BML-111 TREATMENT PREVENTS CARDIAC APOPTOSIS AND OXIDATIVE STRESS IN A MOUSE MODEL OF AUTOIMMUNE MYOCARDITIS

SHORT TITLE: PROTECTIVE CARDIAC EFFECTS OF BML-111 IN AN EAM MODEL

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ABBREVIATIONS:

8-OHdG: 8-hydroxy-2'–deoxyguanosine

AMPKα: AMP-activated protein kinase alpha

ANP: atrial natriuretic peptide

ARE: antioxidant response element

BML: 5(S), 6(R), 7-trihydroxyheptanoic acid methyl ester

BNP: brain natriuretic peptide

BW: body weight

CaMKK2: calcium/calmodulin-dependent protein kinase kinase 2

CVDs: cardiovascular diseases

DCM: dilated cardiomyopathy

EAM: experimental autoimmune myocarditis

EF: ejection fraction

EndoG: endonuclease G

FS: fractional shortening

HO-1: heme oxygenase 1

HR: heart rate

HW: heart weight

IL-1β: interleukin 1 beta

IL-6: interleukin 6

Keap1: Kelch-like ECH associated protein-1

LKB1: liver kinase B1

LPS: Lipopolysaccharide

LVEDD: left ventricle end-diastolic diameter

LVESD: left ventricle end-systolic diameter

Ly6G: lymphocyte antigen 6 complex locus G6D

MDA: malondialdehyde

MMP: metalloproteinase

NES: nuclear exportation sequence

NQO1: NAD(P)H quinone oxidoreductase 1

Nrf2: nuclear factor erythroid 2-related factor 2

PRDX-1: peroxiredoxin-1

RORγT: RAR-related orphan receptor gamma T

ROS: reactive oxygen species

SPM: specialized pro-resolving mediator

TAK1: transforming growth factor beta-activated kinase 1

TGF-β: transforming growth factor beta

TIMP: tissue inhibitor of metalloproteinase

TL: tibial length

TNFα: tumor necrosis factor alpha

UV: ultraviolet

ABSTRACT

Myocarditis is an inflammation of the myocardium that can progress to a more severe phenotype of dilated cardiomyopathy. Three main harmful factors determine this progression: inflammation, cell death and oxidative stress. Lipoxins and their derivatives are endogenous pro-resolving mediators that induce the resolution of the inflammatory process. This study aims to determine whether these mediators play a protective role in a murine model of experimental autoimmune myocarditis (EAM) by treating with the lipoxin A_4 analog BML-111. We observed that EAM mice presented extensive infiltration areas that correlated with higher levels of inflammatory and cardiac damage markers. Both parameters were significantly reduced in BML-treated EAM mice. Concordantly, cardiac dysfunction detected in EAM mice was prevented by BML-111 treatment. At the molecular level, we demonstrated that treatment with BML-111 hampered apoptosis and oxidative stress induction by EAM. Moreover, both *in vivo* and *in vitro* studies revealed that these beneficial effects were mediated by activation of Nrf2 pathway through CaMKK2-AMPKα kinase pathway. Altogether, our data indicate that treatment with the lipoxin derivative BML-111 effectively alleviates EAM outcome and prevents cardiac dysfunction, thus underscoring the therapeutic potential of lipoxins and their derivatives to treat myocarditis and other inflammatory cardiovascular diseases.

Keywords: Myocarditis, lipoxin, inflammation, resolution, oxidative stress.

INTRODUCTION

Myocarditis is an inflammation of the myocardium as a result of infection, autoimmune disease or cardiotoxic agents that can lead to acute heart failure, life threatening arrhythmias and even sudden death (1). Cardiac function may rapidly deteriorate and patients become hemodynamically unstable requiring supportive therapy or cardiac transplantation. Regardless of its etiology, the initial acute inflammation may progress to tissue remodeling, fibrosis, and loss of myocardial architecture and contractile function. The latter chronic damage corresponds to the development of dilated cardiomyopathy (DCM) (2). DCM overall incidence among myocarditis cases is not accurately known, but retrospective studies report that up to 50% of unexplained non-ischemic DCM cases exhibit histological evidence of myocarditis (3). Therefore, it is important to detect the disease in its early stages to find therapeutic targets aiming to prevent the progression to a more advanced and irreversible cardiomyopathy.

Experimental autoimmune myocarditis (EAM) mouse model, which is induced by immunizing mice with myocardial self-antigens, resembles the autoimmunological process of acute myocarditis and has been used to study the pathogenesis of clinical myocarditis. In this model, an inflammatory process is progressively developed, peaking at day 21 after first immunization, when the presence of extensive areas of infiltrated immune cells is maximum (4). These cells migrate to the organ releasing high levels of pro-inflammatory mediators (IL-1β, IL-6, TNFα…) that initiate the inflammatory response (5). Afterwards, the accumulation of these harmful molecules can cause metabolic alterations in cardiac cells as well as increasing the release of reactive oxygen species (ROS) (6). This adverse scenario induces apoptosis on cardiac cells, eventually driving to a mechanic overload in the heart that cells compensate by increasing their size and thus generating a hypertrophic phenotype (7). When this situation is maintained, it drives to organ dysfunction causing the release of cardiac damage mediators like natriuretic peptides —BNP and ANP (8)— or galectin 3 (9). Moreover, some research groups have described the appearance of fibrotic areas in the affected hearts that could aggravate cardiac dysfunction. The onset of fibrosis in EAM model seems to be mainly modulated by transforming growth factor beta-1 (TGF-β1) and collagen overexpression (10).

Current therapies for myocarditis or DCM are mainly based on anti-inflammatory and heart failure treatments, like beta-blockers, angiotensin-converting enzyme inhibitors and

diuretics. However, their outcome is not completely favorable and patients do not exhibit full recovery. Therefore, ongoing research is focusing on the design of more efficient and specific therapies (i.e., Registry of Cell Therapy in Non-Ischemic Dilated Cardiomyopathy (RECORD); *ClinicalTrials.gov*). Specialized pro-resolving lipid mediators (SPMs) are endogenous molecules that represent a promising alternative thanks to their pleiotropic anti-inflammatory actions (11). It has been broadly described that these SPMs limit the recruitment of neutrophils, increase the production of anti-inflammatory mediators and promote the clearance of cell debris (12, 13). Among them, the most studied are lipoxins, which are endogenously released in the inflammatory area to allow for the recovery of tissue homeostasis (14). However, due to its rapid inactivation and short half-life, more stable and effective analogs like BML-111 [5(S), 6(R), 7-trihydroxyheptanoic acid methyl ester] have been designed, exerting anti-inflammatory and pro-resolving effects equivalent to lipoxins in several pathological *in vivo* models with a prolonged action (15). In fact, it has been already demonstrated that BML-111 reduces inflammation in psoriasis (16), ischemia-reperfusion injury (17), acute lung injury (18) or arthritis (15) animal models. Furthermore, ongoing clinical trials are evaluating the protective role of lipoxin analogs in asthmatic children with acute episodes through inhalation (19) or in gingivitis (*ClinicalTrials.gov*: NCT02342691). Despite the broad number of studies analyzing the beneficial effects of SPMs in human pathologies, their role in cardiovascular diseases (CVDs) has only been recently addressed.

Since myocarditis-affected hearts are characterized by exacerbated levels of oxidative stress that eventually lead to cardiac cells apoptosis, the transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2) plays an important role as a major regulator of the expression of antioxidant genes (20). These include heme oxygenase 1 (HO-1), NAD(P)H quinone oxidoreductase 1 (NQO1), catalase and peroxiredoxin-1 (PRDX-1). Nrf2 stability and activation is mainly mediated by Keap1 (Kelch-like ECH associated protein-1), which interacts with Nrf2 and inhibits its nuclear translocation (21). In addition, Nrf2 activation can also be modulated by post-translational modifications, particularly through phosphorylation by different kinases like AMPKα (AMP-activated protein kinase alpha) (22). AMPKα induces nuclear sequestration of Nrf2 by phosphorylating its nuclear exportation sequence (NES) (23). Upstream to AMPKα, three kinases have been described: LKB1, CaMKK2 or TAK1. Although LKB1 has been

tightly associated with cardiac signaling (24), recent studies indicate that CaMKK2 may play a significant role mediating AMPKα activation in response to changes in calcium efflux (25) or during ischemic complications (26). Since these kinases are sensors to cell stress, they could be activated in a cardiac harmful scenario modulating Nrf2 pathway thus controlling oxidative stress and cell death.

Several works have demonstrated the modulation of Nrf2 by lipoxins or their derivatives as one of the main mechanisms behind their beneficial effects in pathological models like intestinal (27) and renal (28) ischemia/reperfusion damage, in LPS-induced mouse acute lung injury (29) or venous thromboembolism (30). Nevertheless, the modulation of this antioxidant pathway in cardiac pathologies remains to be further investigated since it may be responsible, at least in part, for the beneficial effects of SPMs observed in CVDs. In addition, growing evidence indicates that decreased Nrf2 activity contributes to oxidative stress induction in the heart, favoring the pathophysiology of cardiovascular disorders found in obesity, diabetes mellitus, and atherosclerosis (31). Therefore, compounds capable of modulating cardiac Nrf2 activation could be effective in several CVDs. In the present study, we performed an EAM murine model where we investigated the regulatory role of BML-111 in anti-inflammatory and antioxidant cardiac pathways. This study provides new insights for the development of therapeutic targets driven to improve prognosis and treatment of CVDs patients.

2. MATERIAL AND METHODS.

2.1. Animals, immunization and treatment. BALB/c mice from Charles River Laboratories (Wilmington, MA, USA) were housed in the Animal Care Facility from the Instituto de Investigaciones Biomédicas "Alberto Sols" (IIBM, CSIC-UAM) and were provided food and water *ad libitum*, under standard conditions. All experimental procedures were reviewed and approved by the Animal Ethics Committee of the IIBM and Comunidad Autónoma de Madrid (PROEX 144/17) and were in accordance with the European Legislation.

Before the procedure, all mice were anesthetized by inhalation of 2% isoflurane and 2 l/min oxygen flow rate to minimize animal stress. Disease was induced in 8 week-old female mice by subcutaneous immunization in both dorsal flanks of the animal at days 0 and 7 with 350 µg of cardiac myosin previously isolated following the protocol described by Shiverick et al. (32) from BALB/c mice in a 1:1 emulsion with Complete Freund´s

Adjuvant containing *Mycobacterium tuberculosis* H37RA (5 mg/ml). Control groups were injected with physiological saline solution instead of myosin. BML-111 (Enzo Lifescience (NY, USA), 10 mg/ml) or its vehicle (ethanol) were intraperitoneally injected at 1 mg/kg from day 7 to day 21 in the corresponding groups. Thus, four experimental groups were established: Control+Vehicle (Control+Veh), Control+BML-111 (Control+BML), Myosin+vehicle (EAM+Veh) and Myosin+BML-111 (EAM+BML). At day 21, all mice were anesthetized again with 2% isoflurane and 2 l/min oxygen flow rate to perform echocardiographic study. Then, blood was obtained, and mice were sacrificed by cervical dislocation. Body weight and tibial length were recorded. Finally, hearts were carefully removed from the chest, cleaned, weighed and processed.

2.2. Transthoracic echocardiography. M-mode echocardiography was employed to evaluate cardiac function. Mice were anesthetized with 2% isoflurane and 2 l/min oxygen flow rate; the chest of the mouse was shaved, and warm ultrasound transmission gel was used to obtain an optimal image quality. Mouse hearts were analyzed with a high-frequency micro-ultrasound system (Vivid Q, General Electric Healthcare, IL, USA). Parasternal short-axis-view images of the heart were recorded using a 30-MHz RMV scan head in B-mode to allow M-mode recordings by positioning the cursor in the parasternal short-axis view perpendicular to the interventricular septum and posterior wall of the left ventricle. Left ventricle ejection fraction and fractional shortening were determined using the on-site software cardiac package (GE Healthcare (Chicago, IL, USA)).

2.3. BNP levels determination. Plasmatic BNP levels were determined using the RayBio® Immunoassay (Raybiotech, Inc (GA, USA), #EIA-BNP-1) following the manufacturer´s instructions.

2.4. Immunohistochemistry. Sections were fixed in fresh 4% paraformaldehyde and embedded in paraffin, cut in a microtome and stained with hematoxylin/eosin. Histological examination was performed with a Zeiss Axiophot microscope connected to a digital camera. Three sections per mouse were examined blindly by two different investigators and the severity of disease was qualitatively scored according to Dallas classification with grades from 0 to 4 (0=no inflammatory infiltrates; 1=small foci of inflammatory cells between myocytes; 2=larger foci of 100 inflammatory cells; 3=more than 10% of cross-section involved; and 4=more than 30% of cross-section involved) (33).

2.5. Immunofluorescence. Heart sections were dehydrated in a sucrose gradient, fixed in 4% paraformaldehyde and then frozen in OCT reagent. Tissues were then cut in a cryostat; sections were picked up on a glass slide and maintained at -80°C. After blocking with 5% BSA + 10% normal goat serum + 0.3% Triton X-100 for 1 h at room temperature, heart slides were incubated with the corresponding primary antibodies at 4°C overnight: CD68 (Novus Biologicals (Centennial (CO, USA), 100-683), Ly-6G (Novus Biologicals 600-1387), RORγT (Thermo Fisher (Waltham, MA, USA) 14-6988-80), cleaved-caspase 3 (Cell Signaling (Danvers, MA, USA) #9664), α-actinin (Sigma (San Luis, MO, USA) #A7811), 8-OHdG (Abcam, Cambridge, UK #62623), MDA (Abcam #6463) or Keap1 (Thermo Fisher #1F10B6). Samples were incubated with secondary antibodies combined with Alexa Fluor 488, 546, 647 (Molecular Probes (Eugene, OR, USA)) for 2 h 1:500 and then DAPI (Molecular Probes #D1306) 1:500 for 10 min at room temperature, gently washing with PBS1X between incubations. Finally, sections were washed again and coverslips were mounted in ProLong® Gold Antifade reagent (Life Technologies, (Carlsbad, CA, USA)) and examined using a confocal microscope Leica PCS SP5. Values of intensity fluorescence were determined with Image J software.

2.6. TUNEL assay. Heart sections were stained using the *in situ* cell detection kit with fluorescein (Roche (Basel, Switzerland) #11684795910) following the manufacturer´s instructions.

2.7. RNA isolation and qPCR. RNA was extracted from hearts using TRI Reagent® solution (Ambion (Austin, TX, USA)) and 250 ng were reverse-transcribed into cDNA using the High-capacity cDNA reverse transcription kit (Applied Biosystems (Foster City, CA, USA) #4368813). Then, PCR reaction was performed with this template cDNA at 2,5 ng/µl adding *Power* SYBR Green PCR Master Mix (Applied Biosystems #4367659) and the specifics primers (Table 1) in a 7900HT Fast real time PCR system (Applied Biosystems).

Gene	Forward	Reverse
Anp	ATTGACAGGATTGGAGCCCAGAGT	TGACACACCACAAGGGCTTAGGAT
Col1a1	AATGGCACGGCTGTGTGCGA	AGCACTCGCCCTCCCGTCTT
Col3a1	CTGTAACATGGAAACTGGGGAAA	CCATAGCTGAACTGAAAACCACC
Il1b	AGAAGCTGTGGCAGCTACCTG	GGAAAAGAAGGTGCTCATGTCC
Il6	GAGGATACCACTCCCAACAGACC	AAGTGCATCATCGTTGTTCATACA
Tgfb1	CCACCTGCAAGACCATCCAC	CTGGCGAGCCTTAGTTTGGAC
Tnfa	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC
Rplp0	AGATGCAGCAGATCCGCAT	GTTCTTGCCCATCAGCACC

Table 1. Specific primers used in qPCR assays.

2.8. HL-1 cell culture. Cells were maintained in pre-coated flasks with gelatin/fibronectin using complete Claycomb medium (Sigma #51800C) with 10% FBS following the distributor instructions.

2.9. Preparation of whole protein cardiac/cell extracts. Hearts were homogenized in a buffer containing sucrose 320 mM and protease and phosphatase inhibitor cocktails (Sigma) at pH7. Cells were homogenized in a buffer containing 10 mM Tris-HCl pH7.5; 1 mM $MgCl_2$, 1 mM EGTA, 10% glycerol and 0.5% CHAPS with the same inhibitors. Both homogenates were vortexed for 45 min and centrifuged (13,000g, 15 min at 4°C) and supernatants were frozen and stored at −80°C for western blot analysis. Protein concentration was determined by Bradford assay (Bio-Rad (Hercules, CA, USA).

2.10. Preparation of nuclear and cytosolic cell extracts. Cardiac tissue/cells were homogenized in 0.25 M sucrose buffer with protease and phosphatase inhibitor cocktails (Sigma), using a Dounce tissue grinder and maintained 30 min on ice. Solution was centrifuged at 800g 15 min at 4°C to separate cytosolic extract (supernatant) and nuclear extract (pellet). Cytosolic extract was serially centrifuged at 800g 10 min and then at 11,000g 10 min both at 4°C. Supernatant was then precipitated by treating with cold acetone for 1 h at -20°C and centrifuging at 12,000g 5 min at 4°C. Pellet obtained was resuspended in 150 µl (cells) or 500 µl (tissue) of sucrose buffer. Nuclear extract was washed with sucrose buffer and centrifuged first at 500g 15 min and then at 1,000g 15 min, both at 4°C. Pellet obtained was resuspended in 200-500 µl of a buffer containing 20% glycerol and 1% Triton X-100, lysed by passing through a 20G needle, and then centrifuged at 9,000g 30 min at 4°C. Supernatant obtained represented nuclear fraction. Both cytosolic and nuclear extracts were maintained at -20°C for western blot analysis. Protein concentration was determined by Bradford assay (Bio-Rad). Lamin B was used as a nuclear marker to ensure an appropriate purification of each fraction.

2.11. Western blot. Equal amounts of protein (20-60 µg) from each fraction obtained were loaded into 8-12% SDS-PAGE. Proteins were size fractionated, transferred to a PVDF membrane (Bio-Rad) and, after blocking with 5% non-fat dry milk, incubated with the corresponding antibodies (Table 2). Blots were developed by ECL protocol and different exposition times were performed for each blot to ensure the linearity of the band intensities. Values of densitometry were determined using Image J software.

Target	Distributor	Reference
AMPKα	Cell Signaling	5832
P-AMPKα	Cell Signaling	2535
Bcl2	Cell Signaling	2870
CaMKK2	Cell Signaling	16810
Catalase	Millipore (Burlington, MA ,USA)	219010
EndoG	Santa Cruz Biotech. (Dallas, TX, USA)	sc-365359
Galectin-3	Santa Cruz Biotech.	sc-32790
GAPDH	Ambion	AM4300
HO-1	Millipore	AB1284
Keap-1	Thermo Fischer scientific	PA5-34454
Lamin B	Santa Cruz Biotech.	sc-6217
NQO1	Cell Signaling	3187
Nrf2	Homemade Dr.Cuadrado´s lab	----
PRDX-1	Santa Cruz Biotech.	137222
TNFα	Abcam	ab-8348
Vinculin	Cell Signaling	13901

Table 2. Specific antibodies used in western blot assays.

2.12. Statistical analysis. Values in graphs correspond to the mean ± standard deviation (SD). In box and whiskers diagram, boxes represent median and interquartile range and whiskers represent maximum and minimum values. Statistical significance was estimated with two-tailed Student *t* test for unpaired observations and one-way ANOVA for multiple comparisons followed by Tukey´s range test as indicated under each figure. All analyses were performed using GraphPad Prism Software.

3. RESULTS.

3.1. EAM model characterization.

Since EAM is an inflammatory model of cardiac damage, we first determined the presence of immune infiltrates in the heart by histological staining, quantifying them according to Dallas criteria (33) (Fig. 1a). We determined that EAM+vehicle group (hereinafter referred to as EAM) had higher infiltration score than both controls, which was significantly lower in BML-treated EAM group (Fig. 1b). We next characterized the immune cells in the infiltrated area by immunofluorescence. Compared to control groups that present very low levels of immune cells, hearts from EAM mice animals showed higher count of $CD68^+$ macrophages (14.6% of total cell count), $Ly6G^+$ neutrophils

(6.4%) and RORγT^{+} T_H17 lymphocytes (9.8%) (Supp. Fig. 1). These values were significantly reduced by more than half in hearts from BML-treated EAM animals. Since a severe immune infiltration was evident in EAM hearts, we analyzed cardiac mRNA levels of *Il1b, Il6* and *Tnfa* as classic pro-inflammatory cytokines by qPCR. As expected, EAM group exhibited significantly augmented levels of these cytokines that were decreased in BML-treated EAM animals in the case of *Il1b* and *Tnfa* (Fig. 1c). Accordingly, when we determined the protein levels of inflammatory markers in the heart, we detected enhanced protein expression of Galectin-3 and TNFα in EAM group compared to both controls, which was significantly reduced in BML-treated EAM mice (Fig. 1d). These results indicate that BML-111 treatment averted expression of pro-inflammatory mediators and blocked the recruitment of immune cells to the affected hearts.

The inflammatory phenotype associated to EAM produces cardiac dysfunction. Thus, we carried out echocardiographic studies to determine cardiac function in each experimental group (Table 3). Whereas vehicle and BML-treated control groups showed normal values for the analyzed parameters, EAM group exhibited significantly decreased ejection fraction (EF) and fractional shortening (FS) and augmented levels of left ventricle end-systolic diameter (LVESD) compared to control groups. All these functional parameters indicate the appearance of ventricular dysfunction in EAM mice that was significantly prevented in BML-treated EAM mice. Other factors, such as left ventricle end-diastolic diameter (LVEDD) or heart rate (HR) did not show any difference between groups (Table 3).

Experimental groups	EF (%)	FS (%)	LVESD (mm)	LVEDD (mm)	HR (bpm)	N
Control + Veh	70.3 ± 8.7	41.3 ± 7.1	1.5 ± 0.1	2.6 ± 0.6	333.7 ± 43.8	15
Control + BML	67.2 ± 4.9	36.4 ± 10.9	1.9 ± 0.2	2.9 ± 0.6	308.7 ± 31.7	19
EAM + Veh	47.6 ± 3.2 ***	20.9 ± 3.4 ***	2.1 ± 0.1 ***	2.6 ± 0.4	304.1 ± 44.7	22
EAM + BML	66.6 ± 4.9 ###	39.2 ± 13.4 ###	1.6 ± 0.1 ###	2.7 ± 0.7	306.3 ± 36.1	17

Table 3. Cardiac function parameters obtained from echocardiography. Values represent mean ± SD. Statistical significance was determined using one-way ANOVA followed by Tukey´s multiple comparisons test for multiple groups comparisons. ***P≤0.001 vs. Control+Veh; ###P≤0.001 vs. EAM+Veh group.

In this experimental model, cardiac dysfunction could be associated to the development of hypertrophy in the hearts of affected animals. Indeed, we observed that EAM mice exhibited higher heart weight (HW) to body weight (BW) or tibial length (TL) ratios (Fig. 2a, b), being both significantly prevented in BML-treated EAM group. Body weight

values did not show any difference among groups (data not shown). In the same line, we determined increased levels of cardiac damage and hypertrophy markers in EAM mice, concretely plasmatic BNP (Fig. 2c) and cardiac *Anp* mRNA expression (Fig. 2d). Both parameters were significantly lower in BML-treated EAM mice. These results demonstrate that BML-111 treatment is able to prevent cardiac dysfunction in EAM-induced animals in part by reducing their associated cardiac hypertrophy. Besides cardiac hypertrophy, fibrosis development, which can also severely impair cardiac function, has been described to occur in EAM model at day 21 as well (34). In our case, EAM animals presented higher cardiac mRNA levels of the classic fibrotic markers TGF-β1 (*Tgfb1*) or collagen 1 (*Col1a1*) and 3 (*Col3a1*) (Fig. 2e) that were significantly lesser in the case of TGF-β1 and collagen 1 in BML-treated EAM mice.

3.2. Effects of BML-111 on cardiac cells apoptosis.

Cardiac dysfunction can be associated to increased cardiomyocyte death, which eventually debilitates the contractive capacity of the heart. We determined that in EAM group, hearts presented significantly higher percentage of TUNEL-positive nuclei that were reduced upon BML-111 treatment (Fig. 3a). Accordingly, we also detected higher expression of the classic apoptotic effector cleaved-caspase 3 by immunofluorescence in EAM group that were significantly lower in BML- treated EAM mice (Fig. 3b). As these results indicate the activation of cardiomyocytes apoptosis, we next analyzed the levels of apoptotic proteins in cardiac tissue by western blot (Fig. 3c). We observed that the levels of the anti-apoptotic protein Bcl-2 were reduced in EAM animals, and BML-111 treatment maintains higher levels of this protein despite not reaching statistical significance. Conversely, the pro-apoptotic protein endonuclease G (EndoG) levels were increased in EAM animals and were significantly reduced in BML-treated EAM mice. This protein induces apoptosis *via* DNA degradation when translocating from the mitochondria to the nucleus under oxidative stress conditions. Therefore, BML-111 appears to ameliorate EAM outcome in part by mitigating cardiac cell death, thus improving cardiac functionality.

3.3. Effects of BML-111 in the cardiac oxidative stress development: Nrf2 pathway.

Hearts from EAM animals are also exposed to elevated levels of oxidative stress that overwhelm the capacity of antioxidant systems to cope with this severe inflammatory scenario. This stress can exacerbate inflammation aggravating the autoimmune reaction of myocarditis and could represent one of the pathological mechanisms involved in

cardiac remodeling, eventually leading to cardiomyopathies. At the cellular level, this oxidative damage can permanently affect membrane lipids, proteins, and even DNA. Thus, we first characterized the cardiac presence of a broadly described biomarker of oxidative DNA damage, 8-hydroxy-2'–deoxyguanosine (8-OHdG) by fluorescence techniques (Fig. 4a). We detected significantly higher levels of 8-OHdG in EAM mice hearts compared to controls and BML-111 treatment significantly prevented these. We additionally evaluated malondialdehyde (MDA) staining for lipid peroxidation, which also exhibited higher levels in hearts from EAM mice (Fig. 4b). Despite a certain variability, MDA levels also tended to reduce in BML-treated EAM mice (Fig. 4b).

Since it is known that SPMs can induce the activation of the Nrf2 transcription factor, the "master regulator" of oxidative stress resistance, we hypothesized that the antioxidant effect of BML-111 could be based on an induction of the Nrf2 pathway. First, we analyzed the protein levels of the main Nrf2 inhibitor, Keap1, in cardiac tissue by immunofluorescence (Fig. 4c). We observed increased positive staining in EAM mice compared to both controls that was reduced in BML-treated EAM animals. These results were confirmed by western blot analysis of Keap1 protein levels (Fig. 4d). These data suggested that BML-111 treatment could mitigate oxidative stress by activating Nrf2 pathway. To deeply analyze the involvement of Nrf2 in the BML-mediated beneficial effects, we performed a set of experiments inoculating additional mice with BML-111 for shorter periods of time (1-6 h) and quantified Nrf2 nuclear protein levels in cardiac tissue (Fig. 5a). In these experiments, we observed that BML-111 significantly induced the translocation of Nrf2 into the nucleus, particularly at 1 and 6 h. Moreover, longer treatment with BML-111 for 24h significantly upregulated several of Nrf2 main antioxidant targets: HO-1, NQO1, catalase and PRDX-1 (Fig. 5b). All these data demonstrate that the cardioprotective effect of BML-111 is in part based on an induction of the antioxidant response *via* activation of the Nrf2 pathway.

3.4. Molecular analysis of the signaling pathway activated in the heart by BML-111.

To elucidate the molecular pathway involved in this Nrf2 induction, we performed some additional *in vitro* experiments using the murine cardiac cell line HL-1. Once verified that in these cells Nrf2 nuclear levels were also significantly increased after BML-111 treatment, we analyzed upstream signaling *in vitro*. It has been described that stress can directly activate CaMKK2 in cardiac cells (35). This protein is capable of activating AMPKα which in turn directly phosphorylates Nrf2 facilitating its nuclear accumulation

(22). When HL-1 cells were treated with BML-111 for short periods of time, we detected an early induction of CaMKK2 at 5 min followed by an increased phosphorylation of AMPKα from 30 min to 2 h, which agrees with CaMKK2-AMPKα-Nrf2 signaling pathway (Fig. 6a). To further confirm the implication of AMPKα in BML-111-mediated Nrf2 activation in this model, we treated HL-1 cells with compound C, a potent, selective and reversible inhibitor of AMPK activity (36). We confirmed that compound C effectively inhibits AMPKα since phosphorylation of ACC (acetyl-CoA carboxylase), one of the main targets of AMPKα kinase activity, was abolished (data not shown). Then, we observed that Nrf2 nuclear translocation induced by BML-111 treatment was significantly inhibited upon compound C treatment (Fig 6b). In conclusion, these data confirm that the cardioprotective effect of BML-111 appears to be regulated by the sequential activation of the CaMKK2-AMPKα axis.

4. DISCUSSION.

Myocarditis is an inflammatory disease of the myocardium characterized by the presence of immune infiltrates and apoptotic cardiomyocytes in cardiac muscle. Importantly, affected patients often progress to a more severe form of the disease termed DCM that, once developed, it is irreversible in the broad majority of cases. Thus, the design of efficient therapeutic approaches is still needed to reduce the progression from cardiac harmful initial conditions to DCM.

Since inflammation plays an important role in CVDs, SPMs arise as promising endogenous targets to design a new therapy due to their broadly demonstrated anti-inflammatory and pro-resolving properties (37). In recent years, SPMs have been already shown to have protective role in major CVDs in murine models where they are able to improve cardiac function and reduce both efferocytosis and pro-inflammatory cytokines release, improving the pathological phenotype. Accordingly, in the present study we provide experimental evidence that the lipoxin A_4 analog BML-111 decreases EAM severity in mice by reducing both inflammatory cell infiltration and the expression of several pro-inflammatory cytokines. In fact, BML-111 was able to prevent the increase of both IL-1β and TNFα levels. However, it was unable to modify IL-6 levels, possibly due to its role as a pleiotropic cytokine with a dual role in inflammation. There exists evidence that IL-6 can be both protective or pathogenic depending on the kinetics of the host response (38). In fact, it has been showed that this cytokine can modulate both the

inflammatory response and fibrosis development in several autoimmune disease models (39). In our study the maintenance of high cardiac levels of IL-6 after BML-111 treatment can be considered as a protective response since this cytokine protects myocytes against oxidative stress and its signaling induces an anti-apoptotic program (40).

Furthermore, we here demonstrated that BML-111 treatment prevents cardiac dysfunction of EAM mice. In the same line, BML-treated EAM mice exhibited reduced levels of cardiac damage markers like plasmatic BNP, which have been reported to be inversely related to LXA_4 levels in chronic heart failure patients (41), and cardiac ANP expression. Interestingly, our data suggest that despite cardiac inflammation and dysfunction are both evident in EAM mice, fibrosis contribution to the final phenotype is minimal. Indeed, implication of fibrosis in EAM development is still controversial due to the heterogeneity of the data, with some groups reporting the presence of fibrotic areas in EAM hearts at day 21 (34, 42), whereas other groups consider that fibrosis development does not become apparent until day 54 (43, 44). On the other hand, BML-111 treatment also managed to reduce cardiac hypertrophy in EAM mice as observed by the reduction of both HW/BW or HW/TL ratios. These results unveil the ability of SPMs to prevent pathological cardiac hypertrophy and requires further investigation.

The appearance of cell death foci of cardiac cells in affected hearts is another important factor contributing to cardiac dysfunction in CVDs. Several studies have demonstrated that SPMs are capable to modulate cell death pathways, acting as pro- or anti-apoptotic mediators depending on the cell type (45, 46). Indeed, it has been shown that elevated protein levels of LXA_4 can be related to higher cardiomyocyte survival by modulation of PI3K/Akt pathway (47). This pathway has also been related to the anti-inflammatory effects of LXA_4 observed in the EAM model (48). However, we have demonstrated for the first time that, in addition to their anti-inflammatory actions, BML-111 also reduced the number of apoptotic areas in EAM hearts and this effect is associated to a significant reduction in cardiac levels of the apoptotic proteins EndoG and caspase 3. These results are in accordance with Zhao *et al.*, who demonstrated that LXA_4 can regulate cardiac mRNA levels of caspase 12, which acts upstream in caspase 3 activation pathway, in an ischemia-reperfusion model (49). Overall, the ability of BML-111 to reduce cardiac cell death preserves appropriate heart functioning, decreasing the consequent development of fibrotic and non-functional regions that impairs cardiac contractility and seriously compromises both the quality of life and prognosis of affected patients.

Furthermore, oxidative stress, defined as an exacerbated production of ROS affecting cells, tissue and organ homeostasis, has been shown to play an important role in the pathophysiology of CVDs (50). In this context, it has been demonstrated that SPMs possess an antioxidant effect in many pathological scenarios, mainly through an induction of the transcription factor Nrf2, considered as the "redox guardian" of the cells. Nonetheless, little is known about this modulation in the cardiac context, with only Chen *et al*. demonstrating that LXA_4 was able to protect cardiomyocytes against hypoxia/reoxygenation injury mainly by activating the Nrf2 pathway (51). Here we determined that BML-111 is able to reduce the DNA damage associated to oxidative stress in EAM model also through activation of Nrf2 pathway as well as its antioxidant targets. Furthermore, we detected that this BML-associated cardiac induction of Nrf2 is mediated by Keap1, whose modulation by SPMs had been only demonstrated in intestinal (27) or lung injury animal models (52).

Our study demonstrates the contribution of the antioxidant Nrf2 pathway to the improved cardiac phenotype observed in SPMs-treated animals upon EAM induction. Moreover, we have looked into the molecular signaling mediated by SPMs in the heart. By using an *in vitro* model we determined that Nrf2 upstream signaling is regulated by the sequential activation of CaMKK2-AMPKα kinase cascade in cardiomyocytes. The interaction between SPMs and AMPKα signaling had been already proposed in adipose tissue (53), where RvD1 treatment in *db/db* mice increased AMPKα phosphorylation. However, its role in obesity remains controversial as Borgesson *et al.* thereafter showed that, in an obesity-induced adipose inflammatory model, lipoxins can exert their function independently of AMPKα (54). Regarding cardiac tissue, previous studies have described that the flavonoid butin induced the modulation of the Nrf2 antioxidant pathway through AMPKα (55). Interestingly, we have demonstrated for the first time that SPMs are able to activate the same molecular signaling that this flavonoid in cardiomyocytes, highlighting the potent antioxidant role of these pro-resolution mediators.

In summary, myocarditis is an extremely complex immune-mediated process. Despite the growing body of knowledge about its pathogenesis, the specific factors implicated in disease development in patients remain unknown. Indeed, myocarditis progression to DCM is still unpredictable in clinical practice. Advances exist in therapeutics, but still relying in global immunosuppression and unspecific immunomodulation, with

suboptimal results. Future basic and translational studies might provide new insights in the pathogenesis of myocarditis leading to development of better diagnostic tools. Among them, SPMs, which we have demonstrated to have a protective effect on EAM cardiac model attenuating both inflammation, cardiomyocyte death and cardiac oxidative stress, arise as a potential alternative with low adverse effects. Thus, since this harmful scenario is common to the majority of CVDs, these lipid mediators represent a promising targeted therapeutic option in the future, mainly contributing to improve their initial stages and to limit the progression to a more harmful and irreversible phenotype.

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AUTHORS' CONTRIBUTIONS.

P.P., R. I-J, M. F-V designed the study, performed experiments, analyzed data, and wrote the paper. VT, S.S-G, N.C-B, A.V-B. performed experiments. C.Z. carried out all the echocardiographic study and analysed the corresponding data. M. V-C. performed the histological techniques. L.B. designed the study, provided funding and wrote the manuscript.

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Figure Legends.

Figure 1. **Hearts from BML-111 treated-mice presented lower inflammatory levels than EAM-mice and a lesser degree of immune infiltration** (**a**) Representative images of histological analysis of immune cell infiltration in cardiac tissue from mice of each experimental group and the corresponding quantification (**b**) following Dallas criteria. Objective lens 20X. n≥20. Boxes represent median and interquartile range; whiskers represent maximum and minimum values. (**c**) Cardiac mRNA levels of several pro-inflammatory mediators (*Il1b*, *Il6* and *Tnfa*) determined by qPCR. Graphs represent fold induction (FI) of each experimental group using *Rplp0* as endogenous control. n≥7 (*Il1b*), n≥13 (*Il6*), n≥18 (*Tnfa*). (**d**) Representative western blot images (left panel) and their corresponding quantification (right panel) of Galectin-3 and TNFα protein levels in cardiac tissue from all experimental groups. Normalization was performed using GAPDH as reference. n≥7 (Galectin-3), n≥6 (TNFα). Graphs represent mean ± SD. Dots represent individual data points. Statistical significance was determined using one-way ANOVA followed by Tukey´s multiple comparisons test for multiple groups comparisons. ***P*≤0.01 and ****P*≤0.001 *vs.* Control+Veh; #P≤0.05 and ##P≤0.01 *vs.* EAM+Veh group.

Figure 2. BML-111 treatment prevented the hypertrophy and fibrosis phenotype in EAM mice. Heart weight to body weight ratio (HW/BW) (**a**) and heart weight to tibial length (HW/TL) (**b**) determination from all experimental groups. n=24. (**c**) BNP quantification in murine plasma at day 21 from all experimental groups. n=24. (**d, e**) Analysis of *Anp* (**d**) or *Tgfb1*, *Col1a1* and *Col3a1* (**e**) mRNA levels in cardiac tissue from all experimental groups at day 21. Graph represents the fold induction (FI) of each experimental group using *Rplp0* as endogenous control. n≥22 *(Tgfb),* n≥17 (*Col1a1),* n≥16 (*Col3a1*). Dots represent individual data points. Graphs depict mean ± SD. Statistical significance was determined using one-way ANOVA followed by Tukey´s multiple comparisons test for multiple groups comparisons. ***P*≤0.01 and ****P*≤0.001 *vs.* Control+Veh; ###*P*≤0.001 *vs.* EAM+Veh group.

Figure 3. Apoptosis of cardiac cells in EAM was reduced after BML-111 treatment. (**a**) Representative images of TUNEL staining performed in cardiac tissue from all experimental groups. TUNEL-positive nuclei are stained in green and DAPI in blue. Values indicated in each image correspond to the mean of TUNEL-positive nuclei (%) ±

SD. Objective lens 20X. n≥8. (**b**) Representative immunofluorescence images of cardiac tissue from all experimental groups stained with cleaved-caspase 3 in red, α-actinin in green and DAPI in blue. Values indicated in each image correspond to fluorescence quantification in relative fluorescence units (r.f.u.) ± SD. n≥7. Objective lens 63X. (**c**) Representative western blot images (left panel) and their corresponding quantification (right panel) of Bcl-2 and EndoG protein levels in cardiac tissue from all experimental groups. Normalization was performed using GAPDH as reference. n≥8 (Bcl-2), n≥5 (EndoG). Dots represent individual data points. Graphs represent mean ± SD. Statistical significance was determined using one-way ANOVA followed by Tukey´s multiple comparisons test for multiple groups comparisons. *P≤0.05 and ***P≤0.001 *vs.* Control+Veh; #P≤0.05 and ###P≤0.001 *vs.* EAM+Veh group.

Figure 4. Treatment of EAM mice with BML-111 reduced the cardiac oxidative stress by reducing Keap1 levels. (**a, b**) Determination of (**a**) oxidative DNA damage by measuring 8-OHdG fluorescent marker and (**b**) lipid peroxidation by measuring MDA fluorescent staining in cardiac tissue from all experimental groups. n≥5. (**c**) Representative immunofluorescence images of cardiac tissue from all experimental groups stained with Keap1 in red and DAPI in blue. Values indicated in each image correspond to fluorescence quantification in relative fluorescence units (r.f.u.) ± SD. Objective lens 63X. n≥4. (**d**) Representative western blot images (left panel) and their corresponding quantification (right panel) of Keap1 protein levels in cardiac tissue from all experimental groups. Normalization was performed using GAPDH as reference. n≥4. Dots represent individual data points. Graphs represent mean ± SD. Statistical significance was determined using one-way ANOVA followed by Tukey´s multiple comparisons test for multiple groups comparisons. *P≤0.05, **P≤0.01 and ***P≤0.001 *vs.* Control+Veh; #P≤0.05 and ###P≤0.001 *vs.* EAM+Veh group.

Figure 5. BML-111 induced Nrf2 antioxidant pathway activation in the heart. (**a**) Representative western blot images (left panel) and their corresponding quantification (right panel) of Nrf2 nuclear levels in cardiac tissue from mice treated with BML-111 for the indicated times. Normalization was performed using Lamin B as reference. n≥5. Statistical significance was determined using one-way ANOVA followed by Tukey´s multiple comparisons test for multiple groups comparisons. *P≤0.05, **P≤0.01 *vs.* control group. (**b**) Representative western blot images (left panel) and their corresponding quantification (right panel) of HO-1, NQO1, Catalase and PRDX-1 protein levels in

cardiac tissue from mice treated with BML-111 for 24 h. Normalization was performed using Vinculin as reference. n≥4 (HO-1, NQO1 and Catalase) and n≥3 (PRDX-1). Dots represent individual data points. Graphs represent mean ± SD. Statistical significance was determined using unpaired Student´s t test. *P≤0.05, **P≤0.01 *vs.* control group.

Figure 6. Nrf2 upstream signaling is mediated by sequential activation of several kinases in response to BML-111 challenge. (a) Representative western blot images (left panel) and their corresponding quantification (right panel) of CaMKK2, P-AMPKα and AMPKα protein levels HL-1 cells treated with BML-111 for the indicated times. Normalization was performed using Vinculin as reference. n≥8. (**b**) Representative western blot images (left panel) and their corresponding quantification (right panel) of Nrf2 nuclear levels in HL-1 cells treated with BML-111 and/or Compound C for the indicated times. Normalization was performed using Lamin B as reference. n≥4. Dots represent individual data points. Graphs represent mean ± SD. Statistical significance was determined using one-way ANOVA followed by Tukey´s multiple comparisons test for multiple groups comparisons. *P≤0.05, **P≤0.01, ***P≤0.001 *vs.* control group.