Background: While mitochondria play an important role in innate immunity, the relationship between mitochondrial dysfunction and inflammation in heart failure (HF) is poorly understood. In this study we aimed to investigate the mechanistic link between mitochondrial dysfunction and inflammatory activation in peripheral blood mononuclear cells (PBMCs), and the potential anti-inflammatory effect of boosting NAD level.

Methods: We compared the PBMC mitochondrial respiration of 19 hospitalized Stage D HF patients with 19 healthy participants. We then created an in vitro model of sterile inflammation by treating healthy PBMC with MitoDAMP (Mitochondrial Damage-Associated Molecular Patterns) isolated from human heart tissue. Lastly, we enrolled Stage D HF patients and sampled their blood before and after taking 5-9 days of oral nicotinamide riboside, an NAD precursor.

Results: We demonstrated that HF is associated with both reduced respiratory capacity and elevated proinflammatory cytokine gene expressions. In our in vitro model, MitoDAMP-treated PBMCs secreted IL-6 that impaired mitochondrial respiration by reducing Complex I activity. Last, oral NR administration enhanced PBMC respiration and reduced proinflammatory cytokine gene expression in 4 HF subjects.

Conclusion: These findings suggest that systemic inflammation in HF patients is causally linked to mitochondrial function of the PBMC. Increasing NAD levels […]
**Title.** Boosting NAD Level Suppresses Inflammatory Activation of PBMCs in Heart Failure

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The authors have declared that no conflict of interest exists.

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Abstract

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Methods:
We compared the PBMC mitochondrial respiration of 19 hospitalized Stage D HF patients with 19 healthy participants. We then created an in vitro model of sterile inflammation by treating healthy PBMCs with MitoDAMP (Mitochondrial Damage-Associated Molecular Patterns) isolated from human heart tissue. Lastly, we enrolled Stage D HF patients and sampled their blood before and after taking 5-9 days of oral nicotinamide riboside, an NAD precursor.

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We demonstrated that HF is associated with both reduced respiratory capacity and elevated proinflammatory cytokine gene expressions. In our in vitro model, MitoDAMP-treated PBMCs secreted IL6 that impaired mitochondrial respiration by reducing Complex I activity. Lastly, oral NR administration enhanced PBMC respiration and reduced proinflammatory cytokine gene expression in 4 HF subjects.

Conclusion:
These findings suggest that systemic inflammation in HF patients is causally linked to mitochondrial function of the PBMCs. Increasing NAD levels may have the potential to improve mitochondrial respiration and attenuate proinflammatory activation of PBMCs in HF.

Funding
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Introduction

Worldwide, heart failure (HF) is among the top causes of morbidity and mortality. In the United States alone, annual direct medical expenditures for heart failure exceed $30 billion (1-3). It has long been postulated that a chronic sterile inflammatory state in heart failure may be involved in disease progression and may contribute to worse clinical outcomes (4, 5). While proinflammatory cytokines such as TNFA, IL1, IL6, and IL18 have been demonstrated to have a negative-inotropic effects in various experimental models (6), results of anti-TNFA clinical trials in HF were discouraging (7, 8). More recently, the CANTOS Trial of anti-IL1B monoclonal antibody therapy in atherosclerosis showed promising results in the HF sub-group analysis (9). These conflicting results likely reflect the complexity and intricacy of cytokine-mediated mechanisms of HF. A better understanding of the molecular mechanisms by which peripheral monocytes process and respond to proinflammatory signals is imperative to the development of targeted novel therapeutics for HF.

Mitochondrial dysfunction contributes to the development of heart failure via multiple mechanisms (10). Damage-associated molecular pattern (DAMP) released from mitochondria has been implicated to activate the Toll-like receptors (TLRs)/NFκB axis of peripheral monocytes in cardiac injury, leading to cytokine production and systemic inflammation (4, 5, 11). Furthermore, production of reactive oxygen species (ROS) by mitochondria has been proposed as a critical step in the activation of NLRP3 inflammasome and secretion of proinflammatory cytokines (12, 13). To date, it has not been determined whether mitochondrial dysfunction of circulating immune cells contributes to the heightened inflammatory state in heart failure patients.
Recently, nicotinamide riboside (NR), a precursor in the NAD “salvage” pathway (14), has been demonstrated to enhance respiratory function, reduce mitochondrial ROS (mtROS) production, and reduce IL1B production in peripheral blood mononuclear cells (PBMCs) (15). In addition, NR was shown to reduce the production of TNFA and IL6 and upregulate anti-inflammatory molecule, adiponectin, in mouse hepatocytes (16) and reduce circulating of IL5 and IL6 in healthy elderly subjects (17). Lastly, systemic NAD repletion by NR or NMN has been shown to be cardioprotective in multiple murine models of cardiomyopathy (18-20). These observations raise the possibility that targeting NAD metabolism might benefit HF patients by modulating the HF-associated inflammatory state.

To further explore this possibility we: 1) compared PBMC respiratory function and inflammatory cytokine expression of patients with HFrEF (Heart Failure with Reduced Ejection Fraction) and healthy participants, 2) investigated the mechanistic link between mitochondrial dysfunction and inflammatory activation in PBMCs, and 3) explored the possibility that targeting mitochondrial metabolism by increasing NAD level with NR might attenuate PBMC inflammatory activation in vitro and in patients with HFrEF.
Results

A. Baseline characteristics of study participants. A total of 19 Stage D heart failure (HF) patients and 19 healthy participants were recruited. As shown in Table 1, the HF subjects were predominantly male with a mean left ventricular ejection fraction (LVEF) of 20±7%, among which approximately 80% had non-ischemic etiology of cardiomyopathy, and 68% were on inotropic support when their blood samples were obtained. Mean age comparison between healthy and HF groups by Mann-Whitney test showed a P-value of 0.034.

B. PBMCs from HFrEF patients showed reduced respiratory capacity and elevated proinflammatory cytokine gene expression. Purified PBMCs from study participants were subjected to the standard Seahorse Mito Stress Test (Figure 1A) (21). Mean PBMC basal respiration (OCR) trended lower in HF than healthy subjects though not reaching statistical significance. (Figure 1B). The FCCP-induced maximal OCR was significantly lower in HF patients as compared to healthy participants (Figure 1C). Together, these findings suggest that PBMC respiratory capacity is impaired in HFrEF. Furthermore, consistent with previous reports that HFrEF is associated with a proinflammatory state, we found that mRNA levels of NLRP3, a key component of the inflammasome in monocytes and macrophages, as well as proinflammatory cytokines (IL1B, IL18, TNFA) were significantly higher in Stage D HFrEF patients as compared to healthy participants. IL6 showed a similar trend but did not reaching statistical significance (Figure 1D). Although HF subjects had slightly higher monocyte-to-lymphocyte ratio in the PBMCs (Supplemental Table 2), it unlikely accounted for the reduced maximal respiration or increased cytokine expression. It has been shown that basal and maximal OCR per μg protein are
comparable between healthy monocytes and lymphocytes (22) and monocytes are roughly 2 times larger in volume than lymphocytes (23, 24). Thus, a higher fraction of monocytes in PBMCs would increase rather than decrease OCR. Moreover, the IL1B, IL6, and TNFA productions in response to LPS simulation were shown comparable between monocytes and PBMCs (25).

C. Mitochondrial DAMP induces PBMC respiratory impairment and inflammatory cytokine gene expression, which can be attenuated by inhibition of the NLRP3 inflammasome axis. To explore the potential cellular mechanisms linking mitochondrial respiratory function and cytokine production, we sought to create in vitro models to mimic the proinflammatory state in HF. We first treated PBMCs from healthy participants with lipopolysaccharide (LPS), an endotoxin known to trigger proinflammatory activation in peripheral monocytes. 4-hours of LPS treatment resulted in a sharp decline in PBMC maximal OCR (Supplemental Figure 1A) as well as increases in mRNA expression for NLRP3 and proinflammatory cytokines (Supplemental Figure 1B), most notably, IL6.

Mitochondrial content, by virtue of its evolutionary origin, can elicit an immunogenic response independent of heart failure status (26). It was recently reported that plasma levels of mitochondrial DNA is elevated in heart failure patients (27). To test whether the release of DAMP from damaged mitochondria could elicit a sterile inflammatory state in HF, MitoDAMP was extracted by lysing mitochondria isolated from myocardial tissue of end-stage heart failure patients undergoing left ventricular assist device (LVAD) surgeries (Figure 2A). Similar to LPS, MitoDAMP treatment of healthy PBMCs induced a marked elevation of proinflammatory cytokine gene expressions (Figure 2B).
As shown in Figure 2C, MitoDAMP treatment resulted in a surge of mtROS production within 2 hours, which persisted for 6 hours. Mitochondrial maximal OCR was significantly impaired by MitoDAMP (Figure 2D). MtROS generation has been reported to be one of the key activators of the NLRP3 inflammasome (28), thus the role of NLRP3 inflammasome in connecting respiratory impairment and cytokine production was tested. Treating the PBMCs with MitoTempo, a nitroxide-based mitochondrial-specific ROS scavenger, in the presence of MitoDAMP, did not change maximal OCR (Figure 2D) but markedly attenuated cytokine gene expressions (Figure 2E). Moreover, treatment with MCC950, a specific inhibitor of the NLRP3 inflammasome, resulted in a similar attenuation of the MitoDAMP-induced surge on IL6 gene expression (Figures 2E), while maximal OCR was marginally improved (Figure 2F). These findings suggest that MitoDAMP-induced mtROS production and NLRP3 activation contributes to proinflammatory cytokine gene expression in PBMCs. However, inhibition of either mechanism alone appears to minimally affects maximal OCR, suggesting that mtROS generation is downstream of respiratory impairment.

D. Secreted IL6 impairs mitochondrial respiration by reducing Complex I activity. Among the cytokines studied in the in vitro experiments, increases in gene expression levels in response to proinflammatory stimuli were consistently highest for IL6. Further, MitoDAMP stimulation of PBMCs resulted in a surge of secreted IL6 protein within 4 hours (Figure 3A). We therefore hypothesized that the MitoDAMP-induced IL6 secretion mediated the observed mitochondrial respiratory impairment in an autocrine fashion. Concurrent treatment with LMT28, an inhibitor of IL6 receptor B (GP130), prevented the MitoDAMP-induced decline in maximal OCR (Figure 3B). Conversely, treating healthy PBMCs with recombinant IL6 resulted in a dose-dependent decline
of maximal OCR (Figure 3C). A significant reduction of maximal OCR by IL6 was observed at concentrations as low as 0.1 ng/mL, which is comparable to the plasma concentrations of IL6 during acute cardiac decompensation (29), as well as with the level of secreted IL6 achieved at 4 hours following Mito-DAMP stimulation in our *in vitro* experiments (Figure 3A). Consistently, treatment of IL6 decreased the maximal OCR of monocytes isolated from healthy subjects (Supplemental Figure 3). In contrast, recombinant IL1B and IL18 treatments did not result in a significant decrease in PBMC maximal OCR (Supplemental Figure 4). Together, these results suggest that autocrine activation of IL6 is a key mediator of MitoDAMP-induced respiratory impairment.

Next, we searched for the end-effector of IL6-induced reduction of maximal OCR. To this end, we compared changes in OCR in response to various electron transport chain (ETC) complex inhibitors in sequence in order to identify those complex(es) whose activity was affected by IL6 treatment. As shown in Figure 3D, complex I inhibition by rotenone virtually eliminated all FCCP-induced OCR such that subsequent inhibition of Complex III by Antimycin A only resulted in minimal OCR reduction, suggesting Complex II was a minor contributor to the overall ETC activity in our system. Moreover, when TMPD and ascorbate were added to the system as electron donors for cytochrome C, which drives OCR through Complex IV, we observed no difference in OCR between vehicle- and IL6-treated PBMCs. These results suggest that IL6 affected function of Complex I function, but not of Complexes II or IV. The contributions of each Complex to the OCR in our assay system is summarized in Figure 3E. Consistent with these findings, direct measurement of Complex I activity showed that IL6 treatment reduced Complex I enzymatic
activity (Figure 3F). Together, these results suggest that IL6 reduces mitochondrial respiration via reduction of Complex I activity.

E. Increasing NAD- levels with Nicotinamide riboside (NR) suppresses proinflammatory response in PBMCs in vitro. Based on the recent observations that NAD-augmentation by NR enhances respiratory function and reduces mtROS and IL1B productions in PBMCs (15), we hypothesized that NR may ameliorate the reductions in maximal OCR and cytokine production seen with MitoDAMP stimulation. As shown in Figures 4A and 4B, concurrent NR treatment of healthy PBMCs in the presence of MitoDAMP resulted in a reduction of IL6 secretion and proinflammatory cytokine gene expressions, respectively. Importantly, NR treatment partially prevented the MitoDAMP-induced decline of maximal OCR (Figure 4C). A similar effect of NR in enhancing maximal OCR and reducing proinflammatory cytokine gene expressions is also observed in LPS-treated PBMCs (Supplemental Figure 1C).

F. NR enhances mitochondrial respiration and reduces proinflammatory cytokine production in human heart failure. We subsequently tested the effect of NR on the PBMCs of heart failure patients in the absence of MitoDAMP. Incubation with NR in vitro increased the basal and maximal OCR in the PBMCs of both healthy and HF subjects (Figures 6A and B, respectively). However, reductions of NLRP3 and proinflammatory cytokine gene expressions in HF PBMCs were minimal (Supplemental Figure 2), indicating that a 4-hour exposure to NR may be insufficient to revert the inflammatory state. Taking our observations to the bedside, we enrolled Stage D HF patients to take NR orally for 5-9 days (Figure 5), and blood samples were obtained pre- and post- NR administration. As shown in Figure 6C, oral NR administration resulted in
increases of whole blood NAD+ levels, as previously seen in healthy subjects (30). Furthermore, NR treatment resulted in a consistent enhancement of both PBMCs basal and maximal OCR (Figures 6D and 6E), and a up to 30-fold reduction of proinflammatory cytokine gene expressions (Figure 6F). Background information of the study subjects can be found in Supplemental Table 1. No adverse effect associated with NR were observed among the 5 participants.
Discussion

This study demonstrates a critical role of mitochondrial function in the inflammatory activation of peripheral immune cells in HFrEF patients. We found that proinflammatory molecules, e.g. damage associated molecular pattern released from dysfunctional mitochondria, likely via the Toll-like receptor cascade, triggers increased expressions of proinflammatory cytokines, particularly IL6. Secreted IL6 appears to feed back in an autocrine manner to impair mitochondrial respiration by inhibiting Complex I activity and inducing mitochondrial ROS production, resulting in assembly of the NLRP3 inflammasome and increased secretion of proinflammatory cytokines such as IL1B and IL18 (13, 28, 31, 32). Secreted IL1B can feedback to further potentiate the NFKB axis, resulting in a vicious cycle (33). (Figure 7)

Based on the results of our in vitro experiments, we speculate that the effect of NR represents an upstream mechanism which preserves mitochondrial respiration and reduces ROS production in the presence of an inflammatory trigger. On the other hand, mtROS and NLRP3 are downstream of the mitochondrial dysfunction; therefore, their inhibitions do not result in improvement of maximal OCR. Although IL6 mRNA level was reduced by mitochondrial ROS and NLRP3 inflammasome inhibition, the treatment may not be sufficient to normalize secreted IL6 level and remove mitochondrial impairment in our experimental setting. The molecular mediators by which NR treatment improves maximal OCR and reduces cytokine production in PBMCs warrant further investigation.
It has been demonstrated that circulating IL6 is upregulated in both acute myocardial infarction and chronic heart failure; the former was thought to be cardioprotective, but if left-unchecked, chronic IL6 elevation can result in maladaptive hypertrophy and reduced LVEF (34, 35), potentially perpetuating heart failure. To date, there has not been a randomized clinical trial antagonizing IL6 during post-MI remodeling or in chronic heart failure. The present study suggests that IL6 assumes an important signaling role connecting mitochondrial function and inflammation in peripheral immune cells. In cultured adipocytes, in vitro IL6 treatment resulted in a decrease of mitochondrial membrane potential, cellular ATP production, an increase in intracellular ROS, and a reduction of respiratory reserve capacity (36). On the contrary, IL6 was shown to hyperpolarize mitochondrial inner membrane in CD4 cells (37). While the exact mechanism remains elusive, there has been evidence suggesting that a small fraction of STAT3, a downstream effector of the IL6R axis, localizes in the mitochondria to regulate the electron transport chain and ATP production (38). Whether mitochondrial STAT3 is responsible for inhibiting complex I activity during chronic sterile inflammatory states, such as heart failure, is currently underexplored.

Recently, there have been tremendous interests in agents that increase cellular NAD as potential therapeutics for heart failure. Multiple studies demonstrated that augmenting NAD level is cardioprotective in animal models of cardiomyopathy induced by pressure overload (18-20), iron-overload (39), or genetic mutations (19, 40). Most of those studies focus on the effect of NAD in the myocardium, such that boosting myocardial NAD+ leads to activation of NAD-dependent deacetylases (i.e. Sirtuins) or polyADP-ribosylases. Increasing cardiac NAD levels by pharmacological or genetic approaches has been shown to improve myocardial mitochondrial
function and energy metabolism partly via the activity and downstream targets of mitochondrial-specific Sirtuins (18, 19, 41, 42).

Results from this study suggests that NAD augmentation may be protective against cardiomyopathy by reducing systemic inflammation through inhibition of proinflammatory activation of circulating immune cells which, in turn, allows the system to break from the vicious cycle that perpetuates the disease. Mechanistically, NAD+ repletion by NR has been shown to reduce mtROS production across tissue types (15, 43-46), which suppresses NLRP3 inflammasome/caspase I axis and secretions of active IL1B and IL18 (Figure 6) (28). In addition, NAD+-dependent activities of SIRT1 and SIRT2 in the cytosol have been found to inhibit transcription factor NFkB, resulting in blunted proinflammatory cytokine gene expression (47-49). On the other hand, there is evidence that the NAD+-consuming enzyme CD38 is upregulated in human monocyte-derived macrophages during inflammation, and that CD38 promotes cytokine secretion (50).

To our knowledge, this study is the first to examine whether NAD augmentation has anti-inflammatory effects in the context of human heart failure. Although the human efficacy data is currently lacking, the collective actions of NAD repletion on preserving cardiac function and reducing systemic inflammation may provide a molecular basis for a novel heart failure therapy.

Limitations of the study

For our in vitro experiments, whether a 4-hour culture truly reflects the cellular changes in a chronic inflammatory state needs to be substantiated. In addition, in this study, we modeled sterile
inflammation in heart failure using mitochondrial extracts to mimic MitoDAMP. This mixture contains mitochondrial DNA, RNA, proteins, and phospholipids, many of which have been shown to be proinflammatory (11). The specific component(s) in MitoDAMP responsible for triggering PBMC proinflammatory activation are yet to be identified, and quantitative changes of MitoDAMP in peripheral blood of heart failure patients warrant further investigation. Moreover, while the small number (N=4) and short treatment period (5-9 days) in the NR administration of Stage D HF patients experiments (Figures 5 and 6) limit our ability to draw conclusions regarding the efficacy or safety of NR, the results pose promising opportunities for future clinical trials to investigate NR’s potential anti-inflammatory effect. Lastly, since the average age of healthy participants in the study is younger than that of heart failure patients, a potential influence of age on our findings could not be excluded with the small sample size.
Methods

A. Study Participants.
   a. For baseline Seahorse Standard Mito Stress Test, 19 healthy (i.e. with no history of acute or chronic disease) participants and 19 hospitalized Stage D HFrEF patients were enrolled into the study. In the HF group, those requiring temporary mechanical support, such as Impella, intra-aortic balloon pump (IABP), or extracorporeal membrane oxygenation (EMCO) were excluded.
   b. For experiments associated with the oral NR administration (Figures 6C-6F), 5 hospitalized Stage D HF patients undergoing advanced heart failure therapy evaluations were put on escalating doses of NR (250 mg twice a day for Day 1, 500 mg twice a day for Day 2, and 1000 mg twice a day from Day 3 on) for 5-9 days. Fasting blood samples were obtained for PBMC isolation at baseline and post-NR administration. Subject 5 was withdrawn due to a change in clinical course deemed to be independent from NR administration.
   c. The investigation conforms with the principles outlined in the Declaration of Helsinki.

B. PBMC and Monocyte isolation. 30-60 ml of fasting blood was collected into EDTA-containing Vacutainers (BD Mfr # 364606). Blood then was diluted (1:1) with RPMI medium (Corning; Cat #17-105-CV) and applied to Histopaque gradient medium (Sigma-Aldrich; Cat #10771) using SepMate-50 (STEMCELL Technologies), and centrifuged at 1200 g for 10 minutes. Top layer contained the enriched peripheral blood mononuclear cells (PBMCs) were collected followed by centrifugation at 300 g for 10 minutes. The pellet was resuspended with ACK lysis buffer (Gibco;
Cat. A1049201) and incubated at room temperature for 5 minutes to remove residual red blood cells. Next, the enriched PBMCs were washed twice with RPMI medium. All PBMC samples were subjected to the baseline Seahorse Mito Stress Test. When available, the remaining cells were used for cytokine mRNA quantitative PCR and in vitro assays. For monocyte isolation, a magnetic bead-based negative-selection monocyte isolation kit (Miltenyi Biotec, 130-096-537) was used per manufacturer’s instructions. Briefly, antibody-conjugated magnetic bead solution was added to isolated PBMCs resuspended in RPMI containing 0.5% BSA and incubated for 20 minutes at 4°C, followed by magnetic column binding and elution. The procedure was repeated once to improve the purity of monocytes. The monocytes were washed once with RPMI prior to subsequent experiments.

**C. Seahorse Mito Stress Test.** Oxygen consumption rate (OCR) of freshly isolated PBMCs was assessed using a Seahorse XFe24 analyzer (Agilent Technologies). Briefly, purified PBMCs were resuspended in Seahorse base medium (Agilent Technologies, supplemented with 1 mM pyruvate, 2 mM glutamine, and 10 mM glucose, pH 7.4), and added 10⁶ cells/well at equal volume of 500 μL in Seahorse 24-well plate. The cells were maintained in a non-CO₂ incubator and allowed to settle for 30 minutes at 37°C. Next, the plate was centrifuged at 40 g for 10 minutes at room temperature without any application of the brake to help cells attach to the bottom of the plate. Drugs used in the assay: 5 μM oligomycin A (Sigma-Aldrich; Cat. 75351), 3 μM Trifluoromethoxy carbonylcyanide phenylhydrazone (FCCP) (Sigma-Aldrich; Cat. C2920), 1 μM Rotenone (Sigma-Aldrich; Cat. A8674), 1 μM Antimycin A (Sigma-Aldrich; Cat. R8875), 0.5 mM TMPD (Sigma-Aldrich; Cat. T7394) and 10 mM Ascorbate (Sigma-Aldrich; Cat. 95209).
D. PBMC and Monocyte Culture. Freshly isolated PBMCs or Monocytes from healthy or HF subjects were resuspended with RPMI medium plus 10% heat inactivated FBS. PBMCs (10^6 cells/well) were seeded in Seahorse 24-well plates and incubated at 37°C in CO₂ incubator with various proinflammatory triggers (Vehicle, MitoDAMP, LPS) and compounds (NR, MCC 950, MitoTempo, or LMT-28). After 4 hours incubation, the plate was centrifuged at 40 g for 10 minutes at room temperature without no application of the brake to help cells attach to the bottom of the plate. The cells were washed twice and then brought up to 500 µL using Seahorse base medium (Agilent Technologies, supplemented with 1 mM pyruvate, 2 mM glutamine, and 10 mM glucose, pH 7.4).

E. MitoDAMP Isolation. Human heart tissue samples (100-200 mg) from the left ventricular apex were collected at the time of left ventricular assist device (LVAD) implantation when tissue from the LVAD core became available. Fresh tissue was stored in ice-cold sterile PBS prior to mitochondria isolation per prior protocol (19). The purified mitochondria was resuspended in 200 µL of MSE buffer (19), and subsequently stored in -80°C. Mitochondria from 6 donors were pooled and lysed by freeze (in liquid nitrogen) and thaw for 3 times, followed by sonication at 30% intensity with 3-sec-on and 3-sec-off for 3 cycles. The lysed mitochondria were then spun down at 10,000 RPM for 10 minutes. Protein concentration of the supernatant (MitoDAMP) was determined by BCA kit. The supernatant was aliquoted and stored at -80°C for future use. For all MitoDAMP stimulation assays, the final protein concentration of MitoDAMP was 0.5 mg/mL (Figure 2A).
F. IL6 ELISA. Isolated PBMCs (4 x 10⁶ cells per sample) from healthy or HF participants were resuspended in RPMI medium containing 10% heat-inactivated FBS and treated with various proinflammatory triggers (Vehicle, MitoDAMP, LPS) and compounds (NR, MCC 950, MitoTempo, or LMT-28) to final PBMC concentration of 10 x 10⁶ cells per mL. See below for detailed descriptions of the reagents. The samples were incubated in Eppendorf tube at 37°C in 5% CO₂ incubator with caps open for time designated in figure legends. The cultured PBMC samples were centrifuged at 8,000 RPM at room temperature. The supernatant was used for ELISA per manufacture protocol using R&D Systems human IL6 kit (Cat # DY206-05). The cell pellets were stored in -80°C.

G. RNA Isolation and Quantitative PCR analysis. Total RNA was isolated from frozen PBMC pellets using Trizol (Invitrogen) per manufacturer’s instructions, and cDNA was synthesized using iScript™ Reverse Transcription Supermix (Bio-rad) per manufacturer’s instructions. Real-time PCR was performed in the Corbett rotor gene 6000 real-time PCR machine using iQ™ SYBR® Green Supermix (Bio-rad) and expression levels of the indicated genes were calculated using the ∆∆Ct method. Primer sequences are as follows:

NLRP3 forward: GTGTTTCGAATCCCACTGTG; reverse: TCTGCTTCTCAGTACTTTCTG;
IL1B forward: ATGCACCTGTACGATCACTG; reverse: ACAAAGGACATGGAGAACACC;
IL6 forward: CCACTCACCTCTTCAGAACG; reverse: CATCTTTGGAAGGTTCAGGTTG;
TNFA forward: ACTTTGGAGTGATCGGCC; reverse: GCTTGAGGGTTTGCTACAAC;
IL18 forward: CATTGACCAAGGAAATCGGC; reverse: CACAGAGATAGTTACAGCCATACC;
18S rRNA forward: GTAACCCCGTTGAAACCCCATT; reverse: CCATCCAATCGGCTAGCG.
H. Mitochondrial ROS detection in cultured PBMCs. To detect mitochondrial ROS production, the treated PBMCs were incubated with 5 μM MitoSOX Red (Life Technologies) and MitoTracker Green (200 nM, Life Technologies) at 37°C for 30 minutes in RPMI medium supplemented with 10% heat-inactivated FBS, and then washed twice with cold PBS before cells were analyzed by flow cytometry on a Cytek Aurora (Cytek Biosciences).

I. Complex I in vitro assay. This assay was performed using Abcam Complex I Enzyme Activity Microplate Assay Kit (ab109721), per the manufacturer’s protocol. Briefly, following 4-hour treatment with vehicle or IL6, 5 x 10⁶ PBMCs were washed with PBS and lysed by detergent for 30 minutes on ice, followed by centrifugation at 16,000 g for 20 minutes at 4°C. Supernatant was taken for protein concentration determination. 12.5 μg of total protein in a final volume of 200 μL was incubated in microplates pre-coated with anti-Complex I antibody for 3 hours at room temperature. Post-washing step, NADH and dye were added, and optical density (OD) was serially measured at 450 nm over 1 hour. The slope of the curve within the linear range was recorded as Complex I activity.

J. Whole blood NAD+ assays. These were performed as previously described (30). Briefly, 50 μL of thawed blood samples were added to tubes containing 1 μg BMP as the internal standard and immediately followed by 300 μL of 4% trichloroacetic acid to precipitate proteins. Calibration standards were similarly prepared except that the matrix was 30 mg/mL BSA spiked with NAD+ and NMN that had been dissolved in methanol. After protein precipitation and high-speed centrifugation, the supernatants were loaded onto Costar Spin-X 0.22 μm spin filters and subject to high speed centrifugation again. The eluates were injected onto an Agilent 1100 series high
performance liquid chromatograph coupled to an Agilent G1956B single-quadrupole mass spectrometer. The mass spectrometer was operated in electrospray ionization mode with positive polarity.

K. Reagents.

Lipopolysaccharide (LPS) – purified from Salmonella Minnesota R595, was purchased from Enzo life sciences, Cat #ALX-581-008-L001.

MCC 950 – purchased from Sigma Aldrich, Cat #PZ0280-5MG, stock dissolved in RPMI to 100 μM and stored in -80°C.

MitoTempo – purchased from Sigma Aldrich, Cat #SML07375MG, stock solution dissolved in RPMI to 10 mM and stored in -80°C.

LMT-28 - purchased from Sigma Aldrich, Cat #SML1628-5MG, stock solution dissolved in DMSO to 10 mM and stored in -80°C.

Human Recombinant IL6 – purchase from Gibco, Ref # PHC0064, stock solution dissolved in RPMI to 1 μg/mL and stored in -80°C.

Human Recombinant IL1B and IL18 – purchased from R&D Systems, Cat #201-LB and #9124-IL, respectively. Stock solutions dissolved in RPMI to 1 μg/mL and stored in -80°C.

L. Nicotinamide Riboside (NR). For in vitro assays, NR was supplied as powder by the manufacturer (Niagen®, ChromaDex, Irvine, CA). 10 mM or 100 mM stock solutions dissolved in RPMI with 10% heat inactivated FBS were made on the day of the experiment. For oral administration, NR was supplied by the manufacturer as 250 mg capsules, and manufactured in a GMP-compliant facility according to ISO/IEC 18025:2005 standards.
M. Statistical Analyses: Statistical analyses were performed using GraphPad Prism 8 (Version 8.3.0). For analyses of PBMC respiratory function, the primary outcome was defined as the mean difference in basal OCR and maximal OCR (post FCCP injection), determined by Seahorse Mito Stress Test in PBMCs of healthy vs. HFrEF participants. Mean age comparison between subject groups were subjected to unpaired 2-tailed nonparametric test (Mann-Whitney test). For mRNA expression comparisons between healthy and HF participants, P-value was determined by unpaired parametric 2-tailed t-test. In vitro and in vivo pre- vs post- treatment analyses were performed by paired 2-tailed parametric t-test or 1-way ANOVA followed by post-hoc pairwise multiple comparisons, as appropriate. Error bars in all figures represent SEMs.

N. Study Approval:

All studies were approved by the Human Subject Division of University of Washington. For baseline PBMC measurements, blood samples from adult healthy participants were collected under IRB-approved protocol (STUDY00005599), and HFrEF patient blood and myocardial tissue samples were collected under a separate IRB-approved protocol (STUDY00002544). For the experiments pertaining oral NR administration in HFrEF patients, the study was performed under the IRB-approved protocol STUDY0000543, ClinicalTrials.gov identifier: NCT03727646.

Author Contributions

BZ and DW contributed equally to this work. BZ and DW designed and conducted experiments, acquired and analyzed data. DW wrote the manuscript. YQ, YL, and SA acquired data and edited
the manuscript. ASO edited the manuscript and provided reagent. KOB, and RT supervised, designed, conceptualized the project, and edited the manuscript.

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References


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<tr>
<td><strong>Inotropes</strong></td>
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Table 1. Baseline characteristics of study subjects. LVEF: left ventricular ejection fraction. CM: cardiomyopathy. HTN: hypertension. DM: diabetes mellitus. Data shown in means ± SD.
A. 

B. Basal Respiration

C. Maximal Respiration

D. mRNA Levels
Figure 1: Heart failure is associated with a reduced maximal respiration and elevated pro-inflammatory cytokine gene expressions in PBMCs. A. Representative oxygen consumption rate (OCR) plot upon various inhibitor injections in a standard Seahorse Mito Stress Test, comparing PBMCs from healthy and Stage D HF subjects. Oligomycin A: inhibitor of Complex V. FCCP (Trifluoromethoxy carbonylcyanide phenylhydrazone): uncoupling agent by permeabilizing inner mitochondrial membrane. Antimycin A: inhibitor of Complex III. Rotenone: inhibitor of Complex I. B and C. Basal and FCCP-induced maximal respiration of PBMCs from healthy (N=19) and Stage D HF (N=19) subjects, respectively. OCR data normalized via Log₁₀ transformation were subjected to ordinary unpaired 2-tailed parametric test (Welch’s t-test). Normal distribution was assessed by Kolmogorov-Smirnov test. D. Relative mRNA levels of NLRP3 and pro-inflammatory cytokines of PBMCs of healthy and Stage D HF subjects by RT-qPCR. NLRP3 (Healthy N=9, HF N=9), IL1B (N=9, N=9), IL6 (N=12, N=11), IL18 (N=7, N=9), TNFA (N=9, N=9). Mean mRNA level of healthy subjects normalized to 1. mRNA data analyzed with unpaired nonparametric 2-tailed t test. All data shown in means +/- SEM.
Figure 2. MitoDAMP induces PBMC respiratory impairment and inflammatory cytokine gene expression, and the latter can be partially attenuated by inhibition of the NLRP3 inflammasome axis. A. Schematics of the MitoDAMP extracted from mitochondria purified from human myocardial tissue. B. Relative mRNA levels of NLRP3 and pro-inflammatory cytokines of healthy PBMCs post 4-hour treatments of vehicle or MitoDAMP. Vehicle normalized to 1. P-value was determined by paired 2-tailed t-test. N=4. C. Mitochondrial ROS levels of healthy PBMCs post MitoDAMP treatment. 0 hour normalized to 100%. P-value cut off of 0.05 was determined by paired 2-tailed t-test. N=3. D. Maximal respiration of healthy PBMCs post 4-hour treatment of vehicle or MitoDAMP with or without 0.5 mM MitoTempo, N=4. P-value determined by one-way ANOVA with multiple pairwise comparisons. E. Percent change of NLRP3 and cytokine mRNA levels of healthy PBMCs post 4-hour treatment of MitoDAMP with 0.5 mM MitoTempo (N=4) or 1 μM MCC950 (N=5) relative to MitoDAMP alone. P-value was determined by paired 2-tailed t-test. F. Maximal respiration of healthy PBMCs post 4-hour treatments of vehicle or MitoDAMP with or without 1 μM MCC950. N=3. P-value determined by 1-way ANOVA with multiple pairwise comparisons. ns: not significant. All data shown in means +/- SEM.
A. IL6

B. Maximal Respiration

C. Maximal Respiration

D. Complex II

E. Complex Activities

F. Complex I Activity
Figure 3. Secreted IL6 from MitoDAMP stimulation impairs mitochondrial respiration by reducing Complex I activity. A. Secreted IL6 protein level by ELISA of healthy PBMCs post 2-hour (N=3) or 4-hour (N=6) treatment of vehicle or MitoDAMP. P-value determined by paired 2-tailed t-test. B. Maximal respiration of healthy PBMCs post 4-hour treatments of vehicle or MitoDAMP with or without 100 μM LMT28, a specific inhibitor of the IL6 receptor b (GP 130). N=4. C. Maximal respiration of healthy PBMCs post 4-hour treatments of vehicle or increasing concentrations of human recombinant IL6. N=4. B and C analyzed with 1-way ANOVA with multiple pairwise comparisons. D. Representative Seahorse plot of baseline and post-drug treatment OCR of healthy PBMCs post 4-hour treatments of vehicle or IL6 (1 ng/mL). FCCP; uncoupling agent by permeabilizing inner mitochondrial membrane; Rotenone (Rot): Complex I inhibitor; Antimycin A (AA): Complex III inhibitor; TMPD/Ascorbate: exogenous electron donor for Complex IV. N=3. E. Quantitation of panel D. F. Complex I activity of healthy PBMCs post 4-hour treatments of vehicle or IL6 (1 ng/mL). N=5. Vehicle normalized to 1. E and F analyzed by paired 2-tailed t-test. All data shown in means +/- SEM.
A. Protein Levels

B. mRNA Expression

C. Maximal Respiration


P = 0.042

P < 0.0001

P < 0.0001

P = 0.0094
Figure 4. Nicotinamide riboside (NR) attenuates MitoDAMP-induced PBMC respiratory impairment and pro-inflammatory cytokine production in vitro. A. Secreted IL6 protein by ELISA of healthy PBMCs post 4-hour treatment of vehicle or 1 mM NR in the presence of MitoDAMP. P-value determined by paired 2-tailed t test. B. Percent change of NLRP3 and cytokine mRNA levels of healthy PBMCs post 4-hour treatment of MitoDAMP with 1 mM NR relative to MitoDAMP only. P-value was determined by paired 2-tailed t-test. N=4. C. Maximal respiration of healthy PMBC post 4-hour treatment of vehicle, MitoDAMP, or MitoDAMP with 1 mM NR. N=4. P-value determined by ordinary 1-way ANOVA with multiple pairwise comparisons. B and C shown in means +/- SEM.
Enrollment

Assessed for eligibility (n=5)

Excluded (n=0)

NR Intervention (n=5)

Completed study (n=4)
- Oral NR (2000 mg daily) for 6-9 days

Withdrawn from study (n=1)
- Due to change in clinical status
Figure 5. Study design of oral NR administration in heart failure patients.
A. Healthy

Fold change of OCR (NR / Vehicle)

Pre-NR  Post-NR

0  1  2  3  4
Basal  Maximal

P=0.039
P=0.055
P=0.0024

B. Stage D HF

Fold change of OCR (NR / Vehicle)

Pre-NR  Post-NR

0  1  2  3  4
Basal  Maximal

P=0.037
P=0.056
P=0.0405

C. Whole Blood NAD+


D. Basal Respiration

OCR (pmoles/min/10^6 cells)

Pre-NR  Post-NR

P=0.0296
P=0.055

E. Maximal Respiration

OCR (pmoles/min/10^6 cells)

Pre-NR  Post-NR

P=0.0405

F. NLRP3

Relative mRNA expression

Pre-NR  Post-NR

P=0.146
P=0.0648
P=0.0137
P=0.0064

IL1B

IL6

IL18

Subject 1

Subject 2

Subject 3

Subject 4
Figure 6. NR enhances mitochondrial respiration and reduces proinflammatory cytokine production in heart failure. A and B. Ratios of basal and maximal respiration of healthy or HF PBMCs post 4-hour 1 mM NR treatments relative to vehicle, respectively. N=8 and N=10, respectively. C. Whole blood NAD+ level of Stage D HF subjects pre- or post- 5-9 days of oral NR administration. D and E. Basal and maximal respiration of PBMCs of Stage D HF subjects pre- and post- oral NR administration, respectively. F. Relative mRNA levels of NLRP3 and inflammatory cytokines of PBMCs of Stage D HF subjects pre- and post-NR administration. Post-NR mRNA level normalized to 1. N=4. A and B, P-values determined by unpaired two-tailed t-test, and data shown in means +/- SEM. C-F: P-values determined by paired two-tailed t-test.
Figure 7. Model of DAMP-induced monocyte activation. The “priming” signal involves interaction of MitoDAMP with TLRs to stimulate the expression of inflammasome components and pro-inflammatory cytokines via activation of transcription factor NFKB. The secreted IL6 in the priming step feeds back in an autocrine manner to impair mitochondrial respiration by inhibiting Complex I activity and induce mitochondrial ROS production, which leads to the assembly of the NLRP3 inflammasome to active caspase 1. Caspase 1, in turn, cleaves pro-IL1B to IL1B. Secreted IL1B feedbacks to further potentiate the NFKB axis.
Supplemental Figure 1

A. Maximal Respiration

B. Relative mRNA expression

C. mRNA Expression

% Change

-60 -40 -20 0 20 40

NLRP3  IL1B  IL6  IL18

P=0.0365  P<0.0001

P<0.0001

Vehicle  LPS  LPS + NR

P=0.0003

P=0.0001

0.5 1 2 4

Vehicle  LPS

P=0.0021

P=0.0003

0.03125 1 32 1024 32768

Vehicle  LPS

P=0.0003

0.5 1 2 4

Vehicle  LPS

ns
Supplemental Figure 1. A, Maximal respiration of healthy PBMCs post 4-hour vehicle or 10 ng/mL LPS treatment with or without 1mM NR. N=4. Data analyzed with 1-way ANOVA with multiple comparisons. B, Relative mRNA levels of NLRP3 and pro-inflammatory cytokines of healthy PBMCs post 4-hour vehicle or 10 ng/mL LPS treatment. P-value determined by paired 2-tailed t test. N=4. C. Percent change of NLRP3 and cytokine mRNA level of healthy PBMCs post 4-hour treatment of 10 ng/mL LPS with 1mM NR treatment relative to LPS alone. **P-value <0.005 by paired two-tailed t-test. ns: not significant. All data shown in means +/- SEM.
Supplemental Figure 2

mRNA Expression

Fold change (vehicle / NR)

NS

NLRP3
IL1B
IL6
IL18
Supplemental Figure 2. Ratios of mRNA levels of PBMCs from Stage D HF participants post 4-hour treatments of 1 mM NR treatments relative to vehicle. N=8. P-value determined by paired two-tailed t-test.
Supplemental Figure 3

Maximal Respiration

Supplemental Figure 3
Supplemental Figure 3. Maximal respiration of healthy PBMCs post 4-hour treatments of vehicle or increasing concentrations of recombinant human IL1B or IL18. N=4. Data analyzed with 1-way ANOVA with multiple pairwise comparisons against vehicle.
Supplemental Figure 4

Monocyte Maximum Respiration

OCR (pmoles/min/10^6 cells)

P = 0.0261
Supplemental Figure 4. Maximal Respiration of isolated monocytes post 4-hour treatment of vehicle of 1 ng/mL of recombinant human IL6. N=7. P-value determined by paired 2-tailed t-test.
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Supplemental Table 2

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Supplemental Table 2. Monocyte and lymphocyte counts of the 19 Stage D HF subjects by complete blood count (CBC). For 18 out of 19 subjects, the CBC was done within 15 days (mean 7.7 days) prior to the experimental blood sampling. For the remaining subject, the CBC was done 30 days prior to experimental blood sampling.