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Aggravated post-infarct heart failure in type 2 diabetes is associated with impaired mitophagy and exaggerated inflammasome activation

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# Equal contribution

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**Running title:** Post-infarct heart failure in diabetes

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Abstract

Type 2 diabetes mellitus (T2DM) is a major risk factor for heart disease. Mortality rates following myocardial infarction (MI) are significantly increased in T2DM patients due to dysfunctional left ventricle (LV). However, molecular pathways underlying accelerated post-MI heart failure (HF) in T2DM remain unclear. We investigated the underlying mechanisms by inducing MI in a well-established model of T2DM and control mice. Cardiac imaging revealed a significantly decreased global LV ejection fraction (EF) in parallel with increased mortality post-MI in T2DM mice compared to controls. Genome-wide mRNA sequencing, immunoblot, electron microscopy, together with immunofluorescence staining for LC3 and p62 indicated an impaired mitophagy in peri-infarct regions of LV in T2DM mice compared to controls. Furthermore, defective mitophagy was associated with an increased release of mitochondrial DNA, resulting in Aim2 and NLRC4 inflammasome and caspase-I hyperactivation in cardiomyocytes and cardiac macrophages in peri-infarct regions of LV in T2DM mice. Consistent with inflammasome and caspase-I hyperactivation, cardiomyocyte death and IL-18 secretion were increased in T2DM mice.

Our results indicate that T2DM aggravates post-MI HF through defective mitophagy-associated exaggerated inflammasome activation, cell death and IL-18 secretion, suggesting that restoring mitophagy and inhibiting inflammasome activation may serve as novel targets for the prevention and treatment of HF in T2DM.

Nonstandard abbreviations and Acronyms:
T2DM – Type 2 diabetes
IGF-II – Insulin-like growth factor II
LAD – Left-anterior descending artery
LV – Left ventricle
MI – Myocardial infarction
MRI – Magnetic resonance imaging
FC – Fold change
TUNEL – Terminal deoxynucleotidyl transferase dUTP nick end labeling
FTIR – Fourier Transform Infrared Spectroscopy
Introduction

Type 2 diabetes mellitus (T2DM) together with hyperlipidemia and hypercholesterolemia are major risk factors for cardiovascular diseases including coronary heart disease (CHD) contributing to increased morbidity and mortality.\cite{1-3} Diabetic patients are susceptible to myocardial infarction (MI) due to extensive coronary atherosclerosis. In addition, diabetes substantially accelerates the development of heart failure (HF) and mortality in patients and animal models with MI.\cite{4-6} Morbidity, mortality, and re-infarction rates are 2-4 times higher following MI in diabetic than in non-diabetic subjects, with one-year mortality in this population as high as 50%.\cite{7}

Despite recent progress in coronary intervention strategies, mortality rates due to MI in diabetic patients are significantly increased due to dysfunctional left ventricle (LV).\cite{8, 9} MI leads to increased death of cardiomyocytes and initiates inflammatory responses contributing to cardiomyocyte dysfunction and subsequently HF.\cite{10} At the organ level, LV undergoes a complex remodeling process in order to compensate for the reduced cardiac function after MI. Failure to compensate will eventually lead to infarct expansion, global ventricular dilation, myocardial hypertrophy, cardiac fibrosis and finally HF.\cite{11, 12}

The cellular and molecular pathways contributing to maladaptive ventricle remodeling and HF following MI in diabetic patients are poorly understood and uncovering the underlying pathophysiological mechanisms may lead to targeted therapies for diabetic patients with MI.

Herein, we have addressed cellular and molecular pathways underlying post-MI LV maladaptive remodeling in a well-established mouse model of insulin resistance and T2DM.\cite{13} These mice demonstrate typical features of human T2DM including insulin resistance, hyperglycemia and mild hyperinsulinemia. Additionally, these mice show increased atherosclerotic progression and lesion calcification. Previously, using these T2DM mice we have demonstrated an impaired vascular growth in response to hindlimb ischemia as
a consequence of altered macrophage phenotype.\cite{14} Using transcriptional profiling and molecular characterization, we found an impaired mitophagy in cardiomyocytes from peri-infarct zone of LV in T2DM mice. Additionally, impaired mitophagy was associated with an increased release of mitochondrial derived DAMPs (mtDNA) intracellularly as well as extracellularly. Thus, impaired mitophagy and subsequent inflammasome hyperactivation was associated with an increased cardiomyocyte death and proinflammatory cytokine secretion (IL-18) contributing to post-MI maladaptive LV remodeling and HF. Modulation of mitophagy together with inhibition of inflammasome activation may offer a novel therapeutic approach for the prevention of post-MI HF in T2DM.

Materials and Methods

Mouse model

Both male and female mice aged between 12 to 14 months were used in the study (12-15 mice per group/time point). All animal experiments were approved by the Experimental Animal Committee, University of Eastern Finland and procedures were conducted in accordance with approved animal protocols. Mice over expressing IGF-II in pancreatic beta cells in hyperlipidemic (LDLR\textsuperscript{-/-}\textsuperscript{ApoB\textsuperscript{100/100}}) background (IGF-II/\textsuperscript{LDLR\textsuperscript{-/-}\textsuperscript{ApoB\textsuperscript{100/100}}}) with type 2 diabetic features were fed with high fat diet for 12 weeks (TD 88173, Harlan Teklad: Boxmeer, NL, 42% of calories from fat and 0.15% from cholesterol, no sodium cholate),\cite{13} with C57BL/6J mice fed with regular chow-diet (R36, Lactamin, Stockholm, Sweden) serving as controls.

Mouse myocardial infarction

All mice were randomly selected for myocardial infarction (MI) operations. MI was induced by ligation of the left anterior descending coronary artery (LAD) as described previously.\cite{15} Mice were subjected to small left thoracotomy under general anesthesia with isoflurane (2%). The heart was temporarily exteriorized, and a 6.0 silk suture was placed 2 mm below the
origin of the LAD. The heart was replaced immediately into the thoracic cavity, and all thoracic air was evacuated to avoid pneumothorax. Sham operations were performed by passing the suture around the LAD without ligation. Following MI, the animals remained supervised until fully conscious. The researcher performing MI operations was blinded for mice groups.

**Magnetic resonance imaging (MRI)**

Functional cardiac parameters were measured by MRI at baseline, day 7 and 14 post-MI. Mice were anaesthetized with 1.5-2% isoflurane in 70% N₂ : 30% O₂ throughout the imaging procedure. A pneumatic pillow placed on the side of the mouse was used for respiratory monitoring and gating the imaging. ECG needles were placed subcutaneously on the mouse front paws for monitoring the heart rate and triggering the imaging. Temperature was maintained at 35°C to keep body temperature constant. Images were acquired on a 7T horizontal-bore Pharmascan (Bruker Biospin, Ettlingen, Germany) with a 38 mm diameter of surface RF coil. Short-axis of the heart was determined from echo images on three orthogonal planes (transverse, longitudinal and sagittal). Axial images perpendicular to the long axis were chosen for cine imaging. An orthogonal long axis slice and a stack of short-axis slices covering the heart from apex to base were acquired using an ECG and respiratory gating with the following parameters: repetition time (TR) 5.2 ms, echo time (TE) 2 ms, slice thickness 1mm, matrix size 192x192 (in 2.56 x 2.56 cm² field-of-view), 6-8 frames per sequence.

**Image analysis**

Image analysis was performed using Aedes (http://aedes.uef.fi/), on MATLAB (Mathworks Inc.Natwick, CA, USA). Manual segmentation of the endocardium and epicardium of the LV was performed to measure global LV functional parameters [end-diastolic (EDV) and end-systolic (ESV) volume]. Ejection fraction was calculated using the formula EF (%) = [(EDV-ESV)/EDV] X 100.
RNA-Sequencing Library preparation

Total RNA was isolated from cardiac tissue proximal and distal to LAD ligation using RNeasy Fibrous Tissue Mini Kit (QIAGEN Finland, Helsinki, Finland) according to manufacturer's instructions. RNA quantity and quality was assessed using Nanodrop. Poly (A)-RNA was selectively enriched with MicroPoly (A) Purist Kit (Ambion, Austin, TX, USA). RNA was treated with TURBO DNase (Ambion, Austin, TX, USA), fragmented using RNA Fragmentation Reagents (Ambion, Austin, TX, USA) and purified by running through P-30 column (Bio-Rad, Hercules, CA, USA). Fragmented RNA was dephosphorylated with Antarctic phosphatase (New England Biolabs, Ipswich, MA, USA) followed by heat-inactivation. Dephosphorylation reactions were mixed with 2 x Novex TBE-Urea sample buffer (Invitrogen, San Diego, CA, USA), briefly denatured and loaded on a Novex denaturing 15% polyacrylamide TBE-urea gel according to the manufacturer's instructions. Fragments of 50-300 nucleotides in length were gel purified as described previously.[16] Following this original protocol, also the poly (A)-tailing and cDNA synthesis was performed the next day. However, for reverse transcription oligos with custom barcodes (underlined) were used:

5’phosCA/TG/AC/GTGATCGTCGGACTGTAGAACTCT/idSp/CAAGCAGAAGACGGC
ATACGATTTTTTTTTTTTTTTTTTVN-3’. After cDNA synthesis, exonuclease was used to catalyze the removal of excess oligos. Enzyme was inactivated and RNA hydrolyzed by alkaline treatment (100 mM NaOH) and heat (25 min, 95°C). The cDNA fragments of ~150-200 bps were purified on a Novex denaturing 10% polyacrylamide TBE-urea gel (Invitrogen, San Diego, CA, USA). The recovered cDNA was circularized, linearized, amplified for 8 cycles. The final product was ran on Novex 10% TBE gel, gel purified as above and cleaned-up using ChIP DNA clean & Concentrator Kit (Zymo Research
Corporation, Irvine, CA, USA). The library was sequenced for 50 cycles on the Illumina HiSeq 2000 according to the manufacturer’s instructions.

**RNA-sequencing data analysis**

Single-end sequencing reads were quality controlled using the FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) software and subsequently bases with poor quality scores were trimmed (requiring a minimum 97% of all bases in one read to have a min phred quality score of 10) using the FastX toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). The TopHat software (v2.0.9) was used for read alignment to the mouse genome (version mm9), accepting up to two mismatches. A transcriptome index was built from genes.gtf file available from the UCSC mm9 iGenomes database (Illumina, San Diego, California, U.S.A.). The HOMER v4.3 (http://homer.salk.edu/homer) toolkit was used for visualization and transcript quantification based on UCSC Refseq annotations. Transcripts expressed at a level rpkm > 1 in at least three samples were used for statistical analysis with the R/Bioconductor package edgeR. Estimation of dispersion was performed gene-wise using default settings. Differentially expressed transcripts were identified using the glmLRT function. Transcripts with at least 1.5-fold change in expression level and adjusted p value <0.005 (Benjamini-Hochberg method using p-values from moderated t-test) were defined significantly regulated.

**Functional analysis**

Differentially expressed genes in infarct and peri-infarct regions of LV of T2DM mice compared to controls were subjected to Ingenuity pathways analysis program (IPA, QIAGEN, Redwood City, USA). Differentially expressed genes were grouped in to functional categories by combining data available in NCBI gene information and gene expression data in public data sets.

**Histology**
After sacrificing the mice, hearts were perfused with PBS and immersion fixed in 4% PFA/15% sucrose (pH 7.4) for 4 h and then rinsed in 15% sucrose (pH 7.4) overnight. 5 µm-thick paraffin embedded sections were cut transversely for immunohistochemistry as mentioned previously.\(^{[14]}\) Capillaries were stained with rat anti-mouse CD31 (BD Pharmingen, San Jose, CA, USA. Catalog #550274, 1:50 dilution). Capillary density was measured in a blinded manner from 5 different fields from infarct, peri-infarct and remote zones. Avidin-biotin-HRP system with DAB color substrate (Vector Laboratories, Burlingame, CA, USA. Catalog # PK-6100) was used for signal detection. Collagen content was analyzed from picro-sirius red stained sections. The percent positive stained area was calculated in relation to the whole LV area. Photographs of stained histological sections were taken and processed using an Olympus AX 70 microscope (Olympus Optical, Japan) analySIS (Soft Imaging System) and Photoshop (Adobe, CS4) software. The peri-infarct region within the LV was defined as area containing both infarcted and surviving myocardium within the high power field, whereas the remote region was chosen from a myocardial region without any infarction.

**Immunofluorescence microscopy**

All immunofluorescence stainings were performed on paraffin sections. Macrophages were identified using rat anti-mouse F4/80 antibody (AbD Serotec, Oxford, UK. Catalog # MCA497, 1:50 dilution). M1-Macrophages were detected using F4/80 in combination with TNF-α antibody (1:50 dilution, Santa Cruz biotechnology, Santa Cruz, CA. USA. Catalog # Sc-1351) and M2-Macrophages were detected using F4/80 in combination with arginase I antibody (1:50 dilution, Santa Cruz biotechnology, Santa Cruz, CA. USA. Catalog # Sc-18354). Photographs of stained histological sections were taken and processed using an Olympus AX 70 microscope (Olympus Optical, Japan).

**TUNEL assay for apoptosis**
For in situ detection of apoptosis, TUNEL assay (In Situ Cell Death Detection Kit, Fluorescein, Boehringer Mannheim, Germany. Catalog #1684795) was used. Mouse myocardial tissue sections were deparaffinized, digested with Proteinase K and incubated with TdT and fluorescein-labeled dUTP in a humid atmosphere for 60 min at 37 °C. After incubation for 30 min with an antibody specific for fluorescein-conjugated alkaline phosphatase (AP; Boehringer), the TUNEL stain was visualized with a substrate system in which nuclei with DNA fragmentation stain green (Fluorescein substrate system, DAKO, Carpinteria, California). Sections were counterstained with a monoclonal antibody specific for alpha sarcomeric actinin (Sigma-Aldrich, Helsinki, Finland. Catalog # A7811). Tissue sections were examined microscopically at ×400 magnification and TUNEL positive cells were counted in five high power fields.

**Fourier transform infrared spectroscopic (FT-IRS) collagen I mapping**

Collagen I deposition in remodeled cardiac tissues was detected from 5-µm thick paraffin sections using Perkin Elmer Spotlight 300 spectrometer (Perkin Elmer, Shelton, Connecticut, USA). Data acquisition and analysis were performed as described previously.\[17\] The CH₂ side chain vibration (1338 cm⁻¹) was used to create collagen I distribution images, and the collagen-I area in relation to whole LV area was determined from these images.

**Electron Microscopy**

Following sacrifice and PBS perfusion, LV tissue was quickly cut into 1-mm longitudinal and transverse cubes, immersion fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for overnight at 4°C and post-fixed in 1% buffered osmium tetroxide. The specimens were dehydrated and embedded in LX-112 resin and polymerized. Toluidine blue staining of semi thin sections were used to evaluate the orientation of the sections. Ultra-thin sections (~70nm) were double stained with lead citrate and uranyl acetate and visualized with transmission electron microscopy (JEM 1200EX, JEOL Ltd. Japan). Autophagosomes or
autolysosomes were identified by the characteristic structure of a double or multi-lamellar smooth membrane completely surrounding compressed mitochondria or membrane bound electron-dense material.\textsuperscript{[18]}

**Immunoelectron Microscopy**

LV tissues were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, immersed in 2.3 M sucrose in PBS and frozen in liquid nitrogen. Cryo-sections were incubated with Mouse monoclonal antibody against \textit{dsDNA} (Abcam, Cambridge, UK. Catalog # ab27156), followed by incubation with 10nm Gold-conjugated secondary antibody (British Biocell International, Cardiff, UK). Control grids were incubated with the secondary antibody alone or with an irrelevant immunoglobulin G.

**Flow cytometry analysis of cardiac Macrophages**

Flow Cytometry was performed as described previously.\textsuperscript{[14]} Infarcted cardiac muscles were minced and enzymatically dissociated using a cocktail containing 450 U/mL Collagenase I, 125 U/mL Collagenase XI, 60 U/mL DNaseI, and 60 U/mL hyaluronidase (Sigma Aldrich, Helsinki, Finland) for 1 h at 37°C. The cells were then counted and stained for Macrophage antigens after initially blocking with rat anti-mouse CD16/32 mAb (Fc Block, BD-pharmingen. San Jose, CA, USA. Catalog # 553141) for 10 min at 4°C. To identify M2-like Macrophages, we performed staining for Cd45 (Biolegend, San Diego, CA, USA. Catalog # 103131), F4/80 (AbD Serotec, Cambridge, UK. MCA497FB), and MRC1 (Biolegend, San Diego, CA, USA. 141707). Fluorescence minus one (FMO) controls were used to analyze the specific stainings. FACS was performed on FACS AriaIII (BD Biosciences, San Jose, CA, USA) and data was analyzed with FCS express 6 (De Novo software, CA, USA).

**Adult cardiomyocyte isolation**
Mice were killed by rapid neck disarticulation. The thorax was opened and the aorta was cannulated. Adult mouse ventricular myocytes were obtained by enzymatic dissociation as previously described (AfCS Procedure Protocol PP00000125). Briefly, isolated hearts were placed in a Langendorff apparatus for perfusion (37°C, 3 mL/min) with trypsin (Sigma-Aldrich, St. Louis, MO, USA) and liberase (Roche Applied Science, Indianapolis, IN, USA) solution. After perfusion, ventricles were cut into small pieces and gently minced with a pasteur pipette. The supernatants containing dissociated cardiomyocytes was filtered through a nylon mesh (100-µm pore size) and the cardiomyocyte fraction was separated by sedimentation by centrifugation at 100g for 1 min.

**Flow-Assisted Cell Sorting of cardiac Macrophages**

Non-cardiomyocyte fraction from Langendorff perfusion protocol was subsequently labelled with CD31 magnetic beads and separated into CD31+ve and CD31-ve fractions using CD31 magnetic bead enrichment (Miltenyi Biotec, Cologne, Germany. Catalog # 130-097-418). For Macrophage sorting CD31-ve fraction was incubated for 15 min with rat anti-mouse CD16/32 mAb (Fc Block, BD-pharmingen, Catalog # 553141) and stained with FITC conjugated rat anti-mouse F4/80 antibody (AbD Serotec, Oxford, UK. Catalog # MCA497F) for 30 min at 4°C. FACS sorting was performed on FACS AriaIII (BD Biosciences, San Jose, CA, USA) as described previously.

**Quantitative real-time PCR**

Total RNA isolated from cardiac tissue distal to LAD ligation was reverse transcribed to cDNA using M-MuLV Reverse Transcriptase (Fermentas, Helsinki, Finland). First strand cDNA was synthesized from 1 µg of the extracted RNA in a 20-µl reaction volume with the use of random hexamers (Promega Corporation, WI, and USA) as primers. Total RNA was isolated from cardiomyocytes and FACS sorted Macrophages using Arcturus PicoPure RNA isolation Kit (Thermo Fisher Scientific, KIT0204). First strand cDNA was synthesized from
100 ng of total RNA in a 20-µl reaction volume as described above. Gene expression analysis was performed using the Step one plus Real-time PCR system with assays on demand (Applied Biosystems: Foster City, CA).

**Western Blotting**

Left ventricle tissue was homogenized in tissue protein extraction reagent supplemented with protease inhibitors and sodium orthovanadate (Na$_3$VO$_4$). The homogenate was centrifuged at 12000 g for 5 min and the supernatant was collected for downstream analysis. Total protein concentration was measured using BCA kit (Pierce Biotechnology, Rockford, IL, USA. Catalog # 23227). 100µg of total protein was run on 12% pre-cast gels (Biorad, Hercules, CA, USA. Catalog # 4561043) and transferred on to PVDF membrane. The membrane was blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline /0.1% Tween 20 (TBST) for 1 hr at room temperature and then probed with Rabbit Monoclonal LC3 antibody (Cell signaling, Danvers, MA, USA. Catalog # 4599) and Rabbit Monoclonal p62 antibody (Origene, MD, USA. Catalog # TA307334) at 4°C overnight. Proteins were detected using HRP-conjugated Goat anti-rabbit secondary antibody (Thermo Fisher Scientific, Rockford, IL, USA. Catalog # 31460) in TBST for 1 hr at room temperature, followed by thorough washing with TBST and incubation with ECL substrate (Pierce Biotechnology, Rockford, IL,USA. Catalog # 32106).

**Statistical analysis**

Results were expressed as means ±SEM. Statistical significance was evaluated using one-way ANOVA followed by Bonferroni analysis, or by the Student’s t test. All statistical analyses, except RNA-Seq data analysis were performed using GraphPad Prism 5 software (La Jolla, CA, USA). A p <0.05 was considered statistically significant.

**Results**
T2DM accelerates heart failure and mortality post-MI

T2DM mice displayed an increased body weight, plasma insulin and blood glucose levels at baseline. Whereas, heart weight to body weight ratio was comparable between groups (Table 1). MI was induced by LAD ligation in control and T2DM mice. In response to MI, a marked increase in LV chamber dilation was observed in both groups compared to their respective sham controls (Figure 1A). In response to MI, ~60% of all T2DM mice died during the 14 days post- MI, whereas only 2.8 % of mice died in the control group during the same period. No deaths were observed in sham-operated groups (Figure 1B). At baseline conditions, T2DM mice showed a mild yet non-significant decline in EDV compared to controls, whereas ESV volumes were similar in both groups. Following MI, both groups showed a significant increase in LV EDV and ESV compared to baseline values. T2DM mice displayed a robust increase in EDV and ESV compared to controls post-MI (Figure 1, C and D). A significant systolic dysfunction was observed as assessed by LVEF in mice with MI compared to sham controls (p <0.005, MI vs. Sham). However, systolic dysfunction was exacerbated in T2DM mice compared to controls as exemplified by a significant decline in global LV EF (P<0.005 at day 7 and 14 Post-MI, T2DM MI vs Control MI) (Fig. 1E).

Collectively, these data indicate that T2DM adversely influence cardiac function and increase mortality rate in response to MI.

RNA sequencing reveals dysregulation of genes related to cardiac contractility, cell death and metabolism of nucleic acids in T2DM hearts

Since T2DM severely impairs tissue repair after injury and accumulating evidence suggests alterations in inflammation resolution phase of tissue repair, we performed genome-wide mRNA sequencing on peri-infarct and infarct regions of LV tissue collected at day-7 post-MI. LV tissue remote to infarct region served as an internal control. The RNA sequencing
data has been deposited in NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE69201. By using a threshold of fold change >1.5 and an adjusted P-value of <0.005, we found 1609 genes to be differentially expressed in the peri-infarct and infract regions of LV of T2DM compared to controls. Among them, 568 genes were upregulated and 1041 were downregulated as shown in the scatter plots in Figure 2A, volcano plots in Figure 2B and Supplemental Table S1 [20-22] and Supplemental Table S2. Ingenuity functional annotation suggested an increased cell death, necrosis, movement disorders, heart disease, neuromuscular disease, hypertrophy and cardiomyopathy, whereas decreased metabolism of nucleic acid components, metabolism of nucleotides, heart rate, contractility of muscles in T2DM mice compared to controls as shown in Table 2, Supplemental Table S1 [20-22] and Supplemental Table S3. Ingenuity pathway analysis revealed ‘mitochondrial dysfunction’, ‘TCA cycle’ and ‘Fatty acid β-oxidation’ as the most prominent pathways affected by T2DM in post-MI LV as shown in Figure 2C and Supplemental Table S4. Ingenuity upstream regulator analysis suggested an inhibition of PGC-1α regulatory network (Essential regulator of cardiac metabolism and mitochondrial homeostasis) consisting of coregulators PGC-1α, PGC-1β and nuclear receptors ESRRα, ESRRG, PPARα, PPARG, PPARD, and other transcription factors TFAM, HAND2, GATA4, whereas activation of STAT1, NR3C2, NRIP1, SMAD3, IRF3, NFATC2, SPI1 in T2DM mice compared to controls as shown in Table 3 and Supplemental Table S5. Collectively, these data reveal that T2DM dysregulates genes important in cardiac function, mitochondrial homeostasis, cell death and metabolism of nucleic acids in post-MI LV.

**Impaired mitophagy in cardiomyocytes of peri-infarct LV in T2DM hearts**

Genes critical in mitochondrial fusion machinery and regeneration of healthy mitochondria including, Mfn1, Mfn2, Opa1 and genes involved in removal of damaged mitochondria and
autophagy including, Lc3a and Pink1 were significantly downregulated in peri-infarct regions of LV of T2DM mice compared to controls at day 7 post-MI. (Figure 3A, Supplemental Table S1 \cite{20-22} and Supplemental Table S2). In order to unravel cell specificity of differential gene expression, we isolated cardiomyocytes from peri-infarct and infarct regions using Langendorff perfusion procedure. Subsequently, non-cardiomyocyte fractions were used for cardiac Macrophage isolation using a combination of magnetic assisted cell sorting with FACS sorting.\cite{14} Cell specific gene expression showed decreased activity of mitochondrial fusion and autophagy related genes predominantly in cardiomyocytes from peri-infarct regions of LV of T2DM compared to controls (Figure 3B). Additionally, western blotting analysis showed increased LC3-II/I ratio and p62 levels in LV homogenates from T2DM mice compared to controls, indicating impaired mitophagy/autophagy (Figure 3C). Electron microscopy images showed an ordered array of myofibers arranged with mitochondria stacked in between them in cardiomyocytes of control mice. In addition, intramyocellular lipid deposition was seen in between mitochondria in the T2DM mice at baseline, similar to observations in diabetic human hearts. Mitophagy was activated in the peri-infarct regions of LV of both groups at day 7 post-MI. Interestingly cardiomyocytes in peri-infarct regions of LV in T2DM showed increased accumulation of undegraded mitochondria in autophagosomes (Figure 3D). Immuno-electron microscopy analysis using anti-DNA antibody showed localization in autolysosomes, suggesting the release of undegraded mitochondrial-DNA (mtDNA) into cytosol as a consequence of impaired autolysosomal degradation (Figure 3E). Interestingly, our RNA sequencing analysis suggested decreased metabolism of nucleic acids and nucleotides in LV of T2DM corroborating with impaired mitophagy. Additionally, immunofluorescence staining showed increased accumulation of LC3 positive mito/autophagosomes, together with increased p62 levels in peri-infarct regions of LV in T2DM mice compared to controls, further suggesting...
impaired mitophagy / autophagy, as a potential mechanism of release of mitochondrial derived damage associated molecular patterns (DAMPs) intracellularly as well as extracellularly following cell death. (Figure 3F).

**Exaggerated inflammasome activation and Caspase-I activity in T2DM hearts**

Genes involved in cytosolic double-stranded DNA (dsDNA) sensing (Ifi204, pyhin1), inflammasome (Aim2, NLRC4) and caspase-I were significantly upregulated in peri-infarct and infarct regions of LV in T2DM mice compared to controls at day 7 post-MI (Figure 4A, Supplemental Table S1 [20-22], Supplemental Table S2). In order to dissect out the cell specific roles of inflammasome in cardiomyocytes and Macrophages, we isolated these cell populations and looked at Pyhin1, Ifi204, Aim2, NLRC4, Eif2ak2 and Caspase-1 gene expression. Of note, all these genes were significantly upregulated both in cardiomyocytes (Except Eif2ak2) and Macrophages isolated from T2DM mice compared to controls (Figure 4B). Furthermore, immunofluorescence double stainings for cardiomyocyte and Macrophage specific dsDNA sensors (Pyhin1, Ifi204) showed increased activation both in cardiomyocytes and Macrophages from peri-infarct and infarct regions of LV in T2DM compared to controls at day 7 post-MI (Supplemental Figure S1). Similarly, immunofluorescence double stainings for cardiomyocyte and Macrophage specific inflammasome (Aim2, NLRC4) showed abundance of double positive cells in peri-infarct and infarct regions of LV in T2DM compared to controls at day 7 post-MI (Figure 4, C and D).

Inflammasome activation culminates in either caspase-I mediated cell death (Pyroptosis) or cytokine processing into mature active forms (IL-1β, IL-18). In order to understand the consequence of inflammasome activation in this context, we looked at caspase-I activity by immunostainings of LV tissue collected at day-7 post-MI. Similar to Aim2 and NLRC4 stainings, caspase-1 immunostaining showed increased activation in peri-infarct and infarct regions of LV in both groups (Figure 4E). In addition, caspase-1
colorimetric assay using LV tissue homogenates showed increased activity in T2DM mice compared to controls (Figure 4G). Next, we looked into cell death using TUNEL assay, similar to caspase-I stainings we observed an increased number of TUNEL-positive cells in peri-infarct and infarct regions of LV of T2DM mice compared to controls (Figure 4, F and H). In parallel with increased inflammasome and caspase-I activity, we observed increased caspase-1 dependent inflammatory mediator (IL-18) in circulation of T2DM mice compared to controls at baseline and day 7 post-MI (Figure 4I). Of note, increased inflammation as evidenced by increased IL-18, tumor necrosis factor α and circulating immune cells were shown to associate with post-MI remodeling and HF in patients.[23, 24] Collectively, these results indicate that exaggerated activation of Aim2 and NLRC4 inflammasome and subsequent increase in caspase-I mediated pathways in cardiomyocytes as well as Macrophages in peri-infarct and infarct regions of LV of T2DM mice compared to controls.

**Altered cardiac Macrophage phenotypic responses in T2DM hearts**

Double immunofluorescence staining of LV tissue for M2-like Macrophage (anti-F4/80 and anti-Arginase-1) showed decreased numbers of M2-like Macrophage in peri-infarct and infarct regions in LV of T2DM mice compared to controls. On the contrary, stainings for M1-like Macrophage (anti-F4/80 and anti-TNF-α) showed an increase in T2DM mice compared to controls at day 7 post-MI (Figure 5A). Furthermore, we performed flow cytometry analysis for M2-like Macrophages in LV tissues collected at day-7 post-MI as described previously.[14] CD45+F4/80+CD206+ M2-like Macrophages were analyzed from sham and infarcted hearts of control and T2DM mice (Figure 5B). Quantification suggested a significant decline in M2-like Macrophages in LV of T2DM mice compared to controls (Figure 5C). Collectively, these results indicate an altered Macrophage phenotypic response to MI in LV of T2DM mice consistent with exaggerated inflammasome activation.

**Impaired neovascularization and increased fibrosis in T2DM hearts**
Post-MI neovascularization responses were examined using capillary endothelial staining with CD31 (CD31-positive cells). Quantification of CD31 positive cells showed a significantly decreased number of capillaries in peri-infarct and infarct regions of LV in T2DM mice compared to controls at day 7 and 14 post-MI (Figure 6, A and B). Corroborating with altered Macrophage phenotype, capillary stainings demonstrated an impaired post-MI neovascularization in LV of T2DM mice compared to controls.

Infarct size and myocardial fibrosis were evaluated using Sirius-red staining and Fourier transform infrared spectroscopic (FT-IRS) mapping of collagen-I at baseline and day-14 post-MI (Figure 6C). Interestingly, Sirius red staining and Collagen-I mapping by FTIR-S showed similar patterns of collagen distribution in LVs of both control and T2DM mice. Quantification showed significantly increased area of infarction and collagen-I deposition especially in LV of T2DM mice compared to controls (Figure 6D). Increased fibrosis has been suggested to disrupt the excitation-contraction coupling between cardiomyocytes and a subsequent increase in stiffness will lead to decreased ventricular wall compliance, resulting in both systolic and diastolic dysfunction.\(^{[25]}\) Thus, impaired post-MI neovascularization responses coupled with increased fibrosis might further decrease cardiac contractility and LVEF of the post-MI heart in T2DM mice compared to controls.

Discussion

In our previous study, we have characterized a mouse model of insulin resistance and T2DM, which demonstrates typical features of human T2DM including insulin resistance, hyperglycemia and mild hyperinsulinemia.\(^{[13]}\) Additionally, these mice show an increased atherosclerotic progression and lesion calcification. Recently, using these T2DM mice we have demonstrated an impaired vascular growth in response to hindlimb ischemia as a consequence of altered macrophage phenotype.\(^{[14]}\) In the present study, we provide insights
into pathways underlying accelerated post-MI HF in T2DM. Transcriptional profiling revealed an increased cell death, necrosis, myocardial dysfunction and decreased heart rate, cardiac contractility and metabolism of nucleic acids. Integrating transcriptomics and molecular characterization, we show an impaired mitophagy associated with increased accumulation of undegraded mitochondria in autophagosomes in cardiomyocytes from peri-infarct regions of LV. Furthermore, our immuno-gold labelling studies suggested localization of mitochondrial DNA (mtDNA) to autolysosomes in cardiomyocytes of LV, indicating increased release of undegraded mtDNA into cytosol as a result of impaired autolysosomal degradation of dysfunctional mitochondria. Reflecting increased cytosolic mtDNA as a result of impaired mitophagy, cytoplasmic DNA sensors including Pyhin1, Ifi204 and inflammasome, namely AIM2, NLRC4 and Caspase-I, were highly activated in cardiomyocytes and Macrophages in peri-infarct and infarct regions of LV. This was associated with increased cell death and IL-18 secretion, thus linking defective mitophagy to inflammasome activation (Supplemental Figure S2). Moreover, cardiac Macrophages showed an altered phenotype consistent with inflammasome activation in LV of T2DM mice.

Mitophagy is a selective form of autophagy involved in the removal of damaged mitochondria during cellular stress, which prevents the release of mitochondria derived DAMPS to the cytoplasm. Additionally, mitophagy prevents activation of pro-death pathways in favor of adaptation to stress. We observed an increased numbers of autophagosomes containing undegraded mitochondria (mitophagy), but reduced amount of autolysosomes in cardiomyocytes from peri-infarct regions of LV in T2DM mice together with an increased LC3-II/LC3-I ratio and p62 levels. Furthermore, immunofluorescence staining showed an increased accumulation of LC3 positive mito/autophagosomes, together with increased p62 levels further suggesting impaired mitophagy. Given the abundance of mitochondria in cardiomyocytes, it is likely that substantial amount of mitochondrial DNA
would be released extracellularly in the heart upon tissue damage as a result of impaired mitophagy.\cite{27} Consistent with this notion, immunoelectron microscopy analysis using anti-DNA antibody showed localization in autolysosomes. Additionally, our RNA sequencing analysis suggested decreased metabolism of nucleic acids and nucleotides in LV of T2DM supporting the notion that impaired degradation of mtDNA as a result of insufficient mitophagy might lead to increased release of undegraded mtDNA. Thus, impaired mitophagy-associated DAMPs likely contribute to exaggerated inflammasome activation and cell death. Our observations are in line with a recent study showing increased inflammation and cardiac dysfunction in response to transaortic constriction (TAC), as a result of mtDNA release from inefficient degradation by autophagy.\cite{28}

Inflammasome is a multiprotein complex that activates Caspase-1, triggering an inflammatory cell death called pyroptosis. In addition, caspase-1 triggers the maturation and release of proinflammatory cytokines IL-1β and IL-18.\cite{29} Indeed, inflammasome activation in the infiltrated inflammatory cells are shown to enhance myocardial I/R injury. Interestingly, almost all cardiac Macrophages expressed inflammasome adaptor ASC, whereas ≈60% of neutrophils expressed ASC. The expression of caspase-1 was visualized mainly in the infiltrated cells of the ischemic myocardium.\cite{30} NLRP3 inflammasome activation in cardiomyocytes and cardiac leukocytes in response to ATP released from dying cells was shown to play an important role in adverse cardiac remodeling in response to MI under non-diabetic conditions.\cite{31} Thus, inflammasome-dependent caspase-I activation and subsequent release of IL-18 and IL-1β, both in cardiomyocytes and Macrophages seems to lead to HF. Accordingly, inhibition of caspase-I reduced cardiomyocyte death, improved cardiac function and significantly decreased mortality.\cite{32-34} Transgenic mice overexpressing caspase-1 show an increased cardiomyocyte cell death and HF post-MI, independent of increase in IL-1β and IL-18 secretion.\cite{35} Collectively, our findings suggest that defective
mitophagy in T2DM contributes to post-MI HF through exaggerated inflammasome activation, subsequent cell death and cytokine secretion.

Our results suggest an increased inflammasome activation in cardiac Macrophages from T2DM mice compared to control mice. In parallel, immunofluorescence stainings and flow cytometry analysis suggested a decrease in reparative M2-like Macrophages, but an increase in inflammatory M1-like Macrophages in LV of T2DM mice. Consistent with altered Macrophage phenotype, T2DM displayed impaired neovascularization and increased fibrosis in peri-infarct and infarct regions in LV at day-14 Post-MI. While both M1-and M2-Macrophages are critical for resolving myocardial injury, it has become increasingly evident that shifting the balance of Macrophage to an M2-like phenotype is cardioprotective.\[^{36}\] On the contrary, the delayed phenotypic transition of M1 like-Macrophages to M2 like -Macrophages and/or prolonged and increased recruitment of inflammatory monocytes to the heart leads to impaired infarct healing and increased post-MI HF as demonstrated in atherosclerotic mice.\[^{37, 38}\] In line with these observations, we found skewing of cardiac Macrophages towards a pro-inflammatory phenotype in T2DM hearts, associated with impaired neovascularization and increased fibrosis compared to reparative Macrophage phenotype in controls.

We recognize that our study has limitations. Comparisons were made between T2DM mice with C57BL/J controls in order to keep all mice on the same genetic background which can have significant effects on the measured variables. Also, aged mice with HFD were used to aggravate the T2DM phenotype.\[^{13}\] However, we cannot exclude a possibility that other factors like age, insulin resistance or HFD could have contributed to the observed differences in response to MI. Inspite of these limitations, our findings suggest that T2DM significantly contributed to aggravated post-infrac heart failure.
Conclusions

Our results suggest that an impaired mitophagy and exaggerated inflammasome activation together with altered Macrophage phenotypic responses may contribute to post-MI HF in T2DM. Modulation of mitophagy together with inhibition of inflammasome activation may offer a novel therapeutic target.

Acknowledgements

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References


Figure legends

**Figure 1: T2DM accelerates post-MI heart failure and mortality.** (A) Representative short-axis MRI images of sham and infarcted mouse hearts. (B) Post-MI Kaplan–Meier survival curves for control (dotted line; \(n=28\)) and T2DM (thick line; \(n=23\)) during 2 weeks post-MI. ***\(P<0.005\) (Log-rank test). (C-E) Quantification of cardiac parameters including end-diastolic volume (EDV), end-systolic volume (ESV) and Global ejection fraction (EF) at baseline and day 7 and 14 post-MI. For (C-E), data are shown as mean ± s.e.m. (\(n=5\)). Statistical analysis was conducted using one-way ANOVA with Bonferroni’s multiple comparison test. *\(p<0.05\) ***\(p<0.005\) T2DM versus controls.

**Figure 2: Transcriptomics analysis reveals cellular alterations associated with post-MI HF in T2DM.** (A and B) Genome-wide mRNA sequencing analysis of LV tissue at day 7 post-MI. Using a Fold change >1.5 and an adjusted p-value of <0.005, revealed 1609 genes to be differentially expressed (568 upregulated and 1041 downregulated) in the peri-infarct and infarct LV of T2DM mice compared to controls at day 7 post-MI as shown in the scatter plots (A) and Volcano plots (B). (C) Major biological processes altered in peri-infarct and infarct areas from LV of T2DM identified by the Ingenuity pathway analysis.

**Figure 3: Impaired mitophagy in cardiomyocytes from peri-infarct LV post-MI in T2DM.** (A) Expression levels of genes critical in mitochondrial fusion machinery and autophagy in peri-infarct and infarcted LV of T2DM mice compared to controls at day 7 post-MI. \(n=4-5\) (Control) and \(n=4\) (T2DM). Data are shown as mean ± s.e.m, one-way ANOVA with Bonferroni’s multiple comparison test. *\(p<0.05\) T2DM versus controls. (B) Expression levels of genes critical in mitochondrial fusion and autophagy in cardiomyocytes (CM) and macrophages isolated from peri-infarct and infarct regions of LV at day-7 post-MI.
n = 4 for each group. Data are shown as mean ± s.e.m, t-test. *p<0.05 ***p<0.005 ****p<0.0001 T2DM versus controls. (C) Western blotting analysis of autophagy proteins LC3 and p62 in myocardial homogenates at day 7 post-MI. The Quantitative data is shown as mean ± s.e.m, t-test. *p<0.05, n = 4. (D) Representative electron microscopy images showing an ordered array of myofibers arranged with mitochondria stacked in between them in cardiomyocytes of control mice. In addition, intramyocellular lipid deposition can be seen in between mitochondria in the LV of T2DM mice at baseline (Black arrows), whereas no lipid droplets were observed in similar regions in control mice. Mitophagy activation showing abundant vacuoles/autolysosomes containing completely degraded mitochondria (Yellow arrowheads) in cardiomyocytes in peri-infarct regions of LV of control mice, whereas cardiomyocytes from T2DM show an increased accumulation of autophagosomes containing undegraded mitochondria (Red arrowheads). Mitochondria undergoing autophagosomal degradation (Red insets) are shown at higher magnification in the corresponding lower panels. (E) Immunoelectron microscopy using anti-DNA antibody showing localization in autolysosomes (Blue arrowheads). (F) Representative Immunofluorescent images showing accumulation of autophagosomes within cardiomyocytes from peri-infarct areas of LV at day 7 post-MI. LC3 and p62 are shown in red and sarcomeric actin in green. Scale bar, 20µm.

**Figure 4: Inflammasome hyperactivation in cardiomyocytes and macrophages from peri-infarct and infarct LV post-MI in T2DM.** (A) Expression profiles of genes involved in cytosolic dsDNA sensing and inflammasome activation from sham and infarcted LV at day 7 post-MI. n = 5 (Control) and n = 4 (T2DM). Data are shown as mean ± s.e.m., one-way ANOVA with Bonferroni’s multiple comparison test. *p<0.05 ***p<0.005 T2DM versus controls. (B) Expression profiles of genes involved in cytosolic dsDNA sensing and inflammasome activation in cardiomyocytes (CM) and macrophages isolated from peri-
infarct and infarct areas of LV at day-7 post-MI. n = 4 for each group. Data are shown as mean ± s.e.m., t-test. *p<0.05 ***p<0.005 ****p<0.0001 T2DM versus controls. (C) Representative images from immunofluorescence double stainings for cardiomyocyte inflammasomes (Aim2, NLRC4) and (D) Macrophage inflammasome (Aim2,NLRC4) in LV at day 7 post-MI (White arrows). (E, G) Representative images from caspase-1 immunostaining of the LV at day 7 post-MI (Black arrows). In addition, caspase-1 colorimetric assay using LV tissue homogenate is shown. The Quantitative data is shown as mean ± s.e.m, t-test. ***p<0.005, n = 4-5. (F) Representative TUNEL+ apoptotic cardiomyocytes (Green) in peri-infarct and infarct LV at day 7 post-MI (White arrows). (H) Quantification of TUNEL+ cardiomyocytes from border, peri-infarct and infarct regions of LV from sham and infarcted hearts at day 7 post-MI. Data are shown as mean ± s.e.m., one-way ANOVA with Bonferroni’s multiple comparison test. *p<0.05 ****p<0.0001 T2DM versus controls. n = 4-5. (I) Quantification of caspase-1 dependent inflammatory mediator (IL-18) in circulation at baseline and day 7 post-MI. Data are shown as mean ± s.e.m, one-way ANOVA with Bonferroni’s multiple comparison test. *p<0.05 T2DM versus controls. (Panels C, D Scale bars = 20µm. SA = Sarcomeric actin. Panels E, F Scale bars = 50µm).

Figure 5: Altered cardiac Macrophage phenotype in peri-infarct and infarct LV post-MI in T2DM. (A) Double immunofluorescence staining of M2-like Macrophages (anti-F4/80 and anti-Arginase-1) and M1-like Macrophages (anti-F4/80 and anti-TNF-α) in control and T2DM LV at day 7 post-MI (White arrows). Scale bar = 50µm. (B) Leukocytes were gated with CD45 fluorescence versus side scatter-area (SSC). Scatter plots are gated on CD45+ population. M2- Macrophages (CD45+F4/80+CD206+ cells) were analyzed from sham and infarcted hearts of control and T2DM mice. SSC, side scatter. (C) Quantification of M2-Macrophages from LV tissues of sham and infarcted hearts. Data are shown as mean ± s.e.m,
one-way ANOVA with Bonferroni’s multiple comparison test. *p<0.05 ***p<0.005 vs controls. n = 3-4.

Figure 6: Impaired neovascularization and increased infarct and fibrotic area post-MI in T2DM. (A) Representative images showing capillary endothelial staining with CD31 in peri-infarct and infarct regions of ischemic LV at day 7 post-MI. Scale bars = 50µm. (B) Quantification of CD31+ capillaries from remote, peri-infarct and infarct regions of LV of sham and infarcted hearts at day 7 and 14 post-MI. Data are shown as mean ± s.e.m, one-way ANOVA with Bonferroni’s multiple comparison test. *p<0.05 ***p<0.005 vs controls. n = 4-5. (C) Representative images from sirius red staining for collagen deposition in the ischemic LV together with representative images from FTIR-S mapping for collagen-I. Scale bars = 2000µm. (D) Quantification of collagen positive area from whole LV at day-14 post-MI. The Quantitative data is shown as mean ± s.e.m, t-test, *p<0.05 T2DM versus controls, n = 4-5.
Table 1: Baseline metabolic parameters.

<table>
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<th>Parameter</th>
<th>Control</th>
<th>T2DM</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (gms)</td>
<td>29.94 ± 0.9589</td>
<td>41.34 ± 2.482</td>
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<td>Heart/Body weight ratio</td>
<td>0.004961 ± 0.00042</td>
<td>0.004676 ± 0.00031</td>
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<td>Plasma Insulin (ng/mL)</td>
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<tr>
<td>Blood glucose (mmol/L)</td>
<td>7.783 ± 0.8264</td>
<td>18.28 ± 2.932</td>
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ns – not significant.
Table 2: Ingenuity functional annotation of differentially expressed genes in peri-infarct and infracted left-ventricle of T2DM mice compared to controls at day 7 post-MI.

<table>
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<th>Diseases or Functions Annotation</th>
<th>Z-score</th>
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<tr>
<td>Cell Death</td>
<td>2.933</td>
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<tr>
<td>Necrosis</td>
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<td>2.026</td>
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<tr>
<td>Dilated Cardiomyopathy</td>
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<td>Myocardial Dysfunction</td>
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<tr>
<td>Congestive Heart Failure</td>
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<td>Increased</td>
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<tr>
<td>Muscle Contraction</td>
<td>-2.315</td>
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<tr>
<td>Heart Rate</td>
<td>-2.419</td>
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<tr>
<td>Metabolism of Nucleic Acid Component</td>
<td>-1.542</td>
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Table 3: Transcription factors and ligand-dependent nuclear receptors inhibited and activated in peri-infarct and infarcted LV of T2DM mice compared to controls at day 7 post-MI.

<table>
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<th>Transcription regulator</th>
<th>Activation z-score</th>
<th>p-value of overlap</th>
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<td>ESRRA</td>
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A positive z-score indicates activation, whereas a negative score indicates inhibition.
Supplemental Figure Legends

Supplemental Figure 1

Immunofluorescence double stainings for (A, C) cardiomyocyte and (B, D) Macrophage specific dsDNA sensors (Pyhin1, Ifi204) showing abundance of double positive cells (white arrows) in peri-infarct and infarct regions of LV in T2DM compared to controls at day 7 post-MI. (Scale bar, 20µm. SA = Sarcomeric actin, F4/80 = Macrophage marker)

Supplemental Figure 2

Proposed mechanism underlying accelerated post-MI HF in T2DM. Ischemic stress causes extensive mitochondrial damage in cardiomyocytes. The dysfunctional mitochondria need to be removed in order to prevent further damage to neighbouring cells as well as to prevent recruitment of inflammatory immune cells. Mitophagy is a selective form of autophagy, whereby dysfunctional mitochondria is enclosed in a double membrane vesicle (auto/mitophagosome) and subsequently fuse with lysosome to form auto/mitolysosome in which the sequestered mitochondria are degraded by the enzymes of lysosomes. Eventhough auto/mitophagosome assembly was undisturbed in LV of T2DM mice, but subsequent degradation by lysosomes were impaired as suggested by increased LC3-II/I ratio and p62 levels. An activation of cytoplasmic DNA sensors including Pyhin1, Ifi204 and inflammasome, namely AIM2 and NLRC4 and localization double stranded DNA to autolysosomes indicated an increased release of undegraded mtDNA into cytosol as a result of impaired autolysosomal degradation of dysfunctional mitochondria. Thus inflammasome activation as a consequence of defective mitophagy generates caspase-1, which in cardiomyocytes leads to cell death and converts pro-IL-18 to mature active form of IL-18 in macrophages. This vicious cycle leads to increased infarct area and subsequently cardiac dysfunction leading to heart failure.