Resident macrophages and their potential in cardiac tissue engineering

Tissue Engineering

Tissue Engineering Manuscript Central: http://mc.manuscriptcentral.com/ten

Resident macrophages and their potential in cardiac tissue engineering

Journal:	*Tissue Engineering*
Manuscript ID	TEB-2021-0036.R1
Manuscript Type:	Review Article - Part B
Date Submitted by the Author:	13-May-2021
Complete List of Authors:	Suku, Meenakshi; University of Dublin Trinity College Forrester, Lesley; University of Edinburgh MRC Centre for Inflammation Research Biggs, Manus; National University of Ireland Galway Monaghan, Michael; University of Dublin Trinity College,
Keyword:	Cardiac Tissue < Applications in Tissue Engineering (DO NOT select this phrase; it is a header ONLY), IPS Cells < Fundamentals of Tissue Engineering (DO NOT select this phrase; it is a header ONLY), Inflammatory/Immune Response < Fundamentals of Tissue Engineering (DO NOT select this phrase; it is a header ONLY)
Manuscript Keywords (Search Terms):	Resident macrophages, Cardiac macrophages, Myocardial Infarction, iPS Cells, Engineered heart tissue
Abstract:	Many facets of tissue engineered models aim to understand cellular mechanisms to recapitulate in vivo behavior, study and mimic diseases for drug interventions and to provide better understanding towards improving regenerative medicine. Recent and rapid advances in stem cell biology, material science and engineering, have made the generation of complex engineered tissues much more attainable. One such tissue, human myocardium; is extremely intricate, with a number of different cell types. Recent studies have unraveled cardiac resident macrophages as a critical mediator for normal cardiac function. Macrophages within the heart exert phagocytosis and efferocytosis, facilitate electrical conduction, promote regeneration and remove cardiac exophers to maintain homeostasis. These findings underpin the rationale of introducing macrophages to engineered heart tissue, to more aptly capitulate in vivo physiology. Despite the lack of studies using cardiac macrophages in vitro, there is enough evidence to believe that they will be useful in making engineered heart tissues more physiologically relevant. In this review, we explore the rationale and feasibility of using macrophage as an additional cell source in engineered cardiac tissues.

Resident macrophages and their potential in cardiac tissue engineering

Meenakshi Suku[1,2,3], Lesley Forrester[4], Manus Biggs[3], Michael G. Monaghan*[1,2,3,5]

1. Department of Mechanical, Manufacturing and Biomedical Engineering, Trinity College Dublin, D02 PN40, Ireland
2. Trinity Centre for Biomedical Engineering, Trinity Biomedical Science Institute, Trinity College Dublin, D02 PN40, Ireland
3. CURAM SFI Research Centre for Medical Devices, National University of Ireland, Galway, H91 CF50, Ireland
4. MRC Centre for Regenerative Medicine, University of Edinburgh, 5 Little France Drive, Edinburgh EH16 4UU, UK.
5. Advanced Materials for Bioengineering Research (AMBER) Centre, Trinity College Dublin and Royal College of Surgeons in Ireland, D02 PN40, Ireland

*Corresponding author

Monaghan, Michael G - monaghmi@tcd.ie, +353 1 896 8582

Biggs, Manus - manus.biggs@nuigalway.ie, +353 9 149 4328

Forrester, Lesley - L.Forrester@ed.ac.uk, 0131 651 9553

Suku, Meenakshi - sukum@tcd.ie, +353 877201117

Keywords: Resident macrophages, Cardiac macrophages, Myocardial infarction, iPSCs, Engineered heart tissue

Abstract

Many facets of tissue engineered models aim to understand cellular mechanisms to recapitulate *in vivo* behavior, study and mimic diseases for drug interventions and to provide better understanding towards improving regenerative medicine. Recent and rapid advances in stem cell biology, material science and engineering, have made the generation of complex engineered tissues much more attainable. One such tissue, human myocardium; is extremely intricate, with a number of different cell types. Recent studies have unraveled cardiac resident macrophages as a critical mediator for normal cardiac function. Macrophages within the heart exert phagocytosis and efferocytosis, facilitate electrical conduction, promote regeneration and remove cardiac exophers to maintain homeostasis. These findings underpin the rationale of introducing macrophages to engineered heart tissue, to more aptly capitulate in *vivo* physiology. Despite the lack of studies using cardiac macrophages *in vitro*, there is enough evidence to accept that they will be key to making engineered heart tissues more physiologically relevant. In this review, we explore the rationale and feasibility of using macrophages as an additional cell source in engineered cardiac tissues.

Impact statement

Macrophages play a critical role in cardiac homeostasis and in disease. Over the last decade, we have come to understand the many vital roles played by cardiac resident macrophages in the heart, including immunosurveillance, regeneration, electrical conduction and elimination of exophers. There is a need to improve our understanding of the resident macrophage population in the heart *in vitro*, to better recapitulate the myocardium through tissue engineered models. However, obtaining them *in vitro* remains a challenge. Here, we discuss the importance of cardiac resident macrophages and potential ways to obtain cardiac resident macrophages *in vitro*. Finally, we critically discuss their potential in realizing impactful in vitro models of cardiac tissue and their impact in the field.

Mary Ann Liebert, Inc.,140 Huguenot Street, New Rochelle, NY 10801

Introduction

Macrophages are present in almost every vertebrate tissue. They contribute to the maintenance of immunity by phagocyting microbes, clearing senescent cells and promoting homeostasis by repairing and regulating the functions of organs. Initially perceived as a cell population only present in, and originating from the blood, recent studies have brought to light the stark heterogeneity in blood-derived and a pre-natal embryologically derived macrophage populations. Most tissues in the body contain a resident macrophage population exhibiting diverse phenotypes, specific to that tissue and niche[1]. This phenotypic heterogeneity is potentially due to the impact of the resident tissue environment as well as to differences in the macrophage origin[2]. However, despite being best known as the key mediators in tissue defense, the exact role of different subsets of resident macrophages in protecting the body from pathogens or environmental change are still unclear, as they are hard to extract, purify and study in vitro.

The focus of this review, cardiac resident macrophages; originate during embryogenesis and populate the heart prenatally[3]. Only within the past 3-4 years, seminal studies unravelling the functions and the physiological importance of cardiac macrophages have emerged (in mice), identifying these cells as a potential candidate in tissue engineering applications. In addition to performing critical functions in healthy myocardium, cardiac resident macrophages respond uniquely to subtle changes in the homeostatic equilibrium. Although there has been a limited number of studies focusing on the role of resident macrophage populations in the heart, there exists enough groundwork to explore the possibilities of achieving more humanized cardiac tissue models using resident macrophages. Additionally, by incorporating resident macrophages within engineered heart tissues, models can be more physiologically relevant, with applications in regenerative medicine, disease modelling and drug screening. However,

Mary Ann Liebert, Inc.,140 Huguenot Street, New Rochelle, NY 10801

there remain a lot of questions to be answered - what degree of these cells are lost during myocardial disease/ischemia; how do they function in response to injury and disease, and what role do their paracrine factors exert in tissue engineered models, to name but a few. We discuss cardiac macrophages in healthy myocardium, and during myocardial infarction (MI) and post-MI heart failure. We recap on the various engineered heart tissue (EHT) models that have emerged over the years and discuss the potential applications of macrophages within such models in cardiac tissue engineering. Finally, we discuss the challenges of obtaining cardiac resident macrophages *in vitro*, and avenues of obtaining them *in vitro* for tissue engineering - such as using induced pluripotent stem cell (iPSC) derived macrophages (iMacs), opening up the possibility to study genes and genetic pathways involved in their function.

Macrophage Populations in the heart and their functions

The cellular content of human myocardium (in descending order by number) predominantly consists of cardiomyocytes, fibroblasts, pericytes and smooth muscle cells, endothelial cells and immune cells[4]. In the murine heart, macrophage populations can be discerned based on their expression of C-C chemokine receptor type 2 (CCR2), major histocompatibility complex-II (MHC-II), T-cell immunoglobulin and mucin domain containing 4 (TIMD4) and Lymphatic Vessel Endothelial Hyaluronan Receptor 1 (LYVE1). Four populations of macrophages have been identified in the murine heart[5] (Fig.1). The minority are two subsets of $CCR2^{+}MHC\text{-}II^{high}$ macrophage populations derived from hematopoietic stem cells (HSCs), whereas the most dominant subset, cardiac resident macrophages derived from the yolk sac, are $CCR2^{-}MHC\text{-}II^{low}TIMD4^{+}LYVE1^{-}$. There also exists a fourth subset that consists of $CCR2^{-}MHC\text{-}II^{high}TIMD4^{+}LYVE1^{+}$ macrophages which are likely derived from CCR2-$MHC\text{-}II^{low}$ macrophages during postnatal development[5, 6] and partially maintained by $CCR2^{+}MHC\text{-}II^{high}$ macrophages. $CCR2^{+}MHC\text{-}II^{high}$ macrophages populate the heart soon after birth and are maintained through monocyte recruitment and proliferation[7]. Also, under homeostasis, cardiac

macrophages continually self-renew with a turnover period of almost five weeks[8]. Recently this same $CCR2^+$ and $CCR2^-$ macrophage distinction has been identified in humans[9], with the yolk sac-derived $CCR2^-$ human counterpart showing $CD14^+CD45^+CD64^+CCR2^-HLA\text{-}DR^{high}$ expression[5, 9]. In a more recent study, Tucker *et al.* reported two cardiac macrophage subsets within the human heart which were positive for CD163, COLEC12, mannose receptor MRC1, E3 ubiquitin ligase MARCH1 and natural resistance–associated macrophage protein 1 (NRAMP1). While both of the identified subsets express anti-inflammatory (M2) polarization–associated genes, one subset particularly expressed RBPJ, F13A1 and the other expressed transmembrane collagen COL23A1[10].

$CCR2^+$ and $CCR2^-$ macrophage populations are present in specific locations within the heart, performing distinct functions. $CCR2^+$ macrophages are usually found in the trabecular projections of the heart and their role in cardiac development has not been fully delineated. However, they are activated when homeostatic balance is interrupted- such as during a disease[11]. In contrast, $CCR2^-$ macrophages, herein referred to as cardiac resident macrophages, occupy the myocardial wall and play a principal role in normal coronary development and maturation[12]. However, such studies are mostly limited to murine models. In addition to demonstrating robust phagocytic behavior[13], cardiac resident macrophages have demonstrated a role in neonatal cardiac regeneration in mice, salamanders and zebrafish[14-16], potentially through the stimulation of angiogenesis[17].

Furthermore, cardiac resident macrophages interact very closely with cardiomyocytes and form signaling complexes through connexin-43 allowing synchronous depolarization[18] (Fig.2). Hulsmans *et al.* have shown that depletion of either cardiac resident macrophages or connexin-43 effects a delay in conduction and blockage in the atrioventricular node in mice. This study highlights a critical role of cardiac macrophages in coupling with cardiomyocytes to facilitate electrical conduction within the heart. Undoubtedly, the coupling of cardiomyocytes and

cardiac resident macrophages holds a lot of possibilities in regenerative medicine, to be explored in the future.

In addition to their involvement in the conduction system of the heart, cardiac macrophages also play a role in phagocytosis and efferocytosis[19] (Fig.2). Cardiac macrophages express high levels of myeloid epithelial reproductive tyrosine kinase (Mertk), which plays a role in macrophage-mediated efferocytosis. In the absence of this protein, cell debris removal during cardiac repair is severely affected, resulting in increased infarct size and reduced cardiac function[20]. Removal of debris and other materials ejected by cardiomyocytes is crucial in maintaining adequate cardiac function even in non-pathological settings. Similar to how the central nervous system disposes of dysfunctional organelles[21], cardiomyocytes remove dysfunctional subcellular particles with the help of cardiac resident macrophages[22]. Likewise, Nicolas-Avila *et al.* documented that cardiomyocytes, which are continuously contracting and metabolically active cells, release subcellular particles termed "exophers" containing defective mitochondria[22]. These exophers are taken up by cardiac resident macrophages through Mertk to maintain cardiac homeostasis. This maintenance role of cardiac resident macrophages is further demonstrated when they are depleted in the myocardium (by intraperitoneal injection of 10 µg/kg diphtheria toxin in mice)[22], which in turn reduces the presence of these exophers, delineating the role of cardiac resident macrophages in maintaining normal cardiac autophagy. In addition to unveiling this unique role of cardiac resident macrophages, this is the first study to show that myocardial tissue releases exophers (in mice).

Macrophage Populations during MI and post-MI heart failure

In the murine heart, the macrophages and monocytes population in the myocardium following an infarct is dramatically different from that in steady state (Fig.3). The temporal dynamics of leukocyte infiltration following surgically induced MI has been assessed using flow cytometry and determines that neutrophils are among the first leukocytes to reach ischemic heart tissue[23].

This migration of leukocytes to the ischemic site is mediated by C-X-C motif chemokine ligand 2 (CXCL2) and CXCL5 production by $CCR2^{+}$ macrophages[24]. Monocyte recruitment to the infarct site begins as early as 30 minutes depending on CCR2 signaling, from both the bone marrow and the spleen, via MCP-1/CCR2 signaling[25]. This infiltration of monocytes and macrophages to the infarcted tissue occurs in two phases[23]. The Ly-$6C^{high}$ monocytes exhibiting pro-inflammatory phenotype predominate the early days (marking the first phase), peaking on day 3 [23] (Fig.3). Around 40% of the infiltrating Ly-$6C^{high}$ monocytes in the first 24hrs post-MI are from the splenic monocyte reservoir[26]. During this period, also known as the inflammatory phase, macrophages and neutrophils clear dead cells and debris. After proper resolution of the inflammatory phase, there occurs a phenotypic transition from Ly-$6C^{high}$ monocytes to Ly-$6C^{low}$ monocytes and M2 macrophages which exert reparative functions and renew locally[26]. Ly-$6C^{low}$ macrophages predominate the later stages as inflammation resolves (marking the second phase), peaking between days 5 to 7 post-infarct[27] (Fig.3). Similarly, the remote myocardium also undergoes a sequential Ly-$6C^{high}$ to Ly-$6C^{low}$ differentiation pattern, with the Ly-$6C^{low}$ cells peaking 5 days later than at the infarct[28]. The cardiac resident macrophages in the infarct area die shortly after MI[6]. However, more recent studies show that in the post-MI phase, the majority of the cardiac resident macrophages proliferate locally with a minor contribution from blood-derived monocytes[6].

During the inflammatory phase, classically activated (M1) macrophages secrete cytokines such as tumor necrosis factor α (TNFα), interleukin (IL)-1β, IL-6 and matrix metalloproteinases (MMPs) to facilitate extracellular matrix (ECM) breakdown[29]. Phagocytosis mediated by M1 macrophages is crucial for the initiation of the wound healing process. However, prolonged production of these inflammatory cytokines could lead to a number of pathological consequences including cardiomyocyte death, cardiac rupture, fibrosis, dilated cardiomyopathy due to matrix degradation etc.[30]. For example, TNFα (produced by

Mary Ann Liebert, Inc.,140 Huguenot Street, New Rochelle, NY 10801

macrophages, cardiomyocytes and endothelial cells) induces cardiomyocyte hypertrophy which can lead to post-MI heart failure[31]. Over the following days, macrophages promote fibroblast differentiation into myofibroblasts which express smooth muscle actin and lay down collagen, contributing to scar formation[32]. Additionally, vascular endothelial growth factor (VEGF) and transforming growth factor-β (TGF-β) produced by the macrophages facilitate revascularization of tissues[23, 33]. Finally, macrophages also produce MMPs that help in breaking down the ECM and promote tissue remodeling.

Any abnormalities in either of the phases can be detrimental to the cardiovascular system. In particular, macrophage depletion is deleterious to infarct healing and worsens the remodeling process[34, 35]. Despite being merely 2-5% of the total macrophage population in the heart in the initial weeks post-MI, the depletion of cardiac resident macrophages impaired normal infarct healing and adversely affected the remodeling process[5]. Shirishi *et al.* demonstrated the effect of inactivation of alternatively activated (M2) macrophages post-MI by using a mouse model with Trib1 gene deletion[36]. The impaired wound remodeling was rescued by the introduction of M2 macrophages exogenously. Additionally, limiting the number of infiltrating monocytes via CCR2 blockage has shown to decrease the infarct size in MI recovery animal models[37-39], demonstrating the relevance of therapeutic approaches to limit infiltrating monocyte numbers is patients with increased systemic inflammatory activity to improve post-MI recovery.

As mentioned previously, initial fibrosis following MI is necessary to facilitate scar formation and initiate tissue remodeling. However, extensive fibrosis can result in morbid cardiac remodeling and heart failure[40]. The macrophage number in the heart progressively increases from the onset of MI to heart failure, with local proliferation as well as recruitment responsible[41]. While macrophages in the remote myocardium increase, their number depletes at the site of infarct[6]. This local expansion of cardiac macrophages aggravates the progression of heart failure by producing factors that promote fibrosis, such as TGF-β, platelet derived

growth factor and angiotensin-II[42]. Myofibroblasts and cardiomyocytes also further prompt TGF-β production causing adverse remodeling that leads to heart failure. The detrimental effects of recruited monocytes/macrophages are further recognized as splenectomy reduces infiltrating macrophages and improves cardiac function[43]. A recent report also suggested that mechanical strain can trigger macrophage proliferation by activating mitogen activated protein kinase (MAPK) pathway, demonstrating that increased higher mechanical strain in failing heart could stimulate macrophage expansion[6]. These evidences further highlight the ill impacts of infiltrating macrophages in the infarcted heart. It is also of importance to note recent studies suggesting that the opportunity for successful regeneration of the heart after MI is restricted to 3 days post-MI and is potentially mediated by cardiac resident macrophages and fibroblasts[44].

Engineered Heart Tissue Models

Tissue engineering considerably enables our understanding of disease biology and possibilities for therapeutic intervention[45]. As opposed to 2D cell culture models, tissue engineered cardiac models provide the benefit of mimicking *in vivo* myocardium, with the additional functional benefit of tissue contraction, which is inherent to cardiac function[46]. Moreover, it also comes with the ethical benefit of using lesser number of animals. Engineered constructs are typically generated using primary cardiomyocytes (adult, neonatal, fetal), as well as cardiomyocytes obtained through iPSCs or embryonic stem cells (ESCs)[47]. Although engineered heart tissues (EHTs) can be fabricated purely using cardiomyocytes, the contractile function and the complexity of native myocardium is better recapitulated with the addition of non-myocytes such as fibroblasts and endothelial cells[48]. Other non-myocytes that have been explored include cardiac stem cells[49].

Over two decades ago, the first EHT model was fabricated with chick embryonic cardiomyocytes mixed with Matrigel which anchored on to opposite ends of two Velcro coated glass tubes[50] (Fig.4). This culture provided mechanical strain to the engineered tissue and hence

Mary Ann Liebert, Inc.,140 Huguenot Street, New Rochelle, NY 10801

was utilized as a platform for understanding the effects of uniaxial and multiaxial stretching, growth factors, cardiomyocyte maturation etc. This same principle was used to modify the geometry of the EHT to obtain a ring-shaped model which supported mammalian (rat) cardiomyocytes and also facilitated long-term force measurements[51, 52]. Although a number of different scaffold-based cardiac tissue engineered models were developed around this period, the next major modification to the EHT design was miniaturized EHTs made with fibrin, that were adapted to 24-well format, to facilitate drug screening[53]. This study demonstrated the use of video-optical recording for the measurement of contractility of the tissues, which is a commonly used method of contractility measurement at the moment[53]. Shortly after, Boudou *et al.* generated collagen/fibrin matrix-based cardiac microtissues using microfabricated tissue gauges that incorporate microelectromechanical systems (MEMS) cantilevers[54]. This facilitated in simultaneously constraining and reporting forces generated by the tissues in real time[54]. Similar approaches with cantilevers to allow tissue anchorage has also been used by Turnbull *et al.* to facilitate fabrication of potential preclinical *in vitro* myocardium models[55]. Another interesting modification to the EHT came with the use of a surgical suture within PDMS molds to obtain aligned cardiac tissues with striations called 'Biowire'[56]. Following this study, Bian *et al.* demonstrated that fabricating cardiac tissues with longer elliptical pores within the patch networks improve ECM alignment within the patch[57]. In the past 5 years, the field of EHTs further underwent drastic advances where fibrin based EHTs under dynamic culture (using platform rockers) showed improvement in tissue maturation[58]. These EHTs, called 'cardiobundles' were designed to be cylindrically shaped and anchored within porous flexible nylon frames that supported chronic auxotonic loading and free-floating culture of the tissues[58]. More recently, the advanced successor of Biowire, named 'Biowire II', a heteropolar cardiac tissues containing distinct atrial and ventricular ends was generated[59]. This heteropolar tissue formation was achieved using directed cell differentiation and electrical field

Mary Ann Liebert, Inc.,140 Huguenot Street, New Rochelle, NY 10801

conditioning[59]. With over two decades of input, one of the most recent advancement in EHTs is a mesh structured EHT patch of dimension 5 x 7cm, that closely resemble human ventricular tissue[60].

One of the key features of EHTs is their ability of respond to drugs. Most of the different types of EHTs fabricated over the years aim to feature their drug response. For example, over a decade ago, Hansen *et al.* showed that EHTs display a concentration-dependent increase in relaxation time on treatment with chromanol, quinidine, and erythromycin, while doxorubicin treatment resulted in decreased contractile force [53]. Similarly, Boudou *et al.* tested isoproterenol and digoxin on their EHTs to understand the functional consequences of pharmacologic agents[54]. They showed that small doses (10 and 100 nM) of isoproterenol increased the contractility of the EHTs while 1 and 10 mM decreased the contractility[54]. On the other hand, digoxin improved the contractility at 1 nM and 1 mM but was found to be cardiotoxic at 10 mM concentration[54]. Hence, the varied and dose dependent sensitivity of EHTs towards different drugs make them an exciting alternative to animal models in addition to being a useful tool in understanding the effect of various drugs on the myocardium. Despite the proven efficacies of these EHTs, there still remain challenges in making them more humanized, including the addition of right cell types to mimic the complexity of the myocardium. For a more detailed review capturing EHT the reader is referred to the paper by Christian Zuppinger [61]. One of the potential additions to improve the response of EHTs would be cardiac resident macrophages, as they are critical in the normal functioning of the heart.

Macrophages in Tissue Engineering

In recent years, the critical importance of incorporating macrophages into engineered constructs is being recognized[62,63] and aside from the immunological responses of host macrophages, their role has been harnessed in a number of tissue engineering approaches to

Mary Ann Liebert, Inc.,140 Huguenot Street, New Rochelle, NY 10801

improve tissue maturation, repair and regeneration[64]. Understanding macrophage responses during biomaterial design[65, 66], as well as exploring the effects of mechanical cues on macrophage polarization are other areas within tissue engineering that have gained attention[67, 68]. Researchers have studied the potential of immune assisted tissue engineering, by incorporating macrophages into tissue engineered constructs (Table 1). In particular, macrophages have been used to vascularize biomaterials[69], by promoting angiogenesis[70] and vessel formation[71]. Although immune assisted tissue engineering has not been largely investigated, these data show the significance of macrophages in various tissue engineered constructs.

Within cardiac tissue engineering, the specific functional requirement of this tissue includes contraction, which is inherent to cardiac function[46]. As macrophages have been shown to be integral during signal propagation (See Fig.2), their inclusion in engineered tissue could facilitate robust and more physiologically accurate contraction mechanics. Engineered constructs are typically generated using of primary cardiomyocytes (adult, neonatal, fetal), as well as cardiomyocytes obtained through iPSCs or embryonic stem cells (ESCs)[47]. Since the critical role played by macrophages in cardiac regeneration, maintenance of homeostasis and disease progression has been recognized (in mice), cardiac tissue engineering has begun to focus on incorporating macrophages into engineered heart tissues to recapitulate native tissue functions. A pioneering *in vitro* study in which human peripheral blood-derived macrophages and iPSC-derived cardiomyocyte interaction was assessed, attempted to recapitulate critical cellular events during myocardial injury, using a 3D inversion assay. They proposed a bone morphogenetic protein (BMP) mediated crosstalk between these two cell types[72]. Cardiomyocyte-derived BMP promoted pro-inflammatory (M1) macrophage recruitment and M1 macrophage-derived BMP improved cardiomyocyte proliferation and differentiation

potential. This proposed paracrine mechanism of BMPs was new and provided a new insight into the mechanism of interaction between cardiomyocytes and peripheral blood-derived macrophages. Interestingly, a recent study has shown that the presence of macrophages in engineered cardiac tissue facilitates cardiomyocyte de-differentiation and remodeling[73]. In this study, it was observed that during the initial days (up to 3 days), the macrophages in the engineered cardiac tissue were polarized to an M1 state (CD86 positive), followed by a predominance of M2 macrophages (CD206 positive) as the cardiomyocytes de-differentiated. The effects of macrophage activity on the engineered cardiac tissue model could not be explained in this study however, as a heterogenous population of whole cells from rat heart was used to make the engineered cardiac tissue. Nonetheless, future studies into the influence of macrophage populations of different ontological origin on cardiac function will shed light on their potential role in cardiac tissue engineering.

One study, by Wrona et al. explored the response of human hESC-derived cardiomyocytes to peripheral blood derived monocytes, with a focus on investigating the effects of polarized macrophages and their activation inducing cytokine profiles on cardiomyocyte function[74]. In this study the gene expression profile of the macrophages remained unaffected upon exposure to hESC-derived cardiomyocytes while cardiac-specific genes were downregulated on exposure to M1 and M2 polarized macrophages[74]. Tying in with the findings of this work, a study by Hitscherich et al. using murine ESC-derived cardiomyocytes also showed a decrease in the expression of cardiac-specific genes such as cardiac troponin T and sarcoplasmic/endoplasmic reticulum calcium ATPase when cultured with conditioned media from M1 polarized blood-derived murine macrophages[75]. Here a co-culture system was employed, to demonstrates that M1 macrophages promoted a higher Ca^{2+} fractional release and both macrophage subtypes downregulated the store-operated Ca^{2+} entry in murine ESC-derived cardiomyocytes. As discussed previously, EHTs and other scaffold-based tissue engineered

Mary Ann Liebert, Inc.,140 Huguenot Street, New Rochelle, NY 10801

models for the myocardium heavily rely on cardiomyocytes, cardiac fibroblasts and endothelial cells to represent the myocardium. Although literature shows the potential of EHTs in guiding us towards obtaining robust *in vitro* models of human myocardium that has the ability to mature, beat in synchrony and respond to drugs, the addition of macrophages into these models could benefit our understanding on the *in vitro* behavior of macrophages in a complex, multicellular humanized setting. From the available *in vivo* studies (in mice), it could be anticipated that the incorporation of cardiac resident macrophages within an EHT (containing cardiomyocytes) will facilitate improved contractility and electrical conduction of the tissue. Additionally, the tissue could be hypothesized to exhibit improved maturity as there could be constant exophers elimination and maintenance of homeostasis.

Another interesting avenue that can be pursued using an EHT model that incorporates cardiac resident macrophages is screening anti-inflammatory drugs for post-MI treatment. Although clinical studies on drug treatment in MI patients is limited, there are a number of drugs that have been developed for regulating immune response post-MI[76]. This includes non-steroidal anti-inflammatory drugs, glucocorticoids, cyclosporine etc[77]. Despite showing an improvement in mortality, these broad anti-inflammatory drugs often fail to provide a cardio-protective effect[77]. To circumvent this limitation, targeted anti-inflammatory drugs can be used. For example, the CANTOS (Canakinumab Anti-Inflammatory Thrombosis Outcomes Study) trial was the first large cardiovascular clinical trial to target the inhibition of inflammation by using the drug canakinumab, which inhibits IL-1β to subsequently reduce the production of IL-6 and high sensitivity C-reactive protein (hsCRP)[78]. Among the 10,061 patients with previous MI and hsCRP level ≥ 2mg/l, patients treated with 150 and 300 mg of canakinumab experienced a 15% reduction in major adverse cardiovascular events in comparison with placebo[78]. Although canakinumab was not ultimately approved for cardiovascular treatment, CANTOS trial demonstrated that targeting the inflammatory pathway involving IL-1β and hsCRP reduces

Mary Ann Liebert, Inc.,140 Huguenot Street, New Rochelle, NY 10801

adverse cardiovascular events. CANTOS was followed by CIRT (Cardiovascular Inflammation Reduction Trial) in which 4786 patients with stable atherosclerosis with diabetes mellitus or metabolic syndrome were subjected to low-dose methotrexate[79]. Low-dose methotrexate failed to reduce major adverse cardiovascular events, and also failed to reduced plasma levels of IL-1β and hsCRP, further demonstrating the importance of adequate inhibition of IL-1β to IL-6 pathway for long-term cardiovascular benefits[80]. Further large cardiovascular outcome trials targeting the inflammatory pathways that followed includes COLCOT (Colchicine Cardiovascular Outcomes Trial)[81] and LoDoCO2 (Low Dose Colchicine after Myocardial Infarction)[82]. Both of these trials used low doses of colchicine (0.5mg/day), which is a repurposed anti-inflammatory agent. While COLCOT had 4745 patients with recent MI (less than 30 days), LoDOCO2 had 5522 patients with chronic coronary artery disease and both the studies showed reduction in the incidence of adverse cardiac events such as MI, stroke, cardiovascular death etc[81, 82]. However, colchicine being associated with increase in pneumonia, nausea and flatulence poses risk[80]. To that end, the lack of success for most of these drugs could also stem from the fact that they are not thoroughly validated *in vitro* prior to human trial. The trajectory of recent and ongoing cardiovascular outcome trials show an increased interest towards novel anti-inflammatory and anti-cytokine drugs[80]. This further demonstrates the benefit of an *in vitro* model of the myocardium that has innate immune cells (especially macrophages) as they serve as the primary target for these drugs and can potentially fill the gap between the patient and the pharmaceutical industry.

iPSC and ESC-derived macrophages

Obtaining human cardiac macrophages for subsequent *in vitro* studies is challenging. To date, macrophage studies have heavily relied on murine or human blood-derived monocytes but these cells are not ideal for appropriating tissue resident phenotypes due to their vast

Mary Ann Liebert, Inc.,140 Huguenot Street, New Rochelle, NY 10801

transcriptomic and epigenetic variations, as well as difference in origin between blood-derived and resident macrophages[83]. Obtaining primary resident macrophages from vital organs (cardiac macrophages, Kupffer cells, microglia etc.) is not feasible, as acquiring tissue samples from healthy donors is often associated with donor site morbidity, in addition to ethical considerations. To circumvent these limitations, the use of iPSCs and hESCs as macrophage progenitor sources has come to the forefront (Fig.5) (Table 2). Over the last decade, a number of different protocols have been established for stem cell culture and differentiation, to obtain $CD14^{high}CD16^{low}CD163^{+}CD11b^{+}$ macrophages, morphologically similar to blood-derived macrophages[84-88]. Critically, iPSC and hESC-derived macrophage sources have shown therapeutic benefits in ameliorating liver fibrosis[89], acute lung injury[90] and are also under clinical trial for liver cirrhosis[91].

Furthermore, recent studies have shown that iMacs share ontogeny with tissue resident macrophages and are independent of MYB[90, 92, 93], suggesting that iMacs in isogenic co-cultures may offer a facile and effective approach to obtaining physiologically relevant resident macrophages[94]. For example, iMacs in co-culture with neural cells were found to become microglia-like cells[95, 96]. Haenseler *et al.* showed that iMacs in co-culture with iPSC-derived neurons, expressed markers relevant to microglia as well as major neurodegenerative disorders[84]. Similarly, iMacs in co-culture with hepatocytes were found to show Kupffer cell-like characteristics[93].

In addition to co-culture models, the effect of the local biochemical environment on the specialization of tissue-resident macrophages is becoming more evident in regenerative medicine. Certain transcriptional factors regulate the expression of tissue-resident macrophages[97], such as Spi-c for the development of the red pulp macrophages in spleen[98], Sall1 for microglia[99], and Id3 for Kupffer cells[100]. In a more recent study, activation of iMacs

Mary Ann Liebert, Inc.,140 Huguenot Street, New Rochelle, NY 10801

with transcriptional factor KLF1 was found to drive them towards an erythroblastic island like phenotype[101]. These studies demonstrate that physiologically relevant resident macrophages could be obtained *in vitro* by providing tissue specific cues or by activating specific transcriptional factors. Although, cardiac resident macrophages have not yet been derived from iPSCs, research is proceeding in the right direction, paving way to understanding optimal methods of cardiac resident macrophage acquisition *in vitro*, such as using primary cardiomyocyte conditioned media on iPSC-derived macrophages, establishing co-culture models of cardiomyocytes and iPSC-derived macrophages or using bioinformatics tools to determine transcriptional factors for their induction. Taken together, iPSC-derived macrophages offer great potential in understanding and modelling tissue resident macrophages.

Conclusion

From regarding macrophages as a system of clearing dead cells, to appreciating their role in tissue defense, homeostasis, regeneration and organ development, our understanding of macrophages has advanced significantly in recent years. Lineage tracing and fate mapping studies demonstrated that most vital organs are largely populated by resident macrophages at the onset of embryological development and play critical roles in health and disease. Within the heart, resident macrophages ensure homeostatic cardiac function by performing a number of vital functions including phagocytosis, efferocytosis, eliminating exophers, facilitation of electrical conduction etc. Our understanding of cardiac immunology has progressed well enough to regard cardiac resident macrophages a potential candidate to include in engineered heart tissues, to further our understanding of cell interactions in addition to making the engineered tissues more physiologically relevant. But much more still remains to be

understood, such as what degree of these cells are lost during myocardial disease/ischemia; how do they function in response to injury and disease, their paracrine effects in tissue engineered models etc. The next step in cardiac tissue engineering is to unravel the crosstalk between cardiac macrophages and cardiomyocytes *in vitro* and further our understanding the molecular processing involved in their interaction and their potential in regenerative medicine, therapeutics, disease modelling and drug screening. With engineered heart tissues gaining great research interest, it is highly pertinent to include a resident macrophage population to make the tissue more physiologically relevant. By building on our strong foundational knowledge on different macrophage populations and with the assistance of new technologies, we will hopefully see rapid progress in finding new therapeutics using cardiac tissue engineering.

Acknowledgement

Funding: This work was supported by EPSRC and SFI Centre for Doctoral Training in Engineered Tissues for Discovery, Industry and Medicine, Grant Number EP/S02347X/1. This publication has emanated from research supported in part by a grant from Science Foundation Ireland (SFI) and the European Regional Development Fund (ERDF) under grant number 13/RC/2073.

Conflict of Interest Statement

The authors declare no conflict of interest.

Authorship Confirmation Statement

All co-authors have reviewed and approved of the manuscript prior to submission. The manuscript has been submitted solely to this journal and is not published, in press, or submitted elsewhere.

References

1. Williams, J.W., Giannarelli, C., Rahman, A., Randolph, G.J.and Kovacic, J.C. Macrophage Biology, Classification, and Phenotype in Cardiovascular Disease: JACC Macrophage in CVD Series (Part 1). Journal of the American College of Cardiology **72**, 2166, 2018.
2. Gordon, S.and Plüddemann, A. Tissue macrophages: heterogeneity and functions. BMC Biology **15**, 53, 2017.
3. Lavine, K.J., Pinto, A.R., Epelman, S., et al. The Macrophage in Cardiac Homeostasis and Disease: JACC Macrophage in CVD Series (Part 4). Journal of the American College of Cardiology **72**, 2213, 2018.
4. Litviňuková, M., Talavera-López, C., Maatz, H., et al. Cells of the adult human heart. Nature, 1, 2020.
5. Dick, S.A., Macklin, J.A., Nejat, S., et al. Self-renewing resident cardiac macrophages limit adverse remodeling following myocardial infarction. Nat Immunol **20**, 29, 2019.
6. Sager, H.B., Hulsmans, M., Lavine, K.J., et al. Proliferation and Recruitment Contribute to Myocardial Macrophage Expansion in Chronic Heart Failure. Circulation research **119**, 853, 2016.
7. Bajpai, G., Bredemeyer, A., Li, W., et al. Tissue Resident CCR2- and CCR2+ Cardiac Macrophages Differentially Orchestrate Monocyte Recruitment and Fate Specification Following Myocardial Injury. Circ Res **124**, 263, 2019.
8. Heidt, T., Courties, G., Dutta, P., et al. Differential contribution of monocytes to heart macrophages in steady-state and after myocardial infarction. Circ Res **115**, 284, 2014.
9. Bajpai, G., Schneider, C., Wong, N., et al. The human heart contains distinct macrophage subsets with divergent origins and functions. Nat Med **24**, 1234, 2018.
10. Tucker, N.R., Chaffin, M., Fleming, S.J., et al. Transcriptional and Cellular Diversity of the Human Heart. Circulation 2020.
11. Honold, L.and Nahrendorf, M. Resident and Monocyte-Derived Macrophages in Cardiovascular Disease. Circ Res **122**, 113, 2018.
12. Leid, J., Carrelha, J., Boukarabila, H., et al. Primitive Embryonic Macrophages are Required for Coronary Development and Maturation. Circ Res **118**, 1498, 2016.
13. Shigeta, A., Huang, V., Zuo, J., et al. Endocardially Derived Macrophages Are Essential for Valvular Remodeling. Dev Cell **48**, 617, 2019.
14. de Couto, G. Macrophages in cardiac repair: Environmental cues and therapeutic strategies. Exp Mol Med **51**, 1, 2019.
15. Godwin, J.W., Debuque, R., Salimova, E.and Rosenthal, N.A. Heart regeneration in the salamander relies on macrophage-mediated control of fibroblast activation and the extracellular landscape. NPJ Regen Med **2**2017.
16. Lai, S.L., Marín-Juez, R., Moura, P.L., et al. Reciprocal analyses in zebrafish and medaka reveal that harnessing the immune response promotes cardiac regeneration. Elife **6**2017.
17. Pinto, A.R., Godwin, J.W.and Rosenthal, N.A. Macrophages in cardiac homeostasis, injury responses and progenitor cell mobilisation. Stem Cell Res **13**, 705, 2014.
18. Hulsmans, M., Clauss, S., Xiao, L., et al. Macrophages Facilitate Electrical Conduction in the Heart. Cell **169**, 510, 2017.
19. Li, Y., Li, Q.and Fan, G.C. Macrophage Efferocytosis in Cardiac Pathophysiology and Repair. Shock 2020.
20. DeBerge, M., Yeap, X.Y., Dehn, S., et al. MerTK Cleavage on Resident Cardiac Macrophages Compromises Repair After Myocardial Ischemia Reperfusion Injury. Circ Res **121**, 930, 2017.
21. Melentijevic, I., Toth, M.L., Arnold, M.L., et al. C. elegans neurons jettison protein aggregates and mitochondria under neurotoxic stress. Nature **542**, 367, 2017.
22. Nicolás-Ávila, J.A., Lechuga-Vieco, A.V., Esteban-Martínez, L., et al. A Network of Macrophages Supports Mitochondrial Homeostasis in the Heart. Cell 2020.
23. Nahrendorf, M., Swirski, F.K., Aikawa, E., et al. The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. J Exp Med **204**, 3037, 2007.
24. Li, W., Hsiao, H.-M., Higashikubo, R., et al. Heart-resident CCR2(+) macrophages promote neutrophil extravasation through TLR9/MyD88/CXCL5 signaling. JCI Insight **1**, e87315, 2016.
25. Jung, K., Kim, P., Leuschner, F., et al. Endoscopic time-lapse imaging of immune cells in infarcted mouse hearts. Circ Res **112**, 891, 2013.

Mary Ann Liebert, Inc.,140 Huguenot Street, New Rochelle, NY 10801

26. Hilgendorf, I., Gerhardt, L.M., Tan, T.C., et al. Ly-6Chigh monocytes depend on Nr4a1 to balance both inflammatory and reparative phases in the infarcted myocardium. Circ Res **114**, 1611, 2014.
27. Lee, W.W., Marinelli, B., van der Laan, A.M., et al. PET/MRI of inflammation in myocardial infarction. J Am Coll Cardiol **59**, 153, 2012.
28. Leuschner, F., Rauch, P.J., Ueno, T., et al. Rapid monocyte kinetics in acute myocardial infarction are sustained by extramedullary monocytopoiesis. J Exp Med **209**, 123, 2012.
29. Frantz, S.and Nahrendorf, M. Cardiac macrophages and their role in ischaemic heart disease. Cardiovasc Res **102**, 240, 2014.
30. Frangogiannis, N.G. Inflammation in cardiac injury, repair and regeneration. Curr Opin Cardiol **30**, 240, 2015.
31. Dunlay, S.M., Weston, S.A., Redfield, M.M., Killian, J.M.and Roger, V.L. Tumor necrosis factor-alpha and mortality in heart failure: a community study. Circulation **118**, 625, 2008.
32. Frangogiannis, N.G. The inflammatory response in myocardial injury, repair, and remodelling. Nat Rev Cardiol **11**, 255, 2014.
33. Lavine, K.J., Epelman, S., Uchida, K., et al. Distinct macrophage lineages contribute to disparate patterns of cardiac recovery and remodeling in the neonatal and adult heart. Proc Natl Acad Sci U S A **111**, 16029, 2014.
34. Chen, B.and Frangogiannis, N.G. Macrophages in the Remodeling Failing Heart. Circulation research **119**, 776, 2016.
35. van Amerongen, M.J., Harmsen, M.C., van Rooijen, N., Petersen, A.H.and van Luyn, M.J.A. Macrophage depletion impairs wound healing and increases left ventricular remodeling after myocardial injury in mice. Am J Pathol **170**, 818, 2007.
36. Shiraishi, M., Shintani, Y., Shintani, Y., et al. Alternatively activated macrophages determine repair of the infarcted adult murine heart. J Clin Invest **126**, 2151, 2016.
37. Leuschner, F., Dutta, P., Gorbatov, R., et al. Therapeutic siRNA silencing in inflammatory monocytes in mice. Nat Biotechnol **29**, 1005, 2011.
38. Vagnozzi, R.J., Maillet, M., Sargent, M.A., et al. An acute immune response underlies the benefit of cardiac stem cell therapy. Nature **577**, 405, 2020.
39. Zouggari, Y., Ait-Oufella, H., Bonnin, P., et al. B lymphocytes trigger monocyte mobilization and impair heart function after acute myocardial infarction. Nat Med **19**, 1273, 2013.
40. Hulsmans, M., Sam, F.and Nahrendorf, M. Monocyte and macrophage contributions to cardiac remodeling. J Mol Cell Cardiol **93**, 149, 2016.
41. Sager, H.B., Kessler, T.and Schunkert, H. Monocytes and macrophages in cardiac injury and repair. J Thorac Dis **9**, S30, 2017.
42. Leask, A. Getting to the heart of the matter: new insights into cardiac fibrosis. Circ Res **116**, 1269, 2015.
43. Ismahil, M.A., Hamid, T., Bansal, S.S., et al. Remodeling of the mononuclear phagocyte network underlies chronic inflammation and disease progression in heart failure: critical importance of the cardiosplenic axis. Circ Res **114**, 266, 2014.
44. Whitehead, A.J.and Engler, A.J. Regenerative Crosstalk between Cardiac Cells and Macrophages. American Journal of Physiology-Heart and Circulatory Physiology **0**, null.
45. Shafiee, A.and Atala, A. Tissue Engineering: Toward a New Era of Medicine. Annu Rev Med **68**, 29, 2017.
46. Weinberger, F., Mannhardt, I.and Eschenhagen, T. Engineering Cardiac Muscle Tissue: A Maturating Field of Research. Circ Res **120**, 1487, 2017.
47. Rodrigues, I.C.P., Kaasi, A., Maciel Filho, R., Jardini, A.L.and Gabriel, L.P. Cardiac tissue engineering: current state-of-the-art materials, cells and tissue formation. Einstein (Sao Paulo) **16**, eRB4538, 2018.
48. Caspi, O., Lesman, A., Basevitch, Y., et al. Tissue Engineering of Vascularized Cardiac Muscle From Human Embryonic Stem Cells. Circulation Research **100**, 263, 2007.
49. Murphy, J.F., Mayourian, J., Stillitano, F., et al. Adult human cardiac stem cell supplementation effectively increases contractile function and maturation in human engineered cardiac tissues. Stem Cell Research & Therapy **10**, 373, 2019.

Mary Ann Liebert, Inc.,140 Huguenot Street, New Rochelle, NY 10801

50. Eschenhagen, T., Fink, C., Remmers, U., et al. Three-dimensional reconstitution of embryonic cardiomyocytes in a collagen matrix: a new heart muscle model system. The FASEB Journal **11**, 683, 1997.
51. Zimmermann, W.H., Fink, C., Kralisch, D., et al. Three-dimensional engineered heart tissue from neonatal rat cardiac myocytes. Biotechnology and Bioengineering **68**, 106, 2000.
52. Zimmermann, W.H., Schneiderbanger, K., Schubert, P., et al. Tissue engineering of a differentiated cardiac muscle construct. Circ Res **90**, 223, 2002.
53. Hansen, A., Eder, A., Bönstrup, M., et al. Development of a Drug Screening Platform Based on Engineered Heart Tissue. Circulation Research **107**, 35, 2010.
54. Boudou, T., Legant, W.R., Mu, A., et al. A microfabricated platform to measure and manipulate the mechanics of engineered cardiac microtissues. Tissue engineering Part A **18**, 910, 2012.
55. Turnbull, I.C., Karakikes, I., Serrao, G.W., et al. Advancing functional engineered cardiac tissues toward a preclinical model of human myocardium. FASEB journal : official publication of the Federation of American Societies for Experimental Biology **28**, 644, 2014.
56. Nunes, S.S., Miklas, J.W., Liu, J., et al. Biowire: a platform for maturation of human pluripotent stem cell–derived cardiomyocytes. Nat Methods **10**, 781, 2013.
57. Bian, W., Jackman, C.P.and Bursac, N. Controlling the structural and functional anisotropy of engineered cardiac tissues. Biofabrication **6**, 024109, 2014.
58. Jackman, C.P., Carlson, A.L.and Bursac, N. Dynamic culture yields engineered myocardium with near-adult functional output. Biomaterials **111**, 66, 2016.
59. Zhao, Y., Rafatian, N., Feric, N.T., et al. A Platform for Generation of Chamber-Specific Cardiac Tissues and Disease Modeling. Cell **176**, 913, 2019.
60. Querdel, E., Reinsch, M., Castro, L., et al. Human Engineered Heart Tissue Patches Remuscularize the Injured Heart in a Dose-Dependent Manner. Circulation **0**.
61. Zuppinger, C. 3D Cardiac Cell Culture: A Critical Review of Current Technologies and Applications. Front Cardiovasc Med **6**, 87, 2019.
62. Dollinger, C., Ciftci, S., Knopf-Marques, H., et al. Incorporation of resident macrophages in engineered tissues: Multiple cell type response to microenvironment controlled macrophage-laden gelatine hydrogels. J Tissue Eng Regen Med **12**, 330, 2018.
63. Juhas, M., Abutaleb, N., Wang, J.T., et al. Incorporation of macrophages into engineered skeletal muscle enables enhanced muscle regeneration. Nat Biomed Eng **2**, 942, 2018.
64. Saleh, L.S.and Bryant, S.J. The Host Response in Tissue Engineering: Crosstalk Between Immune cells and Cell-laden Scaffolds. Curr Opin Biomed Eng **6**, 58, 2018.
65. Costantino, M.D., Schuster, A., Helmholz, H., et al. Inflammatory response to magnesium-based biodegradable implant materials. Acta Biomater **101**, 598, 2020.
66. Hotchkiss, K.M., Clark, N.M.and Olivares-Navarrete, R. Macrophage response to hydrophilic biomaterials regulates MSC recruitment and T-helper cell populations. Biomaterials **182**, 202, 2018.
67. Sridharan, R., Cavanagh, B., Cameron, A.R., Kelly, D.J.and O'Brien, F.J. Material stiffness influences the polarization state, function and migration mode of macrophages. Acta Biomater **89**, 47, 2019.
68. Sridharan, R., Ryan, E.J., Kearney, C.J., Kelly, D.J.and O'Brien, F.J. Macrophage Polarization in Response to Collagen Scaffold Stiffness Is Dependent on Cross-Linking Agent Used To Modulate the Stiffness. ACS Biomaterials Science & Engineering **5**, 544, 2019.
69. Spiller, K.L., Nassiri, S., Witherel, C.E., et al. Sequential delivery of immunomodulatory cytokines to facilitate the M1-to-M2 transition of macrophages and enhance vascularization of bone scaffolds. Biomaterials **37**, 194, 2015.
70. Jetten, N., Verbruggen, S., Gijbels, M.J., et al. Anti-inflammatory M2, but not pro-inflammatory M1 macrophages promote angiogenesis in vivo. Angiogenesis **17**, 109, 2014.
71. Moore, E.M., Ying, G.and West, J.L. Macrophages Influence Vessel Formation in 3D Bioactive Hydrogels. Advanced Biosystems **1**, 1600021, 2017.
72. Pallotta, I., Sun, B., Wrona, E.A.and Freytes, D.O. BMP protein-mediated crosstalk between inflammatory cells and human pluripotent stem cell-derived cardiomyocytes. J Tissue Eng Regen Med **11**, 1466, 2017.
73. Wang, C., Liu, W., Shen, Y., et al. Cardiomyocyte dedifferentiation and remodeling in 3D scaffolds to generate the cellular diversity of engineering cardiac tissues. Biomater Sci **7**, 4636, 2019.

Mary Ann Liebert, Inc.,140 Huguenot Street, New Rochelle, NY 10801

74. Wrona, E.A., Sun, B., Romero-Torres, S.and Freytes, D.O. Effects of polarized macrophages on the in vitro gene expression after Co-Culture of human pluripotent stem cell-derived cardiomyocytes. Journal of Immunology and Regenerative Medicine **4**, 100018, 2019.
75. Hitscherich, P.G., Xie, L.-H., Del Re, D.and Lee, E.J. The effects of macrophages on cardiomyocyte calcium-handling function using in vitro culture models. Physiol Rep **7**, e14137, 2019.
76. Lawler, P.R., Bhatt, D.L., Godoy, L.C., et al. Targeting cardiovascular inflammation: next steps in clinical translation. Eur Heart J **42**, 113, 2021.
77. Huang, S.and Frangogiannis, N.G. Anti-inflammatory therapies in myocardial infarction: failures, hopes and challenges. Br J Pharmacol **175**, 1377, 2018.
78. Ridker, P.M., Everett, B.M., Thuren, T., et al. Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease. New England Journal of Medicine **377**, 1119, 2017.
79. Ridker, P.M., Everett, B.M., Pradhan, A., et al. Low-Dose Methotrexate for the Prevention of Atherosclerotic Events. New England Journal of Medicine **380**, 752, 2018.
80. Ridker, P.M. From CANTOS to CIRT to COLCOT to Clinic. Circulation **141**, 787, 2020.
81. Tardif, J.-C., Kouz, S., Waters, D.D., et al. Efficacy and Safety of Low-Dose Colchicine after Myocardial Infarction. New England Journal of Medicine **381**, 2497, 2019.
82. Nidorf, S.M., Fiolet, A.T.L., Mosterd, A., et al. Colchicine in Patients with Chronic Coronary Disease. New England Journal of Medicine **383**, 1838, 2020.
83. Hagemeyer, N., Kierdorf, K., Frenzel, K., et al. Transcriptome-based profiling of yolk sac-derived macrophages reveals a role for Irf8 in macrophage maturation. Embo j **35**, 1730, 2016.
84. Haenseler, W., Sansom, S.N., Buchrieser, J., et al. A Highly Efficient Human Pluripotent Stem Cell Microglia Model Displays a Neuronal-Co-culture-Specific Expression Profile and Inflammatory Response. Stem Cell Reports **8**, 1727, 2017.
85. Gutbier, S., Wanke, F., Dahm, N., et al. Large-Scale Production of Human iPSC-Derived Macrophages for Drug Screening. Int J Mol Sci **21**2020.
86. Cao, X., Yakala, G.K., van den Hil, F.E., et al. Differentiation and Functional Comparison of Monocytes and Macrophages from hiPSCs with Peripheral Blood Derivatives. Stem Cell Reports **12**, 1282, 2019.
87. Nenasheva, T., Gerasimova, T., Serdyuk, Y., et al. Macrophages Derived From Human Induced Pluripotent Stem Cells Are Low-Activated "Naïve-Like" Cells Capable of Restricting Mycobacteria Growth. Front Immunol **11**, 1016, 2020.
88. Shi, J., Xue, C., Liu, W.and Zhang, H. Differentiation of Human-Induced Pluripotent Stem Cells to Macrophages for Disease Modeling and Functional Genomics. Curr Protoc Stem Cell Biol **48**, e74, 2019.
89. Haideri, S.S., McKinnon, A.C., Taylor, A.H., et al. Injection of embryonic stem cell derived macrophages ameliorates fibrosis in a murine model of liver injury. NPJ Regen Med **2**, 14, 2017.
90. Litvack, M.L., Wigle, T.J., Lee, J., et al. Alveolar-like Stem Cell-derived Myb(-) Macrophages Promote Recovery and Survival in Airway Disease. Am J Respir Crit Care Med **193**, 1219, 2016.
91. Moroni, F., Dwyer, B.J., Graham, C., et al. Safety profile of autologous macrophage therapy for liver cirrhosis. Nat Med **25**, 1560, 2019.
92. Buchrieser, J., James, W.and Moore, M.D. Human Induced Pluripotent Stem Cell-Derived Macrophages Share Ontogeny with MYB-Independent Tissue-Resident Macrophages. Stem Cell Reports **8**, 334, 2017.
93. Tasnim, F., Xing, J., Huang, X., et al. Generation of mature kupffer cells from human induced pluripotent stem cells. Biomaterials **192**, 377, 2019.
94. Lee, C.Z.W., Kozaki, T.and Ginhoux, F. Studying tissue macrophages in vitro: are iPSC-derived cells the answer? Nat Rev Immunol **18**, 716, 2018.
95. Takata, K., Kozaki, T., Lee, C.Z.W., et al. Induced-Pluripotent-Stem-Cell-Derived Primitive Macrophages Provide a Platform for Modeling Tissue-Resident Macrophage Differentiation and Function. Immunity **47**, 183, 2017.
96. Quarta, A., Le Blon, D., D'Aes, T., et al. Murine iPSC-derived microglia and macrophage cell culture models recapitulate distinct phenotypical and functional properties of classical and alternative neuro-immune polarisation. Brain Behav Immun **82**, 406, 2019.
97. T'Jonck, W., Guilliams, M.and Bonnardel, J. Niche signals and transcription factors involved in tissue-resident macrophage development. Cell Immunol **330**, 43, 2018.

98. Haldar, M., Kohyama, M., So, A.Y., et al. Heme-mediated SPI-C induction promotes monocyte differentiation into iron-recycling macrophages. Cell **156**, 1223, 2014.
99. Buttgereit, A., Lelios, I., Yu, X., et al. Sall1 is a transcriptional regulator defining microglia identity and function. Nat Immunol **17**, 1397, 2016.
100. Mass, E., Ballesteros, I., Farlik, M., et al. Specification of tissue-resident macrophages during organogenesis. Science **353**2016.
101. Lopez-Yrigoyen, M., Yang, C.-T., Fidanza, A., et al. Genetic programming of macrophages generates an in vitro model for the human erythroid island niche. Nature Communications **10**, 881, 2019.
102. Mahon, O.R., Browe, D.C., Gonzalez-Fernandez, T., et al. Nano-particle mediated M2 macrophage polarization enhances bone formation and MSC osteogenesis in an IL-10 dependent manner. Biomaterials **239**, 119833, 2020.
103. Romero-López, M., Li, Z., Rhee, C., et al. Macrophage Effects on Mesenchymal Stem Cell Osteogenesis in a Three-Dimensional In Vitro Bone Model. Tissue Eng Part A 2020.
104. Rose, K.A., Holman, N.S., Green, A.M., Andersen, M.E.and LeCluyse, E.L. Co-culture of Hepatocytes and Kupffer Cells as an In Vitro Model of Inflammation and Drug-Induced Hepatotoxicity. J Pharm Sci **105**, 950, 2016.
105. Nguyen, T.V., Ukairo, O., Khetani, S.R., et al. Establishment of a hepatocyte-kupffer cell coculture model for assessment of proinflammatory cytokine effects on metabolizing enzymes and drug transporters. Drug Metab Dispos **43**, 774, 2015.
106. Pires, L.R., Rocha, D.N., Ambrosio, L.and Pêgo, A.P. The role of the surface on microglia function: implications for central nervous system tissue engineering. J R Soc Interface **12**, 20141224, 2015.
107. Goshi, N., Morgan, R.K., Lein, P.J.and Seker, E. A primary neural cell culture model to study neuron, astrocyte, and microglia interactions in neuroinflammation. Journal of Neuroinflammation **17**, 155, 2020.
108. Ishii, H., Hayashi, S., Hogg, J.C., et al. Alveolar macrophage-epithelial cell interaction following exposure to atmospheric particles induces the release of mediators involved in monocyte mobilization and recruitment. Respir Res **6**, 87, 2005.
109. Hittinger, M., Mell, N.A., Huwer, H., et al. Autologous co-culture of primary human alveolar macrophages and epithelial cells for investigating aerosol medicines. Part II: evaluation of IL-10-loaded microparticles for the treatment of lung inflammation. Altern Lab Anim **44**, 349, 2016.
110. Kletting, S., Barthold, S., Repnik, U., et al. Co-culture of human alveolar epithelial (hAELVi) and macrophage (THP-1) cell lines. Altex **35**, 211, 2018.
111. Zhang, H., Xue, C., Shah, R., et al. Functional analysis and transcriptomic profiling of iPSC-derived macrophages and their application in modeling Mendelian disease. Circ Res **117**, 17, 2015.
112. van Wilgenburg, B., Browne, C., Vowles, J.and Cowley, S.A. Efficient, long term production of monocyte-derived macrophages from human pluripotent stem cells under partly-defined and fully-defined conditions. PLoS One **8**, e71098, 2013.
113. Lachmann, N., Ackermann, M., Frenzel, E., et al. Large-scale hematopoietic differentiation of human induced pluripotent stem cells provides granulocytes or macrophages for cell replacement therapies. Stem Cell Reports **4**, 282, 2015.
114. Douvaras, P., Sun, B., Wang, M., et al. Directed Differentiation of Human Pluripotent Stem Cells to Microglia. Stem cell reports **8**, 1516, 2017.
115. Muffat, J., Li, Y., Yuan, B., et al. Efficient derivation of microglia-like cells from human pluripotent stem cells. Nat Med **22**, 1358, 2016.

Fig.1 Macrophage subsets in human and mouse and their origin. Cardiac macrophages populate the heart prenatally from the embryonic yolk sac, while bone marrow-derived macrophages enter the heart two weeks after birth. In humans, the macrophage populations are distinguished based on the presence of CCR2, as CCR2^{-}HLA-DR^{high} (cardiac macrophages)

and CCR2^{+}HLA-DR^{high} (bone marrow-derived macrophages). The three distinct populations of macrophages in murine heart are, CCR2^{-}MHC-II^{low} (cardiac macrophages), CCR2^{-}MHC-II^{high} (derived postnatally from cardiac macrophages), CCR2^{+}MHC-II^{high} (bone marrow-derived macrophages). *Created with BioRender.com*

Fig.2 Functions of cardiac macrophages in healthy myocardium. In steady state, cardiac macrophages perform several important functions in the myocardium. One of their major functions is to facilitate electrical conduction by coupling with cardiomyocytes through connexin-43 gap junctions. They help in sustaining normal cardiac autophagy by capturing and eliminating exophers (containing defective mitochondria) released by cardiomyocytes, through Mertk. They also perform immunosurveillance and clear dead cells and debris in the myocardium, to maintain homeostasis. The presence of cardiac macrophages is found to be essential in promoting cardiac regeneration. *Created with BioRender.com*

Fig.1 Macrophage origin and function in infarcted heart. After MI, the monocytes that infiltrate the heart originate from bone marrow (~60%) and spleen (~40%). The recruitment of bone marrow-derived monocytes is CCR2 dependent and that of spleen-derived monocytes is angiotensin II-dependent. Cardiac resident macrophages reside, self-renew and carry out a number of functions in the myocardium. However, at the infarct site and remote myocardium, infiltrating macrophages differentiate to M1 and M2 macrophages in response to different cues, and secrete pro-inflammatory and pro-healing factors based on their macrophage phenotype. The number of M1 and M2 macrophages peak in the infarct site and remote myocardium at different pace. *Created with BioRender.com*

Fig.4 Brief overview of the evolution of Engineered Heart Tissues. The very first EHT was generated in 1997 using chick embryonic cardiomyocytes on Velcro coated glass tubes[50]. This model and the geometry of EHTs evolved over the years, from ring-shaped EHTs[51] to mini-EHTs adapted to 24 well plate format[53], to EHTs with fluorescent cantilevers that can track

Mary Ann Liebert, Inc.,140 Huguenot Street, New Rochelle, NY 10801

contractile motions[54], followed by Biowire[56], cardiobundles[58] and Biowire II[59], to finally reach human-scale EHT patch[60]. *Created with BioRender.com*

Fig.5 Macrophage derivation from iPSCs and their applications. iPSCs can be derived from human beings by reprogramming somatic cells (fibroblasts, blood cells etc.). On specifying the mesodermal origin, they can be differentiated into macrophages. Cardiac resident macrophages can potentially be obtained by giving iPSC-derived macrophages tissue specific cues or activating specific transcriptional factors. These macrophages can be used for a number of applications such as disease modelling, cell therapy, genomics (to identify new genes) and drug testing. *Created with BioRender.com*

Tables

Table 1 Examples of macrophage use in tissue engineering

Tissue	Macrophage	Application
Bone	Blood-derived macrophages	Vascularization of scaffold[69]
	Blood-derived macrophages	Osteogenesis[102, 103]
Skeletal muscle	Blood-derived macrophages	Regeneration[63]
Blood vessel	Blood-derived macrophages	Vascularization of scaffold[71]
Liver	Kupffer cells	Hepatotoxicity[104]
	Kupffer cells	Disease-drug interaction[105]
Nerve	Microglia	Regeneration[106]
Brain/spinal cord	Microglia	Understand cellular crosstalk during neuroinflammation[107]
Lungs	Alveolar macrophages	Understanding response to atmospheric particles[108]
	Alveolar macrophages	*In vitro* lung inflammation platform[109]

Mary Ann Liebert, Inc.,140 Huguenot Street, New Rochelle, NY 10801

	Alveolar macrophages	*In vitro* model for air-blood barrier[110]

Table 2. Examples of iPSC-derived macrophage subsets and their respective phenotypic markers

Tissue	Cell-type	Phenotypic markers
Blood	Blood-derived macrophages	CD18+, CD11b+, CD11c+, CD14+, CD16+, CD115+, CD1a-, CD83-, CD3-, CD19-[111] CD14+, CD16+, CD163+, CD86+[112] CD45+, CD11b+, CD14+, CD163+, CD68+[113]
Lungs	Alveolar macrophages	CD80+, SIRPa+, MHCII-, Langerin-[90]
Liver	Kupffer cells	CLEC-4F+, ID1+ and ID3+ [93]
Brain	Microglia	IBA1+, CD11c+, TMEM119+, P2RY12+, CD11b+, and CX3CR1+[114] CD11b+, ITGB2+, CSF1R+, CD45+, IBA1+ and ADORA3+[115]

Mary Ann Liebert, Inc.,140 Huguenot Street, New Rochelle, NY 10801

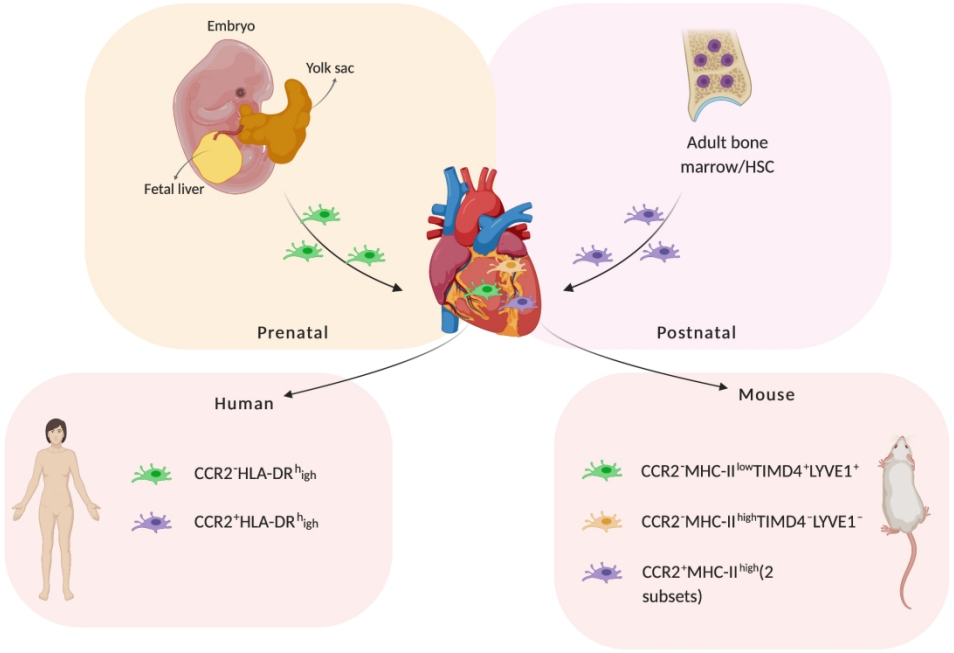

Fig.1 Macrophage subsets in human and mouse and their origin. Cardiac macrophages populate the heart prenatally from the embryonic yolk sac, while bone marrow-derived macrophages enter the heart two weeks after birth. In humans, the macrophage populations are distinguished based on the presence of CCR2, as CCR2-HLA-DRhigh (cardiac macrophages) and CCR2+HLA-DRhigh (bone marrow-derived macrophages). The three distinct populations of macrophages in murine heart are, CCR2-MHC-IIlow (cardiac macrophages), CCR2-MHC-IIhigh (derived postnatally from cardiac macrophages), CCR2+MHC-IIhigh (bone marrow-derived macrophages). Created with BioRender.com

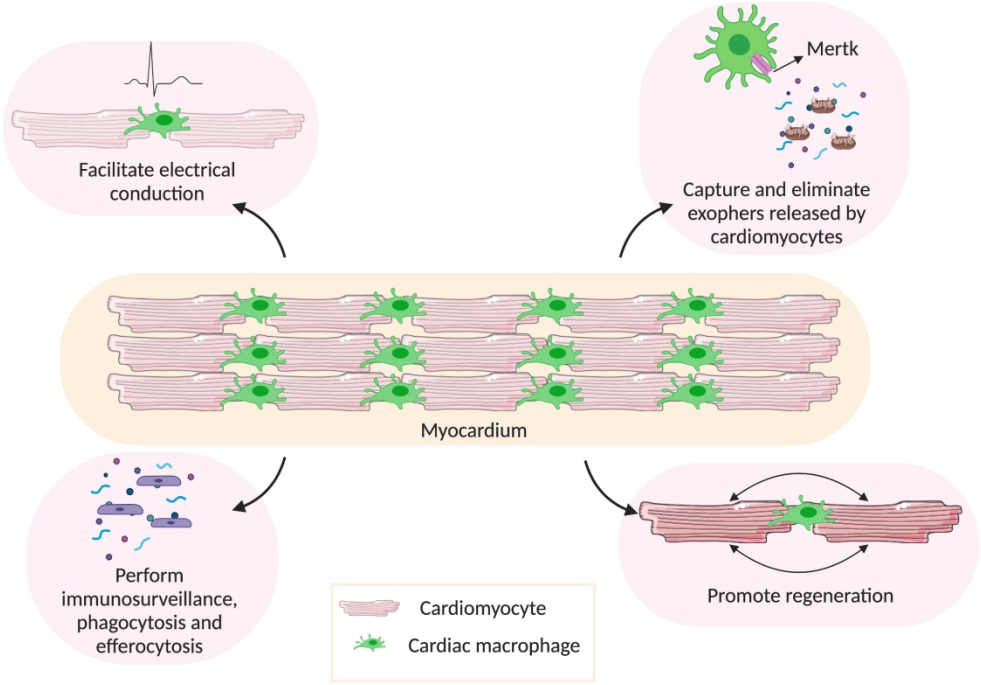

Fig.2 Functions of cardiac macrophages in healthy myocardium. In steady state, cardiac macrophages perform several important functions in the myocardium. One of their major functions is to facilitate electrical conduction by coupling with cardiomyocytes through connexin-43 gap junctions. They help in sustaining normal cardiac autophagy by capturing and eliminating exophers (containing defective mitochondria) released by cardiomyocytes, through Mertk. They also perform immunosurveillance and clear dead cells and debris in the myocardium, to maintain homeostasis. The presence of cardiac macrophages is found to be essential in promoting cardiac regeneration. Created with BioRender.com

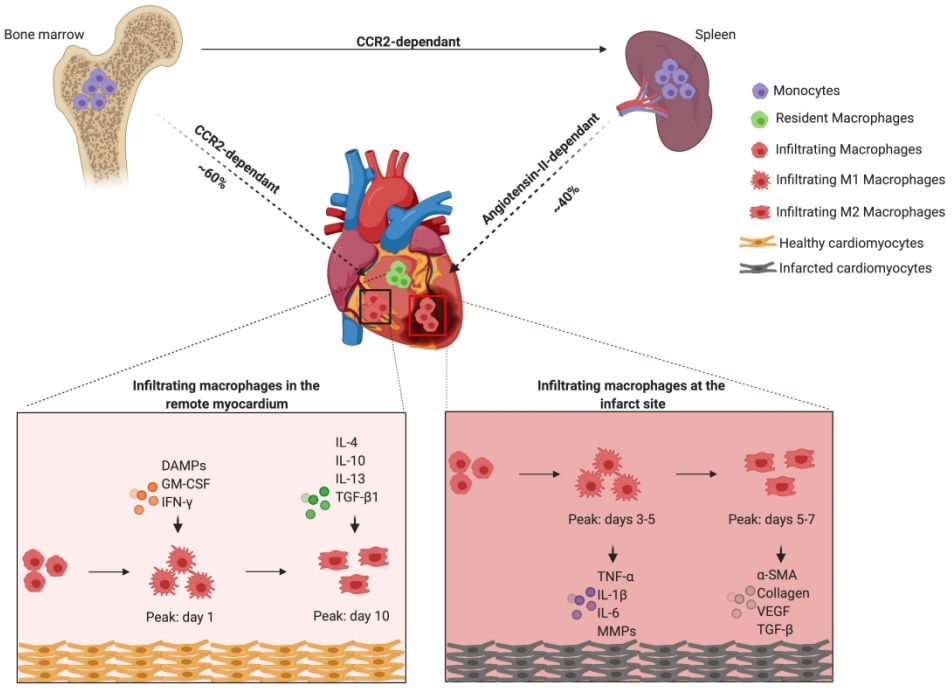

Fig.3 Macrophage origin and function in infarcted heart. After MI, the monocytes that infiltrate the heart originate from bone marrow (~60%) and spleen (~40%). The recruitment of bone marrow-derived monocytes is CCR2 dependent and that of spleen-derived monocytes is angiotensin II-dependent. Cardiac resident macrophages reside, self-renew and carry out a number of functions in the myocardium. However, at the infarct site and remote myocardium, infiltrating macrophages differentiate to M1 and M2 macrophages in response to different cues, and secrete pro-inflammatory and pro-healing factors based on their macrophage phenotype. The number of M1 and M2 macrophages peak in the infarct site and remote myocardium at different pace. Created with BioRender.com

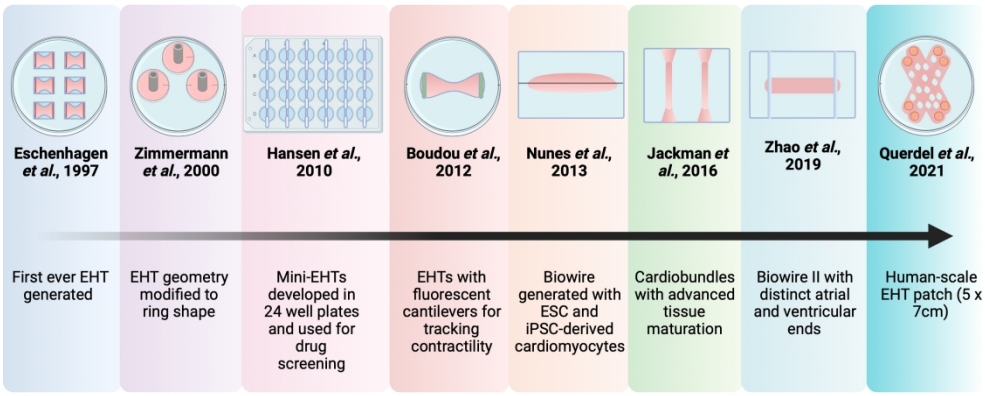

Fig.4 Brief overview of the evolution of Engineered Heart Tissues. The very first EHT was generated in 1997 using chick embryonic cardiomyocytes on Velcro coated glass tubes50. This model and the geometry of EHTs evolved over the years, from ring-shaped EHTs51 to mini-EHTs adapted to 24 well plate format53, to EHTs with fluorescent cantilevers that can track contractile motions54, followed by Biowire56, cardiobundles58 and Biowire II59, to finally reach human-scale EHT patch60. Created with BioRender.com

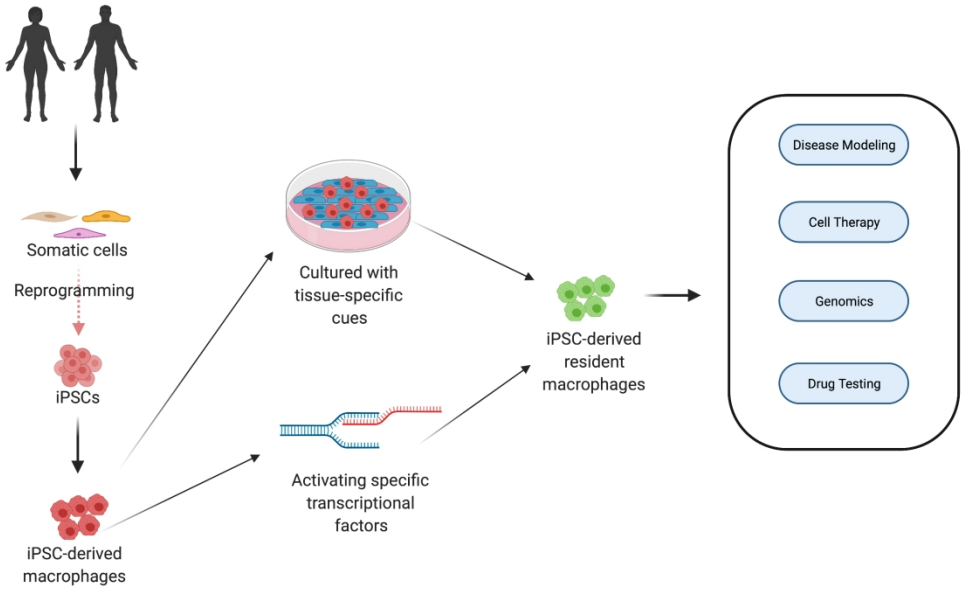

Fig.5 Macrophage derivation from iPSCs and their applications. iPSCs can be derived from human beings by reprogramming somatic cells (fibroblasts, blood cells etc.). On specifying the mesodermal origin, they can be differentiated into macrophages. Cardiac resident macrophages can potentially be obtained by giving iPSC-derived macrophages tissue specific cues or activating specific transcriptional factors. These macrophages can be used for a number of applications such as disease modeling, cell therapy, genomics (to identify new genes) and drug testing. Created with BioRender.com

Table 1 Examples of macrophage use in tissue engineering

Tissue	Macrophage	Application
Bone	Blood-derived macrophages	Vascularization of scaffold[52]
	Blood-derived macrophages	Osteogenesis[55, 56]
Skeletal muscle	Blood-derived macrophages	Regeneration[46]
Blood vessel	Blood-derived macrophages	Vascularization of scaffold[54]
Liver	Kupffer cells	Hepatotoxicity[57]
	Kupffer cells	Disease-drug interaction[58]
Nerve	Microglia	Regeneration[59]
Brain/spinal cord	Microglia	Understand cellular crosstalk during neuroinflammation[60]
Lungs	Alveolar macrophages	Understanding response to atmospheric particles[61]
	Alveolar macrophages	*In vitro* lung inflammation platform[62]
	Alveolar macrophages	*In vitro* model for air-blood barrier[63]

Table 2. Examples of iPSC-derived macrophage subsets and their respective phenotypic markers

Tissue	Cell-type	Phenotypic markers
Blood	Blood-derived macrophages	CD18+, CD11b+, CD11c+, CD14+, CD16+, CD115+, CD1a-, CD83-, CD3-, CD19-[89] CD14+, CD16+, CD163+, CD86+[90] CD45+, CD11b+, CD14+, CD163+, CD68+[91]

Lungs	Alveolar macrophages	CD80+, SIRPa+, MHCII-, Langerin-[78]
Liver	Kupffer cells	CLEC-4F+, ID1+ and ID3+ [81]
Brain	Microglia	IBA1+, CD11c+, TMEM119+, P2RY12+, CD11b+, and CX3CR1+[92] CD11b+, ITGB2+, CSF1R+, CD45+, IBA1+ and ADORA3+[93]

Fig.1 Macrophage subsets in human and mouse and their origin. Cardiac macrophages populate the heart prenatally from the embryonic yolk sac, while bone marrow-derived macrophages enter the heart two weeks after birth. In humans, the macrophage populations are distinguished based on the presence of CCR2, as $CCR2^{-}$HLA-DR^{high} (cardiac macrophages) and $CCR2^{+}$HLA-DR^{high} (bone marrow-derived macrophages). The three distinct populations of macrophages in murine heart are, $CCR2^{-}$MHC-II^{low} (cardiac macrophages), $CCR2^{-}$MHC-II^{high} (derived postnatally from cardiac macrophages), $CCR2^{+}$MHC-II^{high} (bone marrow-derived macrophages). *Created with BioRender.com*

Fig.2 Functions of cardiac macrophages in healthy myocardium. In steady state, cardiac macrophages perform several important functions in the myocardium. One of their major functions is to facilitate electrical conduction by coupling with cardiomyocytes through connexin-43 gap junctions. They help in sustaining normal cardiac autophagy by capturing and eliminating exophers (containing defective mitochondria) released by cardiomyocytes, through Mertk. They also perform immunosurveillance and clear dead cells and debris in the myocardium, to maintain homeostasis. The presence of cardiac macrophages is found to be essential in promoting cardiac regeneration. *Created with BioRender.com*

Fig.1 Macrophage origin and function in infarcted heart. After MI, the monocytes that infiltrate the heart originate from bone marrow (~60%) and spleen (~40%). The recruitment of bone marrow-derived monocytes is CCR2 dependent and that of spleen-derived monocytes is angiotensin II-dependent. Cardiac resident macrophages reside, self-renew and carry out a number of functions in the myocardium. However, at the infarct site and remote myocardium, infiltrating macrophages differentiate to M1 and M2 macrophages in response to different cues, and secrete pro-inflammatory and pro-healing factors based on their macrophage phenotype. The number of M1 and M2 macrophages peak in the infarct site and remote myocardium at different pace. *Created with BioRender.com*

Fig.4 Brief overview of the evolution of Engineered Heart Tissues. The very first EHT was generated in 1997 using chick embryonic cardiomyocytes on Velcro coated glass tubes[50]. This model and the geometry of EHTs evolved over the years, from ring-shaped EHTs[51] to mini-EHTs adapted to 24 well plate format[53], to EHTs with fluorescent cantilevers that can track contractile motions[54], followed by Biowire[56], cardiobundles[58] and Biowire II[59], to finally reach human-scale EHT patch[60]. *Created with BioRender.com*

Fig.5 Macrophage derivation from iPSCs and their applications. iPSCs can be derived from human beings by reprogramming somatic cells (fibroblasts, blood cells etc.). On specifying the mesodermal origin, they can be differentiated into macrophages. Cardiac resident macrophages can potentially be obtained by giving iPSC-derived macrophages tissue specific cues or activating specific transcriptional factors. These macrophages can be used for a number of applications such as disease modeling, cell therapy, genomics (to identify new genes) and drug testing. *Created with BioRender.com*

Tissue Engineering

Author's Response to Decision Letter for (TEB-2021-0036)

Resident macrophages – a potential gamechanger in cardiac tissue engineering?

Dear Dr. Schenke-Layland,

Thank you for your recent communication regarding this manuscript, and the positive decision to consider it further given some revisions.

Many thanks to the reviewers for the time and attention to detail they have given to critique this manuscript. It is gratifying that they deem this work of interest to the readership of Tissue Engineering Part B: Reviews and recognize the effort we have made in organizing this manuscript. Their scrutiny pointed us towards reviewing the manuscript further and the suggested changes have been made. The revised version has greatly benefited from their critiques.

We are pleased to submit the revised manuscript with the titled modified as "Resident macrophages and their potential in cardiac tissue engineering". Thank you for allowing us the opportunity to address these comments of the reviewers. We have highlighted changes to the text in this revision in RED and specified their location.

All authors have read the manuscript and agreed to submit this revision to Tissue Engineering Part B: Reviews.

Thank you in advance for considering our manuscript.

Sincerely,

_____
Michael Monaghan, Ph.D, B.Eng

Response to individual reviewer comments:
Reviewer 1
'This is a review article that describe about role of cardiac residential macrophage. There was no concrete idea how to utilize this macrophage for cardiac tissue engineering.'
As the reviewer rightly points out, the review article does describe critical functions of cardiac resident macrophages in healthy and infarcted myocardium. We did aim to give equal focus in putting forth potential ways of obtaining cardiac resident macrophages in vitro but agree that the initial submission fell short in fully discussing the utilization of macrophages for cardiac tissue engineering.
With this revised manuscript, we have added a significant discussion on cardiac resident macrophages in the maintenance of normal cardiac function and hence their potential to enhance tissue engineered cardiac models. While this focus may may not have been explicit in the original submission; we are confident that our revised manuscript now does this justice.

'In addition, there was no successful report for iPS derived macrophage.'
Derivation of macrophages from iPSCs is a fairly well-established protocol which we had discussed in the original submission. We emphasize this further in the revised manuscript. Specifically, references 84-93 and 95-97 show successful macrophage derivation from iPSCs.

'Title is misleading and should be changed to such as " The role of residential cardiac macrophage"'
We thank the reviewer for this suggestion. The title has been revised.

'The reviewer recommend to add the immuno-histological pictures of this macrophage in healthy heart and in diseased heart.'
We thank the reviewer for this suggestion. However, to our knowledge; there are no immunohistochemical images available of healthy human heart that depicts the presence of resident cardiac macrophages specifically, studies using healthy human heart tissue being fairly limited. There are, however, plenty of immunohistochemical images taken from murine heart where the depletion of resident macrophages in the myocardium can be seen during injury. Indeed, one of the themes of this review is that such biology is predominantly reported in such mouse models whereby the human biology can be entirely different which we discuss to model using iPSC derivatization of human resident macrophages to study this biology.

Reviewer 2

'The review relies on the role of macrophage subpopulations in cardiac tissue physiology and in myocardial infarction, highlighting how interesting this knowledge is to be applied to cardiac tissue engineering. Although the article brings recent findings on macrophages and cardiomyocytes, very little has been explored on the engineering of cardiac tissue.'
We thank the reviewer for acknowledging the interest of using macrophages in cardiac tissue engineering and agree with the reviewer on how little this field has been explored. Their feedback is similar to that of reviewer one, which we have not made significant revisions to address.

'In addition, a review article with a similar proposal was recently published in this journal. Please, see: h (2020). Crosstalk between cardiac cells and macrophages: Insights from in vitro studies. Tissue Engineering Part B: Reviews.'
Many thanks to the reviewer for mentioning this paper by Hitscherich et. al which we do admire- it is very interesting and covers some topics that are similar to our manuscript. Regrettably, we had not come across it during the preparation of our manuscript due to its very recent publishing data. While some elements in our manuscript share a similar focus; we have shifted our theme to enable a clear distinction between the two. Our revised manuscript places strong focus on the different applications of macrophages in tissue engineering, with particular interest in cardiac tissue engineering. Additionally, we discuss the potential of bringing forward induced pluripotent stem cell (iPSC) and embryonic stem cell (ESC) derived macrophages because of their proven ability to differentiate into other tissue resident macrophages such as microglia and Kupffer cells. It is important to note, that the topic of resident macrophages is extremely topical at the moment- with this review, we aim to bring together existing knowledge in this field and how it can be put together to obtain cardiac resident macrophages.

'I suggest that the authors delve deeper into the tissue engineering models that have already been proposed and also which could be proposed in this area, since the tissue engineering with the immune system cell component is not so trivial.'
We thank the reviewer for this valuable suggestion and acknowledge the importance of tissue engineered models incorporating immune cells components. It shares similarities with the previous comments and we have added a significant amount of discussion to address this.

'Acronyms throughout the text that are not described'
Thanks to the reviewer for pointing this out, the revised manuscript addresses this.

'The title of last topic does not match the information'
Thanks to the reviewer for highlighting this, the revised manuscript includes modified subheading.