Supplementary Data

Methods

In vivo *I/R protocol*

Male C57Bl/6 mice (8–12 weeks of age) were obtained from the University Medical Centre (CMU) animal facility, Faculty of Medicine, University of Geneva. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and has been approved by the local and ethics authorities (Geneva Veterinary Office and the Ethic Commission of Animal Experimentation of the University of Geneva). Mice were initially anesthetized with 4% isoflurane and intubated. After starting mechanical ventilation (tidal volume of 150 ml, 120 breaths/min) by supplementation with 100% oxygen, anesthesia was maintained with 2% isoflurane. During surgery, adequacy of anesthesia was monitored by careful visual and tactile control of mouse consciousness (changes in breathing rate and volume, heart rate, sweating, and tearing). A thoracotomy was performed in the left third intercostal space and the pericardium was removed. Ligature of the left anterior coronary artery at the inferior edge of the left atrium was performed using an 8-0 Prolene suture. A small piece of polyethylene tube was used to secure the ligature without damaging the artery (13). After 5 min of ischemia and 12 h after reperfusion onset, FK866 (30 mg/kg, provided by the NIMH Chemical Synthesis and Drug Supply Program) or control vehicle (0.1% DMSO in PBS) were administered by intraperitoneal injections. The dose and the treatment schedule were planned on the basis of recently published experiments in mice and *in vitro* (2, 19) and *in vivo* results on systemic NAD+ depletion in FK866-treated mice (Supplementary Table S1).

After 30 min of ischemia, the occlusion of the left anterior coronary artery occlusion was released and reperfusion occurred. Analgesia was extended with subcutaneous injection of 0.05 mg/kg buprenorphine HCl every 12 h until sacrifice. Reperfusion was confirmed by visible restoration of color to the ischemic tissue. Then, the chest was closed and the ventilator removed to restore normal respiration. Sham-operated animals were submitted to the same surgical protocol as described but without arterial occlusion.

At different reperfusion time points, animals were anesthetized with 4% isoflurane and sacrificed (by intraperitoneal injection of ketamine-xylazine [4 mg/0.2%]) for infarct size determination and immunohistochemical analysis. Given potential circadian oscillation of Nampt and NAD+ previously reported (14), all mice were operated and sacrificed from 8.00 AM to 9.00 PM.

Ex vivo *I/R protocol*

The technique of Langendorff isolated buffer-perfused mouse heart preparation was used (13). Animals were anesthetized with 4% isoflurane and sacrificed by neck dislocation. The heart was rapidly excised and placed in ice-cold Krebs-Henseleit bicarbonate (KHB) buffer consisting of (mmol/L): 118.5 NaCl, 25 NaHCO3, 4.7 KCl, 1.2 MgSO4, 1.2 KH2PO4, 2.5 CaCl2, 5 glucose, and equilibrated with 95% O2/5% CO2 (pH 7.4). The extraneous tissues (pericardium, lung, trachea, etc) were removed. The aorta was cannulated with an 18-G plastic cannula (1.5 cm length; 0.95 mm, inner diameter) for a Langendorff retrograde perfusion at constant pressure and in a system with recirculation perfusion fluid. After stabilization, ligature of the left anterior coronary artery at the inferior edge of the left atrium was performed using an 8-0 Prolene suture. A small piece of polyethylene tubing was used to secure the ligature without damaging the artery and ventricle tissue. After 5 min ischemia, FK866 (0.375 mg/ml) or vehicle (0.05% DMSO in the perfusion buffer) were added within the circulating Langendorff system until the end of the

Supplementary Table S1. Time Course of Nicotinamide Adenine Dinucleotide + Depletion in White Blood Cells, Spleen, Heart, and Abdominal Aorta

Time of administration (hours)	*Vehicle-treated mice* (n = 6)	*FK866-treated mice* (n = 6)	P *value*
White blood cells (nmol/ml)			
1	12.51 ± 0.87	11.17 ± 0.88	0.3605
4	12.11 ± 3.07	12.19 ± 3.69	0.9882
8	11.93 ± 1.23	6.43 ± 0.56	0.0161
Spleen (nmol/g)			
1	69.02 ± 1.72	75.29 ± 10.37	0.5643
4	77.04 ± 2.65	39.34 ± 3.03	0.0001
8	75.45 ± 4.56	19.06 ± 2.46	0.0001
Heart (nmol/g)			
1	635.25 ± 35.26	713.63 ± 23.02	0.0403
4	622.24 ± 15.89	553.86 ± 13.79	0.0087
8	638.62 ± 18.22	587.80 ± 5.32	0.0373
Abdominal aorta (nmol/g)			
1	87.07 ± 9.95	115.75 ± 2.00	0.0522
4	89.62 ± 7.27	58.02 ± 4.53	0.0041
8	88.33 ± 2.69	59.05 ± 3.26	0.0001

In adult male c57b1/6 mice after single i.p. injection of FK866 (30 mg/kg) as compared to vehicle.
Data are expressed as mean ± SEM of nmol/ml of white blood cell suspension or nmol/g of total tissue.

Supplementary Table S2. NAD+ and ATP Production at 24 H of Reperfusion in Infarcted Hearts

Nampt pathway	*Treatments*		P *value*
	Vehicle (n = *4*)	*FK866* (n = *4*)	
NAD^+	171.8 ± 4.6	103.9 ± 17.6	< 0.05
ATP	149.6 ± 28.3	354.8 ± 11.1	< 0.005

Data are expressed as mean ± SEM of nmol/g of myocardial tissue.
ATP, adenosine triphosphate; NAD^+, nicotinamide adenine dinucleotide.

experiment (for additional 25 min of ischemia and 2 h of reperfusion). The concentration of FK866 was calculated on the basis of previous studies (2), averages of mouse blood volume (about 2 ml) and weight (25 grams) (18) and the dose (30 mg/kg) used in the *in vivo* I/R protocol. After 30 min of ischemia, the polyethylene tube was removed and reperfusion was allowed for 2h in a retrograde perfusion system without circulating leukocytes.

Area at risk and infarct size assessment

To assess area at risk (AAR) and infarct size (I) in *in vivo* I/R protocol, mice were anesthetized and sacrificed after 24 h of reperfusion, as previously described (13). The left anterior coronary artery was re-occluded and Evan's blue dye (2%; Sigma) was injected retro-orbitally to delineate the *in vivo* AAR. The heart was rapidly excised and rinsed in NaCl 0.9%. In the *ex vivo* I/R protocol, after 2 h of reperfusion, the suture was re-occluded and Evan's blue dye 2% was injected into the aortic cannula to delineate AAR. Then, hearts from both assays were frozen and sectioned into 2 mm transverse sections from the apex to the base (5–6 slices/heart). The sections were, incubated at 37°C with 1% triphenyltetrazolium chloride (TTC) in phosphate buffer (pH 7.4) for 15 min, fixed in 10% formaldehyde solution, and photographed with a digital camera (Nikon Coolpix) to distinguish continuously perfused tissue (blue), stained ischemic viable tissue (red), and unstained necrotic tissue (white). The different zones were determined using MetaMorph software (version 6.0, Universal Imaging Corporation). AAR and left ventricular infarct zone (I) were expressed as percentage of ventricle surface (AAR/V) and AAR (I/AAR), respectively.

Supplementary Table S3. Intracellular NAD^+, NADH, $NADP^+$, NADPH Levels at 6 H of Incubation in Human Primary Neutrophils

Nampt pathway	*Treatments*		P *value*
	CTL medium (n = *6*)	*FK866* (n = *6*)	
NAD^+	12.70 ± 0.83	3.29 ± 0.21	< 0.0001
NADH	0.96 ± 0.13	0.42 ± 0.09	< 0.0001
$NADP^+$	0.18 ± 0.02	0.12 ± 0.02	< 0.001
NADPH	1.54 ± 0.28	1.05 ± 0.17	< 0.005

Data are expressed as mean ± SEM of nmol/mg of total intracellular proteins.
NAD^+, nicotinamide adenine dinucleotide; $NADP^+$, nicotinamide adenine dinucleotide phosphate.

Supplementary Table S4. Effects of Pre-incubation with FK866 on TNF-α-Induced Superoxide Anion (O_2^-) Production in Human Neutrophils Adherent to Fibronectin

Stimulation	O_2^- *production (n*M *per 5 × 104 cells)*
CTL medium	0.43 ± 0.20
TNF-α (10 ng/ml)	3.93 ± 0.62*
FK866 (100 n*M*)	0.77 ± 0.18
FFK866 (100 n*M*) + TNF-α (10 ng/ml)	3.90 ± 0.84**,†

Data are expressed as mean ± SEM ($n = 3$).
*$p < 0.01$ *vs.* control (CTL) medium.
**$p < 0.05$ *vs.* FK866.
†not significant *vs.* TNF-α.

Justification of the choice of methods to assess the cardiac function

Area at risk (AAR) is defined as the ischemic myocardial region at risk of ischemic death. In both *ex vivo* (Langendorff) and *in vivo* I/R assays, the similar ratio of the AAR on total heart surface (V) in FK866-treated and control vehicle-treated hearts indicates that the ligature of the left anterior coronary artery was performed at the same level in both groups. This represents a good quality control test, directly suggesting that we performed similar heart ischemia in both treatment groups. Therefore, the data on AAR confirm that the reduction in the necrotic zone (I) and in troponin levels observed in the FK866-treated animals were not biased by a reduction in the ischemic zone (AAR), but were due to the activity of the drug.

We also considered measuring cardiac function as a clinical post-infarction endpoint. However, in our hands, 30 min ischemia followed by reperfusion (as applied in the present study) failed to substantially affect left ventricular function as detected with a follow-up of up to 21 days (unpublished observations). In order to obtain a modest reduction in left ventricular ejection fraction (EF%) in infarcted mice, we have to apply a permanent left coronary ligation or a prolonged ischemia (at least 60 min) (4, 12). Thus, given this feature of

Supplementary Table S5. Effects of FK866 on Mouse Neutrophil Migration *in Vivo*

Polycarbonate assay (C.I.)			
	Upper well		
		FK866 (nM)	
Lower well	*CTL*	*10*	*100*
CTL	1.00 ± 0.00	1.07 ± 0.15	1.09 ± 0.17
CXCL1 (200 ng/ml)	3.88 ± 0.90*	3.70 ± 1.07#	3.34 ± 0.69#
CXCL2 (200 ng/ml)	4.59 ± 0.39**	4.36 ± 0.59##	4.29 ± 0.47##

Data are expressed as mean ± SEM ($n = 4$).
*$p < 0.05$ *vs.* control medium (CTL)-stimulated cell migration to CTL.
**$p < 0.01$ *vs.* CTL-stimulated cell migration to CTL.
#Not significant (N.S.) *vs.* CTL-stimulated cell migration to CXCL1.
##N.S. *vs.* CTL-stimulated cell migration to CXCL2.

Supplementary Table S6. Mouse Primers and Probes Used for Real-Time PCR

Gene	*Function*	*Nucleotide sequence*	*Size (bp)*	*Accession number*
Hprt	Fw	5′-GACCGGTCCCGTCATGC-3′		
	Rv	5′-TCATAACCTGGTTCATCATCGC-3′	66	NM_013556
	probe	5′-*FAM*-ACCCGCAGTCCCAGCGTCGTC-*TAMRA-3′*		
Elane	Fw	5′-ATCTGCTTCGGGGACTCTG-3′		
	Rv	5′-CGAAGGCATCTGGGTACAA-3′	109	NM_015779
	probe	5′-*FAM*-ATCCGAGGAGGCTGTGGATCTGG-*BHQ-3′*		

our experimental model, we did not assess left ventricular function as a clinical endpoint in the present study.

Determination of mouse heart content of Nampt and myeloperoxidase

After 24 h of reperfusion, cardiac Nampt and MPO levels were determined in sham-operated and infarcted mice using enzyme-linked immunosorbent assay (ELISA) kits (respectively from CirculexTM [CycLex Co., Nagano, Japan] for Nampt and from Hycult biotech Inc [Plymouth Meeting, PA] for MPO). Hearts were lysed in 1.5 ml of a buffer containing 200 m*M* NaCl, 5 m*M* EDTA, 10 m*M* Tris, 10% glycerin, 1 m*M* PMSF, 1 mg/ml leupeptin, and 28 mg/ml aprotinin (pH 7.4). After two centrifugations (1500 *g* at 4°C for 15 min), supernatants were collected and ELISA were performed following manufacturers' instructions. The limit of detection was 0.75 ng/ml for Nampt and 1.6 ng/ml for MPO. Mean intra- and inter-assay coefficients of variation (CV) were below 6% for both mediators.

Immunostaining

Hearts from animals sacrificed after 1 h, 12 h, or 24 h of reperfusion were frozen in OCT and cut serially from the occlusion locus to the apex in 7 mm sections. Immunostainings for neutrophils (anti-mouse Ly-6B.2 antibody, ABD Serotec), macrophages (anti-mouse CD68, ABD Serotec), and T lymphocytes (anti-mouse CD3 antibody, ABD Serotec) were performed on five midventricular cardiac sections per animal, as previously described (13). Quantifications were performed with MetaMorph software. Results were expressed as number of infiltrating cells on mm^2 of total heart surface area.

Apoptotic cell measurement within infarcted hearts

As ROS are known to mediate apoptosis in the reperfused myocardium (22), we also evaluated apoptosis in ischemic after 24 h of reperfusion. We performed staining of frozen sections with the Dead End™ colorimetric terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) system (Promega, Madison, WI) using diaminobenzidine as the chromogenic substrate (according to the manufacturer's instructions). Results were expressed as percentages of stained area on total heart surface area.

Oxidative stress determination

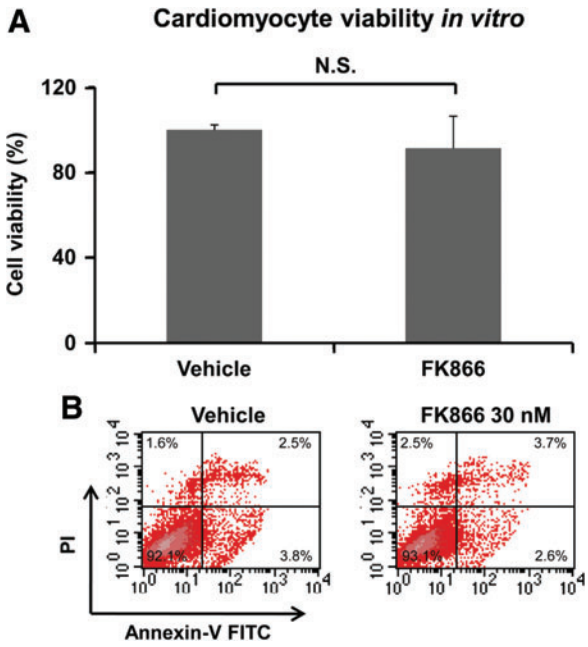
Measurement of superoxide in myocardium submitted to I/R was performed using the superoxide-sensitive dye dihydroethidium (DHE, Molecular Probes). Intracellular oxidative factors oxidize nonfluorescent DHE to fluorescent ethidium and 2-hydroxyethidium. The formation of ethidium and 2-hydroxyethidium, DNA intercalators, can be monitored by measuring its accumulation within the cell nucleus (13). Five frozen midventricular cardiac sections per animal (euthanized after 1 h, 12 h, or 24 h of reperfusion) were stained with 10 μM DHE at 37°C for 30 min in a light-protected and humidified chamber. Then, nuclei were stained with 4′,6-diamidino-2- phenylindole (DAPI). *In situ* fluorescence was assessed using fluorescence microscopy and quantification performed with MetaMorph software. The production of ROS has been assessed by a second histological method assessing their highly toxic product of lipid membrane peroxidation 4-hydroxy-2-nonenal (mouse anti-4- HNE monoclonal antibody at 1 mg/ml, Oxis International Inc, Foster City, CA) (9). Consecutive sections from the same hearts were also immunostained for 3,5- dibromotyrosine (mouse anti-Di bromo tyrosine monoclonal antibody at 10 mg/ml, AMS biotechnology, LTD, Abingdon, UK) Staining within the hearts of these oxidative mediators was performed as previously described (1, 6, 9). To avoid any potential cross-reactivity with mouse heart antigens and to increase the specificity of the primary antibodies, the VECTOR M.O.M Immunodetection kit and the VECTOR VIP substrate kit for peroxidase (Vector Laboratories, Inc. Burlingame, CA) were used, following the manufacturer's instructions. Quantification was performed with MetaMorph software. Results were expressed as percentages of stained area on total heart surface area.

Neutrophil (phagocytic oxidase) versus *cardiomyocyte (mitochondria)-driven ROS formation*

Both neutrophils and ischemic cardiac myocytes have been proposed to be responsible for the release of oxidants within the ischemic hearts (3, 21). Thus, both cell types are expected

Supplementary Table S7. Human Primers Used for Real-Time PCR

Gene	*Function*	*Nucleotide sequence*	*Size (bp)*	*Accession number*
IL8	Fw	5′-TGCTAAAGAACTTAGATGTCAGTGCA-3′		
	Rv	5′-AGTGATTGAGAGTGGACCACACT-3′	98	NM_000584
RN18S1	Fw	5′-GTAACCCGTTGAACCCCATT-3′		
	Rv	5′-CCATCCAATCGGTAGTAGCG-3′	153	NM_003286



SUPPLEMENTARY FIG. S1. Effects of FK866 treatment on rat H2c9 cardiomyocyte viability *in vitro*. (A) Analysis of cell viability rate (sulforodhamine B assay) in cultured cardiomyocytes in the presence of vehicle (1% DMSO) or FK866 (30 m*M*) for 48 h, mean ± SD ($n = 9$ per group); p: N.S. (not significant). **(B)** Representative panel of flow cytometric analysis of annexin V/PI staining of vehicle- or FK866-treated cells at 48 h of incubation.

to participate in the release of these mediators of cardiac injury. To specifically focus on the role of neutrophils, in Supplementary Figure S3, we show a nice co-localization between neutrophils and oxidants in myocardial ischemic areas at 24 h of reperfusion. We believe that together with the positive and significant correlations observed between number of infiltrated neutrophils and amount of oxidants, we might suggest that neutrophils represent a major source of oxidants within the infarcted heart.

However, since oxidant formation was also localized in neutrophil-negative areas (Supplementary Fig. S3), we cannot exclude that ROS might be also released by ischemic cardiomyocytes (9).

Determination of the intracellular NAD+ and ATP levels in mouse hearts

Mouse hearts were flash frozen in liquid nitrogen. Subsequently, hearts were disrupted using a mortar and pestle and immediately lysed with 0.6 *M* perchloric acid (PCA). Samples were neutralized, and NAD+ content was assessed with a sensitive enzyme cyclic assay, which exploits the use of alcohol dehydrogenase to determine NAD+ levels (5). To evaluate the content of ATP, cells were lysed in PCA and the neutralized extracts were analyzed by HPLC. NAD+ and ATP values were normalized to tissue total weight.

Serum Nampt, cardiac troponin I, and chemoattractant level detection

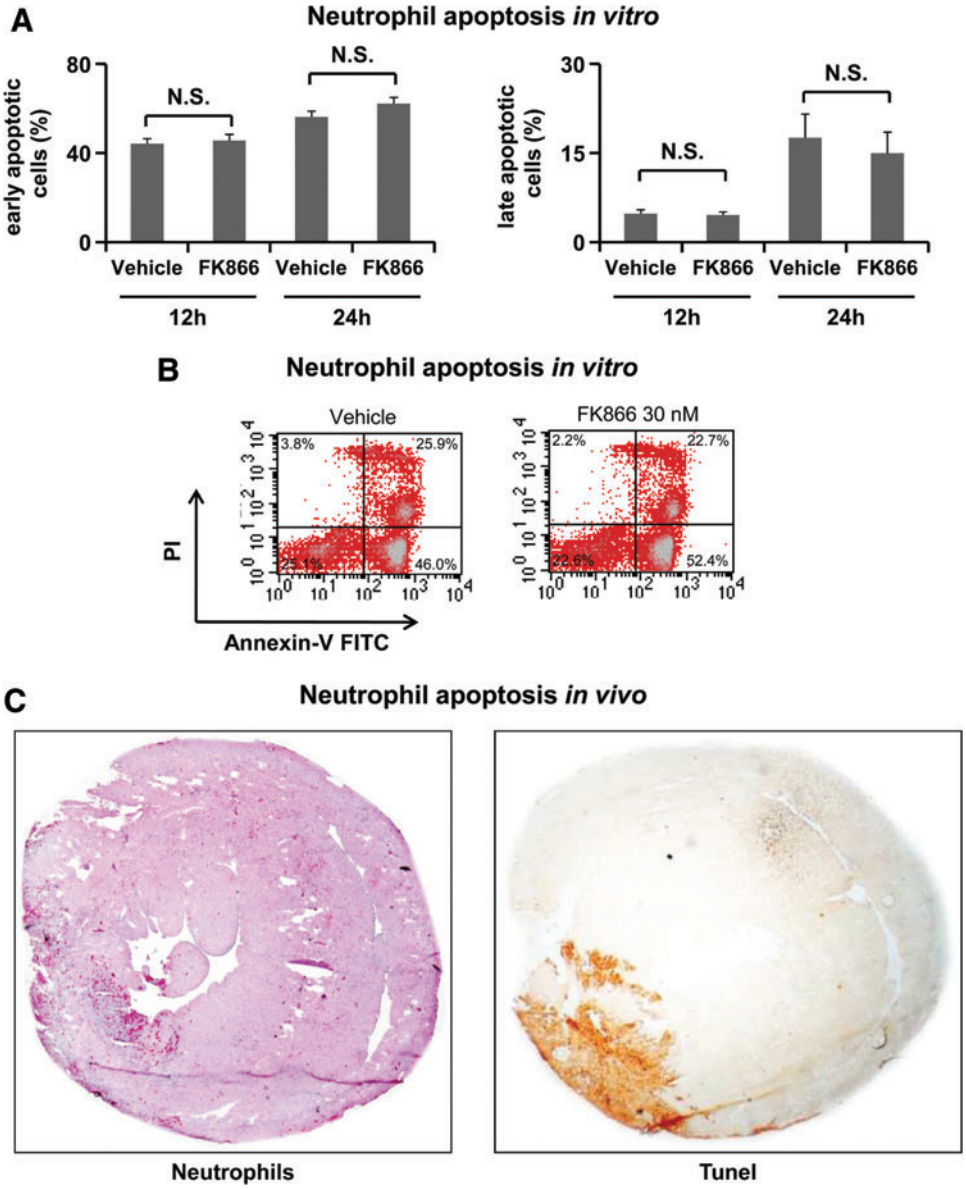
Circulating cardiac troponin I (cTnI) levels in mouse sera after 1 h, 8 h, and 24 h of reperfusion were measured using a high sensitive ELISA kit (Life Diagnostics Inc.). Serum Nampt, CXCL1, CXCL2, and CCL2 were measured in mouse sera after 1 h, 12 h, and 24 h of reperfusion by colorimetric ELISA (respectively, from CirculexTM for Nampt, and from R&D Systems [Minneapolis, Minnesota, USA] for chemokines). All kits were used by following manufacturer's instructions. The limit of detection for cardiac troponin I was 0.156 ng/ml, 0.75 ng/ml for Nampt, 15.6 pg/ml for CXCL1, 7.8 pg/ml for CXCL2, and 7.8 pg/ml for CCL2. Mean intra- and inter-assay coefficients of variation (CV) were below 6% for cardiac troponin I, Nampt, and all chemokines.

Mouse peritoneal neutrophil isolation and migration assay

Mouse neutrophils were obtained as previously described (7, 13). Normal male C57Bl/6 mice without surgery (8–12 weeks of age) were used. The peritoneal lavage fluids from three different mice were pooled for each migration assay after animal sacrifice. Then, neutrophils were resuspended at a density of 106 cells/ml in HBSS containing 1 m*M* CaCl2, 2 m*M* MgCl2 (ICN Biomed), and 0.2% BSA (Sigma Chimica) (mouse chemotaxis medium). Mouse neutrophil chemotaxis was assessed in a 48-well microchemotaxis chamber using a 5 μm pore size, 5 μm thick polyvinylpyrrolidone-free filter (Neuro Probe). 4×105 neutrophils were seeded in the upper wells, while control medium or recombinant CXC chemokines were added to the lower wells. To assess *in vitro* the potential direct effects of mouse serum (at 12 h of reperfusion) from FK866-treated ($n = 4$) or vehicle-treated mice ($n = 4$) on neutrophil migration, cells were also resuspended in different dilutions (respectively 1:1, 1:2, or 1:4) of mouse serum that was added with cells in the upper wells. To assess the direct effect of FK866 on neutrophil migration, cells were pre-incubated 1 h before the test in the presence or absence of FK866 (at 10 or 100 n*M*). Then, without washing, neutrophil migration towards control medium (CTL), 200 ng/ml mouse recombinant CXCL1 (R&D Systems), or 200 ng/ml mouse recombinant CXCL2 (R&D Systems) was assessed. The concentrations of recombinant chemokines were selected on the basis of previous investigations and were comparable to that observed in inflammatory tissues (10, 20). After incubation (60 min, 37°C), the filters were removed from the chambers, washed and stained with Diff-Quick (Baxter, Rome, Italy). The cells of five random oil immersion fields were counted and the chemotaxis index (C.I.) was calculated by dividing the number of cells migrated towards chemoattractants through the number of cells migrated to chemotaxis medium alone.

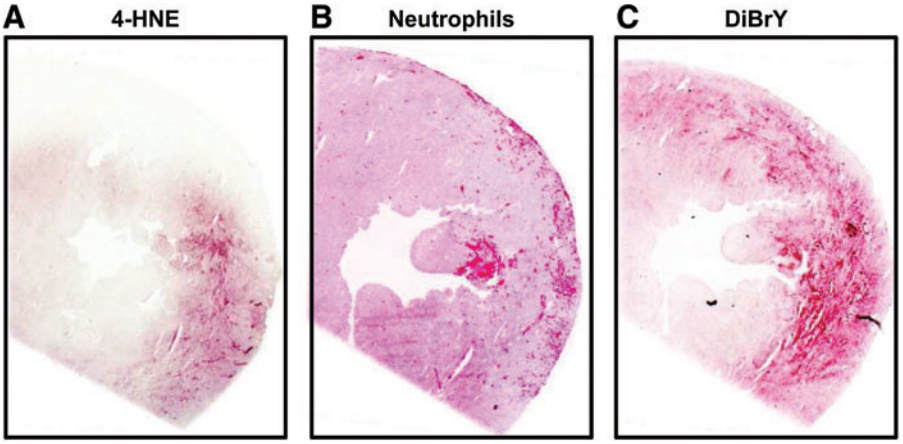
Isolation, culture, and NAD+/NADP+ determination in human primary neutrophils

Neutrophils were obtained from healthy volunteers after signature of an informed consent. The local ethical committee approved the investigation protocol, which conformed to the principles outlined in the Declaration of Helsinki. Human neutrophils were isolated by heparinized venous blood by dextran sedimentation followed by centrifugation on Ficoll–Hypaque density gradient, as previously described (15). Then, cells were resuspended in culture medium (RPMI 1640) and incubated for 6 h at 37°C in the presence or the absence of 100 n*M* FK688. In selective experiments, at the end of the



SUPPLEMENTARY FIG. S2. Effects of FK866 treatment on neutrophil apoptosis *in vitro* and *in vivo*. (A) Quantification of apoptosis in cultured human neutrophils in the presence of vehicle (1% DMSO) or FK866 (30 n*M*) for 12 h or 24 h. Early apoptotic (annexin V+/PI−) and late apoptotic (annexin V+/PI−) cells were enumerated by flow cytometric analysis. Data are expressed as mean ± SEM ($n = 3$ per group); *p*: N.S. (not significant). **(B)** Representative panel of flow cytometric analysis of annexin V/PI staining of vehicle- or FK866-treated cells at 24 h of incubation. **(C)** Representative microphotographs of consecutive cryosections from hearts of FK866-treated mice stained for, respectively, neutrophils (Ly-6B.2+ cells) and tunel (apoptotic cells).

incubation, cells were harvested and lysed with 0.6 *M* perchloric acid (PCA) or 0.1 *M* NaOH, to determine the content of NAD+ or NADH, and NADP+ or NADPH, respectively. The alkaline extracts were incubated at 70°C for 10 min. Both acid and alkaline extracts were neutralized and the intracellular content of the various coenzymes was assessed with a sensitive enzyme cyclic assay, which exploits the use of alcohol dehydrogenase or of G6PD, to determine NAD(H), or NADP(H), respectively. NAD(H) and NADP(H) values were normalized to protein concentrations, determined on an aliquot of the incubation by Bradford's method. In other experiments, tumor necrosis factor (TNF)-α-induced superoxide anion (O_2 -) production from neutrophils (pre-incubated in the presence or absence of 100 n*M* FK866 for 6 h) was measured by superoxide dismutase (SOD)-inhibitable cytochrome c reduction in microplate reader (Titertek Twinreader Plus; Flow Labs, Irvine,UK), as previously described (16). The assays were carried out in 96-well flat-bottomed polystyrene plates (Primaria plates; Falcon, Becton Dickinson, Oxnard, CA). The wells were pre-treated with 50 μl fibronectin (1 μg/well, from Sigma Aldrich) in 5% CO_2 at 37°C for 2 h. Then, after washing, incubation medium with 15 nmol cytochrome c was added to each well and the temperature was brought to 37°C in the microplate reader. Then, 5 × 104 cells were added, followed by control medium alone or TNF-α (10 ng/ml). TNF-α was used to better mimic post-infarction inflammation (characterized by increased circulating levels of TNF-α) (13). Experiments were carried out in triplicate, in the presence or absence of SOD (Sigma Aldrich). The amounts of O_2- produced by neutrophils were determined from the OD_{550} of samples without SOD minus the OD_{550} of matched samples with SOD, using an extinction coefficient of 9.5 m*M*, previously calculated by Leslie and co-workers (8). In all experiments, cell viability was superior to 85% (assessed by flow cytometry [FACS Calibur, Becton Dickinson, BD Italia, Milan, Italy], using propidium iodide [PI] staining). In additional experiments, the effects of FK866 on neutrophil apoptosis were also assessed after 24 h of incubation at 37°C (Supplementary Fig. S2A).



SUPPLEMENTARY FIG. S3. Co-localization between neutrophil infiltration and oxidant production within infarcted hearts at 24 h of reperfusion. Representative microphotographs of consecutive cryosections from hearts of FK866-treated mice stained for, respectively, 4-hydroxy-2-nonenal [4-HNE, **(A)**], dibromotyrosine [DiBrY, **(B)**], and neutrophils (Ly-6B.2+ cells, **(C)**].

Human peripheral blood mononuclear cell isolation and culture

PBMCs (>75% CD3+ lymphocytes) were isolated from blood samples obtained from healthy donors by Ficoll-Hypaque density gradient centrifugation. The local ethical committee approved the investigation protocol, which conformed to the principles outlined in the Declaration of Helsinki. Then, cells were resuspended in culture medium (RPMI 1640 plus FBS 10%, penicillin [10,000 U/ml] and streptomycin [10,000 μg/ml]) for the stimulation. 1.5×106 human PBMCs/well were incubated in 24-well plates in the presence or absence of 30 n*M* FK866. After 24 h, 5 μg/ml phytohemagglutinin-P (PHA), 25 ng/ml phorbol myristate acetate (PMA)/0.5 μ*M* ionomycin (I), 10 m*M* nicotinamide (Nam), or 50 μ*M* sirtinol (Sirt, sirtuin inhibitor) were added where indicated. All these products (PHA, PMA, ionomycin, Nam, and sirtinol) were purchased from Sigma Aldrich Italia, (Milano, Italy). Supernatants and cells were harvested 18 h later to determine CXCL8 concentrations by ELISA (in the supernatants) and for QPCR quantification of *CXCL8* mRNA amounts (in the cells). Cell death at the end of treatment was determined by PI staining and flow cytometry (FACS Calibur, Becton Dickinson) and treatment-specific cell death was calculated with the formula: (experimental death-spontaneous death)/(100-spontaneous death)×100.

Jurkat cell culture and SIRT6 silencing

The T cell leukemia cell line Jurkat was grown in RPMI 1640-based medium supplemented with 10% FBS, penicillin (10,000 U/ml) and streptomycin (10,000 μg/ml). Then, retroviral transgenesis was performed. Empty pRETROSuper (PRS) was obtained from Dr. Thijn Brummelkamp (Whitehead Institute for Biomedical Research, Cambridge, MA); PRS S6-sh1 and S6-sh2 were obtained from Dr. Katrin F. Chua (Department of Medicine, Stanford University School of Medicine, Stanford, CA) (11). Phoenix cells were plated in 4 ml medium in 6 cm-dishes and allowed to adhere for 24 h. Thereafter, cells were transfected with 4 μg plasmid DNA using Transit 293 (Mirus Bio, Madison, WI) according to the manufacturer's instructions. The viral supernatant was harvested 36 h and 48 h later and used to infect Jurkat cells in 24-well plates in the presence of 5 μg/ml protamine sulfate. Successfully infected cells were selected using 1 μg/ml puromycin. Then, 106 Jurkat cells expressing S6-sh1, S6-sh2, or empty PRS were incubated in culture medium (RPMI 1640-based medium supplemented with 10% FBS, penicillin [10,000 U/ml] and streptomycin [10,000 μg/ml]) in the presence or absence of PMA (25 ng/ml)/ionomycin (I, 0.5 μ*M*). 24 h later, supernatants and cells were harvested and used for CXCL8 detection by ELISA (supernatants) and for QPCR determination of *CXCL8* mRNA (cells). Cell death at the end of experiments was determined by PI staining and flow cytometry (FACS Calibur, Becton Dickinson, BD).

Cardiac cell line culture, viability assay, and NAD+ determination

H2c9 cardiomyocytes (a kind gift from Dr. Paola Altieri, Department of Internal Medicine, University of Genoa) were grown at 37°C and 5% CO_2 in DMEM supplemented with 10% FBS, penicillin/streptomycin, 1 m*M* sodium pyruvate, and 4 m*M* L-glutamine. For viability assays, 5×103 cells/well were plated in 96-well plates and allowed to adhere. Vehicle (1% DMSO) or 30 n*M* FK866 was added before cells were imaged and viability was determined at 48 h of incubation. H2c9 cell monolayers were fixed with 3% trichloroacetic acid for 1 h at 4°C and stained with 0.057% sulforodhamine B (Sigma Aldrich) for 30 min, after which the excess die was removed by repeatedly washing with 1% vol/vol acetic acid. Protein-bound dye was dissolved in 10 m*M* Tris base solution for OD determination at 510 nm with a standard plate reader. Cell viability was calculated with the following formula: (χ-background signal)/(untreated-background signal)×100.

Alternatively, for Annexin-V/propidum iodide assays, floating and adhering cells were collected from culture wells by repeatedly washing and by subsequent trypsinization. Subsequently, cells were stained with Annexin-V-FITC (Becton Dickinson Italia, Milan, Italy) and propidium iodide (Sigma Aldrich) according to the manufacturer's instructions and analyzed by flow cytometry with a FACS Calibur (Becton Dickinson). 20,000 events for each sample were acquired. Cells were also imaged at room temperature using with the 10X magnification of a Leica DMI 3000B microscope, a Leica DFC425C camera, and the software Leica Application Suite 3.6.0. A part of cardiomyocytes were immediately lysed with 0.6 *M* perchloric acid (PCA). Samples were neutralized, and NAD+ content was assessed with a sensitive enzyme cyclic assay, which exploits the use of alcohol dehydrogenase to

determine NAD+ levels (5) and analyzed by HPLC. NAD+ was normalized to tissue total weight.

Real time RT PCR

Normal male C57Bl/6 mice (8–12 weeks of age) without surgery were sacrificed and used as controls. Sham-operated and infarcted mice were sacrificed after 8 h of reperfusion. Hearts were excised and total mRNA was isolated with Trireagent (MRC Inc.). Reverse transcription was performed using the Improm II^{TM} (Promega Corporation) according to the manufacturer's instructions. Real-time PCR (StepOne Plus, Applied Biosystems) was performed with the ABsolute^{TM} QPCR Mix (ABgene). Specific primers and probes (Supplementary Table S6) were used to determine the mRNA expression of neutrophils (mouse neutrophil elastase, Elane) and normalized to HPRT gene (housekeeping gene) expression. The fold induction of mRNA levels of Elane in sham-operated and infarcted hearts was calculated by the C_t method comparative on normal mice without surgery. The resultant C_t values were first normalized to the internal control. This was achieved by calculating a delta C_t (ΔC_t) by subtracting the internal control C_t values from the HPRT C_t value. A delta delta C_t ($\Delta\Delta C_t$) was calculated by subtracting the designated control ΔC_t value from the other ΔC_t values. The $\Delta\Delta C_t$ was then plotted as a relative fold change with the following formula: $2^{-\Delta\Delta Ct}$.

Total RNA was extracted from 1.5×10^6 cells using RNeasy kit reagents (Qiagen, Qiagen Italia, Milan, Italy). Total RNA (1 μg) was reverse transcribed using random hexamers in a final volume of 50 μl. 5 μl of the resulting cDNA was used for Q-PCR using a TaqMan 7900 HT Fast Real TimeAB (17). Pre-designed primers and probes for IL8 and RN18S1 were obtained from Applied Biosystems and showed in the Supplementary Table S7. Gene expression was normalized to housekeeping gene expression (RN18S1). Comparisons in gene expression were done using the $2^{-\Delta\Delta Ct}$ method.

Immunoblotting

Cell lysates were generated from 1.5×106 cells by directly resuspending cell pellets in sodium dodecyl sulfate (SDS) sample buffer (Tris-HCl 0.25 M, pH 6.8, SDS 2%, glycerol 10%, β-mercaptoethanol 2%, bromophenol blue 0.005%; Boston Bioproducts, Boston, MA). Cell lysates were immediately boiled at 100°C for 10 min and stored at −20°C for subsequent use. Proteins were separated on an SDSpolyacrylamide gel and electroblotted to a polyvinylidene difluoride (PVDF) membrane (Pall Gelman Laboratory, Ann Arbor, MI). Proteins were visualized by probing the membranes with the following antibodies: anti-SIRT1 (Cell Signaling Technology), and anti-SIRT6 (C-terminal, rabbit polyclonal, Sigma Aldrich).

Supplementary References

1. Braunersreuther V, Pellieux C, Pelli G, Burger F, Steffens S, Montessuit C, Weber C, Proudfoot A, Mach F, and Arnaud C. Chemokine CCL5/RANTES inhibition reduces myocardial reperfusion injury in atherosclerotic mice. *J Mol Cell Cardiol* 48: 789–798, 2010.
2. Bruzzone S, Fruscione F, Morando S, Ferrando T, Poggi A, Garuti A, D'Urso A, Selmo M, Benvenuto F, Cea M, Zoppoli G, Moran E, Soncini D, Ballestrero A, Sordat B, Patrone F, Mostoslavsky R, Uccelli A, and Nencioni A. Catastrophic NAD+ depletion in activated T lymphocytes through NAMPT inhibition reduces demyelination and disability in EAE. *PLoS One* 4: e7897, 2009.
3. Cave AC, Brewer AC, Narayanapanicker A, Ray R, Grieve DJ, Walker S, and Shah AM. NADPH oxidases in cardiovascular health and disease. *Antioxid Redox Signal* 8: 691–728, 2006.
4. Delattre BM, Braunersreuther V, Gardier S, Hyacinthe JN, Crowe LA, Mach F, and Vallée JP. Manganese kinetics demonstrated double contrast in acute but not in chronic infarction in a mouse model of myocardial occlusion reperfusion. *NMR Biomed* 25: 489–497, 2012.
5. Graeff R and Lee HC. A novel cycling assay for cellular cADP-ribose with nanomolar sensitivity. *Biochem J* 361: 379–384, 2002.
6. Kato Y, Kawai Y, Morinaga H, Kondo H, Dozaki N, Kitamoto N, and Osawa T. Immunogenicity of a brominated protein and successive establishment of a monoclonal antibody to dihalogenated tyrosine. *Free Radic Biol Med* 38: 24–31, 2005.
7. Lecut C, Frederix K, Johnson DM, Deroanne C, Thiry M, Faccinetto C, Marée R, Evans RJ, Volders PG, Bours V, and Oury C. P2X1 ion channels promote neutrophil chemotaxis through Rho kinase activation. *J Immunol* 183: 2801–2809, 2009.
8. Leslie RG. Evaluation and improvement of a rapid microassay for measuring superoxide anion production by phagocytes. 1. Spectrophotometric aspects. *J Immunol Methods* 103: 253–259, 1987.
9. Liu YH, Carretero OA, Cingolani OH, Liao TD, Sun Y, Xu J, Li LY, Pagano PJ, Yang JJ, and Yang XP. Role of inducible nitric oxide synthase in cardiac function and remodeling in mice with heart failure due to myocardial infarction. *Am J Physiol Heart Circ Physiol* 289: H2616–2623, 2005.
10. Machado FS, Souto JT, Rossi MA, Esper L, Tanowitz HB, Aliberti J, and Silva JS. Nitric oxide synthase-2 modulates chemokine production by *Trypanosoma cruzi*-infected cardiac myocytes. *Microbes Infect* 10: 1558–1566, 2008.
11. Michishita E, McCord RA, Berber E, Kioi M, Padilla-Nash H, Damian M, Cheung P, Kusumoto R, Kawahara TL, Barrett JC, Chang HY, Bohr VA, Ried T, Gozani O, and Chua KF. SIRT6 is a histone H3 lysine 9 deacetylase that modulates telomeric chromatin. *Nature* 452: 492–496, 2008.
12. Montecucco F, Di Marzo V, da Silva RF, Vuilleumier N, Capettini L, Lenglet S, Pagano S, Piscitelli F, Quintao S, Bertolotto M, Pelli G, Galan K, Pilet L, Kuzmanovic K, Burger F, Pane B, Spinella G, Braunersreuther V, Gayet-Ageron A, Pende A, Viviani GL, Palombo D, Dallegri F, Roux-Lombard P, Santos RA, Stergiopulos N, Steffens S, and Mach F. The activation of the cannabinoid receptor type 2 reduces neutrophilic protease-mediated vulnerability in atherosclerotic plaques. *Eur Heart J* 33:846–856, 2012.
13. Montecucco F, Lenglet S, Braunersreuther V, Pelli G, Pellieux C, Montessuit C, Lerch R, Deruaz M, Proudfoot AE, and Mach F. Single administration of the CXC chemokine-binding protein Evasin-3 during ischemia prevents myocardial reperfusion injury in mice. *Arterioscler Thromb Vasc Biol* 30: 1371–1377, 2010.
14. Nakahata Y, Sahar S, Astarita G, Kaluzova M, and Sassone-Corsi P. Circadian control of the NAD+ salvage pathway by CLOCK-SIRT1. *Science* 324: 654–657, 2009.

15. Ottonello L, Amelotti M, Barbera P, Dapino P, Mancini M, Tortolina G, and Dallegri F. Chemoattractant-induced release of elastase by tumor necrosis factor primed human neutrophils: Auto-regulation by endogenous adenosine. *Inflamm Res* 48: 637–642, 1999.
16. Ottonello L, Morone P, Dapino P, and Dallegri F. Inhibitory effect of salmeterol on the respiratory burst of adherent human neutrophils. *Clin Exp Immunol* 106: 97–102, 1996.
17. Pfaffl MW. A new mathematical model for relative quantification in real-time RTPCR. *Nucleic Acids Res* 29: e45, 2001.
18. Riches AC, Sharp JG, Thomas DB, and Smith SV. Blood volume determination in the mouse. *J Physiol* 228: 279–284, 1973.
19. Rongvaux A, Galli M, Denanglaire S, Van Gool F, Drèze PL, Szpirer C, Bureau F, Andris F, and Leo O. Nicotinamide phosphoribosyl transferase/pre-B cell colony enhancing factor/visfatin is required for lymphocyte development and cellular resistance to genotoxic stress. *J Immunol* 181: 4685–4695, 2008.
20. Wiekowski MT, Chen SC, Zalamea P, Wilburn BP, Kinsley DJ, Sharif WW, Jensen KK, Hedrick JA, Manfra D, and Lira SA. Disruption of neutrophil migration in a conditional transgenic model: Evidence for CXCR2 desensitization *in vivo*. *J Immunol* 167: 7102–7110, 2001.
21. Zhang WJ, Wei H, Tien YT, and Frei B. Genetic ablation of phagocytic NADPH oxidase in mice limits TNFα-induced inflammation in the lungs but not other tissues. *Free Radic Biol Med* 50: 1517–1525, 2011.
22. Zhao ZQ. Oxidative stress-elicited myocardial apoptosis during reperfusion. *Curr Opin Pharmacol* 4: 159–165, 2004.