SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

Study design

Experiments were performed on castrated male Large-White pigs weighing 30 to 40 kg. A total of 40 pigs completed the full protocol and comprised the study population. The study was approved by the Institutional Animal Research Committee and conducted in accordance with recommendations of the Guide for the Care and Use of Laboratory Animals. The study design is summarized in main text Figure 1. Five pigs (Group 1) were sacrificed with no intervention other than baseline CMR, and served as controls. In 20 pigs, reperfused acute myocardial infarction (I/R) was induced experimentally by closed-chest 40-minute left anterior descending coronary artery occlusion. These pigs were sacrificed at 120 minutes (n=5, Group 2), 24 hours (n=5, Group 3), 4 days (n=5, Group 4) and 7 days (n=5, Group 5) after reperfusion. CMR scans, including T2W-STIR and T2-mapping sequences, were performed at every follow-up stage until sacrifice, so that animals sacrificed on day 7 underwent baseline, 120min, 24h, day4 and day7 CMR exams. Another 5 pigs (Group 6) underwent 40min/7 day I/R followed by high-dose steroid therapy starting after the 120min-CMR. A group of 10 pigs underwent ischemia without reperfusion, with sacrifice after 120min-CMR (5 pigs, Group 7) or on day 7 (5 pigs, Group 8). Animals were euthanized immediately after the last follow-up CMR scan, and myocardial tissue samples from ischemic and remote areas were rapidly collected for evaluation of water content and histology.

Myocardial infarction procedure

The I/R protocol has been detailed elsewhere (1). Anesthesia was induced by intramuscular injection of ketamine (20 mg/kg), xylazine (2 mg/kg), and midazolam (0.5 mg/kg), and maintained by continuous intravenous infusion of ketamine (2 mg/kg/h), xylazine (0.2 mg/kg/h) and midazolam (0.2 mg/kg/h). Animals were intubated and mechanically ventilated with oxygen
Central venous and arterial lines were inserted and a single bolus of unfractioned heparin (300 mg/kg) was administered at the onset of instrumentation. The left anterior descending coronary artery, immediately distal to the origin of the first diagonal branch, was occluded for 40 minutes with an angioplasty balloon introduced via the percutaneous femoral route using the Seldinger technique. Balloon location and maintenance of inflation were monitored angiographically. After balloon deflation, a coronary angiogram was recorded to confirm patency of the coronary artery. A continuous infusion of amiodarone (300 mg/h) was maintained during the procedure in all pigs to prevent malignant ventricular arrhythmias. In cases of ventricular fibrillation, a biphasic defibrillator was used to deliver non-synchronized shocks. In the animals allocated to ischemia without reperfusion, the balloon was maintained inflated until the pig was sacrificed and the heart excised (Group 7), or a CMR-compatible coil (MReye® embolization coil, Cook Medical) was inserted in the left anterior descending coronary artery, immediately distal to the origin of the first diagonal branch, to achieve permanent coronary occlusion (Group 8). Total occlusion of the vessel was demonstrated by angiography in all cases within 30 seconds after placing the coil.

High-dose steroid therapy

High-dose steroid therapy in Group 6 was initiated after completion of the 120min-CMR. Pigs were administered with 3 doses of intravenous methylprednisolone (30 mg/kg/dose) within the first 24 hours after I/R and then with single daily doses of intravenous methylprednisolone (30 mg/kg) for the following three days.

Quantification of myocardial water content

Paired myocardial samples were collected within minutes of euthanasia from the infarcted and remote myocardium of all pigs. Tissue samples were immediately blotted to remove surface moisture and introduced into laboratory crystal containers previously weighed on a high-precision scale. The containers were weighed before and after drying for 48 hours at 100°C in a desiccating
oven. Tissue water content was calculated as follows: water content (%) = [(wet weight−dry weight)/wet weight] ×100. An empty container was weighed before and after desiccation as an additional calibration control.

**Histological and immunohistochemical analysis**

Myocardial samples were collected within minutes of euthanasia from the ischemic (anteroseptal) mid-apical ventricular wall. Tissue samples were fixed in 10% neutral buffered formalin for 48 hours and processed by dehydrating the tissue in increasing concentrations of ethanol. Samples were then cleared in xylene, embedded in paraffin wax and cut into 4 micron sections.

For histopathological analysis sections were stained with Hematoxylin and Eosin (H&E) and Masson’s Trichrome. Necrotic tissue was defined upon the presence of typical signs of coagulative necrosis including marginal contraction bands, fading and eventually loss of nuclei and striation in cardiomyocytes. Granulation tissue was defined upon the presence of loose collagen and abundant capillaries with a nearly complete removal of necrotic myocytes by infiltrated macrophages. Finally, lesion area was defined as the sum of necrotic and granulation areas.

For immunohistochemical analysis sections were deparaffinized and antigen unmasking was performed using heat induced epitope retrieval (HIER) with citrate buffer at pH6 (anti-PM1), or proteinase K (anti-F4/80). Before incubation with primary antibodies, endogenous peroxidase was blocked by incubation with H₂O₂ for 5 minutes and endogenous antigens were blocked with fetal bovine serum (FBS) for 20 minutes.

Primary antibodies used were mouse monoclonal anti-PM1 (BMA biomedicals; T-3503) to detect neutrophils, and mouse monoclonal anti-F4/80 (Abcam; ab22506) to detect macrophages. As secondary antibodies we used a HRP-conjugated goat anti-mouse (Dako; P0447) for PM1, and a HRP-conjugated goat anti-mouse/rabbit (EnVision FLEX® SM802) for F4/80. Bound antibody was revealed by staining with diaminobenzidine and nuclei were counterstained with hematoxylin. All immunohistochemical procedures were performed using an automated
autostainer (Autostainer Plus®), Dako). For analysis the images were digitalized with a scanner (Nanozoomer-RS C110730®, Hamamatsu) and examined with image analysis software (Tissuemorph®, Visiopharm) by an experienced veterinary pathologist blinded to group allocation.

**CMR protocol**

Baseline CMR scans were performed immediately before myocardial infarction and scans subsequently repeated at post-infarction follow-up time points until sacrifice. All studies were performed with a Philips 3-Tesla Achieva Tx whole body scanner (Philips Healthcare, Best, the Netherlands) equipped with a 32-element phased-array cardiac coil. The imaging protocol included a standard segmented cine steady-state free-precession (SSFP) sequence to provide high quality anatomical references, a T2-weighted triple inversion-recovery (T2W-STIR) sequence, and T2- turbo spin echo mapping (T2-TSE map). The imaging parameters for the SSFP sequence were a FOV of 280 x 280 mm, a slice thickness of 6 mm with no gap, TR 2.8 ms, TE 1.4 ms, flip angle 45°, cardiac phases 30, voxel size 1.8 x 1.8 mm, and 3 NEX. The imaging parameters for the T2W-STIR sequence were FOV 280 x 280, slice thickness 6 mm, TR 2 heartbeats, TE 80 ms, voxel size 1.4 x 1.95 mm, delay 210 ms, end-diastolic acquisition, echo-train length 16, and 2 NEX. The imaging parameters for the T2-TSE mapping were FOV 300x300, slice thickness 8 mm, TR 2 heartbeats, and ten echo times ranging from 4.9 to 49ms. SSFP and T2W-STIR sequences were performed to acquire 13-15 contiguous short axis slices covering the heart from base to the apex, whereas T2-maps were acquired in a mid-apical ventricular short axis slice corresponding to the same anatomical level in all studies, in order to track T2 relaxation time changes over time.

**CMR data analysis**

CMR images were analyzed using dedicated software (MR Extended Work Space 2.6, Philips Healthcare, The Netherlands) by two observers experienced in CMR analysis blinded to group
allocation. T2-maps were automatically generated on the acquisition scanner by fitting the signal intensity of all echo times to a monoexponential decay curve at each pixel with a maximum likelihood expectation maximization algorithm. T2 relaxation maps were quantitatively analyzed by placing a wide transmural region of interest (ROI) at the ischemic area of the corresponding slice in all studies. Hypointense areas suggestive of microvascular obstruction or hemorrhage were included in the ROI for T2 quantification purposes.
SUPPLEMENTAL REFERENCES