Chemical and mechanical activation of resident cardiac macrophages in the living myocardial slice ex vivo model

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Abstract

Resident cardiac macrophages (rcMACs) are among the most abundant immune cells in the heart. Plasticity and activation are hallmarks of rcMACs in response to changes in the microenvironment, which is essential for in vitro experimentation. The in vivo investigation is confounded by the infiltration of other cells hindering direct studies of rcMACs. As a tool to investigate rcMACs, we applied the ex vivo model of living myocardial slices (LMS). LMS are ultrathin ex vivo multicellular cardiac preparations in which the circulatory network is interrupted. The absence of infiltration in this model enables the investigation of the rcMACs response to immunomodulatory and mechanical stimulations. Such conditions were generated by applying interferon-gamma (IFN-γ) or interleukine-4 (IL-4) and altering the preload of cultured LMS, respectively. The immunomodulatory stimulation of the LMS induced alterations of the gene expression pattern without affecting tissue contractility. Following 24 h culture, low input RNA sequencing of rcMACs isolated from LMS was used for gene ontology analysis. Reducing the tissue stretch (unloading) of LMS altered the gene ontology clusters of isolated rcMACs with intermediate semantic similarity to IFN-γ triggered reaction. Through the overlap of genes affected by IFN-γ and unloading, we identified Allograft inflammatory factor 1 (AIF-1) as a potential marker gene for inflammation of rcMACs as significantly altered in whole immunomodulated LMS. MicroRNAs associated with the transcriptomic changes of rcMACs in unloaded LMS were identified in silico. Here, we demonstrate the approach of LMS to understand load-triggered cardiac inflammation and, thus, identify potential translationally important therapeutic targets.

Keywords Myocardial slices · Resident cardiac macrophages · Inflammation · Mechanical load

Introduction

Cardiac tissue inflammation is a complex pathophysiological process that involves multiple cell types. The reaction and resolution of cardiac inflammation depend on the cross-talk between cardiomyocytes, cardiac fibroblasts, endothelial cells, and immune effector cells [40, 54]. Alterations in intercellular interactions can result in excessive extracellular matrix deposition, tissue remodeling [14, 54], maladaptive cardiac function and increased arrhythmic tendencies [32]. These conditions can eventually lead to heart failure [15]. Besides the role in homeostasis and efferocytosis [3, 48], the contribution of rcMACs during disease state has not been well appreciated. Several recent studies have highlighted their crucial function in tissue repair not only during steady-state, but also in damaged hearts [18, 51]. In line, the depletion of this cell type results in impaired cardiac regeneration [9]. After interacting with immunomodulatory compounds like interferon-gamma (IFN-γ) or interleukin-4 (IL-4), macrophages classically undergo polarization and transition towards an activated state [26, 30]. Macrophages can alter the microenvironment of other cardiac cells [28], play a pro-fibrotic role by stimulating other cells to secrete collagen [19], and influence cardiomyocytes fractional release of Ca²⁺ [16]. A better understanding of rcMACs’ significance and the modalities that regulate their activation attract the scientific community’s interest for their use in discovering novel therapeutic strategies.
To date several factors have limited the investigation of rcMACs. One factor is their relatively small number, estimated to be approximately 7.5% of the total cardiac cells [12]. The second factor is the rapid de-differentiation of rcMACs during in vitro culture and consecutive alterations of their response to various chemical and mechanical cues [29]. This approach, however, has the advantage of a precise cytokine supplementation to the culture media. In vivo, deregulated cytokine production and cytokine injections lead to dynamic recruitment and infiltration of circulating inflammatory cells [12], modulating rcMACs response. This process starts with the early infiltration of neutrophilic granulocytes, followed by a second infiltration wave made of bone marrow-derived macrophages [21, 61]. These factors impede the direct investigation of rcMAC’s role in health and disease. Novel cardiac models for unbiased studies of rcMACs are required. One alternative to the current in vivo [27] or in vitro models are living myocardial slices (LMS) [56]. In LMS, rcMACs can be cultivated in the multicellular context of the myocardium and the accurate cytokine supplementation allows a precise investigation of rcMAC’s response to the chemical stimulation.

LMS are ultrathin (100–400 µm) cardiac tissue sections proposed as an innovative multicellular ex vivo model for advanced cardiac research [58]. The rapid development of novel ex vivo culture methods and devices capable of delaying structural and functional changes associated with chronic ex vivo culture allows scientists to address new scientific questions and investigate specific cell types while cultured in the context of the cardiac multicellular environment. LMS have already been used for drug testing [34, 41], to investigate cardiac fibroblasts [41] and the transmural electrophysiological differences of the myocardium [44]. Alterations of the mechanical load have also been applied and studied using this ex vivo platform [43, 44, 57]. To generate LMS, the heart is removed from the body, and LMS are prepared with a high-precision vibratome. This process disconnects the tissue from the vascular network, implying that new inflammatory cells can not migrate and infiltrate the tissue. In this study, we took benefit of this multicellular platform to investigate the rcMAC’s immunomodulatory response upon defined cytokine supplementation and compare the chemical rcMAC activation with the effects induced by conditions of unload or overload to identify targets that could be tested for therapeutic applications.

Materials and methods

Living myocardial slice preparation and functional evaluation

The heart samples were obtained from 8- to 12-week-old male Sprague Dawley rats. All animal experiments were registered and approved under §4 Tierschutzgesetz by the LAVES (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit) of Lower Saxony under registration number 2017/117. The animals were housed under specific pathogenic-free conditions in the central animal facility of the Medical School Hanover (MHH/ZTL). They were fed ad libitum in controlled humidity, temperature, and available lighting conditions. In this study, LMS were prepared as described in previous publications [58]. Briefly, modified Tyrode’s slicing solution [58] containing 30 mM 2,3-butanediione monoxime (BDM), 140 mM sodium chloride, 12 mM potassium chloride, 0.33 mM Sodium phosphate monobasic dihydrate, 10 mM anhydrous D(+)-glucose, 10 mM HEPES, 1 mM magnesium dichloride, 0.9 mM calcium dichloride, dissolved in water and adjusted to pH 7.4, was prepared. The solution used for cell isolation and tissue processing was filtered with a 0.2 µm bottle-top vacuum filter, and 100 IU of heparin sodium was added prior to heart explantation. The animals were anesthetized with 4% isoflurane at 1L/min O2 until the postural and interdigital reflex expired, and after cervical dislocation, the heart was quickly removed [58]. LMS were prepared with high-precision vibrating microtomes (Model 7000SMZ-2, Campden Instruments LTD) fitted with a temperature-controlled tissue bath filled with 4 °C cold modified Tyrode’s solution. The Z-alignment of the blade vibration was calibrated to be < 1 µm before each experiment. With a vibration amplitude of 2 mm, a blade frequency of 80 Hz, and a cut speed of 0.07 mm/s, 300 µm thick slices were generated from rat myocardium. Slices designated for a prolonged ex vivo culture were trimmed to approximately 6.5 mm × 6.5 mm using a razor blade, accordingly to the cardiac muscle fiber alignment as previously described [58]. 3D-printed plastic rings (polylactic acid) were attached perpendicular to the fiber alignment with surgical histocayl glue (Braun GmbH). The LMS were cultured in cardiac slice culture media which was made up with M199 with 1:1000 ITS 100X liquid media supplement (Sigma-Aldrich) and 3% Penicillin/Streptomycin (Gibco). The LMS were cultured ex vivo with the biomimetic culture system described by Fischer et al. [6]. This culture device allows for constant monitoring of LMS contraction (acquisition rate of 400 Hz), which provides data for both, force production and contraction kinetics. For this study, the parameters considered were force (amplitude), time to peak and time to 90% of relaxation. LabChart (ADInstruments Ltd.) was used for this analysis. For chronic experiments in which the tissue preload was modified, the target load applied was based on the correlation of relative stretch and sarcomere length (SL) described by Watson et al. [57]. With no external forces applied to freshly prepared slices (unloaded condition = 0% stretch), sarcomeres adopted a resting length of 1.8 µm (SL1.8). The physiological load...
(SL = 2.1 µm, SL2.1) corresponded to 12.6% stretch, and overload (SL2.4) corresponded to 27% distension from resting length [57]. To investigate rcMAC response to pro- and anti-inflammatory compounds, recombinant rat IL-4 (20 ng/ml, Peprotech) or recombinant rat IFN-γ (20 ng/ml, Peprotech) were added to the culture medium. The tissue was electrically paced at 1 Hz. The culture device was kept in an incubator at 37 °C, with 5% CO2. Contraction recordings were acquired over 24 h and analyzed at 4 h intervals using Clampfit10.7 (Molecular Devices, LLC) or LabChart 7 Pro (ADInstruments Ltd).

**Immunofluorescence staining and microscopy**

LMS were fixed in 4% paraformaldehyde (PFA) for 20 min to image macrophages in whole myocardial slices. Fixed LMS were stored in phosphate-buffered saline (PBS) at 4 °C. LMS staining and imaging were performed as previously described [41]. Briefly, the slices were permeabilized in 1% Triton X (Roth) diluted in PBS for 4 h at room temperature (RT). LMS were incubated for 4 h at RT in a blocking solution prepared with 10% fetal bovine serum (FBS, Santa Cruz), 5% bovine serum albumin (Sigma) and 10% horse serum (Thermo Fisher Scientific) in PBS. The primary antibodies were diluted in PBS, and samples were incubated at 4 °C overnight. LMS were washed three times for 30 min with PBS and incubated with the secondary antibody solution for 3 h at RT. LMS were labeled with Hoechst33342 diluted 1:1000 in PBS for 15 min, and the samples were washed three times for the duration of 15 min. Used antibodies and concentrations are stated in Table 1. Image acquisition was performed with Leica Inverted 3 confocal microscope using 20× magnification, 1.00 Zoom. Z-series images were acquired and processed using Fiji [49]. The various cell types, positive for DAPI, Vimentin (VIM), CD163, and CD45 were counted. For each picture, at least 150 DAPI positive cells were counted.

Isolated rcMACS (for isolation procedure refer to “Antibody-mediated cell isolation” section) were seeded in 6-well plates (Sarstedt) on 5 mm round glass coverslips (VWR) coated with 0.1% gelatin (Sigma) in 1 ml M199 with 3% P/S and 1:1000 ITS. After 24 h, the cells were fixated with 4% PFA and stained following the same protocol as for LMS. Nikon epifluorescent microscope with 10× magnification was used to acquire the images. The cells were labeled for CD11b/c and CD163 and the total cell number was identified by the nuclei being stained by Hoechst 33342. The ratio of positive cells is expressed in respect to the total nuclei number.

**Enzymatic slice digestion**

Fresh or cultured LMS were enzymatically digested in a two-step protocol using Collagenase A (> 0.15 U/mg, Sigma-Aldrich) diluted in modified Tyrode’s solution. The first solution had 4.9 mg of enzyme dissolved in 3.5 ml of Tyrode’s solution. After 15 min of incubation time on the rocker at 37 °C, the supernatant was removed. The remaining tissue fragments were incubated for 20 min in solution B, made with 4.9 mg Collagenase A dissolved in 5.2 ml modified Tyrode’s solution. The isolated cells were transferred into Dulbecco’s Modified Eagle Medium with 4.5 g/l glucose (DMEM, Gibco) with the addition of 10% FBS and 4% 20 mM ethylenediaminetetraacetate (EDTA, Gibco). The solution containing the freshly isolated cells was filtered with a 40 µm cell filter to remove large tissue fragments, then centrifuged for 5 min at 300 g and 4 °C. The supernatant was then removed, and red blood cell lysis was performed with 300 µl 10 × MACS red cell lysis solution (Miltenyi Biotec) diluted in 2.7 ml pure distilled water (Invitrogen) with an incubation time of 2 min. After

| Table 1 Antibodies |
|--------------------|
| Antibody         | Manufacturer | Antibody number | Concentration | RRID       |
| Rabbit Anti-CD45  | Abcam        | ab10558         | 1:250         | RRID:AB_442810 |
| Mouse Anti-CD11b/c | Abcam        | ab1211          | 1:250         | RRID:AB_442947 |
| Rabbit Anti-CD163 | Abcam        | ab182422        | 1:250         | RRID:AB_2753196 |
| Chicken Anti-Vimentin | Invitrogen | PA1-10003       | 1:2000        | RRID:AB_2216267 |
| Hoechst33342     | Thermo Fisher | H3570           | 1:1000        | RRID:AB_106267 |
| Anti-Rabbit Alexa Fluor 488 | Thermo Fisher | R37118          | 1:2000        | RRID:AB_2556546 |
| Anti-Mouse Alexa Fluor 594 | Thermo Fisher | A-11005         | 1:2000        | RRID:AB_2534073 |
| Anti-Chicken Alexa Fluor 647 | Thermo Fisher | A-11039         | 1:2000        | RRID:AB_142924 |
centrifugation for 5 min, 300 g and 4 °C, the blood cell lysis solution was removed. The cell pellet was resuspended with MACS rinsing solution (Miltenyi Biotec) prepared with 10% FBS. The cells were counted and centrifuged at 300 g, 4 °C for 5 min and resuspended in M199 with 1:1000 ITS and 3% Penicillin/Streptomycin.

**Antibody-mediated cell isolation**

Cell isolation from freshly prepared LMS or from 24 h ex vivo cultured LMS were performed using CD11b/c anti-rat MACS antibody beads, and MS MACS columns fitted with a 20 µm MACS pre-separation filter. These cells were used, respectively, for gene expression analysis of rcMACs seeded onto coverslips and for RNA-seq experiments. In addition, the columns were fitted into a magnetic MACS 8-column MultiStand (all material from Miltenyi Biotec). The cell isolation was performed according to the manufacturer's recommendation, resulting in 1 ml of CD11b/c positive cells and 2.5 ml of CD11b/c negative cells. Up to 1.5*10^5 cells were seeded on 0.1% gelatin-coated 6-well plates (Sarstedt) and stimulated with recombinant rat IL-4, 20 ng/ml (Peprotech) or recombinant rat IFN-γ, 20 ng/ml. Plates were incubated at 37 °C, with 5% CO₂ and 18% O₂ (C 170, Binder GmbH) for up to 48 h.

**RNA extraction**

Whole LMS were homogenized for 2 × 40 s at 5500 RPM in tubes with six 2.4 mm zinc-ceramic balls (Precellys 24) in 700 µl of QIAzol (Qiagen). Cell suspension samples were prepared for RNA extraction with 700 µl of QIAzol lysis reagent. RNA extraction resulted in 30 µl of eluted RNA for whole tissue slices (RNAeasykit Mini, Qiagen) or in 14 µl of eluted RNA for isolated cells (RNAeasykit Micro, Qiagen) with expected low RNA yield. Quantity and purity were measured with a micro-volume plate reading photometer (Synergy HTX/Take3, BioTeK), and samples were stored at − 80 °C.

**qRT-PCR analysis**

Reverse transcription was performed with 125 ng equals of extracted RNA in a total of 13 µl nuclease-free water. A thermocycler performed the cDNA synthesis (TProfessional Trio, Biometra Ltd.) with a reaction containing 4 µl iScript Reaction Mix (Bio-Rad), 2 µl oligo-dT Primer and 1 µl iScript reverse transcriptase (Bio-Rad). The transcription protocol times were 90 min at 42 °C, 5 min at 85 °C and a hold period at 8 °C resulting in 20 µl of complementary DNA (cDNA). The transcribed 1:3 prediluted cDNA was then analyzed with 5 µl SYBR Green reaction (Bio-Rad), 0.025 µl iQ SYBR 20× Precision Blue RT-PCR Dye, 0.05 µl ROX 1:50 and 10 µM of specific high purity salt-free forward and reverse primers (Eurofins) diluted in 2.425 µl of nuclease-free water and with 2 µl cDNA per reaction. The qPCR reaction was done in 45 cycles (95 °C for 3 min pre-dissociation, 95 °C for 15 s, 60 °C for 30 s and 72 °C for 40 s). Primer-specific amplification was confirmed with melt curve analysis and controls with and without reverse transcriptase added, compared to water. Primer sequences are found in Table 2. The changes in gene expression were calculated using the ΔΔ-CT method and expressed as normalized to the mean of two reference genes (GAPDH/ACTB).

**RNA-seq and data analysis**

1 ng of total RNA was used for library preparation with the ‘SMARTer Stranded Total RNA-Seq Kit v2—Pico Input Mammalian’ (#634413; Takara/Clontech) according to conditions recommended in the user manual #063017. Generated libraries were barcoded by a dual indexing approach and were finally amplified with 12 cycles of PCR. Fragment length distribution of generated libraries was monitored using ‘Bioanalyzer High Sensitivity DNA Assay’ (5067–4626; Agilent Technologies). The libraries were quantified using the ‘Qubit® dsDNA HS Assay Kit’ (Q32854; Thermo Fisher Scientific). Equal molar amounts of twenty libraries were pooled for a common sequencing run. Accordingly, each analyzed library constitutes 5% of the overall flowcell capacity. According to the Denature and Dilute Libraries Guide, the library pool was denatured with NaOH and diluted to 2 pM (Document # 15,048,776 v02; Illumina). The denatured pool (1.3 ml) was loaded on an Illumina NextSeq 550 sequencer using a High Output Flowcell for single reads (20,024,906; Illumina). With the following settings, sequencing was performed: Sequence reads 1 and 2 with 38 bases each; Index reads 1 and 2 with eight bases. Using bcl2fastq Conversion Software version v2.20.0.422 (Illumina), BCL files were converted to FASTQ files. Raw data processing was conducted using nfcore/ rnaseq (version 1.4.2), a bioinformatics best-practice analysis pipeline used for RNA sequencing data at the National Genomics Infrastructure SciLifeLab Stockholm, Sweden. The pipeline uses Nextflow, a bioinformatics workflow tool which pre-processes raw data from FastQ inputs, aligns the reads and performs extensive quality control on the results. The genome reference and annotation data were taken from Illumina’s iGenomes platform (http://igenomes.illumina. com.s3-website-us-east-1.amazonaws.com/Rattus_norve gicus/Ensembl/Rnor_6.0/Rattus_norvegicus_Ensembl_ Rnor_6.0.tar.gz). Normalization and differential expression analysis were performed with DESeq2 (Galaxy Tool Version 2.11.40.2). The default settings were used except for “Output normalized counts table”, “Turn off outliers replacement”, “Turn off outliers filtering”, and “Turn off independent
filtering”, all of which were set to “True”. Gene set enrichment analysis (GSEA) was performed using log2(FC) values of significantly deregulated genes \( (p < 0.05) \) with the WEB-based GEne SeT AnaLysis Toolkit (RRID:SCR_006786) [25]. The organism of interest was “Rattus norvegicus”, and the functional database was “geneontology” with “biological processes nonReduntent” or “network” with “miRNA target”. No advanced parameter was changed. The result of the miRNA target prediction was clustered with the “Affinity propagation” option.

### Statistical analysis

Acquired data were processed with GraphPad Prism 9.1.0 (RRID:SCR_002798, GraphPad Software Inc.). Data are shown as mean ± SEM, if not otherwise stated. Data were first tested for normal distribution. A statistical Student’s \( t \)-test, one-way analysis of variance (ANOVA) with post hoc Tukey’s or Dunnett’s for multiple comparisons was performed, if normality was assumed. If normality was not assumed, Mann–Whitney or Kruskal–Wallis tests were used. For the RNA-seq Data, multiple \( t \)-tests with FDR < 0.05 were performed comparing subgroups. Pathway analysis \( p \)-values were adjusted with FDR < 0.05 correction. \( P \)-values ≤ 0.05 were considered statistically significant \( (p ≤ 0.05^*, p ≤ 0.01^{**}, p ≤ 0.001^{***}, p ≤ 0.0001^{****}) \). Venn diagrams were drawn using free online software [17]. G-SESAME online tool (RRID:SCR_005816) was used to measure the semantic similarity of ranked GO-gene sets using the AIC method [55].

## Results

In this study, we identified and quantified rcMACs in rat ex vivo cultured myocardium. LMS were prepared as previously described [58] and cultured ex vivo in biomimetic culture chambers for 24 h [6]. This culture system allowed constant monitoring of tissue contractility combined with electrical pacing (1 Hz) and medium agitation applied by a rocking motor performing 50 oscillations per minute. The LMS were fixed in 4% paraformaldehyde and stained to identify and count the various cell types (Fig. 1A). The cardiac cellular composition has been extensively described in the literature [41, 42]. Therefore, we focused solely on the cardiac immune cell population and rcMACs. Hoechst33342 was used to determine the cell nuclei and vimentin (VIM) to identify stromal cells [7]. CD45 antibody staining revealed hematopoietic cells, whereas ED2/CD163 is a cell marker exclusively expressed by mature tissue macrophages [45]. The quantification showed that after 24 h in culture, 31.9% of all counted cells stained for VIM, hematopoietic cells (CD45 +) were 18% and adult tissue macrophages (CD163 +) 7.6% of the total stromal cell population (Fig. 1B, C). The fraction of CD163-positive cells remained preserved over 24 h of LMS culture (Fig. 1B, right). We conclude

### Table 2: Primer sequences

| Target  | Forward           | Reverse                  |
|---------|-------------------|--------------------------|
| CD68    | 5-AATCAACCTACCGCCCGCCCTC-3 | 3-CTTACAGGAGGTCCGATACCA-5 |
| CD45    | 5-AGACAGAGGGCAAGGAGAC-3   | 3-CCCTGCCCTGTGACAAGAC-5  |
| CD11b   | 5-GGGGCCCCCCTATACACTAGT-3 | 3-ACATCTCCAGCAGACTGCTA-5 |
| ARG1    | 5-ACGTGCGGTGAAAGGAAAGGAAGAG-3 | 3-CTGTTAAGATAGGGCGGCTCCACAA-5 |
| NOS2    | 5-CGGCTGTGTGTTGAAACTTCTGAGC-3 | 3-GTTGACTAGGCTAAGACAGGAC-5 |
| MMP9    | 5-TTACATCGGAGACACCCCTC-3   | 3-GAGGGAGTACCTGCGGCTAC-5  |
| MMP2    | 5-TACTAGGACCTGCAAGGACC-3   | 3-CATAGGTCGCTCCATCCTC-5   |
| DDR2    | 5-AACCTACAGTCGGGAGTGCAA-3  | 3-ACGTTCATGAGGTGCTGAGGT-5 |
| POSTN   | 5-GTTCTCTGTGACGGAGTTCACC-3 | 3-CCGGCAGACATTCTATATGAC-5 |
| ENG     | 5-AACCTAGCCTGGCACCCTAG-3   | 3-CGATGCTGGTGGGCTGACTC-5  |
| Ly6C    | 5-TTCTCTGTCGCGCTACCGCTTCT-3 | 3-GGAAACTTGCGGCGATAGTG-5 |
| MerTK   | 5-ATGCCTCTTCTGGGCTCCTGAG-3 | 3-CTCCCTAGGCTCTGTGGTG-5   |
| IL6     | 5-CTTCCGCAAGAGACTTTCCA-3  | 3-AGTTCCTTCCTCCGAGCTTG-5  |
| DDR2    | 5-AACTACAGTCGGGAGTGCAA-3  | 3-ACGTTCATGAGGTGCTGAGGT-5 |
| COL4A1  | 5-GCAGAGAGGGCAAGATACCTA-3  | 3-3TATGGCCTGCTGCTGGAGCA-5 |
| COL4A1  | 5-GGATCTCCGTTCTGCGGCCCTC-3 | 3-ACCTGGGACCCATCTGGC-5   |
| COL13A1 | 5-GAAACCGCAGAAGAAAGAAGAA-3 | 3-TATGGTGGTGGTAGAACAGCA-5 |
| AIF-1   | 5-GATCAGACAGACCTCTGCTG-3   | 3-GACATAATATCAGAATCTC-5   |
| ACTB    | 5-CTGAGGGACACCCCTGTCGAGT-3 | 3-CGAGAGGATCACACGGAGACCA-5 |
| GAPDH   | 5-GAAGGGCTCATGACCAGGAT-3   | 3-GGATGCGAGGATGATGCTTG-5  |
Fig. 1 A LMS were cultured ex vivo for 24 h in biomimetic culture chambers and electrically paced at 1 Hz. They were fixed, stained and imaged by confocal microscopy. B Pie chart shows (left) the stromal cell composition of fresh rat LMS and after 24 h of ex vivo biomimetic culture. Leukocytes and rcMACs made up 10.4 ± 4.8% and 7.6 ± 3.5% (mean ± SD), respectively, and the remaining cardiac stromal cells were exclusively VIM-positive (82.0%). The comparison of 0 h and 24 h (prepared from n = 2 or n = 5 animals, respectively) LMS did not show a difference in the amount of VIM and CD163 positive cells. C Representative average Z-stack projected pictures of LMS stained with Hoechst33342 (blue), VIM (red), CD45 (yellow) or CD163 (green). Nineteen pictures from 5 myocardial slice sections produced out of five animals were processed. A total of 5702 cells were counted.
that LMS contain a small population of rcMACs, and their number is preserved during ex vivo culture.

Next, the LMS were prepared and biomimetically cultured ex vivo for 24 and 48 h to analyze time-dependent changes in gene expression profiles of various cell types (Fig. 2A). The RNA was isolated from freshly prepared slices and from 24 and 48 h ex vivo cultured slices and used to evaluate the gene expression profile of various cardiac cell population markers (Fig. 2B–K). Ly6c has been used in rat tissue to identify infiltrating cells [10]. The significant decrease in Ly6c expression, combined with the stable pan-macrophage gene marker CD68, confirmed that tissue macrophages were preserved during ex vivo culture despite the progressive withdrawal of infiltrating cells (Fig. 2B–C). CD31 and ENG are adhesion molecules highly expressed by endothelial cells. The downregulation of CD31 and ENG was expected due to the altered vascular homeostasis and the cessation of circulation in the capillaries (Fig. 2F–G). Although attenuated by the biomimetic culture, ex vivo culture conditions progressively activate cardiac fibroblasts [6, 36, 41], which transition to a myofibroblast phenotype [37]. In line with these observations, we also show the increased gene expression of various fibroblast genes such as VIM, POSTN, and CD90, suggesting a progressive activation of fibroblasts and their transition to a myofibroblast phenotype (Fig. 2H, I, K). These results confirmed that some cardiac

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**Fig. 2** A Schematic of the experimental procedure. LMS were cultured ex vivo for 24 h and 48 h in biomimetic culture chambers and electrically paced at 1 Hz. The LMS were then snap-frozen in liquid nitrogen, and RNA was isolated for RT-PCR. B–K: Gene expression was normalized to the housekeeper genes GAPDH and ACTB (ΔΔ-CT). Inflammatory cell markers (Ly6c, CD11b, CD68, CD45; B–E) and endothelial markers (ENG, CD31; F–G) gene expression profiles were monitored over time. Gene expression of activated fibroblasts such as VIM, POSTN, MMP2, and CD90 were also quantified (H–K). Prolonged ex vivo culture altered fibroblast, endothelial and lymphocyte-associated genes, but did not affect macrophage-associated genes. The experiment was repeated three times. 6–10 LMS were analyzed per group. ANOVA was performed with Turkey’s post hoc test or Kruskal–Wallis with Dunn’s post hoc test.
groups to the control. Cells isolated from 4 hearts were analyzed with Dunnett’s post hoc test was performed comparing the treatment data were normalized to the untreated cell group. Unpaired ANOVA ml IFN-γ (red) or 20 ng/ml IL-4 (green) for 48 h. Gene expression cells were cultured ex vivo and treated with recombinant rat 20 ng/ml IFN-γ (red) or 20 mg/ml IL-4 (green) for 48 h. Gene expression was analyzed by qPCR, expressed as ΔΔ-CT relative to GAPDH and normalized to the positive fraction. The two fractions were compared using an unpaired two-tailed Student’s t-test. The RNA was extracted out of 4 separate cell isolations performed from fresh LMS, J–U 1.5 × 10⁵ cells were cultured ex vivo and treated with recombinant rat 20 ng/ml IFN-γ (red) or 20 mg/ml IL-4 (green) for 48 h. Gene expression data were normalized to the untreated cell group. Unpaired ANOVA with Dunnett’s post hoc test was performing comparing the treatment groups to the control.

Next, we wanted to study if rcMACs isolated from LMS were responsive to chemical activation. RcMACs were isolated from freshly prepared LMS by MACS purification, seeded on coverslips and cultured for 24 or 48 h (Fig. 3A). Consider that CD11b/c is a marker that recognize not only macrophages, but also monocytes, dendritic cells, granulocytes, and NK cells [8, 31, 48], the efficiency and purity of the isolated cells were confirmed by immunostaining and RT-PCR. The adherent culture conditions also favored the removal of stromal cell types that prefer suspension cultures such as monocytes, dendritic and NK cells [35]. By immunostaining we showed that, after MACS purification, 91.4% of the total cells were CD11b/c positive and 81.7% were CD163 positive (Fig. 3B and Suppl. Fig. S1A). The qPCR data also indicated that CD11b/c + cells expressed lower Ly6C levels than the CD11b/c− population, and a lower expression level is regarded as typical for rcMACs. RT-PCR demonstrated that these rcMACs population presented higher gene expression for macrophage-specific genes such as pan-macrophage marker CD68, hematopoietic cell marker CD45, and Proto-oncogene tyrosine-protein kinase MER (MerTK) (Fig. 3D–F) than the negative population, which had higher levels of endothelial (ENG) and fibroblast specific genes (DDR2) (Fig. 3H, I).

The isolated rcMACs population viability was measured after isolation at 0 h, 24 h and 48 h showing a reduction in viability to 70% after 48 h culture (Fig. 3C). To monitor the response of rcMACs to pharmacological stimulation, the isolated rcMACs were treated with either IFN-γ or IL-4 for 48 h (Fig. 3J–U). IFN-γ and IL-4 are immunomodulatory compounds reported to be on the opposite spectrum of the inflammatory activation process [38]. They are also commonly used to induce macrophages to acquire a pro- and anti-inflammatory phenotype [29]. The up-regulated gene expression, upon induction by IFN-γ, was linked to pro-inflammatory response, while these genes were down-regulated in IL-4 stimulated rcMACs. IFN-γ induced a dramatic increase (119.4-fold) in NOS2 expression (Fig. 3K). ARG1 was also up-regulated in the IFN-γ group (Fig. 3L). MerTK was also significantly up-regulated by 4.3-fold. IL-4 stimulation, on the other hand, significantly increased MerTK expression by 2.3-fold (Fig. 3S) and ARG1 by 13.2-fold (Fig. 3L), while the expression of IL-6 decreased (Fig. 3J).

ECM-related genes were highly up-regulated in IL-4 stimulated rcMACs. Col3a1 gene expression was also up-regulated (Fig. 3O), as well as collagen sensor receptor DDR2 (Fig. 3U). Vimentin was significantly down-regulated in the IL-4-treated group (Fig. 3P). No differences were observed for Coll1, Coll4a1, CD68, MMP9 and POSTN. These data demonstrate that the MACS isolated rcMAC can respond to the ex vivo stimulation acquiring a pro- or anti-inflammatory phenotype.

Multicellularity is essential in tissue biology, and the interaction between different cell types dramatically affects their response to various chemical and mechanical stimuli. To investigate the reaction of the myocardium to pro- and anti-inflammatory stimulations, LMS were treated with the immunomodulatory compounds IL-4 and IFN-γ for 24 h (Fig. 4A). The LMS were snap-frozen in liquid nitrogen, and the RNA was extracted for RT-PCR. The 24 h treatment with IFN-γ increased the IL-6 gene expression profile by 2.6-fold (Fig. 4B) and NOS2 by 12.7-fold (Fig. 4C). All three collagen genes (Coll1A1, Col3A1, Col4A1) were significantly down-regulated in the rat IFN-γ-treated group (Fig. 4E–G). MerTK expression was also increased compared to the control group (Fig. 4K). The inflammatory stimulation induced by IL-4 did not cause such a dramatic change in the LMS gene expression profile. The only altered genes were POSTN (Fig. 4M) and the extracellular matrix-remodeling gene MMP9, which were down-regulated (Fig. 4I).

The biomimetic culture device used for the ex vivo culture of LMS allows a constant recording of the LMS contractility. Considering the altered gene expression of various inflammatory, collagen, and fibrosis-related genes, we investigated the contraction parameters of the treated LMS (Fig. 4N–P). Force generation and contraction kinetics (time to peak and relaxation time (T90), and decay rate) were unchanged between the three groups suggesting that the tissue contraction was maintained in all the groups and was not affected by the pharmacological stimulation.
significantly deregulated genes were isolated by MACS purification (Fig. 5A). The transcriptomic IRF1 up-regulated genes were, O, S). The analysis identified that the ten most significantly, Col5a3, a pro-inflammatory phenotype. With IFN-γ clearly shifted the endogenous rcMACs toward process and regulation, cytokine-mediated signaling and able to identify several significantly down-regulated pathways, which included immune effector processes, inflammatory response cytokine production and cytokine production (Fig. 6D).

When the LMS was subjected to a high preload (SL2.4), the most significantly altered pathways included leukocyte proliferation, increased defense response to other organisms, inflammatory response and cytokine-mediated signaling. After performing GSEA analysis, we compared the resulting GO-term sets using statistical semantic similarity analysis (Fig. 7B). This test scores similarity between 0 and 1, no similarity and high similarity, respectively. The analysis revealed an intermediate similarity of 0.657 between the GO-term sets of IFN-γ and overload influenced rcMACs. We compared the fractions of CD11b/c and CD163 positive cells from LMS cultured for 24 h at SL2.1 and incubated with IFN-γ vs LMS with SL1.8. The LMS were fixated and stained for CD11b/c and CD163, VIM and Hoechst33342. Our results indicate that rcMACs respond to the increased preload similar as when activated by a chemical stimulus such as IFN-γ, with intermediate similarity.
To conclude our study and understand whether unload had a similar effect to IFN-γ stimulation, we performed a semantic similarity analysis between the two groups (Fig. 7C). The data revealed an intermediate semantic similarity of 0.744 between IFN-γ and mechanical unloading on rcMACs. The mapping of the significantly deregulated genes resulted in the overlap of 376 shared genes (Fig. 7D). One of the genes in the overlap between unloading and IFN-γ stimulation is *Allograft inflammatory factor 1 (AIF-1)*. AIF-1 is expressed by macrophages and was first reported in rat cardiac allografts with chronic immune reactions [53]. By being highly expressed in macrophages, whole LMS could confirm differences in expression. RT-PCR confirmed the significant deregulation of the *ALF-1* gene in immunomodulatory treated LMS (Fig. 7E). Using GSEA analysis to identify miRNA targets in the treated groups (SL1.8 vs SL2.1), we identified four miRNAs (miRNA-346, miRNA-508, miRNA-526b and miRNA-223) (Fig. 7F). Mi-RNA 346 is an enriched target of the SL2.1 transcriptome changes in rcMACs. Mi-RNA 508, miRNA-526b and miRNA-223 are
associated with unloaded rcMACs (Fig. 7F). To summarize, ex vivo culture in an unloaded state induced a response in the isolated rcMACs similar to their response to IFN-γ treatment. We also identified four potential miRNA targets which could be verified and tested to develop new therapies.

**Discussion**

In this study, LMS were used for the first time to investigate the effects of chemical and mechanical chronic stimulation on a specific immune cardiac cell type, the rcMACs, and their response when cultured in the multicellular physiological environment of the myocardium. This approach has the substantial advantage of being multicellular and disconnected from circulation, hereby excluding infiltrating immune cells. It provides a unique research tool to investigate this specific and rare, but essential resident cell type. Our major findings were:

1. The immunomodulatory stimulation influenced the overall gene expression profile without altering tissue contraction.
RcMACs could be reliably isolated from LMS treated with mechanical or chemical stimuli and investigated for transcriptome alterations. Tissue unloading induced rcMACs to acquire a phenotype similar to pro-inflammatory chemical stimulation. Overloading also caused a pro-inflammatory phenotype, but different gene sets were involved.

In silico analysis predicted miRNA targets that could be used for future therapeutic approaches explicitly targeting rcMACs.

In this study, rcMACs were immunostained with various antibodies, including VIM, CD45 and CD163. VIM is a commonly used marker to identify stromal cells; the combination of VIM with the immune markers CD45 and CD163 was used to identify and count the number of rcMACs and leukocytes relative to the stromal cells. CD163 is an immune cell marker exclusively expressed by rat tissue macrophages [45]. In line with the literature, CD163+ cells, corresponding to the rcMACs, were 7.6% of the total stromal cell population [12]. This study performed rcMAC counts by counting cells after images were acquired with confocal microscopy.
Flow cytometry or Cre/loxP reporter lineage tracking are alternatives to this approach. However, although laborious and time-consuming, this approach is simple and effective, as was previously used to quantify other cell types [41]. The imaging and quantification are also limited by antibody penetration depth and, therefore, restricted to the LMS surface. The LMS contractility and gene expression profile quantification confirmed overall high viability and preserved preparation functionality over the 24 h of ex vivo biomimetic culture. As previously reported [6, 57], we also observed some degree of adaptation to the new culture conditions with increased expression of some genes associated with fibroblasts activation, such as Periostin and Vimentin or endothelial cell de-differentiation (CD31 and ENG). The supplementation of humoral stimuli and metabolic substrates to the culture media has been reported to favor the needs of the myocardium in culture and to delay the onset of cell de-differentiation [36]. The LMS were cultured in a serum-free media as recommended in several publications [6, 57] and to be less dependent on batch to batch serum content variability. The most noticeable data were the dramatic decline of Ly6c, which suggest the rapid depletion of the cardiac granulocytes. Notably, CD68 levels were maintained, meaning, together with the confocal quantification, that the rcMACs can survive the ex vivo culture. Rat myocardial slices were used in this study. It has repeatedly been reported that rat tissue undergoes a faster tissue adaptation and remodeling than LMS prepared from larger animal models [6, 41]. These models could provide a more translational aspect to this study and allow for more prolonged ex vivo chronic treatment. These further studies would be relevant to further monitor and understand the role of rcMACs during chronic stimulation.

This study successfully isolated resident macrophages from both cardiac tissue and cardiac tissue slices after 0 h and 24 h using magnetically labeled antigen facilitated cell separation (MACS). This method is a reliable and straightforward approach to isolate specific cardiac populations like macrophages [4]. CD11b/c is present on macrophages and granulocytes [48] and is currently the only available Miltenyi Biotec MACS antibody for rat macrophages, limiting the specific isolation of rcMACs cell population. An alternative to this approach is Fluorescence Activated Cell Sorting (FACS) sorting, combining more antibodies and isolating and analyzing smaller or more specific cardiac sub-populations. The expression of CD11b was significantly reduced after 24 h, which can be caused by cell depletion or cell de-differentiation. Overall, the amount of RNA after isolation was low, but sufficient to be used for Low-input RNA-Seq. This limited the viable post experiment analysis options.

In this study, IFN-γ and IL-4 were chosen as chemical stimuli to activate rcMACs when cultured ex vivo or in the multicellular environment of the LMS. These two cytokines have extensively been used both ex vivo and in vivo [24, 46, 59] to induce a pro- and anti-inflammatory state in the macrophages. However, their effects exclusively on the rcMAC population have never been tested. Our data demonstrate that they are potent activators of rcMACs ex vivo. Mechanical forces are also potent regulators of cell behavior and inter-cellular communication. Alterations to cardiac tissue contraction, such as unload- or overloading conditions, are known to affect both cardiomyocytes and cardiac stromal cells [43]. In such pathological states, cardiac fibroblasts transition to an activated state, and they secrete and deposit interstitial collagen contributing to tissue fibrosis [60]. Macrophage role in tissue fibrosis is still unclear and under intensive investigation. Even less is known about rcMACs and their response to mechanical stimuli. In this study, we investigated and compared both the effects of chemical and mechanical activation of this specific cell population. As the number of rcMACs in the steady state is under ten percent of all stromal cells, marker genes of rcMAC activation measurable in the whole tissue should be identified to ease further investigation. Comparing clusters of deregulated genes, we identified AIF-1 as a possible
marker gene of rcMACs after overlapping inflammation and unloading transcriptomic results. It is known that AIF-J is induced by IFN-γ stimulation and plays a role in warm and cold ischemia–reperfusion injury in the rat liver [20] or is expressed by macrophages in injured skeletal muscles [23]. We now add to the knowledge that AIF-J may be involved as a marker of inflammation in the unloaded heart.

MicroRNAs are novel and potent treatment options for cardiovascular disease and cardiomyocyte regeneration [1] and can be used as potential biomarkers [11]. ReCMACs could be potential therapeutic targets for miRNAs in the heart [48]. MicroRNA-223 was identified as a target in rcMACs affected by unloading the LMS. It has already been reported that MicroRNA-223 is associated with pro-inflammatory macrophage polarization in murine macrophages [5]. MicroRNA-346, on the other hand, has not been associated with resident cardiac macrophages yet and could therefore be an exciting novel target. These newly identified microRNAs will indeed require further ex vivo and in vivo testing to evaluate and understand their effectiveness for therapeutic use.

Conclusion

ReCMACs were studied ex vivo during prolonged tissue culture in their native environment for the first time. This approach also avoided circulating cell infiltration providing a platform to explore this rare but physiologically relevant cell type. We moreover demonstrated for the first time that a specific cell population can be isolated from LMS post ex vivo culture. Our work shows the ability to change culture conditions mimicking inflammation or alterations of the preload, which induced a similar activation of the rcMACs. In addition, we used low input transcriptome analysis to investigate deregulated inflammatory pathways leading to a new application of marker genes and identification of miRNA targets that could be used for future therapies. Our observations support the use of LMS as a platform to better identify rcMAC regulation and to study rcMACs-associated cardiac inflammation and fibrosis mechanisms. LMS can assist in finding new possible treatment options in translational discovery processes.

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Data availability The analyzed datasets are available from the corresponding author upon request.

Declarations

Conflict of interest TT is founder and shareholder of Cardior Pharmaceuticals (outside of this study). TT filed and received royalties from patents about noncoding RNAs (outside of this study). AD is a co-founder and shareholder of InvitroSys GmbH, a company developing specialized tissue culture equipment.

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References

1. Abbas N, Perbellini F, Thum T (2020) Non-coding RNAs: emerging players in cardiomyocyte proliferation and cardiac regeneration. Basic Res Cardiol 115:52. https://doi.org/10.1007/s00395-020-0816-0
2. Balke C, Shorofsky SR (1998) Alterations in calcium handling in cardiac hypertrophy and heart failure. Cardiovasc Res 37:290–299. https://doi.org/10.1016/S0008-6363(97)00272-1
3. Dick SA, Macklin JA, Nejat S, Momen A, Clemente-Casares X, Althagafi MG, Chen J, Kantores C, Hosseinzadeh S, Aronoff L, Hong A, Zaman R, Barbou I, Besla R, Lavine KJ, Razani B, Ghoulou F, Husain M, Cybulska MI, Robbins CS, Epelman S (2019) Self-renewing resident cardiac macrophages limit adverse remodeling following myocardial infarction. Nat Immunol 20:29–39. https://doi.org/10.1038/s41590-018-0272-2
4. Edlow AG, Glass RM, Smith CJ, Tran PK, James K, Bilbo S (2019) Placental macrophages: a window into fetal microglial function in maternal obesity. Int J Dev Neurosci 77:60–68. https://doi.org/10.1016/j.ijdevneu.2018.11.004
5. Essandoh K, Li Y, Huo J, Fan G-C (2016) MiRNA-mediated macrophage polarization and its potential role in the regulation of inflammatory response. Shock 46:122–131. https://doi.org/10.1097/SHK.0000000000000604
6. Fischer C, Milting H, Fein E, Reiser E, Lu K, Stidel T, Schinner C, Schwarzmayer T, Schramm R, Tomasi R, Husse B, Cao-Ehlker X, Pohl U, Dendorfer A (2019) Long-term functional and structural preservation of precision-cut human myocardium under continuous electromechanical stimulation in vitro. Nat Commun 10:117. https://doi.org/10.1038/s41467-018-08003-1
7. Floy ME, Mateyka TD, Foreman KL, Palecek SP (2020) Human pluripotent stem cell-derived cardiac stromal cells and their applications in regenerative medicine. Stem Cell Res 45:101831. https://doi.org/10.1016/j.scr.2020.101831
8. Fu B, Wang F, Sun H, Ling B, Tian Z, Wei H (2011) CD11b and CD27 reflect distinct population and functional specialization in...
human natural killer cells. Immunology 133:350–359. https://doi.org/10.1111/j.1365-2567.2011.03446.x
9. Godwin JW, DeLuque R, Salimova E, Rosenthal NA (2017) Heart regeneration in the salamander relies on macrophage-mediated control of fibroblast activation and the extracellular landscape. NPJ Regen Med 2:22. https://doi.org/10.1038/s41536-017-0027-y
10. Guan Y, Li X, Yu W, Liang Z, Huang M, Zhao R, Zhao C, Liu Y, Zou H, Hao Y, Chen Z (2018) Intravenous transplantation of mesenchymal stem cells reduces the number of infiltrated Ly6C + cells but enhances the proportions positive for BDNF, TNF-1 α, and IL-1 β in the infarct cortices of dMCAO rats. Stem Cells Int 2018:1–14. https://doi.org/10.1155/2018/9207678
11. Gupta SK, Bang C, Thum T (2010) Circulating MicroRNAs as biomarkers and potential paracrine mediators of cardiovascular disease. Circ Cardiovasc Genet 3:484–488. https://doi.org/10.1161/CIRCGENETICS.109.595363
12. Heidt T, Courties G, Dutta P, Sager HB, Sebas M, Iwamoto Y, Sun Y, da Silva N, Panizzi P, van der Laan AM, Swirski FK, Weissleder R, Nahrendorf M (2014) Differential contribution of monocytes to heart macrophages in steady-state and after myocardial infarction. Circ Res 115:284–295. https://doi.org/10.1161/CIRCRESAHA.113.303567
13. Herum KM, Choppe J, Kumar A, Engler AJ, McCulloch AD (2017) Mechanical regulation of cardiac fibroblast profibrotic phenotypes. Mol Biol Cell 28:1871–1882. https://doi.org/10.1091/mbc.e17-01-0014
14. Heusch G, Libby P, Gersh B, Yellon D, Böhm M, Lopaschuk G, Opie L (2014) Cardiovascular remodelling in coronary artery disease and heart failure. Lancet 383:1933–1943. https://doi.org/10.1016/S0140-6736(14)60107-0
15. Hitscherich PG, Xie L, del Re D, Lee EJ (2019) The effects of α, and IL-1 β in the infarct cortices of dMCAO rats. Stem Cells Int 2018:1–14. https://doi.org/10.1155/2018/9207678
16. Hulsen T, de Vlieg J, Alkema W (2008) BioVenn—a web application for the comparison and visualization of biological lists using area-proportional Venn diagrams. BMC Genomics 9:488. https://doi.org/10.1186/1471-2164-9-488
17. Hulsmans M, Clauss S, Xiao L, Aguirre AD, King KR, Hanley A, Hunger WL, Willers EM, Seemann G, Courties G, Iwamoto Y, Sun Y, Savol AJ, Sager HB, Lavine KJ, Fishbein GA, Capen DE, da Silva N, Miquerol L, Wakimoto H, Seidman JG, Sadreyev RI, Mitchell RN, Brown D, Libby P, Weissleder R, Swirski FK, Kohl P, Vinegoni C, Milan DJ, Ellinor PT, Nahrendorf M (2017) Macrophages facilitate electrical conduction in the heart. Cell 169:510-522.e20. https://doi.org/10.1016/j.cell.2017.03.050
18. Hulsmans M, Sager HB, Roh JD, Valero-Muñoz M, Houstis NE, Iwamoto Y, Sun Y, Wilson RM, Wojtkiewicz G, Tritoc B, Osborne MT, Hung J, Vinegoni C, Naxerova K, Sosnovik DE, Zile MR, Bradshaw AD, Liao R, Tawakol A, Weissleder R, Rosenzweig A, Swirski FK, Sam F, Nahrendorf M (2018) Cardiac macrophages promote diastolic dysfunction. J Exp Med 215:423–440. https://doi.org/10.1084/jem.20171274
19. Jiang W, Kong L, Wu X, Pu L, Wang X (2011) Allograft inflammatory factor-1 is up-regulated in warm and cold ischemia-reperfusion injury in rat liver and may be inhibited by FK506. J Surg Res 165:158–164. https://doi.org/10.1016/j.jss.2009.05.038
20. Jiao J, He S, Wang Y, Lu Y, Gu M, Li D, Tang T,Nie S, Zhang M, Lv B, Li J, Xia N, Cheng X (2021) Regulatory B cells improve ventricular remodeling after myocardial infarction by modulating monocyte migration. Basic Res Cardiol 116:46. https://doi.org/10.1007/s00395-021-00886-4
21. Jin H, Li W, Yang R, Ogasawara A, Lu H, Paoni NF (2005) Inhibitory effects of interferon-γ on myocardial hypertrophy. Cytokine 31:405–414. https://doi.org/10.1016/j.cyto.2005.06.013
22. Kuschel R, Deininger MH, Meyermann R, Bornemann A, Yablonska-Reuveni Z, Schlesener HJ (2000) Allograft inflammatory factor-1 is expressed by macrophages in injured skeletal muscle and abrogates proliferation and differentiation of satellite cells. J Neuropathol Exp Neurol 59:323–332. https://doi.org/10.1093/jnen/59.4.323
23. Leopold Wager CM, Hole CR, Campuzano A, Castro-Lopez N, Cai H, Caballero Van Dyke MC, Woźniak KL, Wang Y, Wormley FL (2018) IFN-γ immune priming of macrophages in vivo induces prolonged STAT1 binding and protection against Cryptococcus neoformans. PLoS Pathog 14:e1007358. https://doi.org/10.1371/journal.ppat.1007358
24. Liberale L, Ministrini S, Carbone F, Camici GG, Montecucco F (2021) Cytokines as therapeutic targets for cardio- and cerebrovascular diseases. Basic Res Cardiol 116:23. https://doi.org/10.1007/s00395-021-00863-x
25. Lindsey ML, Bolli R, Canty JM, Du X-J, Frangogiannis NG, Franz S, Gourdie RG, Holmes JW, Jones SP, Klomer RA, Lefer DJ, Liao R, Murphy E, Ping P, Przyklenk K, Recchia FA, Schwartz Longacre L, Ripplinger CM, van Eyk JE, Heusch G (2018) Guidelines for experimental models of myocardial ischemia and infarction. Am J Physiol 314:H812–H838. https://doi.org/10.1152/ajpheart.00335.2017
26. Liu S, Chen J, Shi J, Zhou W, Wang L, Fang W, Zhong Y, Chen X, Chen Y, Sabri A, Liu S (2020) M1-like macrophage-derived exosomes suppress angiogenesis and exacerbate cardiac dysfunction in a myocardial infarction microenvironment. Basic Res Cardiol 115:22. https://doi.org/10.1007/s00395-020-0781-7
27. Martinez FO, Sica A, Mantovani A, Locati M (2008) Macrophage activation and polarization. Front Biosci 13:453–461. https://doi.org/10.2741/2692
28. Ma Y, Mouton AJ, Lindsey ML (2018) Cardiac macrophage biology in the steady-state heart, the aging heart, and following myocardial infarction. Transl Res 191:15–28. https://doi.org/10.1016/j.trsl.2017.10.001
29. Merad M, Sathe P, Helft J, Miller J, Mortha A (2013) The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. Annu Rev Immunol 31:563–604. https://doi.org/10.1146/annurev-immunol-020711-074950
30. Morita N, Mandel WJ, Kobayashi Y, Karagueuzian HS (2014) Cardiac fibrosis as a determinant of ventricular tachyarrhythmias. J Arrhythm 30:389–394. https://doi.org/10.1016/j.joa.2013.12.008
31. Nagpal V, Rai R, Place AT, Murphy SB, Verma SK, Ghosh AK, Vaughan DE (2016) MiR-125b is critical for fibroblast-to-myofibroblast transition and cardiac fibrosis. Circulation 133:291–301. https://doi.org/10.1161/CIRCULATIONAHA.115.018174
32. Nunez-Tolrda R, Kirwin T, Ferraro E, Pitoulis FG, Nicastro L, Bardi I, Kit-Anan W, Gorelik J, Simon AR, Terracciano CM (2022) Mechanosensitive molecular mechanisms of myocardial fibrosis in living myocardial slices. ESC Heart Fail 9:1400–1412. https://doi.org/10.1002/ehf2.13832
33. Ogawa K, Tsurutani M, Hashimoto A, Soeda M (2019) Simple propagation method for resident macrophages by co-culture and subculture, and their isolation from various organs. BMC Immunol 20:34. https://doi.org/10.1186/s12865-019-0314-z
36. Ou Q, Jacobson Z, Abouleisa RRE, Tang X-L, Hindi SM, Kumar A, Ivey KN, Giridharan G, El-Baz A, Brittian K, Rood B, Lin Y-H, Watson SA, Perbellini F, McKinsey TA, Hill BG, Jones SP, Terracciano CM, Bolli R, Mohamed TMA (2019) Physiological biomimetic culture system for pig and human heart slices. Circ Res 125:628–642. https://doi.org/10.1161/CIRCRESAHA.119.314996

37. Pakshir P, Noskovicova N, Lodyga M, Son DO, Schuster R, Goodwin A, Karvonen H, Hinz B (2020) The myofibroblast at a glance. J Cell Sci 133:jcs227900. https://doi.org/10.1242/jcs.227900

38. Paludan SR (1998) Interleukin-4 and interferon-γ: the quintessence of a mutual antagonistic relationship. Scand J Immunol 48:459–468. https://doi.org/10.1046/j.1365-3083.1998.00435.x

39. Pappritz K, Savvatis K, Koschel A, Miteva K, Tschöpe C, van der Linthout S (2018) Cardiac (myo)fibroblasts modulate the migration of monocyte subsets. Sci Rep 8:5575. https://doi.org/10.1038/s41598-018-23881-7

40. Pearce L, Davidson SM, Yellon DM (2021) Does remote ischaemic conditioning reduce inflammation? A focus on innate immunity and cytokine response. Basic Res Cardiol 116:12. https://doi.org/10.1007/s00395-021-00852-0

41. Perbellini F, Watson SA, Scigliano M, Bardi I, Ascione R, Terracciano CM, Bolli R, Mohamed TMA (2019) Physiological biomimetic culture system for pig and human heart slices. Circ Res 125:628–642. https://doi.org/10.1161/CIRCRESAHA.119.314996

42. Pinto-AR, Ilinykh A, Ivey MJ, Kwabarata JT, D’Antoni ML, Debuque R, Chandran A, Wang L, Arora K, Rosenthal NA, Tallquist MD (2016) Revisiting cardiac cellular composition. Circ Res 118:400–409. https://doi.org/10.1161/CIRCRESAHA.115.307787

43. Pitoulis FG, Nunez-Toldra R, Xiao K, Kit-Anan W, Mitzka S, Jobboure RJ, Harding SE, Perbellini F, Thum T, de Tombe PP, Terracciano CM (2022) Remodelling of adult cardiac tissue subjected to physiological and pathological mechanical load in vitro. Cardiovasc Res 118:814–827. https://doi.org/10.1093/cvr/cvab084

44. Pitoulis F, Perbellini F, Harding SE, de Tombe P, Terracciano CM (2019) Mechanical heterogeneity across the left ventricular wall - a study using intact multicellular preparations. Eur Heart J. https://doi.org/10.1002/eurheartj.ehz.746.0336

45. Polfliet MMJ, Fabriek BO, Daniëls WP, Dijkstra CD, van den Berg TK (2006) The rat macrophage scavenger receptor CD163: expression, regulation and role in inflammatory mediator production. Immunobiology 211:419–425. https://doi.org/10.1016/j.imbio.2006.05.015

46. Rogers KJ, Brunton B, Mallinger L, Bohan D, Sevcik KM, Chen J, Ruggio N, Maury W (2019) IL-4/IL-13 polarization of macrophages enhances Ebocha virus glycoprotein-dependent infection. PLoS Negl Trop Dis 13:e0007819. https://doi.org/10.1371/journal.pntd.0007819

47. Rose S, Misharin A, Perlman H (2012) A novel Ly6C/Ly6G-based strategy to analyze the mouse splenic myeloid compartment. Cytometry A 81A:343–350. https://doi.org/10.1002/cyt.a.22012

48. Sansonetti M, Waleczek FJG, Jung M, Thum T, Perbellini F (2020) Resident cardiac macrophages: crucial modulators of cardiac (patho)physiology. Basic Res Cardiol 115:77. https://doi.org/10.1007/s00395-020-00836-6

49. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez J-Y, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A (2012) Fiji: an open-source platform for biological-image analysis. Nat Methods 9:676–682. https://doi.org/10.1038/nmeth.2019

50. Shyu K-G (2009) Cellular and molecular effects of mechanical stretch on vascular cells and cardiac myocytes. Clin Sci 116:377–389. https://doi.org/10.1042/CS20080163

51. Simões FC, Cuhill TJ, Kenyon A, Gavirochukina D, Vieira JM, Sun X, Pezzolla D, Ravaud C, Masmanian E, Weinberger M, Mayes S, Lemieux ME, Barnette DN, Gunadasa-Rohling M, Williams RM, Greaves DR, Trinh LA, Fraser SE, Dallas SL, Choudhury RP, Sanka-Spenger T, Riley PR (2020) Macrophages directly contribute collagen to scar formation during zebrafish heart regeneration and muscle heart repair. Nat Commun 11:600. https://doi.org/10.1038/s41467-019-14263-2

52. Takahashi N, Calderone A, Izzo NJ, Mäki TM, Marsh JD, Colucci WS (1994) Hypertrophic stimuli induce transforming growth factor-beta 1 expression in rat ventricular myocytes. J Clin Investig 94:1470–1476. https://doi.org/10.1172/JCI117485

53. Utans U, Arceci RJ, Yamashita Y, Russell ME (1995) Cloning and characterization of allograft inflammatory factor-1: a novel macrophage factor identified in rat cardiac allografts with chronic rejection. J Clin Investig 95:2954–2962. https://doi.org/10.1172/JCI118003

54. Wagner JUG, Dimmel S (2020) Cell-cell cross-talks in the diseased and aging heart. J Mol Cell Cardiol 138:136–146. https://doi.org/10.1016/j.yjmcc.2019.11.152

55. Wang IZ, Du Z, Payattakool R, Yu PS, Chen C-F (2007) A new method to measure the semantic similarity of GO terms. Bioinformatics 23:1274–1281. https://doi.org/10.1093/bioinformatics/btm087

56. Watson SA, Dendorfer A, Thum T, Perbellini F (2020) A practical guide for investigating cardiac physiology using living myocardial slices. Basic Res Cardiol 115:61. https://doi.org/10.1007/s00395-020-00822-y

57. Watson SA, Duff J, Bardi I, Zabielska M, Atanur SS, Jabbour RJ, Simon A, Tomas A, Smolenski RT, Harding SE, Perbellini F, Terracciano CM (2019) Biomimetic electromechanical stimulation to maintain adult myocardial slices in vitro. Nat Commun 10:2168. https://doi.org/10.1038/s41467-019-10175-3

58. Watson SA, Scigliano M, Bardi I, Ascione R, Terracciano CM, Perbellini F (2017) Preparation of viable adult ventricular myocar dial slices from large and small mammals. Nat Protoc 12:2623– 2639. https://doi.org/10.1038/nprot.2017.139

59. Wu C, Xiang F-L, Fang M, Yutzey KE (2017) Loss of β-catenin in resident cardiac fibroblasts attenuates fibrosis induced by pressure overload in mice. Nat Commun 8:3036–3044. https://doi.org/10.1038/s41467-018-02379

60. Xiang F-L, Fang M, Yutzey KE (2017) Loss of β-catenin in resident cardiac fibroblasts attenuates fibrosis induced by pressure overload in mice. Nat Commun 8:3036–3044. https://doi.org/10.1038/s41467-018-02379

61. Yan X, Anzai A, Katsumata Y, Matsuhashi T, Ito K, Endo J, Funakoshi Y, Yamashita Y, Tezuka K, Takeuchi K, Hirooka K, Nishimura A, Shiraishi Y, Takeuchi K, Takagi M, Hori T, Nakamura T, Shimizu T, Yu Y, Saito K (2013) Temporal dynamics of cardiac immune cell accumulation following acute myocardial infarction. J Mol Cell Cardiol 62:24–35. https://doi.org/10.1016/j.yjmcc.2013.04.023