**Supporting Information**

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**SI Materials and Methods**

**Mice.** Knockout mice for TNFR1 (1), CCR2 (2), and MyD88 (3), as well as mice expressing tamoxifen-inducible cardiac-specific Cre recombinase (4), have been described previously.

**Histology and Immunofluorescence Microscopy.** For paraffin sections, hearts were fixed in buffered formalin, dehydrated, embedded in paraffin, and cut in 3-μm sections on a rotary microtome (Thermo Scientific). Sections were deparaffinized and rehydrated, and then used for conventional stainings (H&E and picrosirius red). Where appropriate, sections were boiled in citrate buffer for 10 min before immune stainings. For cryosections, organs snap-frozen in liquid nitrogen were cut in 4-μm sections on a cryotome (Leica). Sections were warmed and immediately fixed with PBS solution/4% paraformaldehyde for 10 min. Before antibody stainings, cryosections and paraffin sections were blocked with 5% BSA in Tris-buffered saline (TBS) solution for 1 h at room temperature (RT). First antibody incubation was done at a dilution of 1:100 to 1:200 (antibody diluent; Dako) for 1 h at RT, with antibodies from BD (B220, CD4, CD8, CD11b, CD11c, CD19, CD45), ebioscience (F4/80), Sigma [sarcomeric actinin, FITC-labeled α-smooth muscle actin (SMA)], Abcam [human IκB kinase (IKK2), myosin heavy chain (MyHC)], and Santa Cruz Biotechnology (dystrophin). Anti-ISG15 rabbit [sarcomeric actinin, FITC-labeled smooth muscle actin (SMA)], Abcam [human IκB kinase (IKK2), myosin heavy chain (MyHC)], and Santa Cruz Biotechnology (dystrophin). Anti-ISG15 rabbit antibody was provided by Klaus-Peter Knobeloch (University of Freiburg, Freiburg, Germany). Secondary antibodies coupled with Alexa Fluor 488 or 596 were purchased from Invitrogen/Molecular Probes, and were incubated for 1 h at RT. Costaining was performed with DAPI for nuclear staining and fluorescence-conjugated wheat germ agglutinin (WGA; Molecular Probes) for visualization of cell boundaries. For detection of apoptosis, an in situ TUNEL detection kit (Roche) was used. Fluorescent samples were analyzed on an Axiosvert 200M microscope (Zeiss) equipped with a digital camera (AxioCam MR3; Zeiss) and Axiovision software. Other stainings were evaluated on a DMS500B microscope (Leica) equipped with a DFC420C camera (Leica).

**Protein Extracts and Western Blotting.** Tissue was snap-frozen in liquid nitrogen, pulverized, and resuspended in a buffer containing 4% SDS, 100 mM Tris-HCl, and protease/phosphatase inhibitors (Roche). Twenty to 40 μg of protein in buffer was separated on a 4%–12% gradient gel (Invitrogen). Proteins were transferred to a PVDF or nitrocellulose membrane with a semidry blotter (Bio-Rad). Membranes were blocked for 1 h (at RT) in TBS solution, 0.1% Tween-20 with 5% wt/vol nonfat dry milk, in TBS solution, 0.1% Tween-20 with 5% nonfat dry milk or 5% BSA, and then incubated with primary antibody overnight (6 °C) in TBS solution, 0.1% Tween-20 with 5% nonfat dry milk or 5% BSA, and then incubated with HRP-coupled secondary antibody for 1 h (at RT). After application of chemiluminescence reagent, membranes were exposed to X-ray films. The following primary antibodies were used: atrial natriuretic peptide (ANP; ABS4590; Chemicon), α-SMA (F3777; Sigma), cleaved caspase 3 Asp175 (9664; Cell Signaling), c-Rel (4774; Cell Signaling), densin (D8815; Sigma), GADPH (sc-25778; Santa Cruz Biotechnology), IκBα (sc-371G; Santa Cruz Biotechnology), human IκK2 (Y466; Abcam), IKKI/2 (sc-7607; Santa Cruz Biotechnology), ISG15 (sc-166712; Santa Cruz Biotechnology), LC3 (L7543; Sigma), NEMO (611306; BD), p50 (06–886; Upstate), p52 (DB036; Delta Bio labs), RelA (sc-372; Santa Cruz Biotechnology), and RelB (sc-226; Santa Cruz Biotechnology).

**Nuclear Extracts and Electrophoretic Mobility Shift Assay.** For nuclear extracts, tissue was homogenized in a Douncer in buffer A (10 mM Hepes, 1.5 mM MgCl2, 10 mM KCl, 1 mM DTT, 1 mM PMSF, protease inhibitors) and incubated for 10 min. Then, cells were lysed by aspirating 10 times through a 26-gauge needle. Nuclei were pelleted by centrifugation (2,300 × g, 10 min, 4 °C), washed with buffer A, and incubated for 1 h in buffer C (20 mM Hepes, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, protease inhibitors). Nuclear protein extracts (4 μg) were incubated for 30 min at RT in binding buffer [50 mM KCl, 20 mM Hepes, 1 mM EDTA, 4% Ficoll, and 1 mM DTT, supplemented with poly(dI:dC) and BSA], and radiolabeled dsDNA probe containing an Igκ enhancer consensus NF-κB site. The DNA–protein complexes formed were then separated on a native 4% polyacrylamide gel and visualized by autoradiography.

**Immune Complex Kinase Assay.** Ventricular tissue was snap-frozen in liquid nitrogen, pulverized, and lysed in a buffer containing 25 mM Tris-HCl, 150 mM NaCl, 25 mM sodium pyrophosphate, 2 mM EGTA, 2 mM EDTA, 10% glycerol, and 1% Triton X-100, supplemented with 1 mM PMSF, 1 mM DTT, and protease inhibitors. A total of 500 μg of protein lysates was used for immunoprecipitation of IKK complexes with α-NEMO antibody (sc-7330; Santa Cruz Biotechnology). The kinase reaction was performed in a buffer supplemented with 500 ng of recombinant GST-IκBα as substrate and in the presence of 10 μCi [32P]γ-ATP for 30 min at RT. The reaction was stopped by boiling in Laemmli buffer at 95 °C for 5 min. Immunoprecipitates were separated by SDS/PAGE and blotted on a PVDF membrane, which were exposed to films to detect radioactivity. Membranes were then incubated with specific antibodies for further analyses.

**Flow Cytometry.** FACS of digested whole hearts was performed on a FACSCanto II equipped with FACSDiva 6.2 software (BD). Antibodies were from ebioscience (MHC class II, CD11b, Gr-1), BD (CD45R/B220, CD11c), and BioLegend (CD45). DAPI staining (Roche) was used to exclude dead cells.

**Cardiac MRI.** Isoflurane-anesthetized mice were examined on a Pharmascan 7.0 T system (Bruker) equipped with a 300 mT/m gradient system, by using a custom-built circularly polarized birdcage resonator and the Early Access Package for self-gated cardiac imaging (Intragate; Bruker). Measurements were based on the gradient-echo method. Multiple contiguous short-axis slices consisting of six to eight slices were acquired for complete coverage of the left ventricle. MRI data were analyzed with Qmass digital imaging software (Medis).

**Adult Murine Cardiomyocyte Isolation.** Excised hearts were digested by retrograde Langendorff perfusion with a calcium-free buffer supplemented with 6.5 mg/mL Liberase DH (Roche) and 3.5 mg/mL trypsin (Sigma). Digestion was stopped with FCS, and the cell suspension was filtered through a 100-μm mesh and centrifuged. After step-by-step reintroduction of calcium, cells were seeded on laminin-coated six-well culture dishes at a density of 1 × 10⁵ cells per well. Stimulation with IFN-γ (Pestka Biomedical Laboratories) was done at 100 U/ mL for 14 h.

**RNA Extraction and Quantitative PCR.** Heart tissue was snap-frozen in liquid nitrogen, pulverized, and processed with the Qiagen RNeasy Fibrous Tissue kit. The Transcriptor High Fidelity cDNA Synthesis Kit (Roche) was used for cDNA synthesis. Quantitative...
PCR was done on a LightCycler 480 system, using the Universal Probe Library (Roche). Rpl13 was used as reference gene for relative quantification. Primer sequences are available on request.

**Gene Expression Profiling.** RNA quality was checked on the Agilent 2100 Bioanalyzer. Two hundred nanograms of total RNA was amplified and labeled with the Whole Transcript Sense Target Labeling Assay (Affymetrix) using the GeneChip protocol. Labeled samples were hybridized to Affymetrix GeneChip Mouse Gene 1.0 ST Array and further processed. Arrays were scanned with an Affymetrix GeneChip Scanner 3000 7G, and data were analyzed by the RMA algorithm using the Affymetrix Expression Console and the GeneSifter microarray data analysis system.

**Secretome Analysis.** Conditioned serum-free medium containing the secreted proteins was collected after 24 h on cardiomyocytes, filtered through 0.45-μm filters, and concentrated by using a 3,000-Da molecular mass cutoff spin column (Centriprep; Millipore). Protein samples were processed, and MS was performed as described (5) by using an LTQ-Orbitrap (ThermoFisher Scientific) combined with a Proxeon nanoflow HPLC system. The mass spectrometer was operated in the data-dependent mode to automatically measure full MS scans and MS/MS spectra. Peptides were identified by searching against the International Protein Index sequence database (mouse, version 3.54) using the Mascot search algorithm (www.matrixscience.com). Mass spectra were analyzed by MaxQuant software (version 14.10).


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**Fig. S1.** Expression of constitutively active IKK2 in the heart induces cardiomyopathy. (A) The tetO.IKK2-CA construct (Upper) enables expression of human IKK2-CA (IKK) and luciferase (Luc) under a bidirectional promoter (P). This promoter, containing a tetracycline response element (TRE), is activated upon binding of tetracycline transactivator (tTA) in the absence of doxycycline. tTA is expressed via the second construct (MyHC.tTA, Lower) under the control of the cardiomyocyte-specific α-MyHC promoter (P-MyHC). (B) Steadily increasing luciferase activity in IKK<sup>MyHC</sup> mice after doxycycline withdrawal, quantified by in vivo luciferase imaging and normalized to the value of IKK<sup>MyHC</sup> mice kept under doxycycline at time point zero (mean ± SD, n = 4 mice). (C) Immunofluorescent staining of different heart regions with an antibody against IKK and with DAPI. (Scale bar: 200 μm.) (D) Western blot of heart extracts from control and IKK<sup>MyHC</sup> animals to assess the expression levels of NF-κB subunits RelA, p50, RelB, p52, and c-Rel. Western blot against GAPDH shown as loading control. (E) Atrial thrombi in IKK<sup>MyHC</sup> hearts. (Scale bars: Top, 5 mm; Middle, 1 mm; Bottom, 100 μm.) (F) H&E stain of end-organ damage in liver (Upper) and kidney (Lower). (Scale bar: 200 μm.) Body (G) and liver weight (H) of IKK<sup>MyHC</sup> animals shown as a percentage of age-matched controls, weeks after doxycycline withdrawal (decompensated animals excluded), and during decompensated heart failure at any time point (HF; mean ± SD, n ≥ 4 animals per group). (I) Heart weight/body weight ratio in IKK<sup>MyHC</sup> and control mice weeks after doxycycline withdrawal (mean ± SD, n ≥ 4 animals per group).
Fig. S2. Expression of constitutively active IKK2 promotes inflammation and myocyte atrophy, but not cell death. (A) Immunofluorescent staining of ventricular heart cryosections from control and IKK<sup>MyHC</sup> mice for CD4 and CD8, mainly expressed on T lymphocytes. (Scale bar: 100 μm.) (B) FACS analysis shows an increase in cells of hematopoietic origin (CD45<sup>+</sup>) in IKK<sup>MyHC</sup> hearts (Left; mean ± SD, n = 3 mice per group). (C) IKK<sup>MyHC</sup> heart paraffin sections (Left) and cryosections (Middle, Right) were stained against IKK (red), and with WGA and DAPI as indicated. In addition, sections were stained against sarcomeric actinin (Middle) or against MyHC (Right) in the indicated colors. (Scale bars: 100 μm.) (D) TUNEL assay on control and IKK<sup>MyHC</sup> heart sections. DNase-treated heart section (Left) shown as a positive control. (Scale bars: 100 μm.) (E) Western blot for cleaved caspase 3 (apoptosis) and LC3 (autophagy) of IKK<sup>MyHC</sup> and control heart extracts, with GAPDH as loading control. (F) Immunofluorescence against IKK (red) and staining of cell boundaries with WGA (green) in heart tissue expressing dominant-negative IKK2 [IKK<sup>MyHC(DN)</sup>], showing that the phenotype does not occur in the absence of IKK kinase activity. (Scale bar: 100 μm.) (G) Western blot for IKK, ANP, and GAPDH (loading) of heart extracts from mice expressing a constitutively active (CA) or a dominant-negative (DN) version of IKK. ANP is up-regulated only in the presence of a functional kinase domain of IKK2.
Fig. S3. Functional and molecular reversibility of IKK-induced cardiomyopathy. (A) Representative scans of the heart of an IKK-MyHC animal at time points 0 (baseline) and 12 wk (diseased) after doxycycline removal, and 3 mo after doxycycline readministration (reversed) in the long axis (end-diastolic phase) and the short axis (end-diastolic and end-systolic phases). The dilation of the heart visible in the second MRI is rescued in part by administration of doxycycline (third MRI). Ao, aorta; LV, left ventricle; Pa, pulmonary artery; Pm, papillary muscle; RA, right atrium; RV, right ventricle; Sp, septum. (B) Example of reversal of late-stage IKK-induced heart failure with generalized edema by administration of doxycycline (Movie S2). (C) Heart weight as percentage of age-matched control mice 0 and 12 wk after doxycycline removal, and 3 mo after doxycycline readministration (reversed; mean ± SD, n ≥ 5 per group). (D) Staining for CD45 (infiltrating cells) and α-SMA (fetal reprogramming) on cryosections of control and IKK-MyHC hearts in the diseased and reversed states reveals normalization after transgene silencing. (Scale bar: 200 μm.)

Fig. S4. Ex vivo activation of transgene expression in isolated cardiomyocytes. Cardiomyocytes were isolated from mice kept under doxycycline (transgene off) and then cultivated without doxycycline (transgene on). (A) Extracts were prepared 0, 12, 24, 36, and 48 h after isolation and analyzed in a Western blot for IKK and GAPDH expression. (B) Quantitative PCR for the indicated NF-κB target genes in cardiomyocytes 48 h after isolation, shown as fold regulation vs. control cardiomyocytes (mean ± SD; n = 3 biological replicates for controls and n = 6 for transgenic; Mann–Whitney test).
Fig. S5. Chemokine, TNF-α, and MyD88 signaling in IKK-induced cardiomyopathy. (A) Quantitative PCR for mRNA transcripts encoding chemokines CCL2 (MCP-1, ligand of CCR2), CXCL10 (IP-10), and chemokine receptor CCR2 in ventricular tissue in the diseased state (12 wk after doxycycline withdrawal) and after disease reversal (3 mo after doxycycline readministration). Shown is the fold up-regulation vs. control animals at the time point “diseased” (mean ± SD, n ≥ 8 mice per group for diseased and n ≥ 5 mice per group for reversed state). (B) Quantitative PCR for TNF-α precursor mRNA in IKK<sup>MyHC</sup> and control heart tissue. Shown is the fold regulation vs. controls (mean ± SD, n ≥ 7 mice per group). (C) Analysis of heart weight in control and IKK<sup>MyHC</sup> animals in the presence or absence of CCR2, TNFR1, and MyD88. TNFR1-KO animals were analyzed at the time point of decompensated heart failure together with age-matched controls (n ≥ 3 per group for WT and n ≥ 6 per group for KO mice). CCR2 (n ≥ 4 animals per group) and MyD88 (n ≥ 7 animals per group) KO mice were analyzed 8 wk after doxycycline withdrawal (mean ± SD). (D) Western blot for IKK, ANP, and GAPDH (loading) of IKK<sup>MyHC</sup> and control heart extracts in the presence or absence of CCR2, TNFR1, and MyD88. (E) Immunofluorescence analysis for CD45<sup>+</sup> infiltrating cells (red) and cell boundaries (WGA or a-dystrophin as indicated, green) on heart sections of control and IKK<sup>MyHC</sup> mice, in the presence or absence of CCR2, TNFR1, and MyD88. (Scale bars: 100 μm.)
Fig. S6. Mechanisms, consequences and relevance of IKK-induced cardiomyopathy. (A) Quantitative PCR of mRNA isolated from heart tissue (acute disease state) of control and IKK<sup>MyHC</sup> animals in the absence (3M−) or presence (3M+) of IκBα superrepressor for the indicated transcripts (<i>n</i> ≥ 3 mice per group). (B) Quantitative PCR for ISG15 pathway components in IKK<sup>MyHC</sup> cardiomyocytes 48 h after isolation. Shown is the fold regulation vs. control cardiomyocytes (<i>n</i> = 3 mice for control and <i>n</i> = 6 mice for transgenic; <i>P</i> values determined with Mann–Whitney test). Usp18 was not detectable in controls (shown is expression relative to <i>Rpl13</i> mRNA). (C) Staining of heart cryosections against ISG15 (red), and with WGA (green) and DAPI (blue) for identification of cell boundaries and nuclei. 1, Atrophic cardiomyocytes; 2, hypertrophic cardiomyocytes; examples of infiltrating cells are marked with an asterisk to their left. (Scale bar: 100 μm.) (D) Western blot against ISG15, IKK, and GAPDH (loading) in control and IKK<sup>MyHC</sup> cardiomyocytes 72 h after doxycycline withdrawal, with an approximate molecular weight marker to the right (in kDa). (E) Western blot against ISG15 and GAPDH (loading) in heart extracts of control and IKK<sup>MyHC</sup> mice 12 wk after doxycycline withdrawal (transgene on, diseased) and 3 mo after doxycycline readministration (transgene off, reversed). (F) WT and cardiomyocytes with a deleted <i>Ikbcg</i> allele (NEMO<sup>ΔMyHC</sup>) were stimulated with IFN-β for 14 h and analyzed for ISG15 expression by Western blot. (G) Comparison of NF-κB activity [electrophoretic mobility shift assay (EMSA), Top] and ISGylation pattern (Middle) in heart extracts of IKK<sup>MyHC</sup> vs. CVB3-infected animals. GAPDH was used as loading control. Co, control animals.
Fig. S7. Reproducibility of IKK-induced cardiomyopathy in a second, independent transgenic mouse line. (A) Heart weight, body weight, and heart weight/body weight ratio of a second transgenic line (IKK\textsuperscript{MyHC} EE-7) in the diseased state. Mice were kept under doxycycline (0.1 g/L in drinking water) until the age of 6 wk, and analyzed 18 wk after doxycycline removal (mean ± SD, n = 9 animals per group). (B) Dilation of an IKK\textsuperscript{MyHC} EE-7 heart (H&E stain). (Scale bar: 200 μm.) (C) Western blots for IKK and ANP expression of heart extracts from control, IKK\textsuperscript{MyHC} EE-5 (used in all other experiments throughout the manuscript), and IKK\textsuperscript{MyHC} EE-7 hearts; GAPDH was used as loading control. (D) Immunofluorescence on cryosections against CD45 shows infiltration of an IKK\textsuperscript{MyHC} EE-7 heart with cells of hematopoietic origin. Staining against dystrophin (green) for staining of cell boundaries, and DAPI (blue) for nuclear staining. (Scale bar: 100 μm.)

Movie S1. Heart function in an IKK\textsuperscript{MyHC} animal before transgene activation. Orientation and labeling of anatomic structures is shown in Fig. S5A. Video was reconstructed from 10 individual midventricular MRI frames.

Movie S1
Movie S2. Heart function in the same IKK<sup>α<sup>MyhC</sup> animal as Movie S1 at 12 wk after transgene activation (i.e., diseased). Orientation and labeling of anatomic structures is shown in Fig. S5A. Video was reconstructed from 10 individual midventricular MRI frames.

Movie S3. Heart function in the same IKK<sup>α<sup>MyhC</sup> animal as Movie S1 at 3 mo after transgene silencing (i.e., reversed). Orientation and labeling of anatomic structures is shown in Fig. S5A. Video was reconstructed from 10 individual midventricular MRI frames.
Movie S4. IKK<sub>MyHC</sub> and control animal (littermate). The first part of the video shows the fully developed disease with dyspnea and generalized edema, and the second and the third part the obvious clinical improvement of the same IKK<sub>MyHC</sub> animal after the indicated time of doxycycline treatment. The control mouse is shown in only the first and second parts; the mouse in the third part is the IKK<sub>MyHC</sub> animal.

Table S1. Gene expression analysis of cardiomyocytes upon ex vivo activation of IKK2

Cardiomyocytes were isolated from mice kept under doxycycline (transgene off) and then cultivated without doxycycline (transgene on). RNA was prepared 48 h after cell isolation for gene expression analysis. Shown are genes with a differential regulation of more than twofold in both directions (>2, up (red); <0.5, down (green)) and a P value <0.05 (corrected according to Benjamini–Hochberg; n = 3 for control samples and n = 6 for transgenic samples).

Table S2. Secretome analysis by MS of cardiomyocytes upon ex vivo activation of IKK2

Cardiomyocytes were isolated from mice kept under doxycycline (transgene off) and then cultivated without doxycycline (transgene on). The medium was replaced 48 h after cell isolation; the supernatant was harvested for analysis by MS 24 h later. Two biological replicates for control and transgenic samples were analyzed. The column “ratio” gives the ratio between the geomeans of IKK<sub>MyHC</sub> to control samples, “+/ n.d.” means that the protein was only identified in the IKK<sub>MyHC</sub> samples, but not detectable in control samples. The column “peptides” gives the number of peptides identified in total or limited to unique peptides in the different samples. In total, 866 proteins could be identified in the supernatant of cardiomyocytes, of which 166 have been described as extracellular (Gene Ontology). Table is limited to extracellular proteins that were up-regulated at least twofold or were identified in both IKK<sub>MyHC</sub> but none of control samples.