Inflammation is a major factor in heart disease. IκB kinase (IKK) and its downstream target NF-κB are regulators of inflammation and are activated in cardiac disorders, but their precise contributions and targets are unclear. We analyzed IKK/NF-κB function in the heart by a gain-of-function approach, generating an inducible transgenic mouse model with cardiomyocyte-specific expression of constitutively active IKK2. In adult animals, IKK2 activation led to inflammatory dilated cardiomyopathy and heart failure. Transgenic hearts showed infiltration with CD11b+ cells, fibrosis, fetal reprogramming, and atrophy of myocytes with strong constitutively active IKK2 expression. Upon transgene inactivation, the disease was reversible even at an advanced stage. IKK-induced cardiomyopathy was dependent on NF-κB activation, as in vivo expression of IκBα superrepressor, an inhibitor of NF-κB, prevented the development of disease. Gene expression and proteomic analyses revealed enhanced expression of inflammatory cytokines, and an IFN type I signature with activation of the IFN-stimulated gene 15 (ISG15) pathway. In that respect, IKK-induced cardiomyopathy resembled Coxsackievirus-induced myocarditis, during which the NF-κB dimer is released and translocates to the nucleus, where it regulates the transcription of a diverse range of target genes.

Results

Expression of Constitutively Active IKK2 in the Heart Induces Cardiomyopathy. Activation of IKK/NF-κB in cardiomyocytes was achieved by crossing mice expressing the tetracycline transactivator (tTA) under the control of the α-myosin heavy chain (MyHC) promoter (17) with mice bearing a tTA-responsive constitutively active IKK2 (IKK2-CA) allele (18), thus generating double-transgenic IKKMYHC mice (Fig. S1A). The transgenic system was activated in adult animals by removing doxycycline from the drinking water (Fig. S1B). Expression of IKK2-CA was detected in all areas of the heart, but not in other organs (Fig. 1A and B and Fig. S1C). IKK2-specific kinase activity was strongly enhanced in the hearts of IKKMYHC mice and led to a strong increase in NF-κB–specific DNA-binding activity (Fig. 1C and D). Whereas the expression levels of most NF-κB subunits were unchanged, RelB expression was up-regulated (Fig. S1D).

Six to 8 wk after doxycycline discontinuation, IKKMYHC mice began to show signs of heart failure. Hearts were enlarged strongly, with dilation of ventricles and atria (Fig. 1E). In some
cases, intracardiac thrombi were present (Fig. S1E). Many mice experienced dyspnea, generalized edema, and end-organ damage secondary to heart failure (Fig. 1F and Fig. S1 F–H). A third of the animals died or were terminally ill within 12 wk, and two thirds within 20 wk, of doxycycline discontinuation (Fig. 1G). Of those animals that initially survived, the heart weight steadily increased, with IKK\textsuperscript{MYHC} hearts weighing ~60% more than control hearts 12 wk after doxycycline discontinuation (Fig. 1H) and the heart weight/body weight ratio increasing from 5.4 \times 10^{-3} to 8.0 \times 10^{-3} (Fig. S1J). The development of heart failure was reflected by an increasing expression of natriuretic peptides (Fig. 1I), markers also induced in heart failure in human patients.

**IKK\textsuperscript{MYHC} Hearts Show Myocyte Atrophy and Hypertrophy, Inflammation, and Fibrosis.** IKK\textsuperscript{MYHC} hearts showed a mixture of cardiomyocyte atrophy and hypertrophy and an inflammatory infiltrate (Fig. 2A). Staining for inflammatory cell markers CD45, CD11b, and F4/80 was strongly, for T lymphocyte markers CD4 and CD8, focally increased (Fig. 2B and Fig. S2A). Quantification by FACS analysis revealed that the number of hematopoietic (i.e., CD45\textsuperscript{+}) cells was increased more than 11-fold compared with controls, with more than 82% of infiltrating (i.e., CD45\textsuperscript{+}) cells expressing the myeloid marker CD11b (Fig. S2B). A small percentage of CD11b\textsuperscript{+} cells stained positive for dendritic (5%; CD11b\textsuperscript{+}/CD11c\textsuperscript{+}/MHC-II\textsuperscript{+}) and granulocyte cell markers (1%; CD11b\textsuperscript{+}/Gr1\textsuperscript{+}), suggesting that the large majority consisted of macrophages/monocytes. This impression was supported by the up-regulation of \textit{Emr1} (F/80) mRNA, a marker for macrophages (Fig. 2C), mRNA transcripts of intercellular adhesion molecule-1 (ICAM-1), a marker typically seen in inflammatory cardiomyopathy/myocarditis, were up-regulated as well (Fig. 2D). Moreover, IKK\textsuperscript{MYHC} hearts exhibited mild fibrosis (Fig. 2E).

Cardiomyocyte-Specific IKK/NF-\kappaB Activation Induces Fetal Reprogramming, Remodeling, and Myocyte Atrophy. Heart disease is often associated with the reexpression of fetal markers in the adult heart. Indeed, the expression of \(\alpha\)-smooth muscle actin, an isoform of actin not normally expressed in adult striated muscle, was increased (Fig. 3J). We also detected atrial natriuretic peptide (ANP) in ventricles, where it is normally absent in adult mice. Destrin, an actin-depolymerizing enzyme, was enhanced as well, suggesting ongoing remodeling (Fig. 3B). Between the two main isoforms of myosin heavy chain in mice, the \(\alpha\)-isoform (\(\alpha\)-MyHC) predominates in adult mice, whereas the \(\beta\)-isoform (\(\beta\)-MyHC) is strongly expressed in embryos. We observed a down-regulation of \(\alpha\)-MyHC transcripts, whereas \(\beta\)-MyHC transcripts were up-regulated, leading to a strong (16-fold) reduction in the \(\alpha/\beta\)-MyHC ratio (Fig. 3C).

IKK expression in individual cardiomyocytes was variable, and, curiously, cardiomyocyte caliber correlated inversely with transgene expression: cells with high IKK2-CA expression were atrophic, whereas the majority of low- or nonexpressing cells were normal or hypertrophic (Fig. 3D and Fig. S2C). Apoptosis and autophagy were not prominent (Fig. S2D and E). Thus, in addition to the induction of a general inflammatory state and fetal reprogramming, IKK/NF-\kappaB promoted cardiomyocyte atrophy, but not cell death. This atrophy-promoting function of IKK2 was dependent on its kinase activity (Fig. S2F and G).

**MRI Reveals Severely Compromised Heart Function in IKK\textsuperscript{MYHC} Mice.** For functional characterization, a cohort of mice was examined by cardiac MRI. In IKK\textsuperscript{MYHC} mice, global heart function was indeed severely compromised (Fig. 4, Fig. S3A, and Movies S1, S2, and S3). Both ventricles and atria were dilated, with an increase in end-diastolic and especially end-systolic volumes. Systolic heart failure was obvious, with an average left ventricular ejection fraction reduced from 59% to 16% and a decrease in stroke volume from 41 to 16 \muL.

IKK-Induced Cardiomyopathy Is Reversible. Surprisingly, when the transgene was shut off in mice with IKK-induced cardiomyopathy, the disease process was almost completely reversed, with full recovery of heart function (Fig. 4, Fig. S3A, and Movies S1, S2, and S3). As shown in Fig. S3B and Movie S4, mice with an
obvious clinical decompensation could also be treated successfully. The recovery did not only concern heart function, but also molecular parameters: markers of inflammation, fetal reprogramming, remodeling, and heart failure normalized almost completely (Fig. 5 and Fig. S3 C and D).

**Gene Expression Analysis of IKKMyHC Cardiomyocytes.** To gain insight into proximal events after activation of IKK2/NF-κB, we isolated cardiomyocytes and activated the transgene in vitro, thus avoiding interference of other cell types (Fig. S4 A and B). Gene expression analysis revealed up-regulation of transcripts of chemotactic cytokines, adhesion molecules, and a striking number of IFN-regulated genes in IKKMyHC cardiomyocytes (Table S1). In addition, apoptotic and antiapoptotic genes were up-regulated.

**Role of Chemokines, Cytokines, Adhesion Molecules, and MyD88 Signaling in IKK-Induced Cardiomyopathy.** The enhanced expression of chemokines by cardiomyocytes was validated by quantitative PCR (Fig. 6A). In a MS-based secretome analysis, the chemokines CCL7, CXCL10, and CX3CL1, as well as galectin-3–binding protein, a lectin mediating interaction with macrophages, were detected in the supernatant of IKKMyHC cells (Fig. 6B and Table S2). The enhanced expression of chemotactic factors and their receptors was also detected in vivo (Fig. S5 A). A cytokine that has been associated with cardiomyopathy in mice is TNF-α. We suspected that IKK/NF-κB might induce cardiomyopathy at least in part by enhanced expression of TNF-α (Fig. S5B). However, IKK-induced cardiomyopathy was not rescued in mice deficient in CCR2 or in TNFR1 (p55), a critical receptor for TNF-α (Fig. S5 C–E). Another important pathway in inflammatory cardiomyopathy signals via the adapter protein MyD88. IKK-induced cardiomyopathy was modulated by ablation of MyD88, with a significant reduction of heart weight in IKKMyHC/MyD88−/− animals (Fig. S5 C–E). However, these mice still progressed to heart failure.

**NF-κB is the Mediator of IKK-Induced Cardiomyopathy.** We next wanted to determine by which specific pathway activation of IKK2 induces cardiomyopathy in IKKMyHC mice. Expression of an IκBα superrepressor in the hearts of IKKMyHC animals completely abrogated myocarditis and cardiomyopathy, thus clearly establishing that IKK induces its effects via classical NF-κB signaling. Heart weight/body weight ratio, expression of ANP, amount of inflammatory cells in the heart, as well as chemokine, chemokine receptor, collagen, and Myh6 mRNA transcripts were completely normal in IKKMyHC mice with a blocked NF-κB pathway (Fig. 6 C–F and Fig. S6A).

**Fig. 2.** Cardiomyocyte-specific IKK/NF-κB activation induces inflammation and fibrosis. H&E stain (A) and immunofluorescent staining (B) of ventricular heart cryosections from control and IKKMyHC mice show infiltration with inflammatory cells. (Scale bar: 100 μm.) (C) Quantitative PCR for Emr1 f4/80 mRNA in ventricular tissue of IKKMyHC and control hearts as a marker for macrophage infiltration (fold up-regulation vs. control; n ≥ 8 mice per group). (D) Quantitative PCR for mRNAs encoding the inflammatory adhesion molecule ICAM-1 in ventricular tissue of IKKMyHC and control hearts (fold up-regulation vs. control; n ≥ 8 mice per group). (E) Sirius red stain on paraffin sections of ventricular tissue of control (Left) and IKKMyHC (Right) mice for the detection of fibrosis (counterstain, fast green). (Scale bar: 100 μm.)

**Fig. 3.** Cardiomyocyte-specific IKK/NF-κB activation induces fetal reprogramming and myocyte atrophy. (A) Heart cryosections of control and IKKMyHC animals were stained for α-smooth muscle actin (α-SMA) and dystrophin, and with DAPI. (Scale bars: 100 μm.) (B) Western blot of ventricular extracts of IKKMyHC and control mice (8 wk after doxycycline withdrawal) against ANP, α-SMA, desmin, and GAPDH (loading control). (C) Quantitative PCR for Myh6 and Myh7 transcripts encoding the α- and β-isoforms of MyHC (shown are fold regulation vs. age-matched control mice and calculated Myh6/Myh7 ratio; n ≥ 8 mice per group). (D) Control and IKKMyHC heart paraffin sections were stained against the transgene (human IKK2, red), and with wheat germ agglutinin (WGA, green) and DAPI (blue). (Scale bars: 100 μm.)
IKK Induces Strong IFN Response and Activation of ISG15 Pathway in an NF-κB–Dependent Manner. Among the IFN-regulated genes up-regulated upon IKK activation, we analyzed IFN-regulated gene 15 (Isg15) in more detail. Its gene product, ISG15, is a ubiquitin-like modifier, which can be covalently attached to a range of proteins, a process referred to as ISGylation. Isg15 transcripts, as well as those for its E3 ligase and the protease removing ISG15 from other proteins, were increased in cardiomyocytes expressing IKK2-CA (Fig. S6f). On IKKα heart sections, Isg15 was expressed in infiltrating cells and in cardiomyocytes (Fig. S6c). Immunoblot revealed widespread protein ISGylation in cardiomyocytes (Fig. S6 D and E), which was dependent on NF-κB (Fig. 6g). Vice versa, when cardiomyocytes deficient for NEMO (and thus, also classical NF-κB signaling) were stimulated with IFN-β, the induction of Isg15 was reduced significantly, but not abolished (Fig. S6f).

Significance of IKK/NF-κB and ISG15 Pathways in a Mouse Model of Viral Myocarditis. Finally, we wanted to examine whether molecular pathways active in our mouse model were relevant in an established model of virus-induced inflammatory heart disease. As NF-κB and ISG15 are central mediators of the defense against viruses, we analyzed their activation status in acute murine CVB3 myocarditis. In CVB3-infected mice, NF-κB–specific DNA binding was strongly activated in the heart (Fig. 6f and Fig. S6G). Expression of Isg15 protein was enhanced, with strong and widespread ISGylation of target proteins similar to that observed in IKK-induced cardiomyopathy (Fig. 6f and Fig. S6G). Thus, IKK-induced cardiomyopathy recapitulates molecular pathways relevant in other mouse models of cardiac disease.

Discussion

IKK/NF-κB is a master regulator of inflammation in many cell types and organs. We show here that, in the heart, activation of IKK/NF-κB leads to myocarditis and inflammatory dilated cardiomyopathy, which is associated with atrophy in IKK-expressing myocytes and a severe reduction in contractility.

IKK activation has been linked to inflammation in several organ systems, but does not necessarily lead to inflammation in all cell types and tissues: in skeletal muscle, no inflammation was observed upon IKK2 activation (19). In our cardiac model, IKK activation led to strong NF-κB activation and inflammation. Cardiomyopathy was mediated by NF-κB, as disease was prevented in mice expressing an IκBa superrepressor. We suspected that some of the inflammatory effects of NF-κB could be mediated by its target gene TNF-α, which by itself can induce cardiomyopathy (20). However, the phenotype could not be rescued by ablation of TNFR1, the receptor considered the mediator of the deleterious effects of TNF-α (21). Vice versa, NF-κB may still mediate some of the detrimental effects of TNF-α (22, 23). Importantly, KO of TNFR1 improves heart function after coronary artery ligation, which correlates with decreased NF-κB activation (22).

Other target genes of NF-κB encode chemokines, which also were up-regulated in our model. However, ablation of the chemokine receptor CCR2 did not modulate our phenotype, possibly because of the high redundancy of the chemokine receptor system.

IKK-induced cardiomyopathy displayed molecular features of viral myocarditis, an important cause for dilated cardiomyopathy. These comprised the transcriptional up-regulation of Toll-like receptors as well as the induction of IFN type I-dependent gene expression. Downstream of TLR signaling, the adapter protein MyD88 is critical in different types of inflammatory cardiomyopathy (24, 25). Ablation of MyD88 modulated our phenotype by attenuating the excessive gain in heart weight; however, it did not prevent heart failure.
IKK activation alone was sufficient to activate the ISG15 pathway, an important antiviral defense mechanism, and elicited widespread ISGylation of proteins in an NF-κB-dependent manner. This was also found in CVB3-induced myocarditis, which showed a strong activation of the NF-κB and ISG15 pathways. Vice versa, in the absence of the IKK subunit NEMO, the induction of ISG15 by IFN-β was attenuated. These results may hint at an important role of IKK/NF-κB signaling in orchestrating the antiviral response in the heart.

The question whether IKK/NF-κB is protective or damaging in heart disease has been a matter of ongoing debate (3). IKK/NF-κB can be protective by reducing oxidative stress (6, 7). Consistent with that, apoptosis was not prominent upon IKK/NF-κB activation in our model. However, our finding that IKK/NF-κB activation can lead to a dramatically impaired systolic function may be of interest for cardiac diseases with strong activation of IKK/NF-κB. In viral myocarditis, for example, myocyte damage and reduced contractility may not only result from direct viral action, but also from the host response to the virus, which involves IKK/NF-κB activation. Establishing a balance between desired protective functions and unwanted side effects may also apply to the IFN type I response. In a recent study on dermatomyositis, an autoimmune disorder involving skeletal muscle, the ISG15 pathway was associated with atrophy (26). In light of our results, further investigation into the link between type I IFN response and myocyte damage may prove fruitful.

Previous studies have established that NF-κB is required for cardiac hypertrophy, but is dispensable for normal heart structure and function (8, 10, 11, 27). Our finding that activation of IKK/NF-κB in the heart primarily induces cardiomyocyte atrophy rather than hypertrophy is not necessarily a contradiction to these studies, as the biological function of IKK/NF-κB may depend on the strength of activation and/or on context, and on the component of the IKK/NF-κB system that is manipulated. For example, IKK2 and NEMO KO mice showed an increase in oxidative stress and apoptosis when challenged by aortic banding, but hypertrophy was not inhibited (6, 7). Interestingly, an atrophy-promoting function of IKK/NF-κB is well established in skeletal muscle (19, 28).

For therapeutic interventions in heart disease, it is of interest that normal heart function and morphology were almost completely restored in IKK-induced cardiomyopathy by shutting off transgene expression. Obviously, the processes regulated by IKK/NF-κB are remarkably plastic. However, IKK/NF-κB signaling cannot be simply labeled protective or damaging, as it displays antiapoptotic and antioxidative functions as well as proinflammatory, atrophy-promoting effects in heart muscle, and can lead to a dramatic reduction in contractility. This complexity should be kept in mind when considering targeted pharmacological interventions involving IKK/NF-κB in heart disease.

Materials and Methods

Mice. Mice were kept under specific pathogen-free conditions. The tetO.IKK2-CA mouse line EE-5 has been described previously (18); the phenotype was
confirmed in a second mouse line (EE-7, Fig. 57). For most experiments, mice were administered doxycycline (1 g/L in drinking water) until adult age and analyzed at the indicated time point after doxycycline withdrawal. For acute activation of the system (designated as acute disease state), mice were kept under doxycycline (0.1 g/L) until the age of 4 wk, and analyzed after 12 d later.

In Vivo Bioluminescence Imaging. Mice were anesthetized by continuous inhalation of 2% (vol/vol) isoflurane. At 5 min after i.p. injection of 3.5 mg luciferin, bioluminescence was detected and quantified in an IVIS 200 system (Caliper).

Western Blotting. Tissue was snap-frozen in liquid nitrogen and lysed in a buffer containing 4% SDS. Lysates were separated by SDS/PAGE and blotted onto membranes, which were blocked, incubated in primary and HRP-coupled secondary antibodies, and developed with chemiluminescence.

Kinase Assay. A total of 500 μg of protein lysate was precipitated with NEMO antibody. The kinase reaction was performed in the presence of [γ-32P]-ATP and recombinant GST-IκBα as substrate, followed by boiling in Laemmli buffer and SDS/PAGE.

Electrophoretic Mobility Shift Assay. Nuclear extraction was done as described (5). For electrophoretic mobility shift assays (EMSA), 4 μg of nuclear extract was incubated with radiolabeled DNA probes containing a consensus κB or Oct-1 site and separated on a native polyacrylamide gel.

Histology and Immunofluorescence Microscopy. Cryosections and paraffin sections were prepared as described in SI Materials and Methods. First-antibody incubation was done overnight at 4 °C with a diluent (Dako). Secondary antibodies coupled with Alexa Fluor 488 or 596 were incubated for 1 h at room temperature.

Cardiac MRI. Isoflurane-anesthetized mice were examined on a Bruker Pharmascan 7.0 T equipped with a 300 mT/m gradient system. 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).

RNA Extraction and Quantitative PCR. RNA extraction and cDNA synthesis were done with kits (Qiagen and Roche). Quantitative PCR was done on a LightCycler 480 system with use of the Universal Probe Library (Roche). RPL37 was used as reference gene for relative quantification. Primer sequences are available on request.

Gene Expression Profiling. A total of 200 ng total RNA was amplified and labeled with the Whole Transcript Sense Target Labeling Assay (Affymetrix). Labeled samples were hybridized to Affymetrix GeneChip Mouse Gene 1.0 ST Array, processed, scanned, and analyzed (RMA algorithm with Affymetrix Expression Console and GeneSifter microarray data analysis system).

Secretome Analysis. Supernatant (serum-free medium) of cardiomyocytes was collected after 24 h, filtered, and concentrated. Protein samples were processed and mass spectrometry was performed as described in SI Materials and Methods by using an LTQ-Orbitrap (Thermo Fisher Scientific) combined with a Proxeon nanoflow HPLC system.

Statistical Analysis. Values are given as arithmetic mean ± SD. Means of two groups were compared by the Student t test or, when indicated, the Mann-Whitney test. Welch correction was applied for unequal variances. Means of multiple groups were compared by ANOVA. All tests were two-tailed. P values <0.05 were deemed significant. Detailed procedures are described in SI Materials and Methods.

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