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Article

Do Human iPSC-Derived Cardiomyocytes Cultured on PLA Scaffolds Induce Expression of CD28/CTLA-4 by T Lymphocytes?

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Abstract: Currently, many research groups have developed various types of tissue-engineered cardiac constructs. However, the immunological properties of such artificial tissues are not yet fully studied. Previously, we developed microfiber scaffolds carrying human iPSC-derived cardiomyocytes (hiPSC-CM). In this work, we evaluated the ability of these tissue-engineered constructs to activate the expression of CD28 and CTLA-4 proteins on T lymphocytes, which are early markers of the immune response. For this purpose, electrospun PLA microfiber scaffolds were seeded with hiPSC-CM and cultured for 2 weeks. Allogeneic mononuclear cells were then co-cultured for 48 hours with three groups of samples: bare scaffolds, pure cardiomyocyte culture and tissue-engineered constructs, followed by analysis of CD28/CTLA-4 expression on T lymphocytes using flow cytometry. PLA scaffolds and concanavalin A stimulation (positive control) statistically significantly increased CD28 expression on $CD4^+$ T cells (up to 61.3% and 66.3%) and on $CD8^+$ T cells (up to 17.8% and 21.7%). CD28/CTLA-4 expression was not increased when T lymphocytes were co-cultured with cardiac tissue engineered constructs and iPSC-CM monolayers. Thus, iPSC-CM in monolayers and on PLA microfiber scaffolds did not induce T cell activation, which suggest that such cardiac constructs would not be a cause of rejection after implantation.

Keywords: graft rejection, iPSC, differentiation, cardiomyocytes, electrospinning, CD28, CTLA-4, immune response

1. Introduction

Functional repair of damaged myocardium remains a challenging task for cardiac tissue engineering [1]. One promising approach to solve this problem is the use so-called cardiac tissue-engineered constructs, the purpose of which is to deliver new cells to the damaged area [2]. Such constructs can consist of cellular and biomaterial components loaded with bioactive molecules [3]. However,an important issue that still needs to be addressed is the host`s immune response and inflammation, which damage cells and scaffolds and can cause fibrosis.

For a long time, tissue from rats or other animals was used as a source of cardiomyocytes. But these cells were only use in research and could not be applied for treatment

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because of insufficient quantity and strong immune reactions associated with transplantation. This problem has been solved with the emergence of so-called cardiomyocytes differentiated from induced pluripotent stem cells which have been actively developed in the last decade[4]. iPSCs are a unique source for obtaining a sufficient number of cardiomyocytes (CM) when creating cardiac tissue-engineered constructs. To date, there are a number of protocols for directed differentiation of induced human and animal pluripotent stem cells into cardiomyocytes [5, 6].

However, it is inefficient to use hiPSC-CM alone in the development of myocardial damage treatment infarction. It has been shown that direct intramyocardial transplantation of cardiac cells without a matrix leads to poor survival and an insufficient fixation at the injection site [7]. This is due to the fact that cardiomyocytes are anchorage-dependent cells and must be cultured on a substrate for long-term survival. To overcome the problem of poor survival and engraftment, it is useful to use twocomponent tissue-engineered cardiac tissues consisting of a cellular component grown on a polymer matrix.

Typically, scaffolds for tissue engineering are made from natural and synthetic polymers that are further modified to obtain morphology and characteristics corresponding to cardiac tissue. These includes polyglycolic acid (PGA), poly(L)-lactic acid (PLA), poly(DL)-glycolate (PLGA), and polyvinyl alcohol or their derivatives [8, 9, 10]. Among the methods of processing biomaterials,, electrospinning occupies an important place since it allows to obtain fibers with the size of natural extracellular matrix fibers. Other advantages are the ability to reproduce the complex microstructure of natural heart tissues, good reproducibility and the possibility of functionalization. [11]. However, the electrospun scaffold itself can activate the host immune response, which should be taken into account.

The use of polymer fiber matrices as cellular substrates can promote proper development of cardiac tissue, provide its mechanical properties and provoke functional electrophysiological fusion of donor cardiomyocytes and recipient tissues [12]. Based on our earlier results, PLA scaffolds were selected for the creation of cardiac constructs and study of their immunological properties [13].

The development of methods for transplantation of cells and cell-carrying tissue-engineered constructs (TEC) includes the study of the donor cells survival and the associated activation of the T cell response. Prevention of immune-mediated inflammation and subsequent post-transplant degeneration of TEC remains relevant. The recipient's immune system recognizes the donor's MHC class I antigens located on almost all nucleated cells and activates the immune response. Two types of T cells, CD4 and CD8, are most involved in the rejection reaction. Activated $CD4^+$ T cells proliferate, secrete various cytokines, growth factors and activation factors for $CD8^+$ cytotoxic T cells, B cells and macrophages, which cause graft destruction [14]. Achievement of the required level of immune response occurs after transmission of an additional signal from coactivation molecules CD28 and CTLA-4. This results in further activation-differentiation or anergy-apoptosis of T and B lymphocytes. Control of these mechanisms and creation of hypoimmune cell products can become a clinical approach in modulating the allogeneic tolerance in cell transplantation [15, 16]. Therefore, to assess the biocompatibility of TECs carrying hiPSC- CM, we performed experiments to investigate the ability of TECs to induce the expression of regulatory markers CD28 and CTLA-4 on T-lymphocytes in vitro.

2. Materials and Methods

2.1 Generation of cardiomyocytes

A human iPSC (iMA-1L cell line, ICG SB RAS, Russia) applied for directional differentiation into cardiomyocytes [17]. hiPSC were cultured on an LDEV-Free Matrigel™ hESC-qualified matrix (Corning, USA) in Essential-8 medium (ThermoFisher Scientific, USA). Differentiation was carried out in accordance with a previously published protocols [6, 18] based on the activation of the WNT-pathway using CHIR99021 (StemRD, USA) for 48 h and subsequent inhibition with IWP2 (Sigma-Aldrich, USA) in RPMI-1640 (Lonza,

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Germany) with B27-supplement (ThermoFisher Scientific, USA) without insulin. The appearance of spontaneously contracting areas observed on days 8-10 of differentiation. On days 14-18 of differentiation, the cells were dissociated using TrypLE Express (ThermoFisher Scientific, USA) and transferred into 6-well plates coated with with Matrigel™ (Corning, USA) in RPMI-1640 medium supplemented with 20% embryonic bovine serum (Autogene Bioclear, UK) and 10 μM Y-27632 supplement (StemRD, USA). Two-days after the transfer and within 1 week, metabolic cell selection carried out to purify the population of cardiomyocytes. Composition of the metabolic selection medium: RPMI-1640 without D-glucose (ThermoFisher Scientific, USA), 213 μg/ml L-ascorbic acid 2-phosphate (Sigma-Aldrich, USA), 500 μg/ml recombinant human albumin expressed in Oryza sativa (Sigma-Aldrich, USA), and 5 mM DL-sodium lactate L4263 (Sigma-Aldrich, USA). Polymeric microfiber matrices colonized with 25-day-old hiPSC-CM. A part of enriched hiPSC-CM cultured up to 40 days until the immunological studies starting.

2.2 Production of polymer matrices and seeding

Electrospinning performed using a Nanon-01 device (MECC Corp., Japan) according to Chepeleva E.V. et al. [13]. To obtain microfiber matrices a 25 mg / ml solution of poly(L)-lactic acid (MW ~ 700 kDa) in hexafluoroisopropanol (all Sigma-Aldrich, USA) was used. The solution fed using a 3 ml syringe with a 24 G needle at a rate of 0.5 - 2 ml/h. Fibers sprayed onto polydimethylsiloxane rings (Dow Chemical, USA) laid on a flat electrospinning collector. The distance from the needle tip to the collector was 10 cm. The voltage between the needle and the collector was 5 to 7 kV. The electrospinning process was continued until a dense and stable layer of fibers was obtained. Prepared samples were removed from the rings and sterilized on all sides with UV irradiation in a laminar flow hood for one hour. Sterilized matrices were transferred in Petri dishes and stored under sterile conditions for no more than 3 days. Prior to hiPSC-CM colonization, polymer matrices treated with Matrigel™ (Corning, USA) according to the manufacturer`s recommendations. Cells were seeded on the matrix surface with a density of 150,000/cm^2. The formation of CM layer completed on the second day. By the beginning of immunological studies, the age of the CM on TECs reached 40 days.

2.3 Immunological studies and flow cytometry

Blood cells procedures were initiated after obtaining informed consent of volunteer donors (n = 3). Mononuclear cells were isolated from EDTA-stabilized peripheral blood by centrifugation on Histopaque-1077 gradient (Sigma-Aldrich, USA). After 3-fold washing with phosphate buffer (Biolot, Russia) the viability of mononuclear cells (Lym) was more than 98%. Lym were then transferred into 24-well plates (Corning, USA) at 10,000 cells per well with 3 ml of RPMI-1640 culture medium (Sigma-Aldrich, USA) containing 10% fetal serum (Stemcell, Canada), L-glutamine (Stemcell, Canada) and gentamicin (Sintez, Russia). The next day, Lym in each well was resuspended and transferred to new 24-well plates with pre-positioned microfiber scaffolds (PLA scaffold group), forty-days hiPSC-CM (CM group), or hiPSC-CM-on-scaffolds (TEC group). The negative control group included intact Lym in culture medium. For the positive control, Lym was incubated with 10 μg/ml concanavalin A (conA group) (Sigma-Aldrich, USA). Two days later, Lym was collected from the plates and fixed using 2% paraformaldehyde.

Cells were labeled with anti-human CD3-APC-A750, CD4-APC, CD8-APC-A700, CD28-PC5, CD152-PE (CTLA-4) antibodies (Beckman Coulter, USA) at recommended concentrations. The stained Lym after washes were analyzed on a Navios flow cytometer set (Beckman Coulter, USA) using Kaluza software. Samples from each of the five experimental groups were examined twice.

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2.4 Electronic and fluorescence microscopic examinations

PLA samples for SEM were coated with a 10 nm gold layer in a Q150R automatic magnetron sputtering machine (Quorum Technologies, UK). The morphology of the obtained PLA microfiber substrates was studied using a JSM-6510LA scanning electron microscope (JEOL, Japan).

Fluorescent visualization of live CMs at the stages of TEC fabrication was performed using a TMRM assay kit, antibodies to sarcomeric alpha-actin, and Nkx2.5 (all Abcam, UK) according to the manufacturer`s recommendations. Generalized cardiomyocytes contraction on the scaffold was observed using Fluo-8-AM staining (Abcam, UK) and a Ti100 fluorescence microscope (Nikon, Japan) with Imstar software.

2.5 Ethical statement

Experimental protocol was approved by Local Ethics Committee of «E. Meshalkin National Medical Research Center» of the Ministry of Health of the Russian Federation. (approval date 26 Dec 2014, protocol 45).

2.6 Statistical analysis

Statistical analysis performed using Statistica 13 software (TIBCO Software, USA). The data checked for normal distribution by the Kolmogorov-Smirnov test. Descriptive statistics presented as mean ± standard deviation. Ordinary one-way ANOVA with Dunnet`s post hoc test was implemented to identify significant differences between groups. Values of $p < 0.05$ considered statistically significant.

3. Results

A protocol based on the WNT-pathway activation by inhibiting the GSK3 enzyme with CHIR99021, followed by WNT repression (with IWP2) applied to induce iPSC differentiation into cardiomyocytes. The first spontaneous contractile cells detected on day 8 of directed differentiation. During cell cultivation, the number of contractile sites and the intensity of contractions increased. The hiPSC-CM metabolic selection method used provided a pure monolayer cell culture. Fluo-8-AM, a green fluorescent calcium-binding dye, used to visualize ionic currents produced by the contraction of the CM culture (supplementary Video S1).

Upon completion of metabolic selection, CMs seeded onto PLA scaffolds. The electrospinning protocol used made it possible to obtain nonwoven PLA materials with aligned fibers. SEM analysis showed afiber diameter of 0.5-1 μm (figure 1a). The extracellular matrix Matrigel™ provided cell attachment and de novo formation of intercellular connections. TMRM assay used to assess viability after cells transfer to scaffolds by monitoring CM mitochondrial function. Under normal conditions, TMRM accumulates in negatively polarized mitochondria and has emission maxima at 573 nm. In apoptotic or metabolically stressed cells, mitochondrial membrane potential drops, the TMRM separates and spreads throughout the cytosol, resulting in a significant decrease in fluorescence intensity (figure 1b). Immunofluorescence staining of the developed TECs confirmed the presence of characteristic markers of differentiated CM. Simultaneous staining for cardiomyocyte nuclear differentiation factor Nkx2.5 and sarcomeric alpha-actin was performed (figure 1c).

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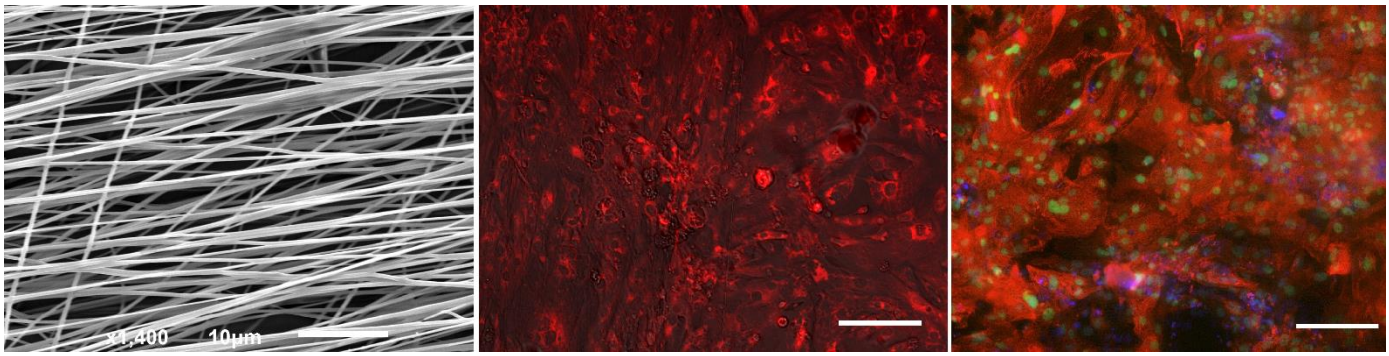

(a) (b) (c)

Figure 1. Bare and seeded PLA scaffolds: (**a**) SEM after electrospinning, bar 10 μm; (**b**) TMRM staining of TECs, bar 50 μm; (**c**) immunofluorescent staining of TECs for Nkx2.5 (green), sacromeric a-actin (red), nuclei (DAPI, blue), bar 50 μm.

On the second day of culturing the reseeded CM, a functioning and pulsiting cardiac construct was obtained (supplementary Video S2).

Recall that hiPSC-CM were 25 days old by the end of metabolic selection. After that, a part of the cells was used for seeding PLA scaffolds, and the other part was cultured in culture medium. In both cases, it took 14 days. Thus, by the beginning of the immunological studies, the age of hiPSC-CM in the CM and TEC groups was the same.

Flow cytometric evaluation of the immune response activation regulators CD28 and CTLA-4 on T lymphocytes carried out 48 hours after co-cultivation of MNCs and experimental samples. Primary selection of lymphocytes from impurities (detached hiPSC-CM, other blood cells) performed according to CD45 and CD3 markers. Analysis of T lymphocyte populationsrevealed no differences in the number $CD3^+/CD4^+$ and $CD3^+/CD8^+$ cells between the experimental groups. Approximately half of the $CD4^+$ T lymphocytes in all groups (55-65%) expressed the activation marker CD28. $CD8^+$ T lymphocytes expressed this marker three times less. CTLA-4 expression was less than 0.5% in experimental groups. Only upon stimulation with conA, the number of $CD4^+/CTLA\text{-}4^+$ T lymphocytes reached 1.2±0,5% (Figure 2).

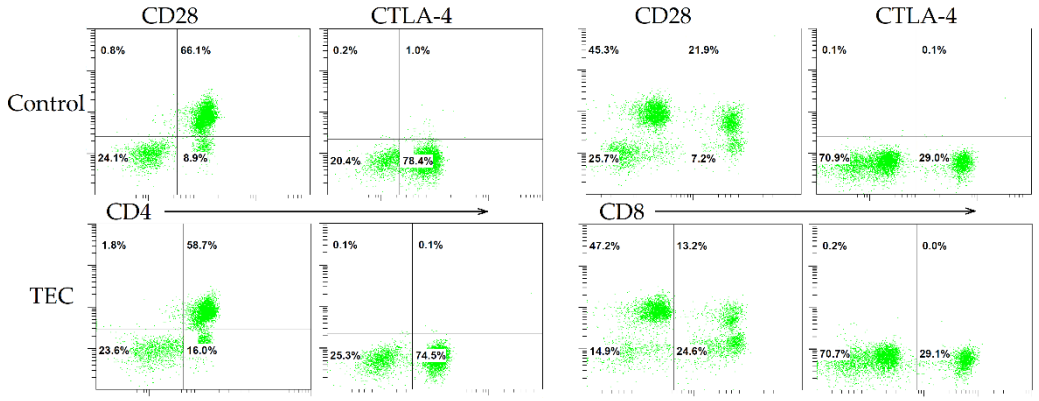

Figure 2. Expression of CD28 and CTLA-4 on $CD4^+$ and $CD8^+$ T lymphocytes induced by concanavalin A (control) or hiPSC-derived cardiomyocytes on PLA scaffolds (TEC)

There was a statistically significant decrease in the expression level of CD28 and CTLA-4 on T-lymphocytes in the CM, TEC and negative control groups compared to the positive control ($p<0,05$). Concanavalin A stimulation slightly increased the expression of CD28 and CTLA-4 on $CD4^+$ and $CD8^+$ T lymphocytes (figure 3, supplementary Table 1).

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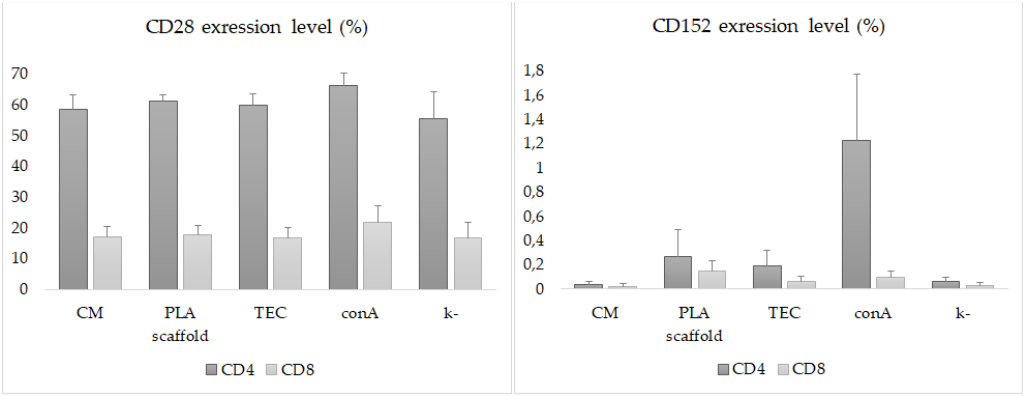

(a) **(b)**

Figure 3. Flow cytometric analysis of CD28 (a) and CTLA-4 (b) expression on $CD4^+$ and $CD8^+$ T lymphocytes: CM – culture of hiPSC-derived cardiomyocytes; PLA scaffold - microfiber scaffold without CM; TEC - hiPSC-derived cardiomyocytes on PLA scaffold; conA – cell stimulation with concanavalin A; k- – intact cells in culture medium.

Thus, our data shown that the developed cardiac constructs and culture of human iPSCs-derived cardiomyocytes did not cause an increase of CD28 and CTLA-4 expression by human T-lymphocytes. On this basis, one can expect the absence of a recipient's immune response to future transplantation of this tissue-engineered construct.

4. Discussion

In this work, we continued our earlier studies on the properties of hiPSC-CM cultured on biocompatible and biodegradable substrates [13, 19]. The proposed methodological approach can be used to obtain spontaneously contracting cardiac cells colonies for further assembly of human myocardium fragments , biological cardiac pacemaker, cell organoids and other electrophysiologically active cell systems for pharmaceutics and regenerative medicine [20].

We used a previously published and standardized protocol for directed differentiation of CM from iPSCs [6]. Purification and enrichment of the CM population was performed using metabolic selection based on the ability of cardiomyocytes to metabolize lactate in the absence of glucose [21]. The selected method allows the production of iPSCs-derived CM that have spontaneous contractile activity and express the main cardiac differentiation markers (Nkx2.5, sacromeric alpha-actin) [13, 21].

TECs were based on PLA scaffolds with unidirectionally oriented fibers arranged rather sparsely. Such filament arrangement was better suited for the cultivationg of contracting cells and assembly of cardiac patches [19]. Other researchers also reported the relationship between fiber alignment and the nature of cardiomyocyte function [22-25]. The study of Parrag I. et al. (2012) showed that aligned electrospinned polyurethane scaffolds lead to anisotropic organization of cardiomyocytes and improve their sarcomere formation [24]. The use of polymer microfiber scaffolds as substrates for the cultivation of iPSC-CM allows controlling the transformation of a group of cardiomyocytes into a consolidated structure [26, 27]. This approach can provides the mechanical strength and functional electrophysiological unity of the cells within the TECs and leads to increase survival of tissue-engineered cardiac constructs cells after transplantation [13].

Transplantation techniques for tissue-engineered constructs carrying live cells are a separate field of research [28, 29]. However, preparation for any transplantation required predicting the probability of development and investigation of the mechanisms of post-transplant reactions both to the entire tissue-engineered construct and to its elements. For this purpose, it is necessary to perform immunological studies.

One of the earliest mechanisms of transplant immunity manifestation is activation of the CD28/B7/CTLA-4 - receptor complex on lymphocytes and antigen-presenting cells (APCs) [30-32]. Increased expression of CD28 and CTLA-4 on T-lymphocytes reflects the

activation of the immune response. It is known that the interaction of the co-stimulatory molecule CD28 with B7 ligands (termed CD80/86) on the APCs leads to T cells activation. But CTLA-4 binding to B7 induces T cell anergy [32]. The prevalence of one or another interaction in the process of antigen presentation determines the outcome: the formation of an active clone of T cells and the activation of transplant rejection mechanism or the formation of recipient tolerance [33]. An imbalance in the expression or hyperstimulation of CD28 has been found to be realized either in the form of transplantation failures or in the development of autoimmune diseases [34, 35]. Expression of CTLA-4 by immune cells reflects the formation of a feedback mechanism that limits the excessive activation of effector T lymphocytes. It is a marker of negative regulation of immune response. [36, 37].

We found that iPSCs-derived cardiomyocytes cultured on PLA scaffolds did not increase the expression of immune response markers CD28 and CTLA-4 on T lymphocytes. Perhaps, the achieved period of directed differentiation is not yet enough to form a full-fledged antigenic phenotype of cardiomyocytes and the triggering of the "friend-or-foe" recognition mechanism by the recipient cells. Although Säljö K. et al. (2017) showed that 26 days were enough for expression of different HLA antigens on the human pluripotent stem cell surface [38]. It is not yet known if the fact that we used a different cell line influenced the result or if there were minor differences in the directed differentiation protocols in our studies. However, another interesting fact should be noted..

Some researchers speculate about the absence of HLA-I class not only on iPSCs and iPSC-derived cardiomyocytes, but also on adult cardiomyocytes [38-40]. This controversial statement, based on the works of the mid-80s, now contradicts the fundamental principles of transplant immunology, experimentally and clinically proven in children and adults [14, 41-46]. We will not disregard this aspect of iPSC immunology and in our next work we will additionally investigate the expression of MHC antigens during the iPSC-derived CM differentiation stages.

5. Conclusions

There remains a high interest in cardiac tissue engineering, which creates scaffolds to control cell behavior, thereby restoring the natural architecture and function of various parts of the heart. The concept of *in situ* tissue engineering represents an innovation for creating a living and immunocompatible scaffold, which significantly reduces the time of implant preparation[47]. In this line of research, the great advances in laboratory studies of iPSCs explain the steady progress in cardiac tissue engineering in recent decades and is probably the next focus in cardiac regeneration.

With this work, we demonstrated some immunological properties of a novel biodegradable microfiber scaffold that allows human iPSCs-derived cardiomyocytes to orient and contract. Neither PLA scaffolds carrying human iPSC-CM, nor forty-day differentiated cardiomyocytes alone induce increased expression of the early T-cell activation markers CD28 and CTLA-4. A continuation of the studies will be transplantation of cardiac TECs into rats and pigs with assessment of the electrophysiological and histological outcomes of the interventions.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Video S1: Oscillation of calcium ions during generalized contraction by hiPSC-CM culture; Video S2: Oscillation of calcium ions by hiPSCs-CM on a PLA scaffold; Table S1: Profile of CD28 and CTLA-4 expression on T-lymphocytes

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