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ORIGINAL CONTRIBUTION

Reduced ambient temperature exacerbates SIRS-induced cardiac autonomic dysregulation and myocardial dysfunction in mice

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Abstract

Sepsis-induced myocardial depression (SIMD) is an early and frequent consequence of the infection-induced systemic inflammatory response syndrome. In homiotherms, variations in ambient temperature (T_a) outside the thermoneutral zone induce thermoregulatory responses mainly driven by a gradually increased sympathetic activity, which may affect disease severity. We hypothesized that thermoregulatory responses upon reduced T_a exposition aggravate SIMD in mice. Mice were kept at neutral T_a (30 ± 0.5 °C), moderately lowered T_a (26 ± 0.5 °C) or markedly lowered T_a (22 ± 0.5 °C), exposed to lipopolysaccharide- (LPS, 10 µg/g, from *Escherichia coli* serotype 055:B5, single intraperitoneal injection) evoked shock and monitored for survival, cardiac autonomic nervous system function and left ventricular performance. Primary adult cardiomyocytes and heart tissue derived from treated mice were analyzed for inflammatory responses and signaling pathways of myocardial contractility. We show that a moderate reduction of T_a to 26 °C led to a 40% increased mortality of LPS-treated mice when compared to control mice and that a marked reduction of T_a to 22 °C resulted in an early mortality of all mice. Mice kept at 26 °C exhibited increased heart rate and altered indices of heart rate variability (HRV), indicating sympathovagal imbalance along with aggravated LPS-induced SIMD. This SIMD was associated with reduced myocardial β-adrenergic receptor expression and suppressed adrenergic signaling, as well as with increased myocardial iNOS expression, nitrotyrosine formation and leukocyte invasion as well as enhanced apoptosis and appearance of contraction band necrosis in heart tissue. While ineffective separately, combined treatment with the β_2-adrenergic receptor (AR) antagonist ICI 118551 (10 ng/gbw) and the inducible nitric oxide synthase (iNOS) inhibitor 1400 W (5 µg/gbw) reversed the increase in LPS-induced mortality and aggravation of SIMD at reduced T_a. Thus, consequences of thermoregulatory adaptation in response to ambient temperatures below the thermoneutral range increase the mortality from LPS-evoked shock and markedly prolong impaired myocardial function. These changes are mitigated by combined β_2-AR and iNOS inhibition.

Keywords Myocardial contractility · Autonomic nervous system · Acute inflammation · Ambient temperature · iNOS

Introduction

Sepsis-induced myocardial depression (SIMD) is a frequent event that corresponds to the severity of sepsis [5, 81]. SIMD occurs early in sepsis, since a considerable cohort of septic patients express features of myocardial dysfunction at intensive care unit (ICU) admission [23, 106]. It may further aggravate the consequences of an already exaggerated systemic host response to infection and finally contribute to sepsis-induced cardiocirculatory shock [2, 88]. Initiation of SIMD is mainly triggered by microbial toxins and proinflammatory mediators, which are excessively released during the innate immune response and lead to activation of pathophysiologically important signaling pathways [59, 61, 80, 113].

Bernadin Ndongson-Dongmo and Guang-Ping Lang contributed equally to this work.

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In contrast to the earlier perception of complete SIMD reversibility in sepsis survivors, suggesting the absence of myocardial damage, recent data indicate that SIMD is in part a result of infection-driven inflammation, oxidative stress, and abnormal calcium homeostasis with subsequent myocardial stunning. Troponin release early after sepsis onset has been verified [107] and histological lesions, possibly indicating stress-induced cardiotoxicity, have been observed in most patients dying from septic shock [83]. Furthermore, the risk for stress-induced (Takotsubo) cardiomyopathy [66] caused by excessive release of catecholamines is increased in bacterial sepsis [20]. Therefore, structural damage and catecholamine-induced cardiomyocyte toxicity may play crucial roles in the complex pathogenesis of SIMD [84, 90].

Thermoregulation is a fundamental homeostatic function of all mammals; it includes afferent thermal sensing, central regulation, and an efferent response with the consequence of tightly controlled body temperature within a narrow species-specific range [64]. Variations of core body temperature (T_c) outside this range trigger autonomic thermoregulatory responses, mainly via a gradually increased sympathetic activity [95]. Clinical data clearly indicate that spontaneous T_c lowering (hypothermia indicating energy exhaustion) is directly correlated with poor outcome of sepsis [85, 111]. It is also known that patients with trauma or burns, who frequently develop accidental hypothermia, exhibit an increased risk of developing sepsis [17, 89] and that the presence of hypothermia increases the risk for subsequent ICU-acquired infection [50]. However, the role of thermoregulatory responses upon exposure to reduced ambient temperature (T_a) in the pathogenesis of acute inflammation-induced myocardial depression has not been studied.

The aim of this study was to examine whether exposure of mice to ambient temperature (T_a) outside their thermoneutral zone (but inside the temperature range of standard practice in preclinical biomedical research [75] and legal recommendations [16, 72]) will affect survival and myocardial dysfunction induced by a systemic inflammatory response in mice. We hypothesized that a reduced ambient temperature deteriorates SIRS-triggered myocardial dysfunction. Survival, the response of the cardiac autonomic nervous system and left ventricular performance were examined after induction of SIRS by intraperitoneal LPS administration in mice exposed to neutral or reduced T_a.

Materials and methods

Animals

Male C57BL/6J mice were used in this study. The animal procedures were performed according to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Experiments were approved by the Thuringian State Office for Food Safety and Consumer Protection.

12- to 15-week-old male C57BL/6J mice were kept at 12-h light and dark cycles with free access to food and water. Animals were allocated to cohorts kept at mouse-specific neutral T_a (30 ± 0.5 °C) [28], moderately lowered T_a (26 ± 0.5 °C) or markedly lowered T_a (22 ± 0.5 °C) during the whole experimental period. After acclimatization for ≥ 5 days at the respective temperatures, mice received LPS (10 µg/g, from *Escherichia coli* serotype 055:B5, Sigma-Aldrich, St. Louis, USA, Lot #032M4082V) as a single intraperitoneal injection. Additionally, saline (500 µl) was injected subcutaneously immediately after LPS administration and after 24 h and 48 h.

Instrumentation/surgical procedures/measurements

Telemetric studies were performed in freely moving mice kept either at neutral ($n = 9$) or moderately lowered ($n = 12$) T_a. For telemetric measurements of body core temperature (T_c), electrocardiography (ECG) and motor activity, an implantable 1.6-g wireless radiofrequency transmitter (ETA-F10, Data Sciences International, St. Paul, MN, USA) was used. For instrumentation, mice were anesthetized with 2.5% isoflurane in oxygen. A midline incision was made on the abdominal wall, the intraperitoneal cavity was gently opened and the transmitter inserted. The leads were transferred through the abdominal wall and the incision closed by a surgical suture. The cathodal lead was looped forward subcutaneously to an area overlying the scapula and attached with a permanent suture. The anodal lead was placed subcutaneously near the heart apex. To maintain body temperature between 36 and 37 °C, a rectal temperature probe and a feedback-controlled warming light were used. Buprenorphine was given against pain on the day of surgery and the following day. Animals were allowed to recover for 10 days from surgical instrumentation [96]. After acclimatization for ≥ 5 days at the respective temperatures T_c, ECG and motor activity were monitored continuously for 8 days. Heart rate variability (HRV) was calculated [1, 96] 1 h before as well as 3 h, 24 h, 3 days and 7 days after LPS administration and indices were determined. For detailed description of data processing and HRV parameter calculation, see online data supplement.

Measurement of left ventricular performance was carried out before as well as 3 h, 1 day, 3 days and 7 days after LPS administration in anesthetized mice kept either at neutral ($n = 30$) or moderately lowered ($n = 25$) T_a. The mice were anesthetized as described above, intubated and

ventilated with a respirator (MiniVent Model 845, Hugo Sachs Elektronik-Harvard Apparatus GmbH, March-Hugstetten, Germany). Body temperature was monitored by a rectal temperature probe, and was maintained throughout the experiment at 37 ± 0.3 °C. A 1.4-F micro-conductance pressure–volume catheter (model SPR-839; Millar Instruments Inc.) was positioned in the left ventricle (LV) via the right carotid artery for continuous registration of LV pressure–volume loops in closed chest animals [76], using a PowerLab system (ADInstruments Ltd., Oxford, UK). Indices of systolic and diastolic cardiac performance were derived from LV pressure–volume data obtained at steady state during brief inferior vena cava occlusion, while ventilation was temporarily turned off. Left ventricular end-systolic elastance (E_{es}) was assessed as the slope of the end-systolic maxima connecting line and considered to be an appropriate variable to determine myocardial contractile state [45, 76]. Arterial blood pressure was measured in the ascending aorta. Cardiac output and ejection fraction were obtained from pressure–volume loops. The intravenous hypertonic saline wash-in technique was employed for assessment of parallel conductance [91]. Conductance catheter calibration was performed by the cuvette calibration method using insulator-type cuvettes (Millar Blood Calibration Cuvette P/N 910-1049; well diameters 1.5, 2.0, 2.5, 3.0, 3.5 mm) filled with heparin-treated blood.

Experimental protocols

For survival analysis and assessment of the clinical status (according to [25]), freely moving mice ($n = 51$) were evaluated daily before and 7 days after LPS administration. Treatment with β_2-AR antagonist ICI 118551 hydrochloride (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany; 10 ng/gbw administered 12 h before and together with LPS injection, i.p.) and the inducible nitric oxide synthase (iNOS) inhibitor 1400W (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany; 5 µg/gbw administered 24 h after LPS injection, i.p.), was performed in mice kept at moderately lowered T_a (26 ± 0.5 °C) (survival study: $n = 20$, hemodynamic/cardiac performance study: $n = 6$).

To obtain tissue samples ($n = 65$), evaluate histology ($n = 56$) and isolate cardiomyocytes for primary cell cultures ($n = 8$), the heart was removed from anesthetized mice after opening the chest cavity. To prepare adult cardiomyocytes, the excised heart was rapidly immersed in Ca^{2+}-free perfusion buffer. The aorta was then positioned onto a cannula and fixed with a silk suture. Immediately afterwards, the heart was mounted on a perfusion system and the coronary blood vessels perfused with an enzyme solution (1 mg/ml collagenase type 2: Worthington; 310 U/mg, prepared in perfusion buffer and supplemented with Ca^{2+} at 12.5 µM) for homogeneous digestion [57, 65].

Measurement of variables

Heart tissue catecholamines

The concentrations of the catecholamines epinephrine (E) and norepinephrine (NE) were assessed in tissue samples obtained from left ventricle. After removal, heart ventricles were washed in ice-cold PBS (pH 4), homogenized in ice-cold PBS using a glass tissue grinder and centrifuged at 1500×*g* for 15 min at 4 °C. The supernatant was immediately taken and kept at − 80 °C until analysis. Measurement of catecholamines was performed by ELISA as per the manufacturer's protocol (Antibodies-online GmbH, Aachen, Germany).

Cytokines

Total cytokine concentrations (TNFα, IL-6, MCP-1) in blood and heart tissue were determined using the BD™ Cytometric Bead Assay (CBA) Mouse Inflammation Kit (Dickinson and Company, San Jose, USA). Blood was obtained via direct heart puncture, collected in a heparinized syringe and immediately centrifuged at 1500×*g* for 10 min at 4 °C. The plasma supernatant was taken immediately and kept at − 80 °C until measurement. For cytokine assessment in the heart tissue, the left ventricle was harvested, rinsed with cold PBS, immediately put in liquid nitrogen and kept at − 80 °C until processing. The heart tissue was then powdered, suspended in ice-cold PBS, homogenized and centrifuged at 1500×*g* for 15 min at 4 °C. The supernatant was kept at − 80 °C until measurement.

Gene expression

Total RNA was extracted from heart tissue using QIAzol Lysis Reagent (#79306) purchased from Qiagen (Hilden, Germany). RNA concentration and quality were checked using the Nanodrop ND-1000 machine (Peqlab, Erlangen, Germany). cDNA was synthesized using RevertAid First Strand cDNA Synthesis kit (#K1612) from Thermo Fischer Scientific (Massachusetts, USA). Gene expression was analyzed using Maxima SYBR Green/ROC qPCR Master Mix (#K0221) from Thermo Fischer Scientific (Massachusetts, USA). The following primer pairs were used: β1-AR forward: ACGCTCACCAACCTCTTCAT and β1-AR reverse: AGGGGCACGTAGAAGGAGAC, β2-AR forward: CCT CATGTCGGTTATCGTCC and β2-AR reverse: GGCACG TAGAAAGACACAATC, β-arrestin 1 forward: AAGGGA CACGAGTGTTCAAGA and β-arrestin 1 reverse: CCC GCTTTCCCAGGTAGAC, β-arrestin 2 forward: GGCAAG CGCGACTTTGTAG and β-arrestin 2 reverse: GTGAGG GTCACGAACACTTTC, GAPDH forward: CATGGCCTT

Table 1 Cytokine content in blood plasma and heart tissue

	Baseline	LPS 3 h	LPS 1 day	LPS 3 days	LPS 7 days
Blood plasma					
TNFα (ng g^{-1})					
NEUTRAL-group	0 (0; 48)	2328 (1834; 3181)*	57 (0.0; 101)	21 (18; 28)	n.d.
COOL-group	n.d.	1892 (1641; 2317)*§	n.d.	14 (13; 31)	n.d.
IL-6 (ng g^{-1})					
NEUTRAL-group	4.2 (3.4; 5.0)	101277 (87079; 167223)*	494 (444; 563)	23 (15; 27)	0 (0; 4.2)
COOL-group	6.0 (3.8; 15.7)	81445 (65051; 130517)*	444 (318; 1276)	42 (25; 56)	0 (0; 8.8)
MCP-1 (ng g^{-1})					
NEUTRAL-group	0 (0; 34.1)	77811 (60376; 103754)*	6468 (6386; 6518)	438 (424; 542)	n.d.
COOL-group	n.d.	49757 (39589; 74604)*§	1733 (1394; 3042)	603 (168; 1260)	n.d.
Heart tissue					
TNFα (ng g^{-1})					
NEUTRAL-group	0 (0; 4.5)	102 (99; 142)*	6.0 (4.8; 7.2)	9.9 (9.4; 11.1)	5.1 (4.5; 7.5)
COOL-group	0 (0; 3.7)	100 (69; 102)*	5.5 (4.5; 9.6)	6.7 (4.8; 10.0)	0 (0; 2.2)
IL-6 (ng g^{-1})					
NEUTRAL-group	1.6 (0; 3.5)	2031 (1933; 4210)*	41 (40; 43)	4.2 (4.2; 4.4)	3.0 (2.6; 4.9)
COOL-group	1.9 (1.5; 2.1)	2475 (2151; 2622)*	47 (26; 95)	17 (13; 31)	2.3 (0.0; 3.1)
MCP-1 (ng g^{-1})					
NEUTRAL-group	27 (27; 42)	3347 (2547; 3468)*	590 (588; 654)*	582 (576; 642)*	41 (36; 50)
COOL-group	34 (23; 37)	2780 (2757; 3019)*	448 (422; 461)*	430 (382; 671)*	37 (31; 39)

Values are given as medians as well as the first quartile and third quartile in parenthesis; $n = 4$ per group and time point, $p < 0.05$, *significant difference between baseline and LPS-stimulated state within each group, §significant differences versus mice kept under neutral ambient temperature

CCGTGTTTCCTA and GAPDH reverse: CCTGCTTCA CCACCTTCTTGAT. All data were normalized to GAPDH as housekeeping gene.

Relative gene expression was calculated using the comparative C_T ($2_T^{-\Delta\Delta C}$) method [56].

SDS-PAGE and Western blotting

Proteins from lysates of cardiac ventricles, including their phosphorylated and/or tyrosine nitrated forms, were analyzed by Western blotting. Lysis was performed in 50 mM Tris–HCl, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 1% Triton X-100 and 0.5% deoxycholate (final pH 7.4), supplemented with protease inhibitor cocktail, and proteins were quantified using the Lowry method. After adding loading buffer (62.5 mM Tris–HCl, glycerol 10%, bromophenol blue 1% wt/vol, SDS 2%, mercaptoethanol 2.5%), lysates were boiled for 5 min at 95 °C (except those for the analysis of tyrosine nitrated proteins, which were kept on ice). Proteins were separated by SDS–PAGE using 5, 7.5, 10 or 15% gels, and then transferred to nitrocellulose membranes. The membranes were blocked with 5% milk in Tris-buffered saline containing Tween-20 (TBST) for 2 h. They were then incubated with the indicated primary antibodies overnight at 4 °C and subsequently with appropriate secondary antibodies. Blots were developed by enhanced chemiluminescence reaction using a LAS4000 (Fuji Photo Film Co., Ltd, Tokyo, Japan) camera.

Histopathology and immunohistochemistry

Hearts were rinsed with PBS and fixated in situ with 4% paraformaldehyde, then immediately excised, post-fixated in vitro in 4% paraformaldehyde at 4 °C for 1 day, embedded in paraffin and cut into 6 µm thick sections. After blockade of non-specific binding sites, antigen retrieval (microwave, 750 W, 11 min, 0.01 mol/l citrate buffer, pH 6) and deparaffinization, slide-mounted tissue sections were incubated with the desired primary antibody in PBS at 4 °C overnight and subsequently with the appropriate secondary antibody at 4 °C for 1 h. Control sections were incubated with goat serum in the absence of the primary antibody.

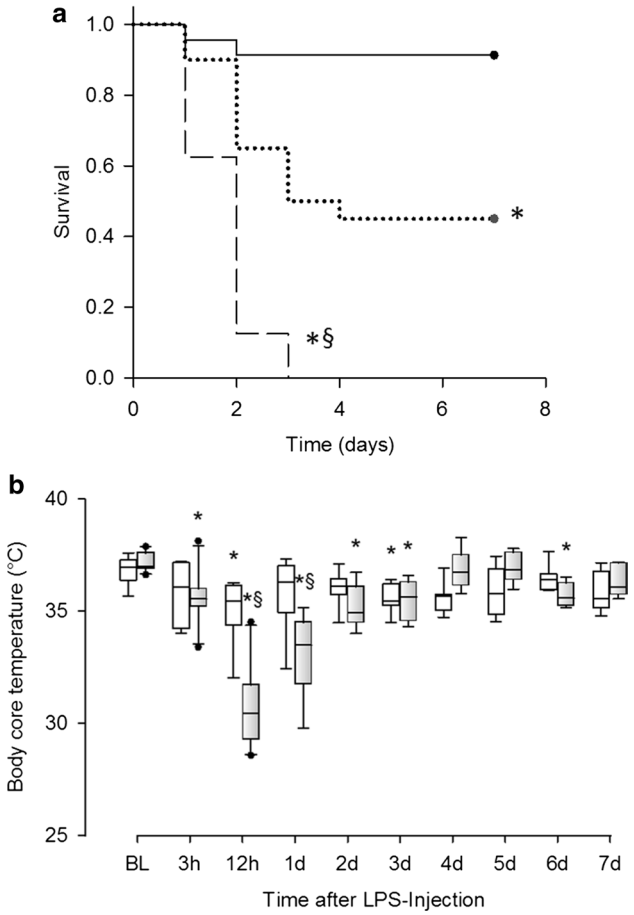


Fig. 1 Effect of ambient temperature on survival and core temperature. **a** Survival analysis. Animals were adapted to the respective ambient temperature (T_a) for ≥5 days before SIRS was induced by intraperitoneal LPS injection (10 µg/gbw). Mice were monitored for 7 days. Increased mortality in mice kept at reduced ambient temperatures (T_a = 26 °C, n = 20, dotted line; T_a = 22 °C, n = 8, dashed line) compared to mice kept at neutral ambient temperature (T_a = 30 °C, n = 23, solid line) is shown. **b** Aggravated hypothermia early after LPS-evoked shock in mice kept under moderately reduced T_a (filled boxplots), compared with mice kept under neutral T_a (open boxplots). **a** *$^{\S}p < 0.05$, *significant difference versus mice kept at neutral T_a, §versus mice kept at T_a = 26 °C (Kaplan–Meier survival analysis; log-rank test followed by Holm–Sidak test for post hoc multiple comparisons was performed). **b** Values are presented as boxplots illustrating medians within boxes from first quartile to the third quartile, whiskers ranging from the 10th to the 90th percentiles and outliners as dots (neutral T_a: n = 9, all survived, reduced T_a: n = 12, four animals survived; non-surviving animals died between 2nd and 4th day after LPS administration; values of dying animals were omitted). *$^{\S}p < 0.05$, *significant difference between baseline (BL) and LPS-stimulated state within each group, §significant differences versus mice kept under neutral T_a at the same experimental state (one-way repeated measures ANOVA, followed by Holm–Sidak test for post hoc multiple comparisons was performed for comparison within the respected group, t test was used for comparisons between both groups with Bonferroni's correction for adjustments of multiple comparisons)

Table 2 Clinical severity score

	Neutral T_a	Moderately reduced T_a	Markedly reduced T_a
Baseline	1.0 (1.0; 1.0)	1.0 (1.0; 1.0)	1.0 (1.0; 1.0)
LPS 3 h	2.0 (1.0; 2.0)	2.0 (2.0; 2.0)	2.0 (1.8; 2.0)*
LPS 1 day	3.0 (3.0; 3.0)*	4.0 (3.8; 4.0)*§	3.0 (3.0; 5.0)*
LPS 2 days	3.0 (2.5; 3.0)*	4.0 (4.0; 5.0)*§	4.5 (3.0; 5.0)*§
LPS 3 days	2.0 (2.0; 2.0)	4.0 (3.0; 5.0)*§	5.0 (5.0; 5.0)*§
LPS 7 days	1.0 (1.0; 1.0)	3.5 (2.0; 5.0)*§	5.0 (5.0; 5.0)*§§

Animals were adapted to the respective ambient temperature (T_a) for ≥5 days before injection. For SIRS induction endotoxin/LPS (10 µg/gbw) was injected intraperitoneally (n = 8 in each group). Increased level of sickness was shown in mice kept at reduced ambient temperatures (moderately reduced T_a = 26 °C, markedly reduced T_a = 22 °C), compared with mice kept at neutral ambient temperature (neutral T_a = 30 °C). Clinical status was assessed according to [25] with the additional score value 5: death. Data are given as medians as well as the first quartile and third quartile in parenthesis. *$^{\S\S}p < 0.05$, *significant difference between baseline and LPS-stimulated state within each group, §significant differences versus mice kept under neutral ambient temperature, $^{\$}$significant differences versus mice kept under moderately reduced T_a at the same experimental state. One-way measures ANOVA or one-way measures ANOVA on ranks and one-way repeated measures ANOVA, followed by Holm–Sidak test for post hoc multiple comparisons was performed for comparison between and within the respected group

Anti-myeloperoxidase (MPO, Dianova, Hamburg, Germany) and fluorescent goat anti-mouse isotype-specific antibodies Alexa Fluor® 488 (Molecular Probes, Inc., Eugene, USA) were used for staining of neutrophils. Methods for hematoxylin and eosin (H&E) staining and detection of fragmented DNA by TUNEL staining are described elsewhere [10]. For TUNEL staining, deparaffinized tissue sections were pretreated with 20 mg/ml proteinase K, washed in PBS and a commercially available kit was applied according to the manufacturer's protocol (in situ cell death detection kit "AP", Boehringer Mannheim, Germany). Following incubation of tissue sections with fluorescein-conjugated digoxigenin-UTP and terminal deoxynucleotidyltransferase at 37 °C for 1 h, DNA fragmentation was visualized using converter-alkaline phosphatase, NBT/BCIP and counterstaining with Kernechtrot.

Statistics

All data are presented as boxplots, illustrating medians within boxes from first quartile (25th percentile) to the third quartile (75th percentile) and whiskers ranging from the 10th to the 90th percentiles (extreme values are marked outside). Numbers of animals are given in figure legends for each group and time point. Comparisons between groups were made with one-way or two-way analysis of variance, if appropriate. In case of repeated measurements, one-way and

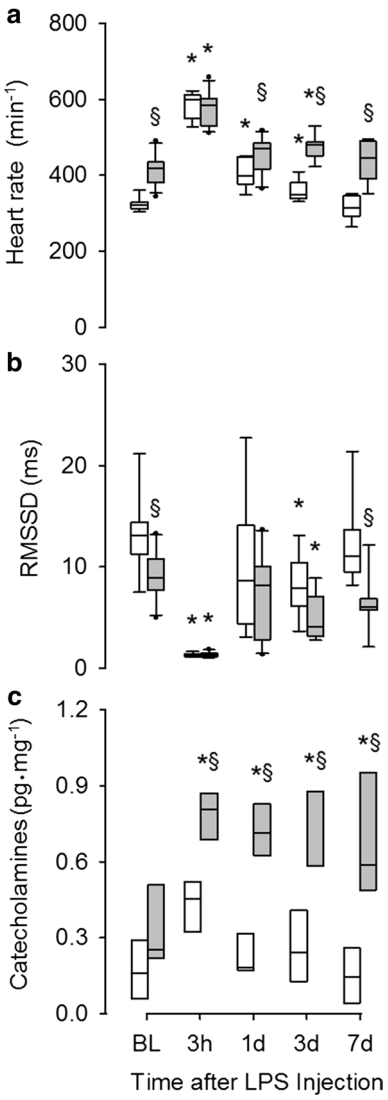


Fig. 2 Autonomic nervous system control of heart function measured by telemetric assessment. Depicted are **a** heart rate, **b** square root of the mean square successive differences between successive normal intervals (RMSSD) as an indicator of heart rate variability (HRV) and, **c** catecholamine concentration in heart tissue before (BL) and at several time points after LPS-evoked shock. Of note, increased heart rate and myocardial catecholamine concentration, as well as reduced HRV in mice kept under moderately reduced T_a (filled boxplots), is seen. In contrast, mice kept under neutral T_a (open boxplots) showed complete recovery of autonomic heart control during the chosen observation period. Values are presented as boxplots illustrating medians within boxes from first quartile to the third quartile and whiskers ranging from the 10th to the 90th percentiles (**a**, **b** neutral T_a: $n=9$, moderately reduced T_a: $n=12$; **c** $n=4$ per group and time point. $^{*\S}p<0.05$, *significant difference between baseline (BL) and LPS-stimulated state within each group, §significant differences versus mice kept under neutral T_a at the same experimental state (two-way ANOVA, followed by Holm–Sidak test for post hoc multiple comparisons)

two-way analyses of variance with repeated measures were used, if appropriate. Post hoc comparisons were made with the Holm–Sidak test or *t* tests with Bonferroni's correction for adjustment of multiple comparisons. Differences in frequencies were assessed by the Fisher Exact Test and were considered significant at p values <0.05. The SigmaPlot program for Windows Version 13.0 Build 13.0.0.83 (Systat Software GmbH, Erkrath, Germany) was applied for all statistical analyses. Additional information can be found in the Online Supplement.

Results

Impact of ambient temperature on SIRS-induced systemic responses: survival, thermoregulation, autonomic regulation

Intraperitoneal LPS administration (10 µg/g) to mice induced a rapid and transient manifestation of SIRS, as demonstrated by an increase of cytokine concentrations in blood plasma and cardiac tissue, which was similar in cardiac tissue under neutral (30 °C) or moderately reduced (26 °C) T_a (Table 1). However, cytokine concentrations in blood plasma were reduced in mice kept under moderately reduced T_a. In contrast, mortality was increased by about 40% in mice kept at 26 °C compared to those kept at 30 °C, and occurred predominantly after the proinflammatory cytokine burst had passed (Fig. 1a). The surviving animals showed an enlarged and prolonged elevation of the clinical severity score when kept at 26 °C compared to 30 °C (Table 2). When accommodated at a T_a of 22 °C, usually used for housing and breeding of laboratory mice, all animals died in response to LPS (Fig. 1a).

To explore the mechanisms underlying the different clinical courses under differing ambient temperatures, we performed telemetric analyses in mice kept under neutral or moderately reduced T_a. Telemetric T_c monitoring revealed that baseline body temperatures in mice kept at 30 °C or 26 °C, respectively, were not different. Nevertheless, mice are clearly subjected to cold stress when kept under reduced T_a, as indicated by a temperature-dependent upregulation of the uncoupling protein 1 (UCP-1) in the epididymal adipose tissue (Supplemental Figure S1). Under baseline conditions, reduction of T_a to 26 °C was accompanied by increased heart rate and altered HRV indices indicating altered sympathovagal balance (Fig. 2, Supplemental Table 1). Transient hypothermia occurred soon after onset of LPS-evoked shock, which was more pronounced and longer lasting in animals kept at 26 °C (Fig. 1b, Supplemental Figure S2). During early LPS-evoked shock, heart rate and HRV indices were similarly changed in mice kept at 30 °C and 26 °C, whereas myocardial catecholamine concentration was already increased in mice kept at 26 °C. These alterations persisted throughout the observation period only in animals kept under moderately reduced T_a (Fig. 2).

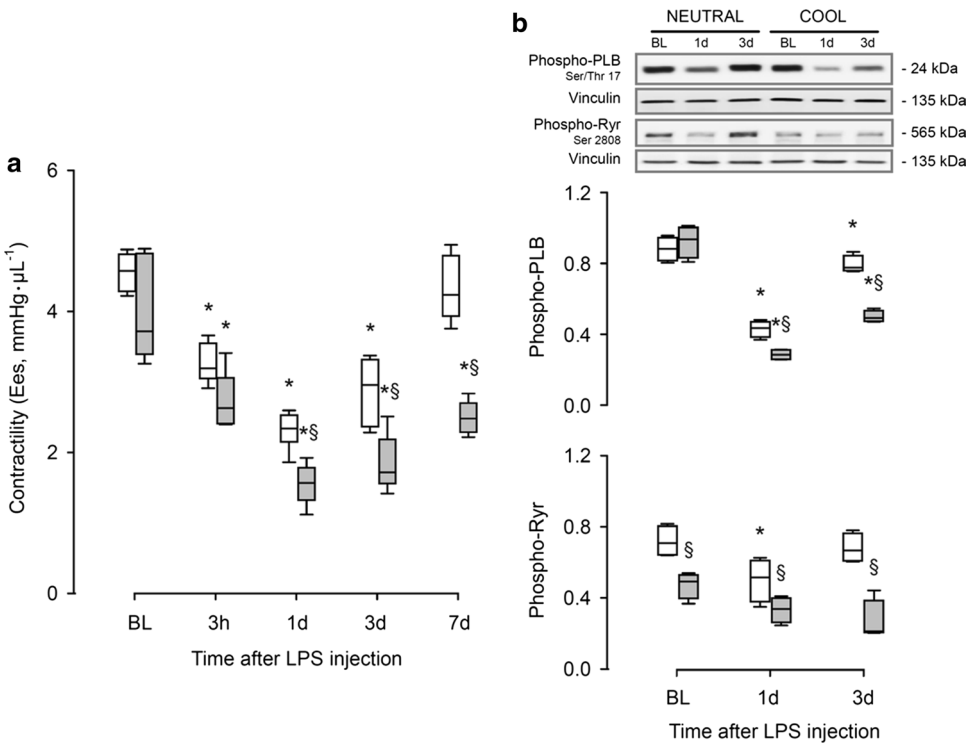


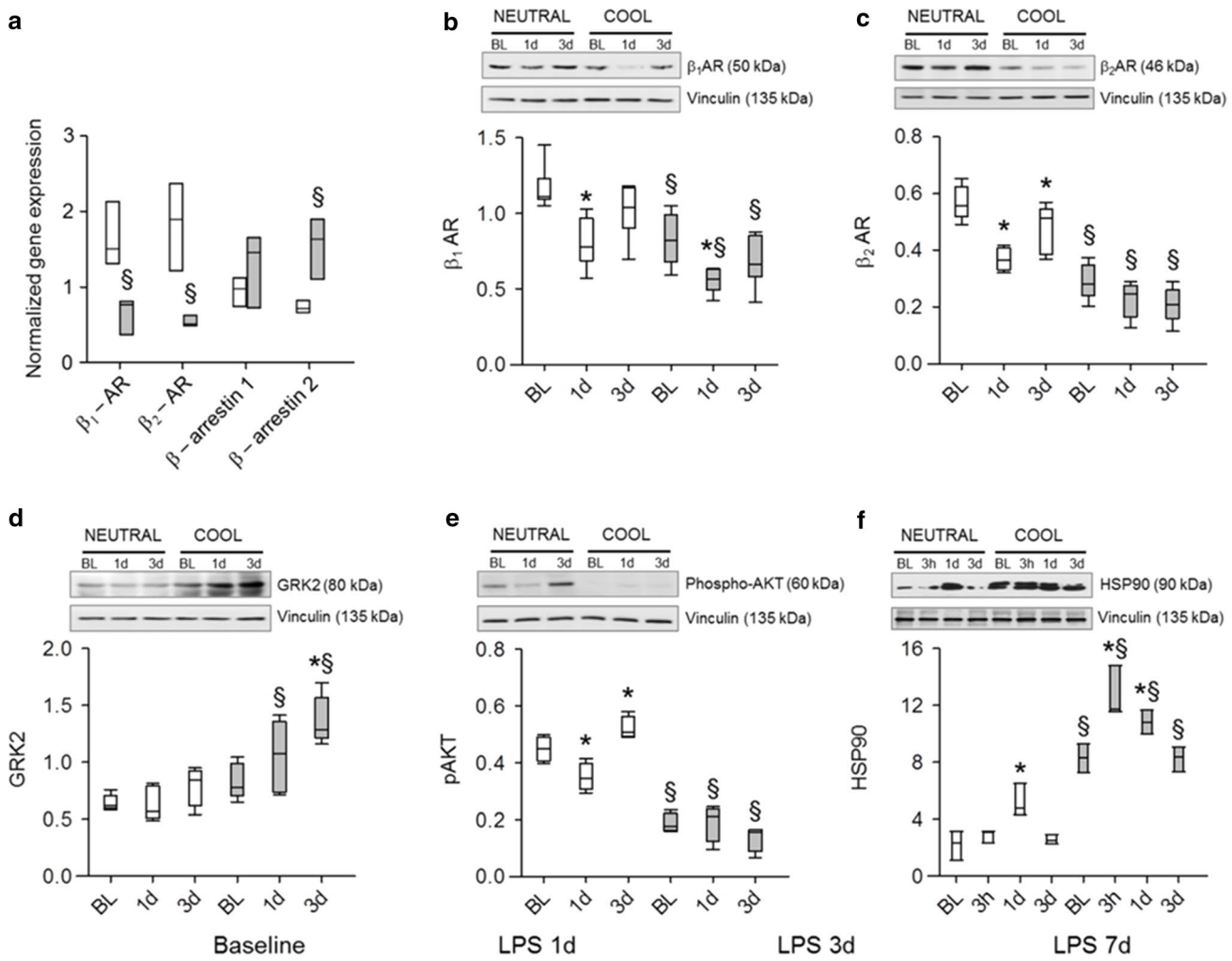
Fig. 3 Effect of ambient temperature on left ventricular function and sarcoplasmic reticulum calcium signaling. **a** Aggravated and sustained myocardial depression in mice kept under moderately reduced T_a (filled boxplots, $n=5$ per time point) after LPS-evoked shock is seen. In contrast, mice kept under neutral T_a (open boxplots, $n=6$ per time point) showed complete recovery of myocardial contractility after less pronounced myocardial depression early after LPS administration. **b** Reduced phosphorylation of the ryanodine receptor (Ryr) and phospholamban (PLB) is observed in heart tissue obtained from mice kept under reduced T_a (filled boxplots) after LPS-evoked shock. In contrast, Ryr and PLB phosphorylation in heart tissue obtained from mice kept under neutral T_a (open boxplots) showed almost complete recovery 3 days after LPS administration. Representative Western blots and densitometry analysis are shown. Values are presented as boxplots illustrating medians within boxes from first quartile (25th percentile) to the third quartile (75th percentile) and whiskers ranging from the 10th to the 90th percentiles. **b** $n=4$ per group and time point (top panel: representative Western blots, bottom panel: densitometric quantification. $^{*\S}p<0.05$, *significant difference between baseline (BL) and LPS-stimulated state within each group, §significant differences versus mice kept under neutral T_a at the same experimental state (**a** one-way ANOVA, followed by Holm–Sidak test for post hoc multiple comparisons was performed for comparison within the respected group, *t* test was used for comparisons between both groups with Bonferroni's correction for adjustments of multiple comparisons, **b** two-way ANOVA, followed by Holm–Sidak test for post hoc multiple comparisons). Densitometric data represent the relative amounts of the indicated proteins in relation to the loading control vinculin

Aggravated LPS-induced myocardial depression at moderately reduced T_a

To evaluate left ventricular function, the pressure–volume conductance catheter technique was applied. Figure 3a shows a considerable decrease of myocardial contractility at 3 h after LPS administration in mice kept at 30 °C. SIRS-induced myocardial depression reached a maximum on day 1 after LPS administration and was accompanied by corresponding changes in hemodynamic variables (Supplemental Table 4). In line with this, phosphorylation of the ryanodine receptor (Ryr) and of phospholamban (PLB) was markedly reduced, indicating a decreased myocardial excitation–contraction coupling (Fig. 3b). Of note, myocardial depression and reduction of Ryr and PLB phosphorylation were more pronounced in mice kept at 26 °C compared to 30 °C. Moreover, impaired cardiac performance and accompanying hemodynamic consequences persisted throughout the whole observation period in mice kept under moderately reduced T_a, whereas cardiovascular functions recovered completely in those kept under neutral T_a. A reduction in T_a to 22 °C led to a decrease in Ryr and PLB phosphorylation, similar to that seen at 26 °C (Supplemental Figure S3A).

Manifestation of SIRS at moderately reduced T_a provokes suppression of adrenergic signalling and aggravates the myocardial inflammatory response

We studied β-adrenergic signaling and inflammatory responses in heart tissue, to understand the mechanisms leading to reduced myocardial performance and enduring



		Baseline	LPS 1d	LPS 3d	LPS 7d
Number of hearts with damage	NEUTRAL	0	0	0	0
	COOL	0	0	2	5 §
Number of damaged areas per heart section	NEUTRAL	0	0	0	0
	COOL	0	0	1-2	4-7 §

◂**Fig. 4** Gene expression and protein levels of key mediators of adrenergic signaling and associated disturbances of structural integrity in the heart. Mice were kept under neutral (open boxplots, NEUTRAL) or moderately reduced T_a (filled boxplots, COOL) before and after LPS-evoked shock. **a** Reduced gene expression of β_1- and β_2-adrenergic receptors (β_1-AR, β_2-AR), as well as enhanced gene expression of β-arrestin 2 in heart tissue obtained from mice kept under moderately reduced T_a after LPS-evoked shock, compared with mice kept under neutral T_a at 24 h after LPS administration (data are normalized with respective data obtained from untreated animals). **b**, **c** Temporal pattern of β_1-AR and β_2-AR protein content in heart tissue obtained from mice kept under neutral and moderately reduced T_a after LPS-evoked shock. Representative Western blots and densitometry analysis are shown. A reduction of both β_1-AR and β_2-AR appeared at baseline and after LPS. A temporary reduction by LPS can be observed for β_1-AR in both groups and for β_2-AR only for the NEUTRAL group. **d** Upregulation of G protein-coupled receptor kinase 2 (GRK2) in heart tissue obtained from mice kept under moderately reduced T_a after LPS-evoked shock, indicates enhanced β_1-AR and β_2-AR internalization as well as disturbed structural integrity. **e** Reduced AKT activation in heart tissue obtained from mice kept under reduced T_a. **f** Increased HSP90 protein content in heart tissue obtained from mice kept under reduced T_a under baseline conditions and further enhanced in LPS-evoked shock. **d**–**f** Representative Western blots and densitometry analysis are shown. **g** Histologic appearance of contraction band necrosis (white arrows) in heart tissue obtained from mice kept under moderately reduced T_a, at later time periods after LPS-evoked shock (HE staining, magnification × 20, bar 50 µm). Values are presented as boxplots illustrating medians within boxes from first quartile (25th percentile) to the third quartile (75th percentile) and whiskers ranging from the 10th to the 90th percentiles. **a** $n=3$ per group. **b** $n=7$; **c**, **d** $n=5$; **e** $n=4$; **f** $n=4$; **g** $n=7$; per group and time point, each. Top panel: representative Western blots, bottom panel: densitometric quantification. $^{*\S}p<0.05$, *significant difference between baseline (BL) and LPS-stimulated state within each group, §significant differences versus mice kept under neutral T_a at the same experimental state (**a** *t* test was used for comparisons between both groups, **b**–**f** two-way ANOVA, followed by Holm–Sidak test for post hoc multiple comparisons, **g** Exact Fisher test). Densitometric data represent the relative amounts of the indicated proteins in relation to the loading control vinculin

myocardial depression in LPS-evoked shock in mice kept at 26 °C, despite an elevated sympathetic input. The evaluation of β-adrenergic receptor (β-AR) expression revealed that β_1- and β_2-AR, the major isoforms in myocardial cells, were downregulated in hearts obtained from mice kept at 26 °C (Fig. 4a). A T_a reduction to 22 °C prompted a further decrease in the myocardial expression of these receptors (Supplemental Figure S3B). β-Arrestin 2 and G protein-coupled receptor kinase 2 (GRK2) were upregulated in heart tissues of these mice, suggesting an enhanced desensitization/internalization of β-ARs induced by moderate reduction of T_a. In addition, AKT activation was reduced and heat shock protein 90 (HSP90) expression was increased under baseline conditions in myocardium obtained from mice kept at 26 °C. HSP90 alteration was aggravated when these mice were challenged with LPS (Fig. 4b–f). In line with this, myocardial tissue from mice kept at reduced T_a exhibited an enhanced apoptosis rate throughout the observation period, compared to tissues from those kept at 30 °C. This was evidenced by positive TUNEL staining and increased content of cleaved Poly (ADP-ribose) polymerase (PARP) (Fig. 6b, c, Supplemental Figure S4A). A disturbed structural integrity at later time periods of LPS-evoked shock was confirmed by the appearance of contraction band necrosis in heart tissue obtained from mice kept at 26 °C (Fig. 4g).

Since compromised myocardial performance is known to be associated with inflammatory processes, we also studied inflammatory variables in the heart tissue. A profound up-regulation of iNOS expression and a corresponding occurrence of nitrotyrosine formation were found in heart tissues of mice kept at 26 °C, a phenomenon which further increased when mice were kept at 22 °C (Fig. 5b, c, Supplemental Figure S4A). This was driven by enhanced abundance of the transcription factor HIF-1α in hearts of these mice as a result of LPS-evoked shock (Fig. 5a). Cell culture studies on primary cardiomyocytes verified that temperature lowering as well as norepinephrine administration led to a HIF-1α accumulation already after 24 h (Supplemental Figure S5). Furthermore, a massive leukocyte invasion occurred at later times of LPS-evoked shock in hearts from mice kept under moderately reduced T_a (Fig. 6d) confirming an inflammatory response. Notably, mice kept at moderately reduced T_a showed a mild increase of autophagy in heart tissue under baseline conditions, which was aggravated by LPS-evoked shock as indicated by an increased conjugation of the autophagy-related protein LC3B (Fig. 6a), suggesting the induction of adaptive stress responses.

Pharmacological blockade of β_2-AR and iNOS ameliorated mortality and myocardial function at reduced T_a

A pharmacological approach was applied to verify the relevance of altered sympathetic input and an enhanced inflammatory response in mice kept under moderately reduced T_a. As illustrated in Fig. 7, combined inhibition of β_2-AR and iNOS pathways improved survival as well as clinical course and ameliorated myocardial function (Tables 3, 4). Heart tissue analyses revealed that the treatment with β_2-AR antagonist and iNOS inhibitor provoked an almost complete rescue of LPS-triggered reduction of Ryr and PLB phosphorylation and myocardial β_1- and β_2-AR downregulation. In addition, the increase in iNOS expression was reduced and the increase in nitrotyrosine formation and PARP cleavage was almost completely prevented (Fig. 8).

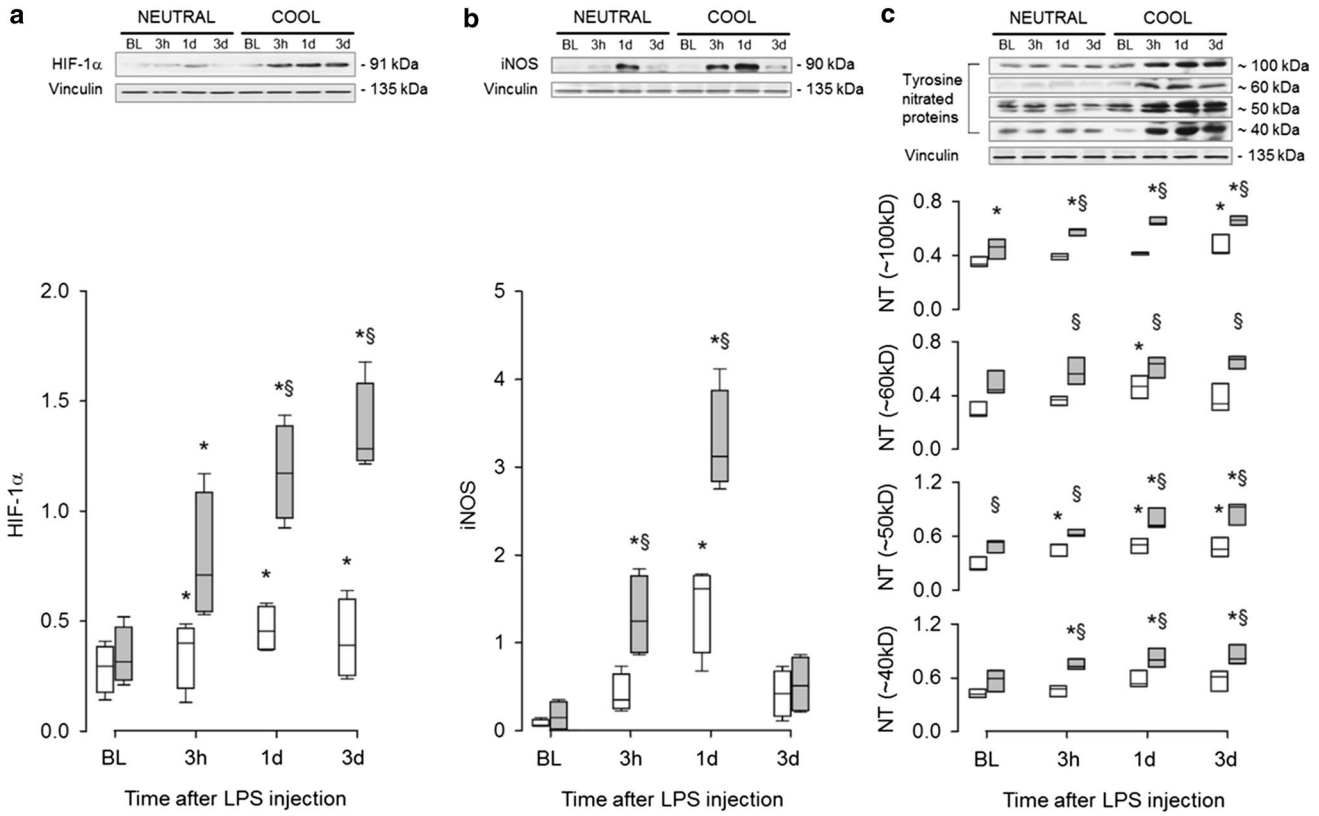


Fig. 5 HIF-1α, iNOS and nitrotyrosine levels in heart tissue. HIF-1α, iNOS and nitrotyrosine are upregulated in heart tissue from mice kept under moderately reduced T_a (filled boxplots, COOL), partly at baseline and always after LPS-evoked shock. **a**, **b** Marked upregulation of HIF-1α and iNOS in heart tissue of LPS-treated mice kept under moderately reduced T_a compared with iNOS protein content in heart tissue of LPS-treated mice kept under neutral T_a (open boxplots, NEUTRAL). **c** Increased nitrotyrosine formation in heart tissue from mice kept under moderately reduced T_a. Representative Western blots and densitometry analysis are shown. Densitometric values are presented as boxplots illustrating medians within boxes from first quartile to the third quartile and whiskers ranging from the 10th to the 90th percentiles ($n=4$ at each group and time point). *§$p<0.05$, *significant difference between baseline (BL) and LPS-stimulated state within each group, §significant differences versus mice kept under neutral T_a at the same experimental state (two-way ANOVA, followed by Holm–Sidak test for post hoc multiple comparisons, each). Densitometric data represent the relative amounts of the indicated proteins in relation to the loading control vinculin

Discussion

Our study identifies ambient temperature as a major factor for the extent and clinical course of LPS-evoked shock and concomitant myocardial depression in mice. We studied LPS-evoked shock at the thermoneutral zone (T_a of 30 °C) [26] and at the upper and lower edge of the recommended standard housing temperatures for laboratory mice, e.g. at a T_a of 26 °C and 22 °C [16, 72]. Importantly, recent guidelines for preclinical studies in cardiovascular research [9, 43, 55], with the emphasis to improve reproducibility and translational impact did not consider that their recommended baseline housing temperatures for small rodents cause chronic 'cold' stress [32, 41]. We show for the first time that a reduction in T_a of only 3°–4° below the thermoneutral zone for mice [27] increased the mortality rate from SIRS by about 40% and worsened the clinical course of the surviving animals. Moreover, induction of SIRS at 22 °C, the lower range of recommended standard housing temperature for laboratory mice, led to an extreme mortality (Fig. 1). Similar outcomes have been described in previous experiments performed under analogous conditions [14]. ECG analysis revealed a progressive sinus bradycardia in the last minutes before death (as compared to heart rates in the last hours before death), which ended in asystole in all animals (Supplemental Figure S6). Therefore, our data clearly show that the clinical course and the disease outcome of severe inflammatory challenges such as LPS-evoked shock are substantially influenced by responses to the respective ambient temperature.

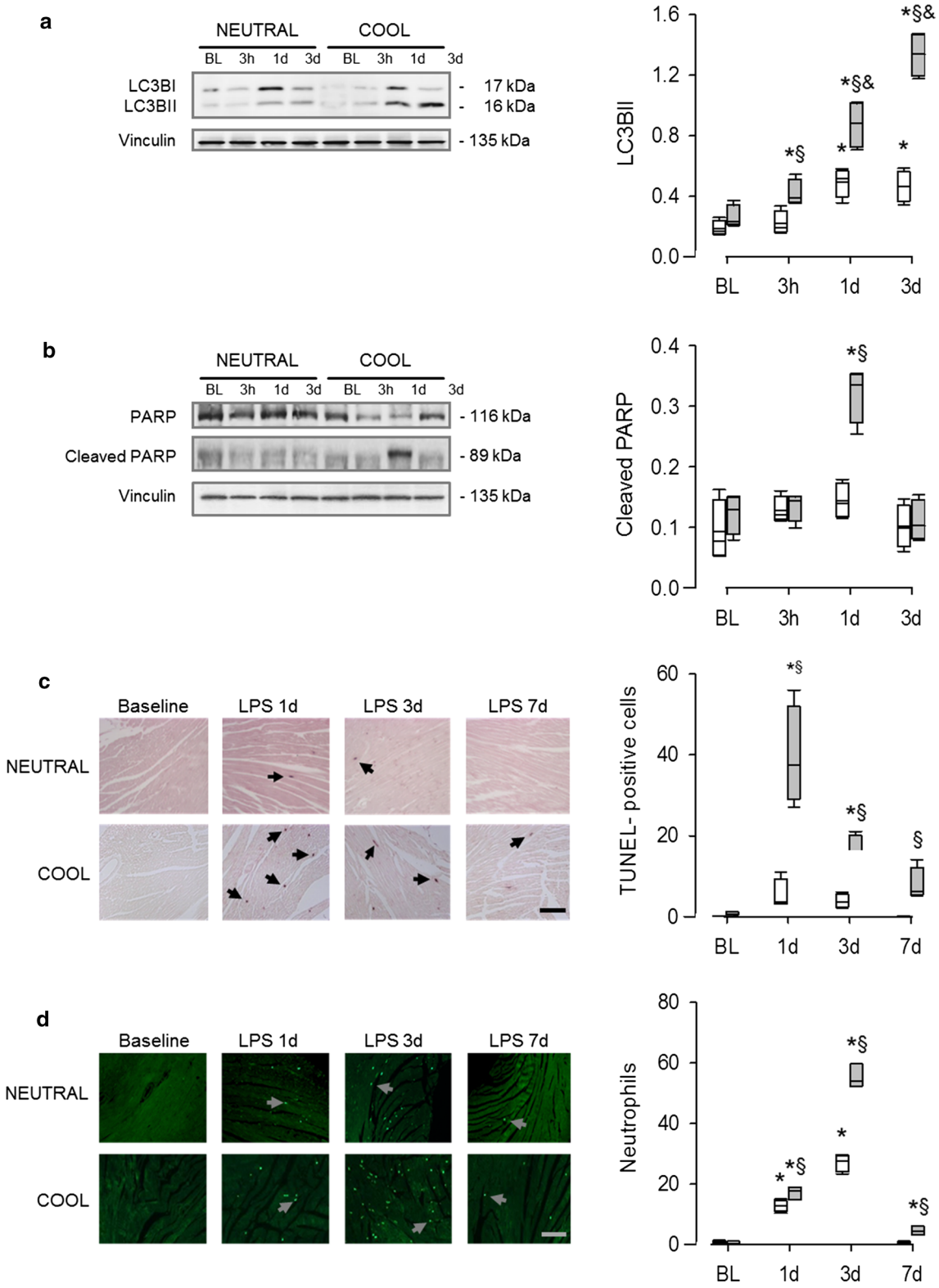
To verify mechanisms responsible for the effects of T_a lowering on the outcome of LPS-evoked shock, we compared systemic and cardiac functions in surviving mice kept at thermoneutral or moderately reduced T_a. Directed telemetric analysis of freely moving mice clearly revealed that a mild reduction of T_a to 26 °C and the resulting cold

adaptation triggered an autonomic nervous system readjustment. Cold exposure stimulates transient receptor potential (TRP) channels in peripheral sensory neurons, thereby initiating a central activation of the sympathetic nervous system (SNS). This generates increased baseline heart rate and altered HRV indices, indicating a sustained sympathetic overstimulation of the heart. At thermoneutral temperatures, i.e., the range of T_a at which energy expenditure to maintain body temperature is lowest [26, 93], vagal tone dominates autonomic control of mouse heart function. When T_a decreases below the thermoneutral zone, a rise in the metabolic rate by adaptive heat production in brown adipose tissue (BAT) and by diminished heat emission due to reduced skin blood flow is induced via relatively selective sympathetic noradrenergic activation [24] to maintain stable T_c in the mice. In line with this, we found a gradual increase of UCP-1 expression in epididymal adipose tissue (Supplemental Figure S1), suggesting the development of a brown-like phenotype of white adipose tissue (WAT) in mice kept at reduced T_a. This finding indicates that a T_a of 26 °C triggers cold adaptation by sustained noradrenergic stimulation [46], even if WAT cell-autonomous effects of temperature itself can directly activate a thermogenic gene program [110]. Healthy mice are capable of controlling such a challenge and maintain their core temperature through an appropriate increase of their metabolism–energy demand is increased by about 50% at T_a of 22 °C compared with thermoneutral conditions [27]. Under disease conditions, however, reduced T_a considerably affects the outcome of disease, as shown here for cardiomyopathy induced by systemic inflammation. Thus, the environmental conditions ubiquitously used in mouse husbandry may compromise the utility of mice as disease models and merit consideration when interpreting experimental data [44].

LPS induced hypothermia (T_c reduction) in mice, whose extent and duration were clearly dependent on T_a (Figs. 1b, S2). Mice kept at moderately reduced T_a developed an exacerbated and prolonged hypothermia, although they exhibited increased heart rate and altered HRV, suggesting sympathovagal imbalance. This might be related to a stronger impairment of thermoregulation, with torpor-like traits induced by LPS-evoked shock [94] in addition to LPS-induced inhibition of brown adipose tissue thermogenesis [73]. Our data are in apparent contradiction of the general opinion that mild hypothermia is cardioprotective [12, 100], which is mainly based on observations of decreased cardiac infarct size/cardiomyocyte injury and attenuation of the myocardial ischemia/reperfusion injury [19, 74] under hypothermia. Nevertheless, in contrast to sickness-related spontaneous T_c lowering, the artificially induced mild therapeutic hypothermia is applied with sedation and/or anesthesia and probably paralysis, which prevents increase of SNS activity and attenuates thermoregulatory responses [79] that otherwise would compromise protective effects of therapeutic hypothermia [97, 101]. Protective mechanisms of hypothermia share characteristics of ischemic preconditioning [34] including ATP pool conservation [68] and mitochondrial permeability stabilization [39, 99], as well as alleviation of excess ROS formation [98] and include the same protective signal transduction pathways—such as the reperfusion injury salvage kinase (RISK) [67, 109] and survivor activating factor enhancement (SAFE) [40] pathways. Under conditions of severe systemic inflammation, however, the SNS-driven thermoregulatory mechanisms override cardioprotective effects of hypothermia with detrimental consequences for myocardial function. Intriguingly, the extent of hypothermia appears not to be directly responsible for increased mortality seen in mice kept at moderately reduced T_a as these mice died after T_c had been recovered (Fig. 1a, b). Rather, the combined consequences of cold adaptation and LPS-induced myocardial inflammation are responsible for exacerbated myocardial depression, since combined treatment with a β_2-AR antagonist and an iNOS inhibitor led to a restitution of myocardial functions. In these experiments, the β_2-AR antagonist and the iNOS inhibitor were administered at different time points before or after LPS injection because elevation of the catecholamines with subsequent β_2-AR downregulation appeared to be an early response to LPS (Figs. 2c, 4a, c); while clear iNOS elevation and associated nitrotyrosine formation in heart tissue appeared to be a consequence of LPS-evoked shock (Fig. 5b). It was noted that single treatment with the β_2-AR antagonist or the iNOS inhibitor did not lead to a significant reversal of mortality and myocardial function (data not shown).

We have shown in a previous study that a complete reversibility of myocardial depression after LPS-evoked shock resulted from PI3Kγ-mediated control of the overstimulated β-adrenergic input, via supporting cAMP degradation and suppression of iNOS-mediated myocardial proinflammatory response [65]. These studies were performed under thermoneutral T_a of 30 °C. The data obtained in the current study suggest that the aggravated myocardial depression observed in mice kept at 26 °C during LPS-evoked shock results from a combination of suppressed adrenergic signaling and an intensified LPS-induced proinflammatory response.

Albeit not verified in this study, sustained microcirculatory disturbances [31, 59] and impaired myocardial oxygen utilization could have contributed to aggravated myocardial depression in response to LPS at moderately lowered T_a, while macrocirculatory coronary blood flow is not likely to be compromised during SIMD [18]. Nevertheless, it has been shown that LPS-induced impaired myocardial performance is accompanied by preserved myocardial high-energy phosphates [ATP, creatine phosphate (CrP)] [104], pointing toward an adaptive myocardial depression at least early after onset of SIRS-induced SIMD, reflecting short-term



myocardial hibernation in a non-ischemic disease state [52], possibly mediated by reduced calcium responsiveness [37]. Furthermore, the sustained myocardial depression in surviving animals treated with LPS at reduced T_a in our study goes along with upregulation of proinflammatory mediators (e.g,. β-AR signaling, iNOS and HIF-1α) with ambiguous effects on myocardial surviving pathways [37], as discussed below. In addition, LPS-induced myocardial

◂**Fig. 6** Enhanced activities of autophagy and apoptosis as well as leukocyte invasion in heart tissue of mice kept under moderately reduced T_a. **a** Increased LC3BII content indicative for enhanced autophagy activity. **b**, **c** Increased content of cleaved PARP and elevated apoptosis rate assessed by TUNEL staining (black arrows), in heart tissue from mice kept under moderately reduced T_a (filled boxplots, COOL; open boxplots, NEUTRAL). **d** Increased number of invading leukocytes (gray arrows) into heart tissue obtained from mice kept under moderately reduced T_a after LPS administration. **a**, **b** Representative Western blots and densitometry analysis are shown. Values are presented as boxplots illustrating medians within boxes from first quartile to the third quartile and whiskers ranging from the 10th to the 90th percentiles (**a**–**d**: $n=4$, at each group and time point. $^{*\S\&}p<0.05$, *significant difference between baseline (BL) and LPS-stimulated state within each group, §significant differences versus mice kept under neutral T_a at the same experimental state, $^{\&}$indicates significant differences versus 3 h within each group, two-way ANOVA, followed by Holm–Sidak test for post hoc multiple comparisons, each. In **c**, **d** magnification × 20, bars 200 µm). Densitometric data represent the relative amounts of the indicated proteins in relation to the loading control vinculin

depression under moderately reduced T_a occurred despite increased myocardial catecholamine concentrations and altered HRV, suggesting a shift of sympathovagal balance toward a sympathetic predominance [1]. Hemodynamic parameters, as well as myocardial systolic and diastolic contractile parameters, remained impaired throughout the observation period, and under these conditions, a markedly attenuated cardiac excitation–contraction coupling was observed indicated by diminished PLB phosphorylation [6]. Apparently, a sustained autonomic nervous system alteration with elevated myocardial catecholamine release in mice due to cold adaptation, which was markedly enhanced during the early period of LPS-evoked shock, was responsible for aggravated myocardial depression. The verified suppression of adrenergic signaling by distinct reduction of β_1- and β_2-AR expression may in part be due to increased GRK2 and β-arrestin 2-mediated desensitization and internalization of the receptors [51, 82]. However, since mRNA levels were also reduced, other mechanisms must also contribute. This process may also contribute to the increased mortality rate and severity of disease in mice kept under reduced T_a. In line with this, early partial inhibition by β_2-AR blockade (together with iNOS inhibition) ameliorated myocardial function and reduced the degree of sickness. Unlike β_1- and β_2-ARs, β_3-ARs are stimulated at high catecholamine levels, and thus may contribute more significantly to βAR signaling in heart pathologies linked to excess of catecholamines [42], such as LPS-mediated SIMD. β_3-AR stimulates the cGMP/nNOS/nitric oxide (NO) pathway for preservation of cardiac function [102] and may additionally promote the sphingosine-1-phosphate (S1P)/S1P receptor 1 pathway for cardioprotection [11, 36].

In addition to suppressed β_1- and β_2-adrenergic signaling, a pronounced proinflammatory response—reflected by similar cytokine concentrations in cardiac tissue early after LPS administration—seems to contribute to enhanced myocardial depression at moderately reduced T_a. Heart tissue from mice kept at 26 °C showed an enhanced iNOS expression and a resulting increase in nitrotyrosine formation during LPS-evoked shock. The latter points to higher levels of peroxynitrite, which may decrease β-AR-stimulated myocardial contraction by promoting PLB dephosphorylation through protein phosphatase 2A activation [48]. These results confirm our and other reports on LPS-induced myocardial iNOS upregulation [22, 65, 92] by signaling cascades, including β_1-AR stimulation and leading to nuclear factor-κB (NF-κB) activation [7, 84]. In addition, early after onset of LPS-evoked shock, myocardial eNOS, which participates in improvement of myocardial contractility under physiological conditions [4], may add to increased NO formation [15, 105] and myocardial dysfunction [103]. Increased eNOS activity has been observed in an ovine model of sepsis [49].

Enhanced iNOS expression may be triggered by a synergistic induction of HIF-1α transcriptional activity by T_a reduction and LPS [8, 63]. HIF-1α is known to mediate inflammatory responses in addition to its role as master regulator of transcriptional responses to hypoxia [3, 86] and contributes to the complex cardioprotective signal transduction program [21, 34, 60], considering ambiguous interactions with adenosine [33, 34, 87]. Here, we show for the first time that HIF-1α is upregulated in cultured cardiomyocytes and in the myocardium by non-hypoxic signals, including thermoadaptation and inflammatory mediators [62, 78], with detrimental effects on myocardial functions. Our findings extend previous data showing that HIF-1α upregulation in bone marrow-derived macrophages contributes to cytokine activation, disease symptoms and lethality of LPS-induced sepsis [62, 78].

The importance of iNOS upregulation was confirmed by the protective effects of iNOS inhibition, which contributes (together with β_2-AR blockade) to prevention of mortality and myocardial dysfunction at reduced T_a. The pronounced proinflammatory response induced by SIRS under moderately reduced T_a was underlined by enhanced PMN invasion, although the reason for increased PMN homing into cardiac tissue is hitherto uncertain. Of note, myocardial cytokine release was similar in mice kept under neutral and moderately reduced T_a.

LPS-evoked shock resulted in stress responses and structural damage in heart tissue obtained from mice kept at moderately reduced T_a. We found that autophagic activity, which was already increased under basal conditions in hearts of mice kept at 26 °C, was further increased after LPS administration. Previous studies reported an increase of cardiac autophagy in response to long-lasting cold exposure ($T_a=4$ °C) associated with myocardial

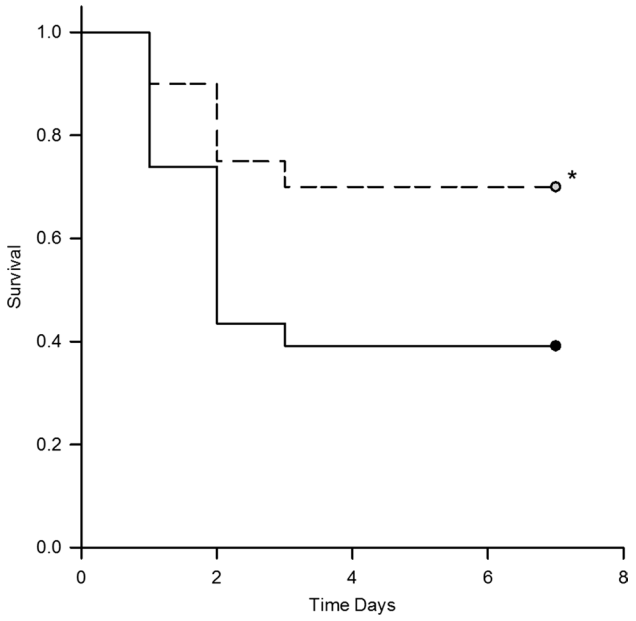


Fig. 7 Effects of combined β_2-AR antagonism and iNOS inhibition on survival from LPS-evoked shock in mice kept at reduced ambient temperature of 26 °C. Animals were adapted to an ambient temperature of 26 °C for ≥ 5 days before injection. For SIRS induction LPS (10 µg/gbw) was injected intraperitoneally. Survival analysis. Mortality was gradually reduced after combined β_2-AR antagonism (ICI 118551 hydrochloride, 10 ng/g b.w. administered 12 h before and together with LPS injection, i.p.) and iNOS inhibition (1400 W, 5 µg/g b.w. administered 24 h after LPS injection, i.p., dashed line, $n = 20$) in mice kept at moderately reduced T_a compared to untreated mice kept at moderately reduced T_a ($n = 20$, solid line, same cohort as shown in Fig. 1a). *$p < 0.05$, *significant difference versus untreated mice kept at moderately reduced T_a, Kaplan–Meier survival analysis; log-rank test followed by Holm–Sidak test for post hoc multiple comparisons was performed

hypertrophy [54, 58]. Our findings indicate that even a mild drop of the neutral T_a threshold provokes cardiomyocyte stress response. This may be related to the sustained increased heart rate and altered HRV, suggesting sympathovagal imbalance and β_2-AR downregulation, which may attenuate β_2-AR-dependent repression of proteolysis [112]. Also, activation of myocardial autophagy by LPS-evoked shock as well as by septic cardiomyopathy has been reported previously [38, 53]. However, the pathophysiological role of increased autophagy remains elusive; autophagy may be either protective or detrimental in the myocardium, depending on the respective disease conditions [29]. Under physiological conditions or in response to mild stress, autophagy provides cellular quality control to promote survival and is therefore seen as an adaptive process in cardiomyocytes [69]. Under severe or chronic stress, excessive or inadequate autophagy may cause massive self-degradation, which is maladaptive and may eventually provoke cell death [30, 70, 71].

Table 3 Clinical severity score of mice kept at moderately reduced ambient temperature and treated with β_2-AR antagonist and iNOS inhibitor

	Without medication	Combined treatment with β_2/iNOS inhibitor
Baseline	1.0 (1.0; 1.0)	1.0 (1.0; 1.0)
LPS-6 h	2.0 (2.0; 3.0)*	2.0 (2.0; 3.0)*
LPS-24 h	4.0 (3.0; 4.0)*§	2.0 (2.0; 3.0)*
LPS-2d	4.0 (3.0; 4.0)*§	2.0 (1.5; 2.0)*
LPS-3d	3.0 (2.0; 3.25)*§	1.0 (1.0; 1.0)
LPS-4d	2.0 (1.75; 3.0)*§	1.0 (1.0; 1.0)
LPS-5d	2.0 (1.0; 2.25)*§	1.0 (1.0; 1.0)
LPS-6d	2.0 (1.0; 2.0)*§	1.0 (1.0; 1.0)
LPS-7d	1.5 (1.0; 2.0)	1.0 (1.0; 1.0)

Animals, $n = 20$, combined treated with the β_2-AR antagonist (ICI 118551 hydrochloride, 10 ng/gbw administered 12 h before and together with LPS injection, i.p.) and the iNOS- inhibitor (1400 W, 5 µg/gbw administered 24 h after LPS injection, i.p.) displayed a markedly improved and accelerated recovery from sickness symptoms after LPS injection until end of the observation period. Mice without medication: $n = 20$. Clinical status was assessed according to [25] with the additional score value 5: death. Data are given as medians as well as the first quartile and third quartile in parenthesis. *§$p < 0.05$, *significant difference versus baseline, §significant difference to animals treated with β_2- and iNOS inhibitors; two-way repeated measures ANOVA, followed by Holm–Sidak test for post hoc multiple comparisons was performed

Our data indicate that cellular stress persists in the heart of mouse survivors kept at 26 °C. Obviously, sarcomere turnover was facilitated as indicated by markedly increased chaperone activity and increased autophagy [108]. Parameters of an increased apoptosis in heart tissue of mice kept at 26 °C during LPS-evoked shock, such as positive TUNEL staining and PARP cleavage, were also seen (Fig. 6). This may be related to the marked GRK2 and HSP90 upregulation, indicating enhanced activation of stress-induced mitochondrial-dependent pro-apoptotic signalling [13]. AKT activation, an important survival signal in cardioprotection [35], was reduced, possibly mediated by cytosolic GRK2 or as a consequence of sustained β_2-AR downregulation. Cardiac apoptosis is known to be initiated in response to several harmful stimuli, including reactive oxygen species and catecholamines [47], leading to stimulation of β-adrenoceptors [77].

In conclusion, our results clearly underline the importance of ambient temperature as a frequently neglected environmental condition in mouse studies of inflammatory/infectious disease. The major significance of the data herein presented is that—despite preadaptation on discrete ambient climate conditions—a moderate variation of an easily controllable variable, i.e., ambient temperature, had a serious impact on the clinical course of LPS-induced SIMD and the survival of mice—variables which are

Table 4 Hemodynamic parameters and indices of systolic and diastolic function of mice kept at reduced ambient temperature and treated with β_2- and iNOS inhibitors

	Baseline	without medication	Combined treatment with β_2/iNOS-inhibitor
Mean arterial blood pressure (mmHg)	75.6 (74.9; 78.0)	73.7 (72.0; 77.7)	82.6 (80.7; 87.1)
Cardiac output (ml min^{-1})	16.5 (15.0; 17.5)	11.1 (9.0; 12.2)*	13.8 (13.5; 14.5)
Ejection fraction (%)	70.0 (58.7; 71.6)	45.5 (44.6; 45.8)*	63.7 (57.7; 66.3)§
Tau (ms)	6.53 (6.23; 7.16)	13.11 (10.98; 13.43)*	8.5 (7.1; 9.1)§
Ees (mmHg μl^{-1})	3.71 (3.52; 474)	2.47 (2.34; 2.55)*	4.5 (4.4; 4.6)§
Heart rate (min^{-1})	534 (510; 582)	403 (366; 439)*	416 (403; 452)*

Values are given as medians as well as the first quartile and third quartile in parenthesis, $n = 5$–6 per treatment groups. *$^{\S}p < 0.05$, *significant difference between baseline and values at day 7 after LPS-induced SIRS within each group, §significant differences versus mice without medication

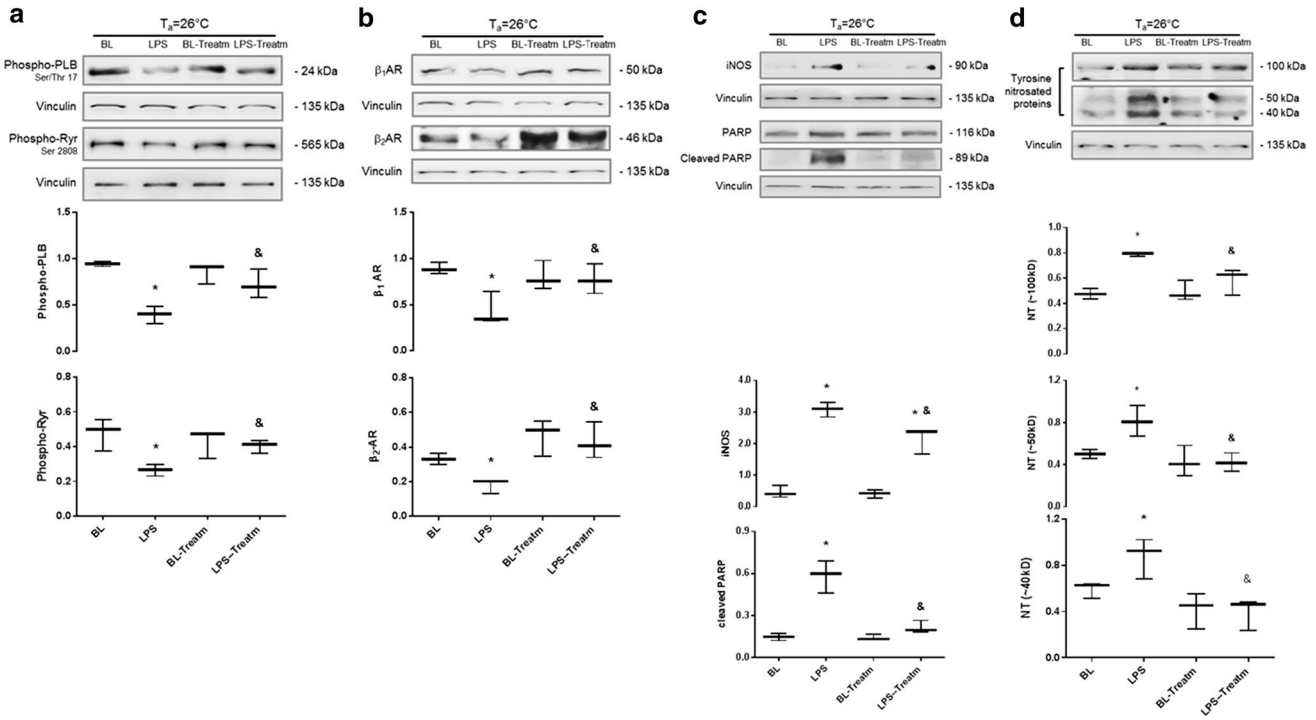


Fig. 8 Effects of combined β_2-AR antagonism and iNOS inhibition (+ Treatm) on sarcoplasmic reticulum calcium signaling (ryanodine receptor (Ryr) and phospholamban (PLB) phosphorylation) as well as iNOS, cleaved PARP and nitrotyrosine levels in heart tissue extracts from mice kept under moderately reduced T_a at baseline (BL) and 24 h after LPS-evoked shock (LPS). Representative Western blots and densitometry analysis are shown. Note an almost complete prevention of **a** reduced Ryr and PLB phosphorylation and **b** reduced myocardial β_1-AR and β_2-AR expression resulting from LPS-evoked shock. Furthermore, a reduction in **c** SIRS-induced iNOS expression and PARP cleavage and **d** nitrotyrosine formation occurred after treatment with β_2-AR antagonist and iNOS inhibitor. Values are presented as medians and first as well as third quartile (**a**–**d** $n = 3$, at each group and time point). *$^{\&}p < 0.05$, *significant difference between baseline (BL), $^{\&}$significant difference versus LPS, two-way ANOVA, followed by Holm–Sidak test for post hoc multiple comparisons. Densitometric data represent the relative amounts of the indicated proteins in relation to the loading control vinculin

usually used as endpoints in translational animal research. Our findings challenge the significance of previous studies which were performed at room temperature, or where information on the prevalent temperatures was not given. Further, our findings clearly demonstrate that enhanced endogenous adaptive thermoregulatory mechanisms are responsible for prolonged and exacerbated myocardial disturbances, in response to systemic inflammation outside the thermoneutral range of T_a. Consequently, therapeutic interventions aimed at maintaining thermoneutral ambient temperatures may improve outcome of inflammatory and infectious diseases and open translational avenues.

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Compliance with ethical standards.

Conflict of interest The authors declare that they have no competing interest.

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