaries and coronary vessels. ECs exhibit heterogeneity in their phenotype, function and genetic composition depending on the compartments in which they localize (Kumar et al., 1987; Turner et al., 1987). Mural cells like pericytes and smooth muscle cells are important part of the vasculature and are involved in the maintenance of vessel wall integrity, stability and function (Gaengel et al., 2009).

To understand vasculature-organ crosstalk in maintaining the oxygen and nutrient levels for cellular health, removing waste products and the response to tissue damage, it is useful to have tractable experimental models to study these processes. Mice are invaluable for investigating blood vessel development and function and vascular biology in general in health and disease. Their genetic composition can be easily manipulated allowing study of the direct impact on genetic changes or mutations on vessel behavior. In addition, physiological changes such as flow rate and vascular permeability associated with developmental defects or disease phenotypes can be addressed in mouse models (Korshunov and Berk, 2003). They are also cost-efficient, although in some jurisdictions they can be expensive, but this is the reason that mice (rather than rats or other larger mammals) are one of the most used animal

models for pre-clinical drug testing. However, physiological differences especially in their heart such as higher beat rate (±500 beats per minute versus ±60 in humans) and different hemodynamic properties, make translating results to humans challenging (Janssen et al., 2016). In this case, other animal models like pigs, dogs and non-human primates may be more useful in translation of some results to humans. However, larger animal models are more expensive, afre more challenging to genetically modify and ethical concerns around these models has driven the search by scientists for more suitable, easily accessible but realistic, human *in vitro* alternatives for some types of research question. This is in part the subject of this thesis.

Immune System

The immune system is composed of resident immune cells like cardiac-resident macrophages (CRMs) and infiltrating immune cells like blood-circulatory monocytes. CRMs regulate the electrical conduction in the myocardium through cellular crosstalk with CMs (Hulsmans et al., 2017; Harari et al., 2017; Tirziu et al., 2010). Besides electrical conduction, CRMs are involved in maintaining homeostasis, by removing mitochondrial waste of CMs, for example (José Nicolá s-A et al., 2020). They also take part in other physiological processes such as inflammation, tissue repair and regeneration upon injury, and angiogenesis. In particular, upon injury CRMs assist the complex cascade of events (Simões et al., 2020). For example, they first engulf and clear the dead cell debris, through the process called phagocytosis. They are involved in modulation of inflammation response to prevent prolonged inflammatory activity which can be detrimental. Furthermore, by releasing certain growth factors and other signaling molecules like cytokines that can help with proliferation and differentiation of cardiac cells, CRMs initiate the tissue repair and regeneration. They also support existing vessels (Moore et al., 2017) in the formation of new blood vessels (angiogenesis) which can supply oxygen and nutrients to the regenerating tissue. The final stage of the tissue regeneration is the formation of functional scar tissue which prevents the tissue collapse and heart rupture. On the other hand, blood circulatory monocytes are mostly involved in the inflammatory response itself (Bajpai et al., 2018).

Initial studies on macrophages claimed that their primary origin is bone marrow derived circulatory blood monocytes (van Furth and Cohn, 1968). However later studies showed more evidence that the macrophage ontogeny is more complex (Hoeffel and Ginhoux, 2015)(Figure 2). It is now clear that CRMs arise from the hemogenic endothelium of the yolk-sac during embryonic development (Yona et al., 2013; Ginhoux and Jung, 2014). CRMs mainly populate the atrioventricular node and in the neonatal heart, they can self-renew via local proliferation (Epelman et al., 2014; Hashimoto et al., 2013). It is thought that due to the high numbers of CRMs, neonatal heart is more regenerative. However, throughout adulthood, CRMs lose some of their self-renewal capacity and the population of CRMs is partially replaced by blood monocytes (Epelman et al., 2014; Dick et al., 2019). Due to this replacement of regenerative CRMs and the pro-inflammatory nature of blood monocytes, the adult heart becomes more pro-inflammatory than the neonatal heart. Although this

switch from regenerative- to pro-inflammatory phenotype is beneficial for tissue repair, excessive inflammatory response can be detrimental. It might lead to (1) apoptosis of cells, which results in further tissue damage and loss; (2) excessive scar tissue formation which makes the heart stiffer and results in cardiac arrest; (3) interference with the mechanical and electrical function of the heart, which can cause conditions like arrythmias. Therefore, maintaining the balance between the pro-inflammatory and anti-inflammatory responses is critical for proper tissue regeneration. Understanding CRM involvement in this balance is critical to harness their regenerative capacity and has potential for future therapeutic approaches to cardiac injury.

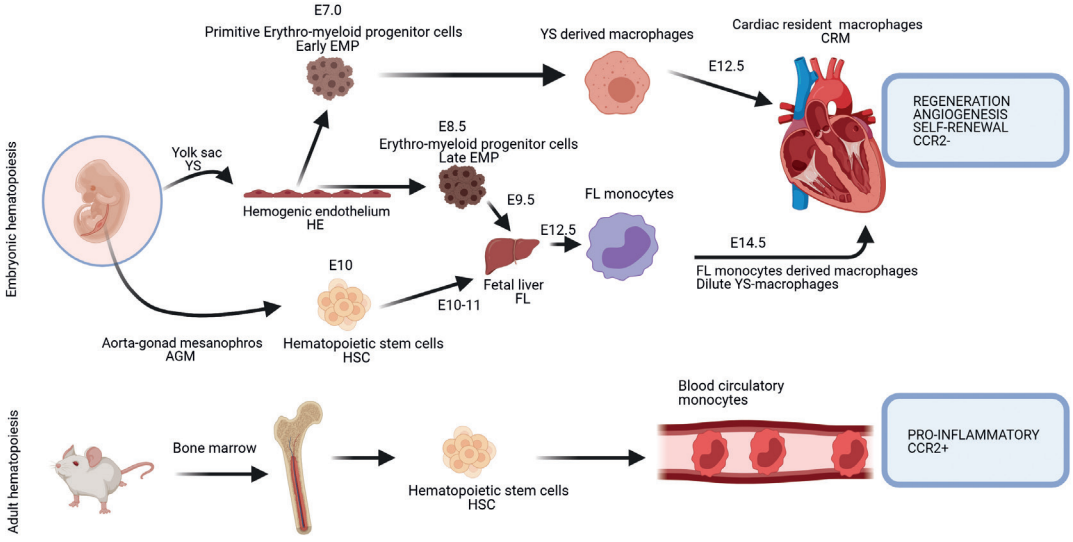

Figure 2: Schematic overview of the macrophage ontogeny in mice. *E, embryonic day.*

In vitro heart models to study the crosstalk in the heart

Crosstalk in the heart can be either between cells or between ECM and cells. Some ECM proteins are critical for cell adhesion, regulating ion channels and mechanical transduction especially in CMs by integrin interactions (Kim et al., 2000; Contard et al., 1991). Cellular crosstalk through direct cell contacts or secreted small molecules and proteins between EC-CM (Segers et al., 2018; Narmoneva et al., 2004; Brutsaert, 2003), CF-CM (Camelliti et al., 2004; Weber, 1989; Ieda et al., 2009; Giacomelli et al., 2020), CRM-CM (Hulsmans et al., 2017; Bajpai et al., 2018) and neural cardiac junctions (Li et al., 2023; Winbo et al., 2023) contributes to cardiac development and function in health and disease.

The discovery of human pluripotent stem cells (hPSCs) (Takahashi et al., 2007; Thomson, 1998) and their ability to differentiate into many different cell types has brought countless opportunities to study heart cells. The heart does not contain a stem cell population so it is not possible to derive and culture them from heart biopsies (Kretzschmar et al., 2018).

Human induced pluripotent stem cells (hiPSC) derived by reprogramming somatice cells from donors, are especially important because they carry the genome of individuals they are derived from; they present options for more precision medicine approaches for disease modelling and drug testing.

One of the first cardiac cell types that was differentiated from hPSCs were CMs (Mummery et al., 2003; Kehat et al., 2002). Patient-derived hPSC-CMs have proven useful over many years to model disorders like channelopathies as they express all ion channels of CMs (Davis et al., 2012; Braam et al., 2010; Denning et al., 2016; Liang et al., 2013; Moretti et al., 2010). However, hPSC-CMs often exhibit an immature phenotype in their genetic profile, structure and function, resembling fetal heart at around 16 weeks of gestation more than adult (Birket et al., 2015; Birket and Mummery, 2015; Ribeiro et al., 2015).

Several groups reported improved maturation in hPSC-CMs when they were cultured in 3D microenvironment (Beauchamp et al., 2015; Desroches et al., 2012; Soares et al., 2012). In addition, 2D hPSC-CMs lack cellular crosstalk with non-CMs, and thus fail to model more complex disorders. Co-culturing these cells with other cell components such as ECs and CFs in 3D tissue microenvironment improved their maturation even further (Beauchamp et al., 2020; Giacomelli et al., 2020; Mills et al., 2019; Richards et al., 2020). Multi-cellular 3D tissue cultures enabled integration of cellular, mechanical and electrical cues in a more controlled microenvironment mimicking the *in vivo* counterpart. These types of cultures can be scaffold-free or engulfed in a scaffold and formed by integrating either (1) hPSCs and differentiating them in 3D using growth factors (cardioids (Drakhlis et al., 2021; Hofbauer et al., 2021; Silva et al., 2020)) or (2) predifferentiated hPSC-derived cells (microtissues/spheroids (Giacomelli et al., 2017; Richards et al., 2020; Beauchamp et al., 2015; Mills et al., 2017)). Engineered heart tissues (EHT) can also integrate biomaterials and microenvironmental cues that can mimic structural properties of tissues like elasticity and organization (Eschenhagen et al., 1997; Zimmermann et al., 2002; Huebsch et al., 2016; Nunes et al., 2013; Dostanić et al., 2020; Mannhardt et al., 2017).

CM, EC and CFs are the most thoroughly studied cardiac cell types in 2D and 3D heart models. However, some studies suggest the important roles of CRMs (Simões et al., 2020; Farache Trajano and Smart, 2021; Dutta and Nahrendorf, 2015) in heart development and pathophysiology. Developing more complex models that can integrate more cell types like immune cells, complex disease such as MI and consequent fibrosis (Figure 3), where the crosstalk between vasculature and immune components is critical, can be better recapitulated.

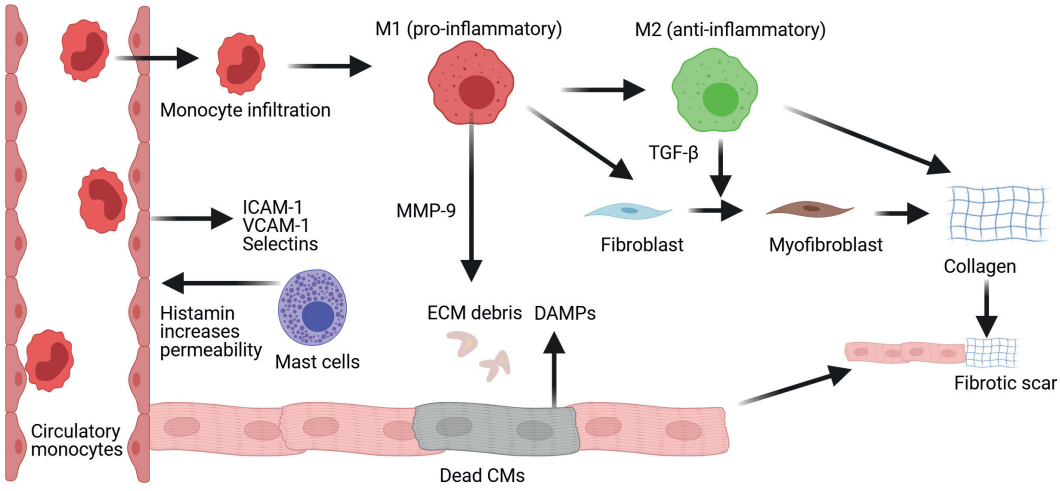

Figure 3: Schematic overview of MI and fibrosis showing the complex cascade of events taking place. *CM death releases damage associated molecular patterns (DAMPs). Mast cells activate the ECs by histamine release. With the increase in ICAM-1, VCAM-1 and Selectins as well as permeability, monocytes circulate to the injured area. First, monocytes are polarized towards the M1 macrophage phenotype which is pro-inflammatory. They clear the ECM debris by the secretion of matrix metallopeptidase (MMP-9). In the meantime, anti-inflammatory M2 macrophages activate myofibroblasts by TGF-β secretion which then deposits collagen to scar area. Macrophages also contribute to collagen deposition in this process (Simões et al., 2020). Consequently, the fibrotic scar is formed.*

Although some of these 3D models integrate ECs, which pre-vascularize these tissues by self-organization into a microvascular networks (Cakir et al., 2019; Homan et al., 2019; Vargas-Valderrama et al., 2020; Giacomelli et al., 2020), most of these models are maintained in static culture conditions. Therefore, they lack functional vasculature. Perfusion is one of the most important components for vessel stability and integrity as without it vessels regress over time (Ryan et al., 2021). Initial strategies to vascularize 3D tissue cultures involved transplanting them into living organisms where the host circulatory system invades the transplanted tissue and connects with its microvascular network (Mansour et al., 2018; Takebe et al., 2013; Ryan et al., 2021). Although this principle generated functional vasculature, translating the research into humans was challenging as inter-species differences lead to altered behavior and pattern of vascular structures (Brady et al., 2023).

Microfluidic Organ-on-Chip systems

Organ-on-chip (OoC) platforms offer alternatives to the static platforms described above, as they provide perfusable engineered microchannels. The first microscale platforms emerged in the early 2000s by microfabricating a hollow channel in the size of a small lung airway to detect changes in acoustic signals during liquid flow (Huh et al., 2007). Following a similar strategy, the design of the platform was later changed to include two parallel channels which allow compartmentalization of different cell types (Domansky et al., 2010; Huh et al., 2010). It also allowed the introduction of two variables in different channels, for example, perfusion

of liquid in EC channel and air in epithelial lung alveolar cell channel, recreating liquid-air interface (Huh et al., 2010). This simple two channel platform was later adapted by many groups to accommodate other tissue types including heart-on- chip models (Agarwal et al., 2013; Marsano et al., 2016; Mathur et al., 2015). These models introduced perfusion in the systems. However, they did not recapitulate the vascularized organ physiology as the flow was not through a vasculature. As a result, the tissues were exposed to the culture medium directly via passive diffusion through gel. More recent strategies like co-culturing of CM, ECs and CFs in an organ-on-chip platform (King et al., 2022) led to formation of perfusable vessels that are surrounded by CMs. However, the model requires high cell numbers, and it is challenging for some labs to upscale to these cell numbers. In addition, complete self-organization of the cells might result in less control in the cell distribution which might lead to heterogeneity in structure and function of the model in one channel. The enclosed chip environment further complicates the study of cellular crosstalk and its effect which is especially important when the immature hiPSC-derived CMs are seeded in the gel. On the other hand, following a similar strategy but integrating organoid/microtissue models in which these variables can be controlled better might offer an alternative.

Aim and the scope of this thesis

Based on the potentials and limitations of current *in vitro* 3D cardiac models that were discussed above, the development of a more complex culture system which can mimic more aspects of the heart *in vivo* would be of value.

This thesis consists of eight chapters and describes the importance of the heart microenvironment and ways to integrate more components of it in cardiac models. The overall aim is to develop cardiac models that incorporate (1) immune; and (2) vascular components, such as functional vasculature, in order to provide cellular crosstalk and study the impact on organ function and response to external stimuli.

In **chapter 2**, we shortly reviewed the current 3D cardiac models and the possible refinement strategies in their future use in the cardiac field.

In **chapter 3**, we reviewed the current cardiac microphysiological systems in a broader manner with their applications and readouts and discussed our future perspectives for their further implementation in academia and industry.

In **chapter 4**, we established optimal conditions to generate 3D cardiac microtissues that integrate hiPSC- derived macrophages (CMECFM0). We first showed that macrophages migrated outside of CMECFM0s in the standard V-bottom 96-well plates. Using ultra-low attachment plates eliminated this problem and macrophages remained in CMECFM0. We further described the effect of culture conditions and hiPSC-macrophages on different cell subsets in the microtissues. We showed that using macrophage colony-stimulating factor (M-CSF) increased the macrophage numbers in CMECFM0s in the early culture period.

However, it did not affect the number in later time period. Macrophages did not significantly alter vessel density and contraction parameters. Finally, we investigated the pro- and anti-inflammatory phenotypes of integrated macrophages, highlighting the importance of culture conditions on the macrophage identity.

In **chapter 5**, we generated a vascularized and perfusable hiPSC-derived 3D cardiac microtissues on a chip platform (VMToC) by combining prevascularized cardiac microtissues with additional vascular cells in fibrin hydrogel. We characterized the vessel parameters and showed that contraction of microtissues did not affect the vascularization process in chips. We further established flow conditions and showed that continuous perfusion improves the vessel density inside and outside the MTs. This work is a foundation of chapter 6 and 7.

In **chapter 6**, we established the utility of VMToCs in terms of the crosstalk between hiPSC-endothelial cells and cardiomyocytes. We first characterized the effect of vasculature on sarcomere organization and contraction parameters during spontaneous or paced beating. We showed that vascularization did not significantly alter sarcomere organization. However, vascularized tissues had significantly longer contraction duration and peak-to-peak times. We then challenged the crosstalk between hiPSC- endothelial cells and hiPSC-cardiomyocytes using (1) nitric oxide synthase inhibitor and (2) pro- inflammatory cytokine stimulation. Only vascularized tissues responded to these stimuli, highlighting the enhanced crosstalk in VMToC model and its effect on the functionality.

In **chapter 7**, we described a detailed protocol to generate VMToC. We then described protocols to characterize VMToCs (1) functionally by applying bead perfusion assay, showing the vessels were lumenized and perfusable, and by pacing; and (2) structurally by fixing and immunofluorescent staining.

In **chapter 8**, the findings of this thesis are summarized and a critical discussion provided of the limitations and advantages of the models described. Finally, we consider future perspectives for these types of vascularized 3D models in the field.

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