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Atuação do *Toll-like receptor 4* (TLR4) no músculo cardíaco de camundongos em diferentes modelos de exercício físico

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Versão corrigida

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Orientador: Prof. Dr. Adelino Sanchez Ramos da Silva

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“Foi o tempo que perdestes com a tua rosa que a fez tão importante.”[1]

(Antoine Saint-Exupéry, 2015, p.74)

VICENTE, LG. **Atuação do *Toll-like receptor 4* (TLR4) no músculo cardíaco de camundongos em diferentes modelos de exercício físico.** 2021. 94f. Tese (Doutorado) - Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, 2021

RESUMO

O *Toll-like receptor 4* (TLR4) é o membro da família dos *Toll-like receptors* com maior expressão no coração. Evidências sugerem que este receptor está relacionado a doenças cardiovasculares e que ele é o principal ativador de eventos desencadeados pelo exercício físico no coração como inflamação, estresse do retículo endoplasmático (RE) e apoptose. Além disso, sua baixa modulação em indivíduos treinados pode estar associada aos efeitos benéficos causados pelo treinamento físico. Assim, o objetivo do presente estudo foi avaliar a real participação do TLR4 na via de sinalização da inflamação, apoptose e estresse do RE no miocárdio desencadeados pelo exercício físico agudo (EFA), e também avaliar se o exercício físico crônico (EFC) é capaz de reduzir a expressão de TLR4 no coração, comparando camundongos *knockout* de TLR4 (KO) com camundongos *wild-type* (WT) As técnicas de RT-qPCR, *immunoblotting* e ecocardiograma foram utilizadas para analisar o miocárdio de camundongos em dois tipos de experimentos, no experimento 1 foram comparados camundongos sedentários a camundongos submetidos a três diferentes intensidades de EFA (45%, 60% e 75% da potência máxima definida pelo teste de carga incremental). No experimento 2 foram comparados animais sedentários (grupo controle) a camundongos submetidos a um protocolo de EFC de 4 semanas, além das análises realizadas no experimento 1, no experimento 2 também foram realizados testes de *performance*, calorimetria indireta e histologia. Os principais resultados do experimento 1 foram: nos animais submetidos ao EFA a deleção de *Tlr4* reduziu a expressão de genes associados a inflamação e ao estresse do RE no estado basal, e sob as mesmas condições de exercício, também reduziu a glicemia e a expressão de genes e proteínas relacionados a apoptose, inflamação e estresse do RE, assim como aumentou a expressão de outros genes relacionados a apoptose, inflamação e estresse do RE. Os principais resultados do experimento 2 foram: no grupo controle a deleção de *Tlr4* reduziu a ativação das vias do estresse do RE e da apoptose e aumentou o gasto energético, e no grupo de EFC, a deleção de *Tlr4* inibiu a melhora da *performance* e da função cardíaca causada pelo treinamento físico, porém o treinamento não reduziu o conteúdo de TLR4 no coração dos camundongos WT. Esses resultados sugerem que a deleção de *Tlr4* atenuou os efeitos das diferentes intensidades de EFA no coração dos camundongos TLR4-KO e que em contraste com a hipótese inicial o EFC não é capaz de reduzir a expressão de TLR4 no coração. No entanto, o TLR4 parece desempenhar um importante papel nas adaptações benéficas secundárias a prática regular de exercício físico.

Palavras-chave: *Toll-like receptor 4*, exercício físico, miocárdio

VICENTE, LG. **Toll-like receptor 4 (TLR4) actuation in mice cardiac muscle in different physical exercise models.** 2021. 94f. Tese (Doutorado) - Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, 2021.

ABSTRACT:

The Toll-like receptor 4 (TLR4) is the Toll-like receptors' family member with the highest expression in the heart. Evidence suggests that this receptor is related to cardiovascular disease and that it is the primary activator of events triggered by physical exercise in the heart, such as inflammation, endoplasmic reticulum (ER) stress, and apoptosis. In addition, its low modulation in trained individuals may be associated with the beneficial effects caused by physical training. Thus, the present study aimed to evaluate the actual participation of TLR4 in the inflammation, apoptosis, and ER stress signaling pathway in the myocardium triggered by acute physical exercise (APE), and also to assess whether chronic physical exercise (CPE) is able to reduce TLR4 expression in the heart, comparing TLR4 knockout mice (KO) with wild-type mice (WT). The RT-qPCR, immunoblotting, and echocardiogram techniques were used to analyze the myocardium of mice in two types of experiments. In experiment 1, sedentary mice were compared to mice subjected to three different intensities of APE (45%, 60% and 75% of the maximum power defined by the incremental load test) In experiment 2, sedentary animals (control group) were compared to mice submitted to a 4-week CFE protocol, in addition to the analyzes performed in experiment 1, in experiment 2, performance tests, indirect calorimetry and histology were also performed. The main results of experiment 1 were: in animals submitted to APE, the *Tlr4* deletion reduced the expression of genes associated with inflammation and ER stress in the basal state, under the same exercise conditions, it also reduced blood glucose and expression of genes and proteins related to apoptosis, ER stress, and inflammation, as well as, increased the expression of others genes related to apoptosis, ER stress, and inflammation. The main results of experiment 2 were: in the control group the *Tlr4* deletion reduced the activation of the ER stress and apoptosis pathways and increased energy expenditure, and the EFC group, the *Tlr4* deletion inhibited the improvement in performance and cardiac function caused by training, but training did not reduce the TLR4 content in the heart of WT mice. These results suggest that the *Tlr4* deletion attenuated the effects of different APE intensities in the heart of TLR4-KO mice, and that in contrast to the initial hypothesis, the CPE cannot reduce TLR4 expression in the mice heart. However, TLR4 seems to play an essential role in the beneficial adaptations secondary to regular physical exercise.

Keywords: Toll-like receptor 4, physical exercise, myocardium

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AGNE- ácidos graxos não esterificados

ATF4- *activating-transcription factor 4*

ATF6- *activating-transcription factor 6*

BAX - *BCL-2 associated protein X*

BIP- *binding protein*

BNP- *B-type natriuretic peptid*

CD36 – conjunto de diferenciação 36

CHOP- *C/EBP- Homologous Protein*

CK- creatina quinase

CKMB- creatina quinase MB isoenzima

CT- controle

EFA- exercício físico agudo

EFA-45%- exercício físico agudo abaixo da máxima fase estável de lactato (intensidade 45%)

EFA-60%- exercício físico agudo na máxima fase estável de lactato (intensidade 60%)

EFA-75%- exercício físico agudo acima da máxima fase estável de lactato (intensidade 75%)

EFC- exercício físico crônico

EFC- CT- exercício físico crônico grupo controle

eIF2-α- *eukaryotic translation initiation factor 2A*

IL-1β- Interleucina 1 beta

IL-6- Interleucina 6

IL-17- Interleucina 17

IRAK1- *interleukin -1 receptor-associated kinase 1*

IRAK4- *interleukin -1 receptor-associated kinase 4*

KO- *knockout* de TLR4

LDH- desidrogenase láctica

LPS- lipopolissacarídeo

MyD88- *Myeloid differentiation primary response 88*

NF-κB- Fator Nuclear kappa B

PCT- Proteínas de choque térmico

PERK- quinase do retículo endoplasmático semelhante à proteína quinase do pâncreas

P38MAPK- p38 *mitogen-activated protein kinase*

RE- retículo endoplasmático

RNAm-RNA mensageiro

TCI- Teste de carga incremental

TIR - *toll* /IL-1 receptor

TIPAP- *TIR domain containing adaptor protein*

TLR4- *toll-like receptor 4*

TNFα- Fator de necrose tumoral alfa

TRAM-*TRIF related adaptor molecule*

TRIF- TIR-domain-containing adaptor protein inducing IFNb.

TTG- Teste de tolerância a glicose

UPR- Resposta a proteína não dobrada

VE- Ventrículo esquerdo

XBP1- *X-box-binding protein*

WT- *Wild-type*

SUMÁRIO

1. INTRODUÇÃO E JUSTIFICATIVA

É amplamente difundido o conceito de que a prática regular de exercício físico acarreta benefícios para todo o corpo, em especial para o coração devido ao seu efeito de proteção do músculo cardíaco[2]. Quando praticado de forma individualizada, o exercício físico pode ser utilizado como uma poderosa estratégia não farmacológica de tratamento e prevenção para diversas doenças crônicas, além de participar como agente redutor da morbimortalidade de grande parte da população[3]. No entanto, quando as recomendações básicas para a prescrição do exercício não são seguidas, uma sessão de exercício pode ser o principal desencadeador de eventos cardíacos indesejados como o infarto agudo do miocárdio e a morte súbita[4].

Uma única sessão de exercício físico, também chamada de exercício físico agudo (EFA), com duração e intensidade excessivas pode causar ajustes transitórios no coração como a redução do diâmetro do ventrículo esquerdo durante a diástole e redução do volume sistólico e diastólico final[5]. Também são reportadas associações entre o EFA e a elevação de marcadores de lesão cardíaca como a creatina kinase MB isoenzima (CK-mb), troponina e *B-type natriuretic peptid* (BNP)[6]. Ao avaliarem os efeitos cardíacos do EFA exaustivo em ratos Wistar, Oláh e colaboradores[7] encontraram aumento de enzimas cardíacas (creatina kinase/CK, troponina T, desidrogenase láctica/LDH e aspartato transaminase), redução da contratilidade e da eficiência cardíaca e aumento da atividade pró-apoptótica e fibrótica. Além disso, durante a análise histopatológica do ventrículo esquerdo, os autores também observaram fragmentação esporádica das fibras miocárdicas, infiltração leucocitária, edema do tecido e eosinofilia citoplasmática.

O EFA também pode provocar aumento de citocinas pró-inflamatórias no coração como a interleucina 1 beta (IL-1β)[8], a interleucina 6 (IL-6)[8,9] e o fator de necrose tumoral alfa (TNFα)[9], aumento este que provavelmente é mediado pelo Fator Nuclear kappa B (NF-κB), cujo papel na regulação ascendente das citocinas pró-inflamatórias já está bem estabelecido[9,10]. Evidências sugerem que uma única sessão de exercício é capaz de elevar a ativação do NF-κB no miocárdio[9] de uma maneira dependente da intensidade[11] e a ativação deste importante fator de transcrição pode ser induzida pelo *Toll like receptor 4* (TLR4)[12] inclusive durante o EFA[13].

O TLR4 se tornou conhecido por seu relevante papel na resposta imune e pode ser encontrado em vários tipos de células do corpo humano como em células do sistema imune, hepatócitos, adipócitos[14], astrócitos[15] e inclusive em cardiomiocitos[16,14,16,17]. Trabalhos publicados demonstram sua participação em desordens cardiovasculares como: isquemia do miocárdio[18], hipertensão[19], aterosclerose e redução da contratilidade dos cardiomiócitos[20; 21]. Recentes estudos apontam o papel do TLR4 como desencadeador de eventos lesivos a célula como inflamação[13], apoptose[22,23] e estresse do retículo endoplasmático (RE)[28,24], fenômenos que podem ser iniciados pela realização de uma sessão extrema de exercício aeróbio[25].

O EFA é capaz de desencadear maior ativação do TLR4[13], o que pode induzir à alterações deletérias no coração como redução da função ventricular sistólica e diastólica[26] e até mesmo danos ao miocárdio, tornando-se um risco em potencial para pessoas portadoras de doenças cardiovasculares[5]. No entanto, apesar de haver algumas especulações, os mecanismos pelos quais o EFA provoca a ativação do TLR4 foram pouco explorados até o presente momento. Basicamente, foram descritas duas vias de sinalização relacionadas ao TLR4: a via MyD88-dependente e a via TRIF-dependente, também conhecida como via MyD88-independente[27]. Embora em outros órgãos essas

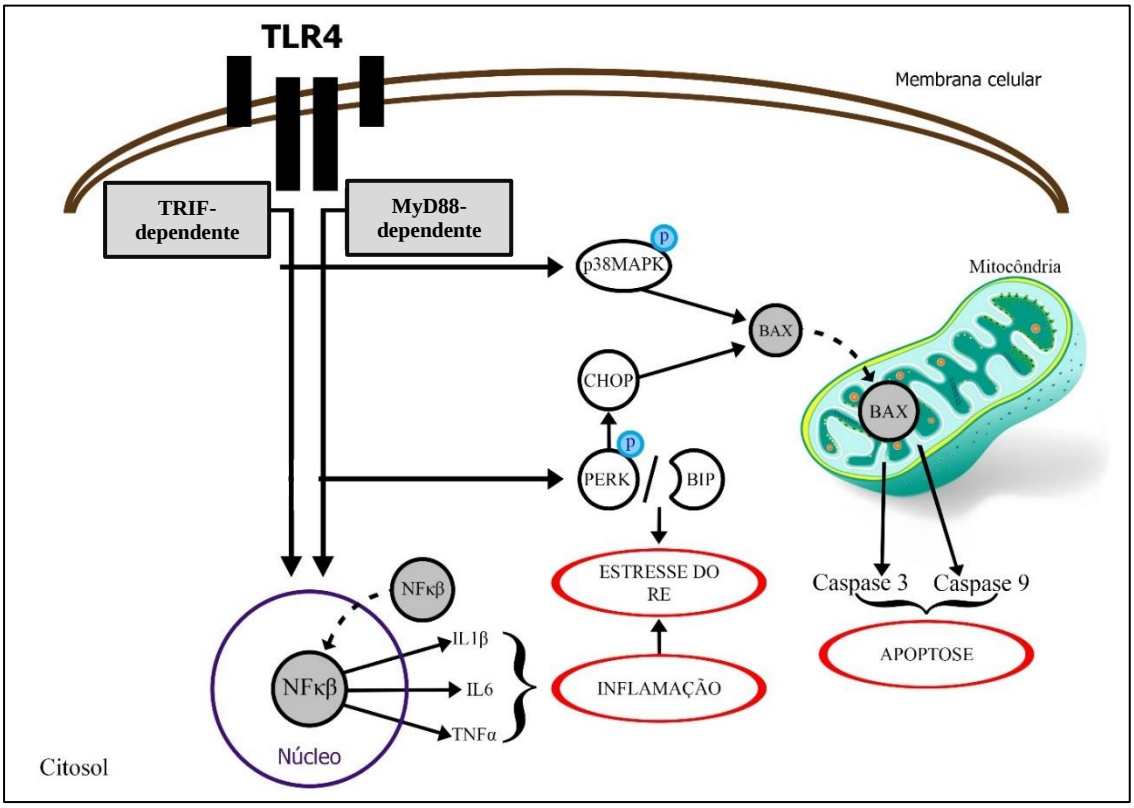
duas vias possam atuar de maneira independente, no miocárdio suas ações ocorrem em conjunto, acarretando na translocação do NF-κB para o núcleo que leva a transcrição de genes que posteriormente promovem a tradução de proteínas[28] como as citocinas pró-inflamatórias IL-1β, IL-6 e TNFα[21; 27; 29] que contribuem para a inflamação do miocárdio. A inflamação causada pela translocação do NF-κB pode levar ao estresse do RE, como comprovado por pesquisadores que, ao administrarem um inibidor de NF-κB em ratos, observaram uma redução do estresse do RE desencadeado pelo TLR4[30]. Uma ativação duradoura da via do TLR4 induz ao estresse do RE, o qual por sua vez promove a síntese de novos TLR4, indicando uma integração de sinais entre a via TLR4 e o estresse do RE[29].

Afrazi e colaboradores[30] verificaram que ratos com deleção seletiva de TLR4 são protegidos do estresse do RE e da apoptose comprovando que o TLR4 é essencial para essas vias de sinalização. Ainda com a finalidade de observar por qual via específica o TLR4 desencadeia o estresse do RE, esses autores induziram este evento através da administração de lipopolissacarídeo (LPS; um ativador de TLR4) em ratos com deleção seletiva das proteínas *activating-transcription factor 6* (ATF6), sua adjacente *X-box-binding protein* (XBP1) e a quinase do RE semelhante à proteína quinase do pâncreas (PERK). Os pesquisadores constataram que apenas os roedores com deleção seletiva de PERK apresentaram atenuação significativa do estresse do RE. Neste mesmo estudo, os autores comprovaram que a *C/EBP- Homologous Protein* (CHOP), proteína diretamente ativada pela PERK, faz parte da via do estresse do RE que é controlada pelo TLR4 e que o eixo PERK/CHOP, além de participar da via do estresse do RE, também participa da via da apoptose[30]. Contudo, somente a via MyD88-dependente parece ter influência sobre a fosforilação da PERK[30], embora outros estudos comprovem que via TRIF-dependente também participa da via do estresse do RE[31].

Sabe-se que o estresse do RE é acionado por uma resposta adaptativa conhecida como resposta a proteína não dobrada (UPR). Uma das proteínas responsáveis por ativar este mecanismo em situações de estresse é a PERK, que se autofosforila e ativa a UPR ao se desligar da chaperona *binding protein* (BIP)[32]. A ativação crônica da PERK acarreta em uma maior expressão da CHOP por meio de vários genes relacionados a UPR. O aumento da CHOP induz a translocação da *BCL-2 associated protein X* (BAX) do citosol para a mitocôndria, retendo o ciclo celular e gerando a apoptose[33].

Além do eixo PERK/CHOP presente na via do estresse do RE, o TLR4 também participa diretamente da geração de apoptose no tecido cardíaco através da mediação da proteína p38 *mitogen-activated protein kinase* (p38MAPK)[23; 34]. A ativação da via de sinalização do TLR4 induz a fosforilação da p38MAPK que provoca a translocação da BAX do citosol para a mitocôndria, a qual aumenta a apoptose dependente da mitocôndria promovendo a clivagem das caspases 3 e 9[22]. A BAX é primordial para a geração da apoptose, pois tem o papel de controlar diretamente a abertura dos poros de transição de permeabilidade mitocondrial. Além disso, sua translocação do citosol para a mitocôndria contribui para a liberação de citocromo C no citosol, que é importante para a ativação da via de sinalização da apoptose[35]. A figura 1 representa de forma resumida as relações entre a ativação do TLR4, inflamação, estresse do RE e apoptose.

Figura 1: Resumo dos mecanismos envolvidos na inflamação, estresse do RE e apoptose desencadeados pelo TLR4.



Legenda: BAX- BCL-2 *associated protein X*; BIP- *binding protein*; CHOP- C/EBP- *Homologous Protein*; IL-1β- Interleucina 1 beta; IL-6- Interleucina 6; MyD88- *Myeloid differentiation primary response 8*; NF-κβ- Fator Nuclear kappa beta, p-fosforilada; PERK- Quinase do retículo endoplasmático semelhante à proteína quinase do pâncreas, P38MAPK- *p38 mitogen-activated protein kinase*; TLR4- *Toll-like receptor 4;* TRIF- TIR *(Toll/interleukin-1 receptor)-domain-containing adaptor protein inducing IFNb.*

Fonte: Acervo pessoal.

O TLR4 também pode interferir na expressão de certos biomarcadores e na conformação do tecido cardíaco durante o exercício físico. Ao submeterem ratos a um modelo de estresse, no qual um dos componentes era uma sessão de natação, Wang e colaboradores[36] apontaram elevações nas concentrações séricas de CK e LDH, enquanto os animais que receberam um bloqueador do TLR4 (eritoran) não apresentaram alterações significativas nas concentrações dessas enzimas cardíacas. Os autores também verificaram edema, hemorragia e infiltração inflamatória no miocárdio do

grupo de ratos submetidos ao estresse de maneira crônica, enquanto que o grupo que recebeu eritoran não demonstrou nenhuma alteração histopatológica.

Também neste estudo os autores avaliaram as respostas cardíacas e do TLR4 a exposição crônica a esse mesmo modelo de estresse. As concentrações dos biomarcadores cardíacos atingiram um pico na primeira semana e foram caindo gradualmente até a quarta semana, atingindo inclusive concentrações menores que o grupo controle. O mesmo ocorreu com o TLR4 que alcançou o pico dos seus níveis de RNAm na primeira semana, estendendo-se até a terceira semana, e caiu na quarta semana[36].

Em relação ao exercício físico crônico (EFC) estudos comprovam que, diferente do EFA, o EFC provoca uma redução da expressão do TLR4[37; 38] por meio de uma resposta de tolerância desenvolvida pelo TLR4 ao seu ligante[39]. Esta resposta pode ser a responsável pelos efeitos anti-inflamatórios do exercício[39; 40], pelo seu papel no controle da diabetes *mellitus* tipo 2[38; 41] e prevenção da aterosclerose[41] . Contudo, algumas alterações na conformação do tecido cardíaco provocadas pelo exercício podem persistir mesmo após reduções nos níveis de TLR4 (pelo menos até a quarta semana de treinamento) e podem estar relacionadas ao pico de TLR4 durante as fazes inicias do protocolo de exercício[36], uma vez que o TLR4 também está envolvido no remodelamento do miocárdio e no surgimento da disfunção contrátil[42]. Porém, não se sabe ainda se essas alterações são passageiras e por quanto tempo elas irão persistir.

O efeito anti-inflamatório do EFC foi demonstrado no estudo de Chen e colaboradores[43], que após submeterem ratos com cardiomiopatia isquêmica a exercício aeróbio por 12 semanas, observaram redução das citocinas pró-inflamatórias IL1-β, IL6, IL17 e TNF-α. Essas citocinas estão intimamente ligadas a insuficiência cardíaca congestiva, que é caracterizada por remodelamento cardíaco e fibrose progressivos, e

quando elevadas são indicativos da patologia[44]. Assim como a IL1-β, IL6 e TNF-α a IL17 também parece ser regulada pelo TLR4. O TLR4 participa da regulação das células Th17, que produzem a IL17, e a falta de TLR4 resulta em redução da geração de Th17[45] e consequentemente de IL17.

A grande maioria dos estudos sobre o mecanismo de atuação do TLR4 no miocárdio é relacionada a doenças cardiovasculares, sendo limitado o número de trabalhos que abordaram a atuação do TLR4 no músculo cardíaco de organismos submetidos ao exercício físico. Além disso, em sua maior parte, esses poucos artigos sobre TLR4 e exercício investigam somente via da inflamação, sendo ainda mais escassos trabalhos que abordaram as repercussões da ativação da resposta inflamatória como o estresse do RE que pode provocar alterações no processamento das proteínas e, quando persistente, pode levar a célula a apoptose. Assim, existe uma carência na literatura científica acerca do papel do TLR4 no estresse do RE e apoptose durante o EFA e EFC. Além disso, a ativação do TLR4 no EFA pode estar relacionada à intensidade do exercício, uma vez que a ativação do NF-κB é dependente dessa variável[11].

1.1. Hipótese

A hipótese do presente estudo é que o TLR4 pode ser modulado pelo exercício físico, tanto agudo como crônico, e que a ocorrência de eventos como inflamação, apoptose e estresse do RE induzidos pelo exercício são desencadeadas pela ativação do TLR4. Além disso, acreditamos que existe uma relação direta entre a ativação do TLR4 com a intensidade do exercício que pode ativar o TLR4 de maneiras diferentes.

1.2. Revisão da literatura

Os receptores do tipo *Toll* foram originalmente descobertos na mosca *Drosophila* e descritos como proteínas transmembranares[46]. Isso significa que estes receptores são proteínas que se localizam na membrana celular, atravessando a sua bicamada lipídica. Por serem proteínas receptoras, quando associados a um ligante específico, sofrem alterações estruturais que desencadeiam uma "transmissão de sinal" do meio extracelular para o intracelular, permitindo interação entre esses dois meios[47].

O homólogo humano destes receptores foi encontrado em 1997 por Medzhitov e colaboradores[12], sendo coletivamente chamado de *Toll like receptor* (TLR). O TLR possui três diferentes domínios estruturais: um domínio de reconhecimento de ligantes, rico em leucina C-terminal extracelular, um domínio transmembranar central e um domínio citoplasmático que é homólogo ao receptor de IL-1 conhecido como TIR (*toll* /IL-1 receptor)[28]. Atualmente foram identificados 11 tipos de TLRs em humanos[48] , sendo todos proteínas transmembranares, onde alguns estão presentes na superfície das células (TLR1, TLR2, TLR4, e TLR6) e outros no interior das células (TLR3, TLR7, TLR8, e TLR9)[16].

O primeiro TLR humano a ser descoberto e o mais estudado até hoje é o TLR4[49]. Sua ativação leva a translocação do fator de transcrição NF-κB do citoplasma para o núcleo que promove a transcrição de genes que traduzem proteínas associadas a efeitos deletérios para o organismo, inclusive para o miocárdio[50].

1.2.1 TLR4 e a via de ativação do NF-κB

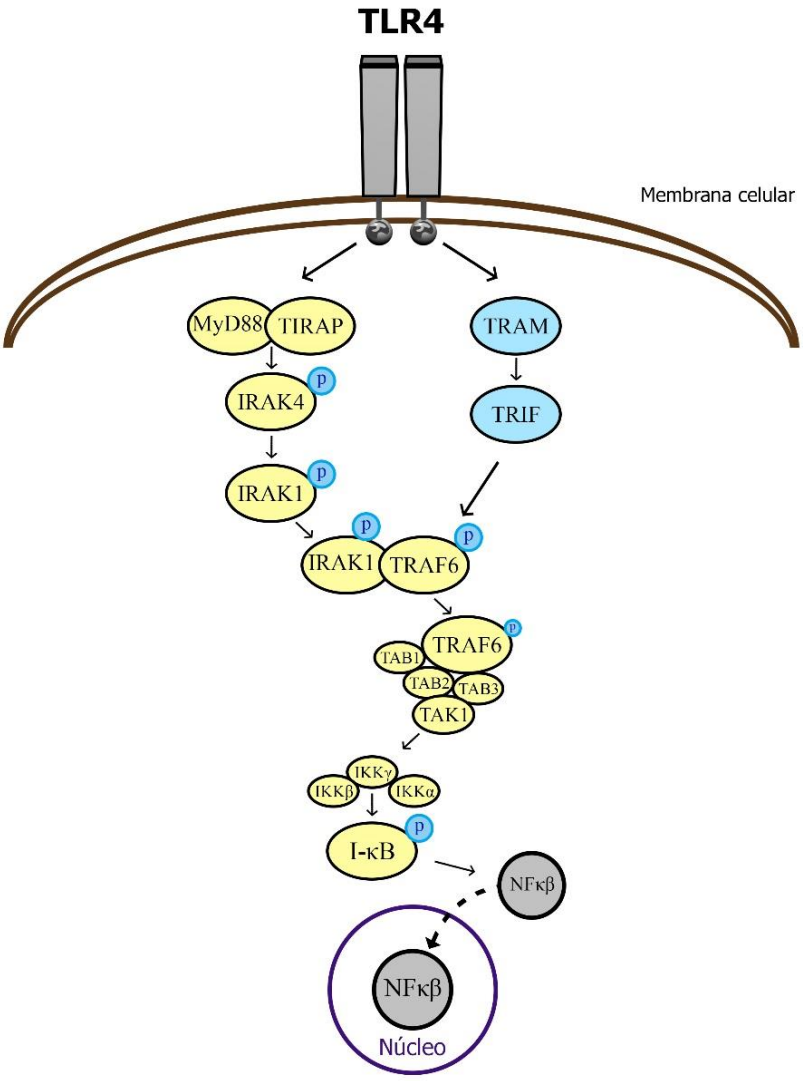
O TLR4 pode ser ativado por diferentes tipos de ligantes incluindo substâncias liberadas durante o exercício físico[39; 51; 52]. Uma vez associado ao seu ligante, esta proteína sofre uma alteração estrutural chamada de dimerização, em que o domínio TIR

das caudas citoplasmáticas da proteína se aproxima e forma um homodímero (que tem duas subunidade idênticas)[53], recrutando o domínio TIR onde estão os adaptadores de proteínas *TIR domain containing adaptor protein* (TIRAP também conhecido como *MyD88 adapter-like-* Mal) e *TRIF related adaptor molecule* (TRAM) que ativam respectivamente a via MyD88-dependente e a via TRIF-dependente[28].

Quando se ativa a via MyD88-dependente, o complexo MyD88/TIRAP ativa a *interleukin-1 receptor-associated kinase 4* (IRAK4) que induz a fosforilação da IRAK1. Uma vez fosforilada, a IRAK1 associa-se a *TNF-Receptor-Associated Factor-6* (TRAF6) e também promove a sua fosforilação[28]. A TRAF6 fosforilada se desliga do seu receptor e se associa a outro complexo de membrana composto pelas proteínas: TAK1 (*transforming growth fator b-activating kinase*), TAB1 (*TAK1-binding protein*), e a TAB2/3. Quando ativada, a TAK1 fosforila o complexo de quinases IKKs (*Inhibitor of κ Light Polypeptide Gene Enhancer in B-Cells Kinase*) composto por IKK-α /IKK-β/ IKK-γ[20]. Este complexo promove a fosforilação da I-κB (*inhibitor of kappa B*), uma proteína responsável por manter o NF-κB no citoplasma e fora do núcleo da célula[54]. A fosforilação I-κB ativa a sua ubiquitinação e degradação, permitindo a translocação do NF-κB para o núcleo[28].

Com relação a via TRIF-dependente, ao sofrer dimerização, o TLR4 libera a proteína adaptadora TRAM que recruta a TRIF. A TRIF então ativa a TRAF6 e desencadeia a mesma cascata de ativação da via My88-dependente a partir da TRAF6 até a degradação da I-κB, que permite a translocação do NF-κB do citoplasma para o núcleo da célula[28]. A figura 2 mostra de forma resumida a via de sinalização que ativa o NF-κB por meio do TLR4.

Figura 2: Resumo da via de sinalização que ativa o NF-κB por meio do TLR4.



Legenda: IKK - *Inhibitor of κ Light Polypeptide Gene Enhancer in B-Cells Kinase* (β /α / γ); I-κB- *inhibitor of kappa B*; IRAK1/4- *interleukin -1 receptor-associated kinase* 1/4; MyD88- *Myeloid differentiation primary response 88;* NF-κB- Fator Nuclear kappa B; p-fosforilada; TAB1/2/3-*TAK1/2/3-binding protein;*TIRAP- *TIR domain containing adaptor protein*; TLR4- *Toll-like receptor 4* TRAM- *TRIF related adaptor molecule* TRAF6- *TNF-Receptor-Associated Factor-6;* TRIF- TIR (Toll/interleukin-1 receptor)-domain-containing adaptor protein inducing IFNb.
Fonte: acervo pessoal.

1.2.2 TLR4 e o exercício físico

Estudos sugerem que o exercício físico, tanto agudo como crônico, tem o potencial de modular o TLR4[13; 37; 38]. No entanto, pouco se sabe ainda sobre os mecanismos que

levam a esse fenômeno e como isso ocorre no músculo cardíaco. Em seu estudo de revisão, Francaux[51] apontou que os ácidos graxos não esterificados (AGNE), também conhecidos como ácidos graxos livres, atuam como um ligante do TLR4 e possivelmente são a principal substância responsável pela sua ativação em decorrência do exercício físico. Outro ativador de TLR4 muito estudado é o LPS. Cientistas do mundo todo utilizam essa substancia como forma de ativar farmacologicamente o TLR4. O LPS é um componente da parede celular de bactérias gram-negativas[55] e o seu reconhecimento pelo TLR4 é fundamental para sua participação na resposta imunológica, uma das primeiras funções descobertas do TLR4. O componente crucial do LSP para ativação do TLR4 é a sua subunidade lipídio A, que é totalmente composta por ácidos graxos. Dessa maneira, Francaux[51] considerou que os aumentos nas concentração plasmáticas de AGNE durante o exercício físico poderiam também ativar o TLR4. Para confirmar essa hipótese, o autor citou dados não publicados de uma pesquisa que submeteu camundongos WT e KO de TLR4 a um protocolo de exercício de *endurance*. Ambos os camundongos apresentaram aumento nas concentrações plasmáticas de AGNE, no entanto, somente os camundongos WT apresentaram maior ativação de proteínas reguladas pelo TLR4. O mesmo aconteceu quando a liberação de AGNE foi estimulada com heparina[51].

Realmente, ambos os tipos de exercício físico, agudo e crônico, promovem aumento das taxas de AGNE, tanto das concentrações plasmáticas como no tecido cardíaco. Em condições aeróbias, como durante o exercício de *endurance,* os AGNE são a principal fonte de energia do coração, e devido a sua alta taxa de oxidação de ácidos graxos, alterações na concentração ou no perfil lipídico dos AGNE no plasma afetariam diretamente a composição lipídica do miocárdio[56], o que levaria a maior ativação TLR4. Contudo, embora esteja estabelecido que os AGNE são um ativador de TLR4, um

elegante estudo de Lancaster e colaboradores[57] comprovou por meio de múltiplas análises que os ácidos graxos de cadeia longa (AGCL) não são agonistas diretos do TLR4, ou seja, não ativam diretamente a via do TLR4. Os resultados do estudo indicam que, diferente do que se havia descrito, o TLR4 participa como um "preparador" da célula promovendo alterações do metabolismo celular e de lipídios, da expressão de genes e da composição dos lipídios na membrana, e são todas estas alterações que permitem que os AGCL ativem vias de sinalização como por exemplo a via da inflamação.

Outro ligante endógeno que supostamente estaria envolvido na ativação do TLR4 pelo exercício físico são as Proteínas de choque térmico (PCT), em especial a PCT60[39] e a PCT70[52; 58]. As PCT tem a capacidade de ativar o TLR4 e provocar a transmissão do sinal que leva a translocação do NF-κB do citoplasma para o núcleo[59]. E, embora o mecanismo de como essa interação entre o TLR4 e a PCT ainda não esteja totalmente esclarecido, durante e após o exercício físico existe um aumento da concentração dessas proteínas[52] que cronicamente podem levar a uma baixa regulação do TLR4[39].

Em pessoas treinadas, o RNAm e a expressão do TLR4 na superfície celular é menor quando comparado à pessoas sedentárias[60] e um possível responsável por este fenômeno seria a PCT60[39]. O exercício físico provoca a liberação de ligantes endógenos que interagem com o TLR4. A liberação constante desses ligantes (particularmente da PCT60), que ocorre em pessoas que praticam exercício regularmente (EFC), desencadeia uma resposta de tolerância, também conhecida com *downregulation*, que atenua a resposta do TLR4 ao seu ligante e reduz a densidade do receptor na superfície celular e/ou altera a sua via de sinalização. Além disso, essa exposição crônica pode reduzir a resposta do TLR4 não somente ao ligante ao qual ele está sendo exposto, mas

também a outros ligantes secundários. Dessa forma, o exercício físico crônico pode provocar uma baixa regulação de TLR4, e este seria um dos mecanismos pelo qual a prática regular de exercício físico promove um efeito anti-inflamatório[39].

As vias moleculares envolvidas na interação entre o exercício físico e o TLR4 não foram totalmente elucidadas. No entanto, comprovadamente essa interação existe, sendo necessários estudos adicionais para compreender e aprofundar melhor nesses mecanismos de interação.

2. OBJETIVO

O presente estudo tem como principal objetivo avaliar se o TLR4 possui participação nos eventos que podem ser prejudiciais ao miocárdio durante o EFA como inflamação, apoptose e estresse do RE. Além disso, objetivamos verificar se a participação do TLR4 é mantida durante o EFC, o que influenciaria as adaptações cardíacas secundárias ao exercício. Para tanto, comparamos os efeitos de diferentes intensidades de EFA e EFC no coração de camundongos *knockout* de TLR4 (KO) com camundongos *wild-type* (WT).

3. METODOLOGIA

3.1. Animais experimentais:

Este estudo recebeu aprovação do Comitê de Ética da EEFERP (CEUA-EEFERP processo nº: 2016.5.82.90.7). Foram utilizados 80 camundongos machos C57BL/6, sendo 40 camundongos *wild-type* (WT) e 40 camundongos *knockout* total de TLR4 (KO), com 8 semanas de vida provenientes do Biotério Central do Campus de

Ribeirão Preto-USP. Os animais foram mantidos em gaiolas de polietileno em trios, com temperatura controlada (22 ± 2 ° C), ciclo claro-escuro invertido (12/12h), com livre acesso à água e ração convencional. Os camundongos foram divididos entre os 12 grupos descritos na tabela abaixo:

Tabela 1: Grupos experimentais

Sigla	Tipo	N	Protocolo de exercício
CT (Controle)	WT	5	Sedentários (sacrificados com 9 semanas)
CT-KO (Controle *knockout*)	KO	5	Sedentários (sacrificados com 9 semanas)
EFA-45%	WT	5	EFA abaixo da máxima fase estável de lactato
EFA-45%-KO	KO	5	EFA abaixo da máxima fase estável de lactato
EFA-60%	WT	5	EFA na máxima fase estável de lactato
EFA-60%-KO	KO	5	EFA na máxima fase estável de lactato
EFA-75%	WT	5	EFA acima da máxima fase estável de lactato
EFA-75%-KO	KO	5	EFA acima da máxima fase estável de lactato
EFC-WT-CT	WT	10	Sedentários (sacrificados com 14 semanas)
EFC-KO-CT	KO	10	Sedentários (sacrificados com 14 semanas)
EFC-WT	WT	10	Exercício físico crônico
EFC-KO	KO	10	Exercício físico crônico

Legenda: CT-controle; EFA- exercício físico agudo; EFC- exercício físico crônico; KO- *knockout*, WT-*wild-type*. **Fonte:** arquivo pessoal.

3.2. Teste de carga incremental (TCI)

Para prescrever as intensidades dos protocolos de exercício físico, o TCI foi utilizado. Os animais foram adaptados ao exercício em esteira rolante durante 5 dias, 10min/dia na velocidade de 6m/min. Conforme padronizado por Ferreira e colaboradores[61], o TCI foi iniciado na velocidade de 6m/min, com 0% de inclinação e incrementos de 3m/min a cada 3min até a exaustão voluntária dos camundongos, que ocorreu quando eles encostaram 5 vezes no final da esteira no intervalo de 1min. Foram utilizados estímulos físicos para encorajar os animais, e quando eles ficaram exaustos, a

velocidade de exaustão foi corrigida como proposto por Kuipers e colaboradores[62] para definir a potência máxima (Pmax) que posteriormente foi utilizada para a prescrição dos protocolos de exercício físico realizados 7 dias após o TCI.

3.3. Protocolos de exercício físico agudo (EFA)

3.3.1 Protocolo de EFA abaixo da máxima fase estável de lactato (EFA-45%)

A máxima fase estável de lactato (MFEL) representa a maior carga de trabalho que pode ser mantida ao longo do tempo sem acúmulo contínuo de lactato no sangue e tem sido extensivamente utilizada como padrão ouro para a determinação da intensidade de exercício correspondente à transição metabólica aeróbia-anaeróbia em roedores[61; 63; 64; 65; 66; 67]. As intensidades de exercício inferiores a MFEL são associadas ao domínio moderado de exercício físico em que ocorre equilíbrio entre a produção e remoção do lactato sanguíneo[64; 68; 69].

Ferreira e colaboradores[61] verificaram que a intensidade de 60% da Pmax corresponde a MFEL em camundongos machos C57BL/6. Dessa maneira, o protocolo de EFA abaixo da máxima fase estável de lactato foi composto por uma sessão de corrida realizada em esteira rolante com intensidade correspondente a 45% da Pmax (ou seja, 15% abaixo da intensidade correspondente a MFEL) durante 30 minutos resultando em uma carga de treino total (produto entre a intensidade e o volume) de 1350 unidades arbitrárias (u.a).

3.3.2 Protocolo de EFA na máxima fase estável de lactato (EFA-60%)

O protocolo de EFA na máxima fase de lactato foi composto por uma sessão de corrida em esteira rolante com intensidade correspondente a 60% da Pmax durante 22 minutos e 30 segundos, totalizando a mesma carga de treino utilizada no protocolo de EFA abaixo da máxima fase estável de lactato.

3.3.3 Protocolo de EFA acima da máxima fase estável de lactato (EFA-75%)

O protocolo de EFA acima da máxima fase estável de lactato foi composto por uma sessão de corrida em esteira rolante adaptada com intensidade de 75% da Pmax até a exaustão voluntária dos animais. A exaustão foi considerada quando o camundongo tocou ao final da esteira 5 vezes no intervalo de 1 minuto. A carga total de treino foi obtida através da multiplicação da intensidade (75% da Pmáx) pelo tempo em que os roedores permanecerem na esteira. Os valores de glicemia basais e após o EFA foram coletados por um sistema de monitorização de glicemia (modelo *Accu-Chek® Active, Roche*).

3.4. Protocolo de exercício físico crônico (EFC)

O protocolo de EFC foi realizado durante 4 semanas e foi adaptado ao estudo de Wakako Tsumiyama e colaboradores[70]. Participaram deste protocolo 20 camundongos (10 WT e 10 KO) com 10 semanas de vida. Cada semana experimental foi composta por 3 dias de exercício intercalado com 48 horas de repouso. A tabela 2 apresenta as características do protocolo de treinamento.

Tabela 2. Protocolo de exercício físico crônico.

Semana	Intensidade (%Pmax*)	Volume (min)	Sessões diárias	Inclinação (%)	Intervalo entre as sessões (h)
1	60	15	1	0	48
2	60	30	1	0	48
3	60	45	1	0	48
4	60	60	1	0	48

***** Potência máxima
Fonte: Arquivo pessoal.

3.5. Análise do desempenho físico

As avaliações do desempenho físico ocorreram antes do início do protocolo de EFC e 48h após a última sessão do protocolo na quarta semana, e foram compostas pelo teste do rotarod[71; 72; 73], teste de carga incremental[71; 72; 74; 75] , e teste exaustivo[71; 72; 74].

3.5.1 Teste do rotarod

Após 48 horas da última sessão do protocolo de EFC, o teste do rotarod foi realizado para avaliar a coordenação motora dos animais. Nesse teste, os camundongos foram posicionados sobre uma barra rotatória de um aparelho programado para girar a barra a uma velocidade inicial de 1rpm e final de 40rpm, alcançada 300s após o início do movimento. A aceleração durante todo teste foi constante, sendo realizadas três tentativas por animal, com intervalos de 10s entre cada tentativa, registrando-se o tempo em segundos até a queda de cada animal[72; 74]. Após 4h da realização do teste do rotarod, os roedores realizaram o teste de carga incremental, conforme descrito no item 3.2 do presente estudo para definir as velocidades dos protocolos de exercício.

3.5.2 Teste exaustivo

O teste exaustivo que é um parâmetro de resistência cardiorrespiratória, foi realizado após 24 horas da realização do TCI. Os camundongos correram em uma esteira rolante na velocidade de 36m/min com inclinação de 8% até exaustão voluntária, que ocorreu quando eles encostaram 5 vezes no final da esteira no intervalo de 1min[72; 74], registrando-se o tempo decorrido até a exaustão dos animais.

3.6. Ecocardiograma

O ecocardiograma foi realizado nos animais em seu estado basal antes do início dos protocolos de exercício e nos animais submetidos ao protocolo de EFC foi realizado também 48 horas após o término da análise do desempenho físico. O exame foi

executado com um sistema de ultrassom da marca Vevo 2100® com um transdutor de 30Mhz em uma plataforma aquecida de manuseio de camundongos com detectores de eletrocardiograma e monitoramento dos sinais vitais (FAPESP 2009/54010-1). Os animais foram anestesiados com o anestésico inalatório isoflurano a 3% do volume da câmara, e a perda do reflexo podal foi o parâmetro para avaliar a eficácia da anestesia.

Após a anestesia, os animais foram posicionados em decúbito dorsal com as patas amarradas ao lado do corpo na plataforma aquecida, inalando isoflurano enriquecido com um fluxo de 5 l/min de oxigênio por meio de um tubo posicionado na região do focinho, depois foram tricotomizados na região torácica para que fosse possível a passagem do transdutor de ultrassom na parte anterior do tórax do animal junto com um gel condutor para melhor acoplamento do transdutor. Foram gravadas imagens bidimensionais (2D) e no modo-M (M-Mode) no eixo longo paraesternal, tendo como referência o centro do ventrículo esquerdo. As variáveis mensuradas no modo M foram: massa ventricular esquerda, diâmetro sistólico e diastólico interno do ventrículo esquerdo, fração de ejeção do ventrículo esquerdo, espessura do septo interventricular na diástole e na sístole, encurtamento fracional e espessura da parede posterior do ventrículo esquerdo na diástole e na sístole. No modo bidimensional, as variáveis analisadas foram: frequência cardíaca, débito cardíaco, volume sistólico e diastólico e volume de ejeção.

3.7. Teste de tolerância a glicose (TTG)

Os camundongos do grupo controle do protocolo de EFC foram submetidos ao TTG na sua décima quarta semana de vida. Após 12 horas de jejum, foi coletada uma gota de sangue da extremidade da cauda do animal e a glicemia foi quantificada por um sistema de monitorização de glicemia (modelo *Accu-Chek® Active, Roche*) para

obtenção do valor da glicemia basal do animal. Posteriormente, foram injetados 2g/kg de glicose na região intraperitoneal do animal, e a glicemia foi mensurada novamente após 15, 30, 60 e 120 minutos[76]. Com os valores obtidos, foi calculada a área sobre a curva usando o princípio trapezoidal[77]

3.8. Calorimetria indireta

O gasto energético dos roedores foi mensurado através da calorimetria indireta nos camundongos do grupo controle do protocolo de EFC quando os mesmos estavam com 12 semanas de vida. Foi utilizado um calorímetro Sistema Oxylet, Panlab®, Espanha. Os animais foram acomodados em caixas de acrílico com 3 camundongos em cada, durante 48 horas, com temperatura controlada em 22 ± 2 ° C. Nas primeiras 24 horas, os camundongos foram adaptados as caixas e nas outras 24 horas determinou-se as seguintes variáveis: gasto energético total (EE); volume de oxigênio consumido (VO_2), volume de gás carbônico exalado (VCO_2) e o coeficiente respiratório (RQ) que é determinado pela relação VCO_2 / VO_2. O RQ é um parâmetro para determinar qual tipo de substrato energético está sendo utilizado como fonte de energia, sendo que os valores de RQ em indivíduos normais variam entre 1, que representa a oxidação pura de carboidratos, e entre 0,7, que representa a oxidação pura de lipídios, assim valores de RQ mais próximos de 1 indicam que os carboidratos estão sendo a principal fonte de energia e o mesmo acontece com os lipídios quando os valores de RQ estão mais próximos de 0,7[78].

3.9. Extração do músculo cardíaco

Duas horas após a realização dos protocolos de EFA[10], e uma semana após o término do protocolo de EFC, os animais foram anestesiados através da administração

intraperitoneal de xilazina (10 mg/kg peso corporal) e quetamina (100 mg/kg peso corporal) misturadas na mesma seringa. O controle da anestesia foi avaliado pela perda do reflexo podal[79]. Posteriormente, os corações foram retirados, 5 amostras de cada grupo de EFC foram armazenadas em formaldeído para análise da geometria e morfologia do músculo cardíaco pela técnica de histologia, e nas amostras restantes os ventrículos esquerdos foram isolados e armazenados a -80°C para análise subsequente da expressão gênica pela técnica de *Real Time- Polymerase Chain Reaction* (RT-PCR) e do conteúdo proteico pela técnica de *immunoblotting* e da geometria e morfologia

3.10. Avaliações sanguíneas

As amostras de sangue foram coletadas por punção cardíaca. Em seguida, o sangue foi centrifugado a 3.500rpm por 15 minutos e o soro foi armazenado em freezer a -80°C para posterior determinação das concentrações das enzimas cardíacas CK, CK-mb e LDH que foram mensuradas utilizando kits de ensaios convencionais comercialmente disponíveis (Analisa, Belo Horizonte, Minas Gerais, Brazil) no espectrofotômetro-UV/Vis (modelo Libra S12, Biochrom, Cambridge, Reino Unido). As citocinas pró-inflamatórias IL-1β, IL-6, IL-17 e TNFα foram mensuradas no aparelho Luminex MAP200 pelo método LuminexTM multiplex (Millipore, ST Charles, MO), e analisadas com o 3.1 xPONENT System.

3.11. *Real Time- Polymerase Chain Reaction* (RT-PCR)

O RNA total do ventrículo esquerdo foi extraído com Trizol (Invitrogen, Carlsbad, CA) e posteriormente quantificado no espectrofotômetro NanoDrop2000c (Thermo Scientific). O cDNA foi sintetizado com 600 ng de RNA total utilizando o High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) de acordo com

as instruções do fabricante. Em seguida, foi realizado o PCR em tempo real no equipamento ViiA7 Real-Time PCR System (Applied Biosystems) para análise da expressão relativa do RNAm dos seguintes genes para as amostras do protocolo de EFA: *Il1b*, *Tnf*, *Nfkb*, *Eif2ak3*, *Hspa5*, *Ddit3*, *Casp3*, *Casp9*, *Bax*, *Mapk14* e *Tlr4*. E para as amostras do protocolo de EFC foram analisados os seguintes genes: *Il1b*, *Tnf*, *Ddit3*, *Casp3*, *Bax* e *Tlr4*. O desenho dos primers utilizados encontra-se em apêndice 1.

Para as amostras do protocolo de EFA as reações de amplificação (volume final 10 uL) foram realizadas em triplicata com os seguintes reagentes: 5uL 2X Power Sybr Master Mix (Thermo Fisher Scientific), 1uL primer forward, 1uL primer reverse, 1uL cDNA diluído 1:6 e 2uL de H_2O. O *Gapdh* e *Actb* foram os genes de referência para a normalização dos dados. Cada reação de amplificação ocorreu na ciclagem *Standad* nos seguintes ciclos: 10 minutos a 95ºC e mais 40 ciclos de 15 segundos a 95ºC e 1 minuto a 60ºC. Para as amostras do protocolo de EFC as reações de amplificação (volume final 10 uL) foram realizadas em duplicata com os seguintes reagentes: 2µL HOT FIREPol® EvaGreen® qPCR Supermix (Solis Biodyne, Tartu, Estonia), 1µL primer forward, 1µL primer reverse, 2µL cDNA diluído em 1:6 e 4µL de H2O. O gene de referência para a normalização dos dados foi o *Actb*. Cada reação de amplificação ocorreu na ciclagem *Standad* nos seguintes ciclos: 12 minutos a 95ºC e mais 40 ciclos de 15 segundos a 95ºC, 20 segundos a 60ºC e 20 segundos a 72ºC. A quantificação relativa das reações de amplificação das amostras dos protocolos e EFA e EFC foram calculadas pelo método $2^{-\Delta\Delta CT}$ utilizando o Thermo Fisher Cloud Software, RQ version 3.7 (Life Technologies Corporation, Carlsbad, CA, USA).

3.12. *Immunoblotting*

As amostras do ventrículo esquerdo foram homogeneizadas com *Tissue-Tearor (model 985370 variable speed; Biospec products, inc)* em tampão de extração (1% de Triton X-100, 100 mM Tris, pH 7,4, contendo 100 mM de pirofosfato de sódio, fluoreto de sódio 100 mM, EDTA 10 mM, vanadato de sódio 10 mM, 2 mM de PMSF e 0,1 mg.ml-1 aprotinina) a 4°C. Os extratos foram centrifugados a 9900rpm a 4°C durante 40min para remover o material insolúvel, e os sobrenadantes destes homogeneizados foram utilizados para quantificação de proteínas de acordo com o método de Bradford[80].

A desnaturação das proteínas ocorreu por fervura em tampão de amostra de *Laemmli*[81] contendo DTT 100mM, realizado em gel SDS-PAGE e então foram transferidas para membranas de nitrocelulose (*GE Healthcare, Hybond ECL*, RPN303D). Por meio da coloração das bandas com *Ponceau* verificou-se a eficácia da transferência das proteínas para as membranas. Posteriormente, bloqueamos as membranas com solução salina tamponada com Tris (TBS) com 5% de BSA e 0,1% de Tween-20, pelo período de 1 hora na temperatura de 4º C. Para as amostras do protocolo de EFA as membranas foram incubadas com os seguintes anticorpos: phospho(p)BAX (Ser184; orb4658) da Biorbyt Ltd. (Cambridge, Cambridgeshire, Inglaterra); PERK (SC13073), pPERK (Thr981; SC32577), BIP (SC33757) e BAX (SC7480) da Santa Cruz Biotechnology (Santa Cruz, CA, EUA); TNF-alpha (60291-1-Ig) da Proteintech (Rosemont, IL, EUA); p-p38MAPK (T180/Y182; #9211s), Caspase 9 (#9508s) e beta-actina (#4967s) da Cell Signaling Technology (Cell Signaling Technology, MA, EUA) e p38MAPK (OPA1-10080) da Thermo Fisher Scientific (Waltham, MA, EUA). Para as amostras do protocolo de EFC as membranas foram incubadas com os seguintes anticorpos: CHOP (15404-1-AP) da Proteintech (Rosemont, IL, EUA); pBAX (Ser184; orb4658) da Biorbyt Ltd. (Cambridge,

Cambridgeshire, Inglaterra); BAD (SC8044), Caspase 3 (SC7272), EIF2alpha (SC11386), pEIF2alpha (Ser52; SC101670), IKK (SC34674), pIKK (Ser176; SC-21661), JNK (SC572), pJNK (Thr183/185; SC6254), PTP1B (H-135) (SC14021), TLR4 (SC293072), TRB3 (SC390242) e α-Tubulina (SC-32293) da Santa Cruz Biotechnology (Santa Cruz, CA, EUA); IRE-1 (AB37073) e pIRE-1 (Ser724; AB104157) da Abcam (Cambridge, Reino Unido) e p-BAD (Ser 112; #9291s), ATF4 (#11815) da Cell Signaling Technology (Cell Signaling Technology, MA, EUA).

Após incubação de 16h com anticorpo primário, as membranas foram lavadas 5 vezes durante 5min com TBS contendo 0,1% de tween-20, depois as membranas foram incubadas com anticorpo secundário pelo período de 1h na temperatura de 4ºC . Por meio de quimioluminescência (*GE Healthcare, ECL Plus Western Blotting Detection System, RPN2132*) detectou-se as bandas imunorreativas específicas. As imagens foram adquiridas pelo *C-DiGit*^{TM} *Blot Scanner (LI-COR*^{R}*, Lincoln, Nebraska, USA)* e a quantificação foi realizada com o *software Image Studio para C-DiGit Blot Scanner*.

3.13. Análise histopatológica

Foram separados para as análises histopatológicas 5 amostras do ventrículo esquerdo (VE) de cada grupo experimental dos animais submetidos ao protocolo de EFC. Para análise morfológica do tecido, as amostras foram previamente depositadas em paraplastico. Em um micrótomo (Jung CM 1800, Leica, Alemanha), foram realizados cortes com 5 µm de espessura, que foram posteriormente corados com hematoxilina e eosina (H&E), picrosirius red e periodic acid Schiff (PAS) [Kit PAS 3X (cod KP08169SO), Êxodo Cientifica, Sumare, SP, Brasil]. Com um fotomicroscópio (AxioCam 2, Carl Zeiss, Jena, Alemanha) e uma câmera fotográfica digital (Axiocam-

HR, CarlZeiss, Jena, Alemanha), foram conduzidas as análises e a documentação fotográfica dos cortes já corados com aumento de 40 vezes.

Para quantificar a reatividade, determinou-se o percentual de intensidade das colorações Picrosirius Red e PAS, realizando uma análise da intensidade da cor por meio do software ImageJ (versão 1.50i) através do plugin IHC-toolbox. Para realizar a análise esterológica, o software Stepanizer (disponível em http://www.Stepanizer.com/) foi utilizado. Ambas as análises foram realizadas em 10 diferentes secções aleatórias das fibras transversais e longitudinais, de cinco camundongos por grupo experimental, chegando-se ao percentual para totalização das variáveis

Analisamos a espessura do VE por meio de coloração H&E. O VE de cada camundongo foi seccionado em 3 cortes transversais, com aumento de 12x no microscópio estereoscópico (Microscópio ZEISS SteREO Discovery V12). Posteriormente, a quantificação foi realizada no programa Image-J 1.5, com apoio do Line Selection Tool, no qual avaliou-se todo o perímetro ventricular com o delineamento de cinco linhas transversais ao longo do miocárdio, resultando em 15 medidas por camundongo.

3.14. Análise dos resultados

Os resultados foram expressos em média ± erro padrão médio (SEM). De acordo com o teste de *Shapiro-Wilk*, verificou-se a distribuição normal dos grupos, e o teste de *Levene* foi utilizado para avaliar a homogeneidade da variância. Quando nenhum desses pressupostos foi atendido, testes não paramétricos foram utilizados. As comparações entre os animais KO e WT foram realizadas com o teste T de Student para amostras independentes e as comparações entre os períodos anteriores e posteriores aos protocolos de exercício foram realizadas com o teste T de Student pareado.

Para examinar os efeitos dos protocolos de EFA na expressão genética de *Tlr4* nos grupos de animais WT, utilizou-se o teste *one-way* ANOVA. Para avaliar as diferenças nos casos em que os animais poderiam sofrer influência do exercício e/ou da composição genética (ser WT ou KO), utilizou-se o teste *two-way* ANOVA. Quando houve significância estatística, o *post hoc* de *Bonferroni* foi utilizado.

Para examinar os efeitos do protocolo de EFC e da deleção de TLR4 no coração dos camundongos, utilizou-se o teste *two-way* ANOVA. E para avaliar as variáveis do ecocardiograma foi realizado o teste estatístico ANOVA mista. Em casos com indicação de interação, o teste *post-hoc* de *Sidak* foi realizado. Todas as análises foram bilaterais e o nível de significância fixado em $p \leq 0,05$. As análises estatísticas foram realizadas utilizando o software *SPSS* v.20.0 para Windows (IBM, Chicago, IL)

4. RESULTADOS E DISCUSSÃO:

Os resultados do estudo foram divididos em dois artigos, um contendo os dados relacionados as análises com os protocolos de EFA e o outro com os dados referentes ao protocolo de EFC.

4.1. A deleção de *Tlr4* atenuou os efeitos das diferentes intensidades de EFA no coração dos camundongos KO.

Contrário à nossa hipótese inicial, não houve influência da intensidade do exercício na expressão de *Tlr4* no coração dos camundongos WT. Essa expressão parece estar mais relacionada ao volume de exercício do que a intensidade. No entanto, no estado basal, os camundongos KO apresentaram redução da expressão de genes associados a inflamação e ao estresse do RE e aumento da concentração sérica de CK, IL-17 e TNF-α. Com as mesmas condições de exercício, os camundongos KO

apresentaram menor glicemia e concentração sérica de CK, IL-17 e TNF-α, assim como menor expressão de genes e proteínas relacionados a apoptose, inflamação e estresse do RE. Porém, também houve aumento da expressão de outros genes relacionados a apoptose, inflamação e estresse do RE, assim como das concentrações séricas de CK-mb e LDH. Esses achados mostraram que a deleção de *Tlr4* atenua os efeitos das diferentes intensidades de EFA no coração. Os resultados e a discussão sobre as análises com os protocolos de EFA podem ser encontrados na íntegra no artigo publicado na revista Life Sciences disponível em anexo 1.

Anexo 1:

DE VICENTE, L.G. PINTO, A.P; MUÑOZ, V.R; ROVINA, R.L; DA ROCHA, A.L; GASPAR, R.C; DA SILVA, L.E.C.M.; SIMABUCO, F.M; FRANTZ, F.G; PAULI, J.R; DE MOURA, L.P; CINTRA, D.E, ROPELLE, E.R; DA SILVA, A.S.R. Tlr4 participates in the responses of markers of apoptosis, inflammation, and ER stress to different acute exercise intensities in mice hearts. Life sciences, v. 240, p. 117107, 2020.

4.2. O TLR4 é importante para via de sinalização do estresse do RE e da apoptose, assim como para as adaptações favoráveis causadas pelo EFC.

No grupo controle, a deleção de *Tlr4* reduziu a ativação das vias do estresse do RE e apoptose, mas aumentou o gasto energético. Em contrapartida a nossa hipótese inicial, o EFC não reduziu o conteúdo de TLR4 nas células cardíacas dos camundongos WT. No entanto, os camundongos KO, diferente dos camundongos WT, não apresentaram melhora da *performance* nos testes físicos e nem da função cardíaca no ecocardiograma, ressaltando a importância do TLR4 nas adaptações favoráveis provocadas pela prática regular de exercício físico. Os resultados e a discussão sobre as

análises com os protocolos de EFC podem ser encontrados na íntegra no artigo submetido a publicação e exposto em apêndice 2.

Apêndice 2:

DE VICENTE L.G.; MUÑOZ V.R., PINTO A.P.; ROVINA R.L.; DA ROCHA A.L.; TAVARES A.E.A.; TEIXEIRA G.R.; FERRARI G.D.; ALBERICI L.C.; FRANTZ F.G.; SIMABUCO F.M.; ROPELLE E.R.; MOURA L.P.; CINTRA D.E.; PAULI J.R.; DA SILVA A.S.R. TLR4 deletion increased basal energy expenditure and attenuated heart apoptosis and ER stress but mitigated the training-induced cardiac function and performance improvement. Manuscrito submetido para publicação

5. CONSIDERAÇÕES FINAIS:

Diferente da nossa hipótese inicial, os resultados do presente estudo mostraram que não existe uma correlação entre as diferentes intensidades de EFA e a expressão de *Tlr4* no coração, porém a deleção de TLR4 atenuou os efeitos das diferentes intensidades de EFA no coração dos camundongos TLR4-KO e provocou alterações significativas na expressão gênica e no conteúdo proteico associados à via da inflamação, apoptose e estresse do RE. Com relação ao EFC, também em contraste com a nossa hipótese inicial, não houve redução da expressão de TLR4 no coração dos camundongos associados ao exercício físico regular, porém os camundongos TLR4-KO apresentaram uma inibição basal das vias do estresse do RE e apoptose. Diferentemente dos camundongos WT, o grupo TLR4-KO não apresentou melhora da função cardíaca e da *performance* no grupo treinado, o que indica que o TLR4 desempenha um importante papel nas adaptações benéficas secundárias ao exercício físico regular.

6. LIMITAÇÕES DO ESTUDO

É importante citar que poderíamos ter utilizado camundongos com deleção de TLR4 específica no coração, assim evitaríamos possíveis interferências das adaptações de outros sistemas a deleção de TLR4, entretanto, somente os camundongos com deleção total de TLR4 no corpo estavam disponíveis para serem utilizados no estudo. Outra limitação foi o período de uma semana decorrido entre a última sessão de exercício do protocolo de EFC e o sacrifício, é possível que quando os animais foram sacrificados eles já estivem começando a fase de destreinamento, contudo, esse período foi necessário para realizarmos todas as análises de desempenho físico e garantirmos que essas análises não iriam interferir nos resultados.

8. ATIVIDADES REALIZADAS

Completei 42 dos 40 créditos exigidos pelo programa para o depósito da tese de doutorado. Ministrei, junto com outros alunos de mestrado e doutorado do presente laboratório, um curso de difusão para alunos do 2° e 3º ano da Escola de Educação Física e Esporte, no intuito de que eles conhecessem como funciona a parte prática do laboratório, assim como a linha de pesquisa do mesmo.

Colaborei, participei e executei projetos realizados no presente laboratório, que resultaram na publicação de nove artigos (dois como autora principal) e na submissão de um artigo, no qual eu sou a autora principal.

17152 - 10233788/1 - Larissa Gaioto de Vicente

Sigla	Nome da Disciplina	Início	Término	Carga Horária	Cred.	Freq.	Conc.	Exc.	Situação
RDF5716-1/3	Intervenções Fisioterapêuticas nos Sistemas Tegumentar e Neuromuscular	06/03/2017	27/04/2017	60	4	100	A	N	Concluída
RDF5704-3/2	Tópicos Especiais em Prática Docente e Pesquisa	03/04/2017	15/05/2017	60	4	100	A	N	Concluída
RDF5723-1/1	Estudos Avançados em Fisiologia Molecular do Exercício	21/04/2017	16/06/2017	60	4	100	A	N	Concluída
EFR5006-1/6	Estatística Aplicada à Educação Física e Esporte	26/04/2017	05/07/2017	60	4	100	A	N	Concluída
RDF5712-3/3	Ética em Pesquisa	15/03/2018	23/05/2018	60	4	100	A	N	Concluída
RDF5725-1/1	Seminário I	01/03/2019	30/06/2019	30	2	100	A	N	Concluída
RDF5726-1/2	Seminário II	01/08/2019	15/11/2019	30	2	100	A	N	Concluída
RBP5799-1/1	Introdução do Processamento e Análise de Imagens	16/03/2020	03/04/2020	60	0	-	-	N	Turma cancelada
EFR5003-1/5	Metodologia do Ensino Superior	19/03/2020	27/05/2020	60	4	100	A	N	Concluída
RDF5707-2/2	Postura e Equilíbrio: Avaliação e Intervenção no Idoso	29/04/2020	09/06/2020	60	4	100	A	N	Concluída
EFR5005-2/1	Métodos Invasivos e Não Invasivos de Avaliação, Prescrição e Monitoramento do Treinamento Aeróbio e Anaeróbio	07/08/2020	30/10/2020	90	6	100	A	N	Concluída
RDF5709-2/2	Neurobiologia e Plasticidade do Comportamento Motor	06/10/2020	16/11/2020	60	4	100	A	N	Concluída

Curso de difusão:

ESCOLA DE EDUCAÇÃO FÍSICA E ESPORTE DE RIBEIRÃO PRETO

USP EEFERP GEFIME

PROGRAMAÇÃO

TEMA	DATA	HORÁRIO
Aula 1 – Apresentação e introdução a biologia molecular. Palestrante: Alisson (sala 05)	09 de agosto de 2017	Quarta feira das 16h às 17:15h
Aula 2 – Exercícios de *endurance*, suas vias moleculares e sua aplicabilidade na saúde e no esporte. Palestrante: Larissa (sala 05)	09 de agosto de 2017	Quarta feira das 16h às 17:15h
Aula 3 – Exercícios de resistência, suas vias moleculares e sua aplicabilidade na saúde e no esporte. Palestrante: Luciana (sala 05)	16 de agosto de 2017	Quarta feira das 16h às 17:15h
Aula 4 – Exercício físico concorrente, suas vias moleculares e sua aplicabilidade na saúde e no esporte. Palestrante: Gustavo (sala 05)	23 de agosto de 2017	Quarta feira das 16h às 17:15h
Aula 5 – Protocolos de exercício aeróbio, força e concorrente. Palestrante: Ana Paula (sala 05)	30 de agosto de 2017	Quarta feira das 16h às 17:15h
Aula 6 – *Immunoblotting* e outros métodos de análise. Palestrante: Ana Paula (sala 05)	06 de agosto de 2017	Quarta feira das 16h às 17:15h
Aula 7 – Overtraining em modelo animal – a história do nosso grupo de pesquisa. Palestrante: Alisson (sala 05)	13 de setembro de 2017	Quarta feira das 16h às 17:15h
Aula 8 – Overtraining em modelo animal – a história do nosso grupo de pesquisa. Palestrante: Alisson (sala 05)	20 de setembro de 2017	Quarta feira das 16h às 17:15h
Aula 9 – Fim de curso e confraternização	27 de setembro de 2017	Quarta feira das 16h às 17:15h

*Sujeito a alteração de horário e data

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Artigo submetido:

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Apêndice 1- Desenho dos primers

Actb _ Forward: GACTCATCGTACTCCTGCTTG

Actb _ Reverse: GATTACTGCTCTGGCTCCTAG

*Bax*_Forward: TTTGCTACAGGGTTTCATCCAG

*Bax*_Reverse: GTCCAGTTCATCTCCAATTCGC

Casp3_ Forward: AGCTTGGAACGGTACGCTAA

Casp3_ Reverse: CCAGAGTCCACTGACTTGC

Casp9_ Forward: ATATTCAGCAGGCAGGATCTGG

Casp9_ Reverse: GTGTCCTCTAAGCAGGAGATGA

*Ddit3*_ Forward: ATCTTGAGCCTAACACGTCGAT

*Ddit3*_ Reverse: GACCAGGTTCTCTCTCCTCAG

*Eif2ak3*_ Forward: AAAGCAGTGGGATTTGGACG

*Eif2ak3*_ Reverse: AAGTTTTGTGGGTGCCCTCT

*Hspa5*_ Forward: GTGTGTGAGACCAGAACCGT

*Hspa5*_ Reverse: GCAGTCAGGCAGGAGTCTTA

*Il1b_* Forward: TGCCACCTTTTGACAGTGATG

*Il1b_* Reverse: GCTCTTGTTGATGTGCTGCT

*Mapk14_* Forward: GGACTGTGAGCTGAAGATCCTA

*Mapk14_* Reverse: CGCATCCAATTCAAGATGACCT

*Nfkb1_* Forward: GATTCCGGGCAGTGACG

*Nfkb1_* Reverse: GATGAGGGGAAACAGATCGTCC

*Tlr4_* Forward: GTTCTCTCATGGCCTCCACT

*Tlr4_* Reverse: GGAACTACCTCTATGCAGGGAT

*Tnf_* Forward: CCACCACGCTCTTCTGTCTA

*Tnf_* Reverse: CCACTTGGTGGTTTGTGAGT

Apêndice 2:

DE VICENTE L.G.; MUÑOZ V.R., PINTO A.P.; ROVINA R.L.; DA ROCHA A.L.; TAVARES A.E.A.; TEIXEIRA G.R.; FERRARI G.D.; ALBERICI L.C.; FRANTZ F.G.; SIMABUCO F.M.; ROPELLE E.R.; MOURA L.P.; CINTRA D.E.; PAULI J.R.; DA SILVA A.S.R. TLR4 deletion increased basal energy expenditure and attenuated heart apoptosis and ER stress but mitigated the training-induced cardiac function and performance improvement. Manuscrito submetido para publicação

ABSTRACT

Strategies capable of attenuating TLR4 can attenuate metabolic processes such as inflammation, endoplasmic reticulum (ER) stress, and apoptosis in the body. Physical exercise has been a cornerstone in suppressing inflammation and dysmetabolic outcomes caused by TRL4 activation. Thus, the present study aimed to evaluate the effects of a chronic physical exercise protocol on the TLR4 expression and its repercussion in the inflammation, ER stress, and apoptosis pathways in mice hearts. Echocardiogram, RT-qPCR, immunoblotting, and histological techniques were used to evaluate the left ventricle of wild-type (WT) and *Tlr4* knockout (TLR4 KO) mice submitted to a 4-week physical exercise protocol. Moreover, we performed a bioinformatics analysis to expand the relationship of *Tlr4* mRNA in the heart with inflammation, ER stress, and apoptosis-related genes of several isogenic strains of BXD mice. The TLR4 KO mice had higher energy expenditure and heart rate in the control state but lower activation of apoptosis and ER stress pathways. The bioinformatics analysis reinforced these data. In the exercised state, the WT mice improved performance and cardiac function. However, these responses were blunted in the KO group. In conclusion, TLR4 has an essential role in the inhibition of apoptosis and ER stress pathways, as well as in the training-induced beneficial adaptations.

Keywords: Bioinformatics; Knockout model; Molecular signaling pathways; Morphofunctional characteristics; Regular exercise.

INTRODUCTION

Toll-like receptors were first discovered in the *Drosophila* fly, and their human counterparts were described in 1997 by Medzhitov *et al.* [82], becoming known as the Toll-like receptor (TLR). The TLRs are a family of transmembrane proteins whose role in the immune response has already been well established. Until today, eleven types of TLRs have been found in humans [83], and among them, the TLR4 (Toll-like receptor 4) has the most significant expression in the heart [84]. Recently published articles have described that TLR4 participates in harmful processes to cells such as apoptosis [85], inflammation [86; 87], and endoplasmic reticulum stress (ER stress) [24]. In addition, it is known that TLR4 can modulate genes and proteins associated with these signaling pathways in mice hearts after performing acute physical exercise [88].

Regular physical exercise benefits the whole body, including cardioprotective effects [89]. Moreover, regular physical exercise has been used as a non-pharmacological strategy for treating and preventing cardiovascular diseases [90]. Some of the regular exercise-induced positive impacts are related to TLR4 downregulation, such as its anti-inflammatory effect [91], type 2 diabetes *mellitus* control [92; 93], and atherosclerosis prevention [92]. Several studies have demonstrated that physical exercise modulates TLR4 [87; 88; 94; 95; 96] by two different ligands. The non-esterified fatty acids are more related to TLR4 acute activation, whose signaling cascade causes the activation of proteins such as p38 mitogen-activated protein kinase (p38MAPK) and c-Jun NH2-terminal kinase (JNK) [97]. The other ligand is the heat shock proteins (HSPs), particularly HSP60 [91] and HSP70 [98], which are more related to TLR4 chronic activation.

People who exercise regularly have both lower TLR4 content on the cell surface and *Tlr4* mRNA [99], which probably occurs due to the constant release of TLR4 ligands

(mainly HSP60) [91] after each exercise session, triggering a tolerance response to this receptor (also known as downregulation) [91]. Thus, we hypothesize that cardiac TLR4 downregulation would be responsible for the beneficial effects of regular physical exercise. By reducing the TLR4 activation, the activation of pathways such as apoptosis, inflammation, and ER stress, which cause harmful consequences for the heart, would also be mitigated. Therefore, the present study aimed to evaluate the effects of a chronic aerobic exercise protocol on TLR4 expression/activation and its repercussion in the pathways of apoptosis, inflammation, and ER stress in the heart.

METHODS

Experimental animals

Eight-week-old male C57BL/6 $Tlr4^{wt/wt}$ and $Tlr4^{-/-}$ mice [100] were maintained in a cage (three or two animals per cage) in a ventilated rack (INSIGHT™, Ribeirão Preto, SP, Brazil) under controlled temperature (22 ± 2 °C), on a 12:12-h light-dark inverted cycle (light: 6 PM to 6 AM, dark: 6 AM to 6 PM). Water and food (QUIMTIA, Nuvilab® CR1; Sogorb Indústria e Comércio Ltda, Colombo, PR, Brazil) were provided *ad libitum*. The diet composition was 63% carbohydrates, 26% proteins, and 11% lipids. The experimental procedures were performed following the Brazilian College of Animal Experimentation (COBEA) and were approved by the Ethics Committee of the University of São Paulo (I.D 2016.5.82.90.7).

The mice were randomly divided into four groups: control - WT (WT-CT, sedentary WT mice sacrificed with 15-week-old, n=10); control - KO (KO-CT, sedentary KO mice sacrificed with 15-week-old, n=10); exercise - WT (WT-EX, WT mice that performed the chronic physical exercise/CPE protocol, n= 10) and exercise - KO (KO-EX, KO mice that performed the CPE protocol, n= 10). The body weight of the experimental groups was recorded weekly using an analytical balance (Toledo, São

Bernardo do Campo, SP, Brazil). The experimental design of the present study is summarized in Figure 1.

Performance evaluations

The mice were acclimatized to the exercise on a treadmill (INSIGHT™, Ribeirão Preto, SP, Brazil) for five days, 10 min/day, at 6 m/min without inclination. The performance evaluation was applied 48 hours after the acclimation and 48 hours after the chronic physical exercise (CPE) protocol. The following tests were used: 1) the rotarod test; 2) the incremental load test (ILT), performed 4 hours after the rotarod test; 3) the exhaustive test, performed 24 hours after the ILT. These tests have already been described previously [101; 102]. The animals in the WT-CT and KO-CT groups performed these evaluations at the same age that the animals in the chronic exercise groups.

Echocardiogram

One week before applying the CPE protocol and 48 hours after the end of the CPE protocol, the exam was applied by a Vevo 2100® ultrasound system (Visual Sonics, Toronto, ON) with a 30Mhz transducer on a heated platform for handling mice with electrocardiogram detectors and monitoring of vital signs. The mice were anesthetized with an isoflurane inhalation anesthetic at 3% of the chamber volume. As soon as the loss of pedal reflexes confirmed the effect of anesthesia, the rodents were positioned with the legs fixed to the side of the body on the heated platform and inhaled isoflurane enriched with a flow of 5 L/min of oxygen. Two-dimensional (B-Mode) and M-mode (M-Mode) images were recorded on the parasternal long axis, with the center of the left ventricle (LV) like a reference.

Chronic physical exercise (CPE) protocol

The aerobic exercise protocol was adapted from the study of Morais *et al.* [103; 104]. In the CPE protocol, each experimental week consisted of 3 days of exercise intercalated with 48 hours of rest. Table 1 summarizes the CPE protocol characteristics.

Table 1. Chronic physical exercise protocol.

Week	Intensity (%EV*)	Volume (min)	Daily sessions	Slope (%)	Interval between sessions (h)
1	60	15	1	0	48
2	60	30	1	0	48
3	60	45	1	0	48
4	60	60	1	0	48

* Exhaustion velocity in the incremental load test.

Indirect calorimetry

Only the mice from both CT groups were submitted to the indirect calorimetry. The exam was performed using the Panlab Oxylet System (Panlab, S.L., Barcelona, Spain). During 48 hours, the rodents were accommodated in acrylic cages (three animals per cage) with a controlled temperature (22 ± 2 °C). The mice were adapted to the apparatus in the first 24 hours. In the other 24 hours, the following variables were measured: oxygen consumption (VO_2), carbon dioxide production (VCO_2), total energy expenditure (EE), and respiratory quotient (RQ), which was determined by the VCO_2/VO_2 ratio.

Glucose tolerance test (GTT)

Only the mice from both CT groups were submitted to the glucose tolerance test. To obtain the baseline glycemia values, we collected a drop of blood from the end of the animal's tail after 12 hours of fasting. After that, we quantified the blood glucose by a blood glucose monitoring system (model Accu-Chek® Active, Roche). Subsequently, 2g/kg of glucose was injected into the animal's intraperitoneal region, and blood glucose

was measured again after 15, 30, 60, and 120 minutes. With these values, the area on the curve was calculated using the trapezoidal principle [105].

Blood collection, heart extraction, and left ventricle (LV) isolation

One week after the end of the CPE protocol or 72 hours after the GTT for the CT groups, fasted mice were anesthetized with an intraperitoneal injection of xylazine (10 mg/kg body weight) and ketamine (100 mg/kg body weight). As soon as the loss of pedal reflexes confirmed the effect of anesthesia, blood was collected by cardiac puncture, and serum was separated by centrifugation (1100 ×g) for 15 min at 4 °C and stored at −80 °C. After each mouse heart was removed, the left ventricle was isolated and washed with saline solution. Five left ventricles of each experimental group were divided into two samples and designated for reverse transcription-quantitative polymerase chain reaction (RTq-PCR) and immunoblotting techniques. At the same time, the other five left ventricles were designated for histopathological analysis.

Serum analysis

The serum concentrations of creatine kinase (CK), creatine kinase MB isoenzyme (CK-mb), and lactate dehydrogenase (LDH) were measured using conventional assay kits according to the instructions of the manufacturer (Analisa, Belo Horizonte, Minas Gerais, Brazil), as well as interleukins 1beta, 6 and 17 (IL1β, IL6, and IL17) and tumor necrosis factor-alpha (TNFα) using Luminex ™ multiplex method (Millipore, St Charles, MO, USA). For the analysis of cytokines, samples were pipetted on the Luminex MAP200 instrument and were analyzed using the 3.1 xPONENT System.

Reverse transcription-quantitative polymerase chain reaction (RTq-PCR)

Total RNA from the left ventricle was extracted using Trizol (Invitrogen, Carlsbad, CA, USA). The cDNA was synthesized with 600 ng of total RNA using the

High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR was performed on the ViiA7 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) for the relative mRNA expression of the following genes associated with the proteins in parentheses: *Il1b* (Il-1beta), *Tnf* (TNFα), *Ddit3* (CHOP), *Casp3* (Caspase 3), *Bax* (BAX), *Tlr4* (TLR4) and for data normalization the *Actb* (β- actin) was the reference gene. Primer designs are described in Table 2.

The quantitative real-time PCR was performed with a final volume in the amplification reactions of 10µL, pipettes in duplicate with the following reagents: 2µL HOT FIREPol® EvaGreen® qPCR Supermix (Solis Biodyne, Tartu, Estonia), 1µL primer forward, 1µL primer reverse, 2µL cDNA diluted in 1:6, and 4µLof H2O. The reaction was performed using the HOT FIREPol® EvaGreen® qPCR Supermix (Solis Biodyne, Tartu, Estonia), manufacturer instructions. Relative quantification was calculated using the Thermo Fisher Cloud Software, RQ version 3.7 (Life Technologies Corporation, Carlsbad, CA, USA) by the 2 −ΔΔCT method.

Table 2. Primer sequences.

Gene	Forward	Reverse
Actb	GACTCATCGTACTCCTGCTTG	GATTACTGCTCTGGCTCCTAG
Bax	TTTGCTACAGGGTTTCATCCAG	GTCCAGTTCATCTCCAATTCGC
Casp3	AGCTTGGAACGGTACGCTAA	CCAGAGTCCACTGACTTGC
Ddit3	ATCTTGAGCCTAACACGTCGAT	GACCAGGTTCTCTCTCCTCAG
Il1b	TGCCACCTTTTGACAGTGATG	GCTCTTGTTGATGTGCTGCT
Tlr4	GTTCTCTCATGGCCTCCACT	GGAACTACCTCTATGCAGGGAT
Tnf	CCACCACGCTCTTCTGTCTA	CCACTTGGTGGTTTGTGAGT

Actb: actin, beta; *Bax*: BCL2-associated X protein; *Casp3*: caspase 3; *Ddit3*: DNA-damage inducible transcript 3; *Il1b*: interleukin 1 beta; *Tlr4*: toll-like receptor 4; *Tnf*: tumor necrosis factor.

Immunoblotting

The immunoblotting technique was performed to determine the protein concentration in the left ventricle as previously described [101; 102]. The antibodies used were BAD (SC8044), Caspase 3 (SC7272), eIF2alpha (SC11386), peIF2alpha (Ser52;

SC101670), IKK (SC34674), pIKK (Ser176; SC-21661), JNK (SC572), pJNK (Thr183/185; SC6254), PTP1B (H-135) (SC14021), TLR4 (SC293072), TRB3 (SC390242) and α-Tubulin (SC-32293) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); p-BAX (Ser184; orb4658) from Biorbyt Ltd. (Cambridge, Cambridgeshire, England); CHOP (15404-1-AP) from Proteintech (Rosemont, IL, USA); IRE-1 (AB37073) and pIRE-1 (Ser724; AB104157) from Abcam (Cambridge, UK) and pBAD (Ser 112; #9291s), ATF4 (#11815) from Cell Signaling Technology (Cell Signaling Technology, MA, USA).

After the band intensities were measured, all proteins (phosphorylated or non-phosphorylated) were normalized by their respective Ponceau S-stain [106]. Then, the ratio phosphorylated/non-phosphorylated or phosphorylated/control protein (alpha-tubulin) was calculated. When two membranes were used for each protein, the comparison of different gels was allowed by applying a previously published calibration method [107]. All Western blot experiments are available in supplementary file 1.

Histological analysis

Samples of the LV were previously stored in paraplast to analyze the general morphology of the tissue. The histological sections (5 µm thick) were obtained in a microtome (Jung CM 1800, Leica, Germany) and stained with hematoxylin and eosin (H&E), picrosirius red, and periodic acid Schiff (PAS) [Kit PAS 3X (cod KP08169SO), Exodo Cientifica, Sumare, SP, Brazil]. The colored sections were used to analyze and photo documentation (400x magnification) of the experimental groups. The images were obtained in a photomicroscope (AxioCam 2, Carl Zeiss, Jena, Germany) with a digital camera (Axiocam-HR, CarlZeiss, Jena, Germany).

Quantification of reactivity: The percentage of intensity of the PAS and Picrosirius Red staining was evaluated by the intensity of the color, considering five animals per group, examined in 10 random fields per animal, using the ImageJ software (version 1.50i) by the IHC-toolbox plugin. The stereological analysis was adapted using a Weibel reticle of 144 points [108] by the Stepanizer software (available on http://www. stepanizer.com/), examined in 50 random fields per group. Thus, the percentage for totalization of variables was analyzed in 10 different sections of the transversal and longitudinal fibers per group.

To assess the thickness of the left ventricle, we used H&E staining. Three cross-sections of the left ventricle per animal were obtained, five animals per group, with 12x magnification (ZEISS SteREO Discovery V12 Microscope). For the quantification, we used the ImageJ software (version 1.50i), with the help of the Line Selection Tool, where five transverse lines were drawn by cutting the myocardium to analyze the entire ventricular thickness, totaling 15 measurements per animal. The ventricle thickness was expressed in millimeters (mm).

Bioinformatics analysis

To expand the role of TLR4 in the cardiac responses to physical training, we utilized transcriptome analysis using the BXD mice database [109]. Bioinformatics analysis was used to explore the correlations of *Tlr4* mRNA in the heart with apoptosis, inflammation, and ER stress genes in cardiac tissue in several BXD mice strains. The correlation analyses were performed using a data set from heart *Tlr4* (EPFL/LISP BXD CD Heart Affy Mouse Gene 2.0 ST Gene Level [Jan 14] RMA) of genetically diverse BXD mice, as previously published [109]. The five strains with the highest *Tlr4* values (i.e., BXD73, BXD75, BXD81, BXD44, and BXD43) and the five strains with the

lowest *Tlr4* values (i.e., BXD68, BXD100, BXD65, BXD69, and BXD66) were selected to perform the correlation with the apoptosis, inflammation, and ER stress genes. All data are available on Genetwork (http:// www.genenetwork.org) [110]. We accessed the online platform on 04/02/2021. The heatmap graph was done in the Gene-E software (Morpheus, https://software.broadinstitute.org/morpheus).

Statistical analysis

Results are expressed as the mean ± standard error of the mean (SEM). The normality and homogeneity were checked using the Shapiro–Wilks W test and Levene's test. The unpaired Student's t-test was used to analyze the difference between WT and KO mice. Two-way analysis of variance (ANOVA) was used to analyze the effects of CPE protocol and the TLR4 deletion in the heart. Mixed ANOVA was used to analyze the echocardiogram parameters. When indicated interaction, Sidak's post hoc test was performed. The analyses were bilateral, and the level of significance was fixed at $P \leq 0.05$.

RESULTS

TLR4 knockout mice had increased VO2, VCO2, and EE

Figures 2A and 2B show that the KO had higher VO2, VCO2, and EE than WT in light and total (light and dark cycles). However, these groups did not present significant differences for the respiratory quotient and GTT (Figures 2C and 2D). All groups had a statistical increase in body weight from the 8th to 15th week of the study; however, there were no significant differences between the experimental groups (Figure 2E).

TLR4 knockout mice did not improve the performance parameters in response to the chronic aerobic training protocol

There were no differences in the performance (incremental load and exhaustive tests) between WT and KO mice at the basal state. However, compared to the basal state, only the WT mice improved the performance of these tests after the CPE protocol. Also, for both evaluations, the performance of KO was lower compared to WT after the CPE protocol (Figures 3A and 3B). In the rotarod test (Figure 3C), the KO had a lower performance than WT at the basal state, which was maintained after the CPE protocol. Regardless of genetic background, the CPE protocol increased the IL6 and decreased CK values (Figures 3D and 3E). Moreover, the CK-mb was lower for KO than WT after the CPE protocol (Figure 3E).

TLR4 knockout mice had no improvement in cardiac function in response to the chronic training protocol

According to table 3, after 4 weeks, the IVS;d of the exercised KO group was lower than the control KO and exercised WT groups. After 4 weeks, the IVS;s of the exercised WT group was higher than the control WT group, while the IVS;s of the exercised KO group was lower than the control KO group. Also, the IVS;s and LVID;d of the control KO group increased after 4 weeks. After 4 weeks, while the LVID;s of the exercised WT group was lower than the control WT group, the LVPW;s, ejection fraction, and fractional shortening of the exercised WT group were higher than the control WT group. Also, after 4 weeks, the LVPW;s, ejection fraction, and fractional shortening of the exercised WT group were higher than the exercised KO group. The cardiac output and heart rate of the control WT group increased after 4 weeks. Also, at the basal state, the heart rate of the control KO group was higher than the control WT group. The heart rate of the control KO group increased after 4 weeks.

Table 3. Echocardiogram parameters were measured in the basal state and after the four weeks of chronic physical exercise protocol for the wild-type (WT) and TLR4 knockout (KO) mice.

Parameters		***WT***		***TLR4 KO***	
		Control	**Exercised**	**Control**	**Exercised**
M-Mode					
IVS;d (mm)	Basal state	0.95 ± 0.05	0.98 ± 0.04	0.93 ± 0.04	0.87 ± 0.05
	After 4 weeks	0.95± 0.01	0.98 ± 0.03	1.03 ± 0.04	0.92 ± 0.32[f,g]
IVS;s (mm)	Basal state	1.32 ± 0.05	1.47 ± 0.05	1.28 ± 0.40	1.34 ± 0.07
	After 4 weeks	1.35 ± 0.04	1.5± 0.04[e]	1.43 ± 0.05[b]	1.27 ±0.04[f]
LVID;d (mm)	Basal state	3.8 ± 0.12	3.77 ± 0.09	3.75 ± 0.13	3.66 ± 0.14
	After 4 weeks	3.88 ± 0.1	3.74± 0.07	3.96 ± 0.13 [b]	3.66 ± 0.08
LVID;s (mm)	Basal state	2.79 ± 0.11	2.42 ± 0.1[a]	2.65 ± 0.14	2.44 ± 0.14
	After 4 weeks	2.77 ± 0.13	2.38± 0.07[e]	2.90 ± 0.13	2.61 ± 0.10
LVPW;d (mm)	Basal state	0.78 ± 0.03	0.86 ±0.05[a]	0.85 ± 0.04	0.76 ± 0.06
	After 4 weeks	0.83 ± 0.05	1.03± 0.06[c]	0.79 ± 0.5	0.92 ± 0.05[d]
LVPW;s (mm)	Basal state	1.05 ± 0.05	1.3 ± 0.09[a]	1.16 ±0.04	1.05 ± 0.07
	After 4 weeks	1.16 ± 0.05	1.5±0.08[c,e]	1.10 ± 0.09	1.22 ±0.06[g]
Ejection fraction (%)	Basal state	52.42± 2.38	66.04± 1.9[a]	56.83 ± 3.43	63.09 ± 1.97
	After 4 weeks	55.87± 2.65	66.5±2.04[e]	52.66 ± 2.11	56.2 ±2.43[d,g]
Fractional shortening (%)	Basal state	26.53± 1.50	35.88 ±1.4[a]	29.54 ±2.28	33.59 ± 1.35
	After 4 weeks	28.83± 1.69	36.24±1.6[e]	26.74 ± 1.35	28.92 ±1.67[g]
LV Mass (mg)	Basal state	96.39± 4.65	105.2± 7.83	98.03 ± 5.16	85.02 ± 6.98
	After 4 weeks	104.2 ± 5.22	117.18±7.4	111.1 ± 7.25	99.42 ± 4.83
		Control	**Exercised**	**Control**	**Exercised**
B-Mode					
Cardiac output (mL/min)	Basal state	13.43± 1.21	18.44± 3.10	15.59 ± 2.18	12.97 ± 1.34
	After 4 weeks	18.73±0.95[a]	16.61± 0.89	19.87 ±2.92	14.62 ±1.45
Stroke volume (uL)	Basal state	33.83± 1.39	35.45± 2.30	37.73 ± 4.10	30.77 ± 2.63
	After 4 weeks	39.38± 2.19	35.92± 1.80	39.71± 6.29	29.51 ± 2.28
Diastolic volume (uL)	Basal state	63.53± 3.28	61.64± 1.7[d]	63.01 ± 6.45	48.44 ± 3.41[b]
	After 4 weeks	67.53± 2.86	58.35±2.25	69.24 ±2.23	57.43 ± 4.14 [d]
Systolic volume (uL)	Basal state	29.70± 2.84	26.19± 1.2[d]	25.28 ± 3.01	17.67 ± 1.18[b]
	After 4 weeks	28.14± 2.06	22.43± 1.76	29.53 ± 3.30	27.92 ± 2.86 [d]
Heart Rate (bpm)	Basal state	396.6±23.4	510.3±60.4	406.7±19.6[a,f]	420.50 ± 22.86
	After 4 weeks	476.75±10.2[a]	463±10.59	506.7±16.2	490,53± 17,23

a= $p < 0.05$ vs. Control WT group at basal state.
b= $p < 0.05$ vs. Control KO group at basal state.
c= $p < 0.05$ vs. Exercised WT group at basal state.
d= $p < 0.05$ vs. Exercised KO group at basal state.
e= $p < 0.05$ vs. Control WT group after 4 weeks.
f= $p < 0.05$ vs. Control KO group after 4 weeks.
g= $p < 0.05$ vs. Exercised WT group after 4 weeks.
Data correspond to mean ± standard error of n=6-8 mice**.** IVS;d - Interventricular septum thickness at end-diastole; IVS;s - Interventricular septum thickness at end-systole; LV - Left ventricle; LVID;d - Left ventricular internal dimension at end-diastole; LVID;s - Left ventricular internal dimension at end-systole. LVPW;d - Left ventricular posterior wall thickness at end-diastole. LVPW;s - Left ventricular posterior wall thickness at end-systole; TLR4 - Toll-like receptor 4.

Exercised mice decreased Il1b gene expression

Figure 4A shows the *Tlr4* gene expression was not modulated in response to the training protocol for the WT group. Also, the gene expressions of *Bax*, *Casp 3*, *Ddit3*, and *Tnf* were not different between the experimental groups (Figure 4B). Regardless of genetic background, the *Il1b* expression in the exercised state was lower than in the control state.

TLR4 knockout mice had lower phosphorylation of proteins related to apoptosis and unfolded protein response activation in control and exercised states

According to figure 5A, the protein content of TLR4 was higher for the WT-EX compared to the WT-CT. Regardless of genetic background, the phosphorylation of BAD and IRE 1 in the exercised state was lower than in the control state. The phosphorylation of BAD, BAX, and IRE1 of KO was lower than WT in control and exercised states (Figures 5B and 5D). The protein contents of Caspase 3, ATF4, pIKK, pJNK, CHOP, peIF2α, PTP1B, and TRB3 were not different between the experimental groups (Figures 5B-5D).

TLR4 knockout mice displayed elevation of glycogen and nucleus percentage in the exercised state, but a reduction of collagen in the control state

Morphological analysis of cardiac tissue from each experimental group shows that the intracellular glycogen stain of the KO-EX group was higher than WT-EX and KO-CT groups in the longitudinal and transversal sections (Figures 6A, 6D, 7A, and 7C). The nucleus, fiber, and connective tissue in the longitudinal sections (Figures 6B and 6E-6G), as well as the fiber and connective tissue in the transversal sections (Figures 7E and 7F), were not different between the experimental groups. In the longitudinal sections of KO mice (i.e., control and exercised states), the cardiac collagen stain was reduced compared to the WT group (i.e., control and exercised states) (Figures

6C and 6H). The stereology analysis demonstrates that the nucleus percentage in the KO-EX group was higher than the WT-EX and KO-CT groups in the transversal sections (Figures 7B and 7D). According to figures 8A and 8B, the ventricular thickness was not different between the experimental groups.

Bioinformatics analysis expanded the relationship of Tlr4 mRNA in the heart with apoptosis, inflammation, and ER stress genes in cardiac tissue in several BXD mice strains

Bioinformatics analysis was performed using one of the most prominent and best-categorized families of isogenic strains, providing a broad set of data for metabolic disorders investigations [109]. First, we visualized the distribution of heart *Tlr4* mRNA levels in 42 strains of isogenic BXD mice, highlighting the five strains with lower (BXD68, BXD100, BXD65, BXD69, and BXD66) and the five strains with higher (BXD73, BXD75, BXD81, BXD44, and BXD43) levels of *Tlr4* mRNA in the heart (Figure 9A). The heatmap emphasized that the genes related to apoptosis and inflammation pathways were downregulated in the heart of the strains with lower *Tlr4* levels (Figure 9B). Using the two factors analysis graphs and Pearson's correlation, we verified that the *Tlr4* gene expression was correlated positively and/or negatively with several genes related to ER stress (Figures 9C), inflammation (Figure 9D), and apoptosis (Figure 9E).

DISCUSSION

The main findings of the present study were: 1) In the control state, the KO mice had higher energy expenditure and heart rate, but lower rotarod performance, pBAD, pBAX, pIRE1, and cardiac collagen; 2) In the exercised state, the KO mice had no improvements in performance and cardiac function, lower CK-mb serum levels,

LVPW;s, ejection fraction, fractional shortening, pBAD, pBAX, and pIRE1, but higher glycogen and nucleus contents; 3) In the exercised state, the WT mice improved performance and cardiac function, but increased TLR4 protein contents. These data did not support our initial hypothesis that cardiac TLR4 downregulation would be responsible for the beneficial effects of regular physical exercise.

To the best of our knowledge, this is the first investigation describing the indirect calorimetry data of the TLR4 KO mice, which presented higher basal levels of VO_2, VCO_2, and energy expenditure. Therefore, we confirmed the hypothesis of Pierre and coworkers [111], who justified the protection of body weight gain and glucose intolerance induced by a high-fat diet in the TLR4 KO model by possible higher energy expenditure. Following previously published findings [88; 112], we did not verify significant differences in body weight and weight gain between WT and KO mice.

Regarding the chronic physical exercise protocol, the KO group did not improve the performances in the incremental load and exhaustive tests, which may be related to blunted adaptations in skeletal muscle. Indeed, Ali *et al*. [113] recently demonstrated that muscle-specific deletion of TLR4 impaired the significant increases in mitochondrial enzyme activities, as well as fatty acid and glucose oxidation, after a 4-week voluntary wheel running exposition. The KO mice showed lower scores in the rotarod test, which may be linked to motor coordination impairment [114].

Although the increased concentrations of IL6 in the exercised WT and KO mice are not in agreement with the literature [115], this result cannot be linked to a proinflammatory state since the IL1β and TNFα were not up-regulated. The decrease in CK levels for WT and KO groups in response to training may be related to adaptation to handling stress since deletion of sarcomeric mitochondrial CK did not impair physical performance [116]. The lower levels of CK-mb in the KO-EX compared to WT-EX may

be considered a regular exercise positive adaptation once the contrary was observed in response to a high-intensity acute exercise session [88].

While we verified the heart rate of the KO was higher than WT at the basal state, Hu and Zhang [117] did not find significant differences in the echocardiogram parameters between these groups. After four weeks, the WT mice presented higher cardiac output and heart rate in the control state, highlighting an aging effect and not only an exercise adaptation. For the first time, we described the training impact on cardiac geometry and function of TLR4 KO mice. Interestingly, only WT mice improved cardiac function after exercise. Therefore, the current lack of training improvements in the cardiac function of KO mice may also have influenced their poor performance.

In agreement with the elevation of heart *Tlr4* mRNA expression after acute exercise [87; 88], the training protocol increased the TLR4 protein content. Although we did not observe a significant alteration in *Tlr4* mRNA expression, several factors regulate mRNA during translation, explaining the lack of linearity with the protein content [118]. Since mRNA expression alterations range from 3 to 12 h after exercise stimulus cessation [119] and heart extraction occurred one week after the training period, the protein findings are more robust for the phenomena interpretation.

Despite the control or exercised states, the KO group displayed a downregulation in apoptosis (i.e., phosphorylation of BAD and BAX) and ER stress (i.e., phosphorylation of IRE1) pathways, reinforcing previously published data [88]. These relationships were confirmed using the BXD mice families. For the WT and KO groups, the cardiac proapoptotic BAD phosphorylation was downregulated in response to chronic exercise, which was not observed in trained hypertensive rats [120]. The decreased IRE1 phosphorylation in both WT-EX and KO-EX groups reinforces the

beneficial effects of the current training program once mice performing excessive training did not modulate this protein [121].

The increased glycogen contents of KO than WT in the exercised state may be related to the higher phosphorylation/inactivation of glycogen synthase kinase-3β, as previously described by Hua *et al*. [122]. Even with higher glycogen and nucleus after training, the KO group did not improve cardiac function and performance. Mice performing excessive exercise also displayed elevated heart glycogen contents and performance impairment [123]. The lower collagen of KO than WT in control states reinforces the findings of Dong and coworkers [124] but was not influenced by the chronic exercise protocol. Figure 10 summarizes the main findings of the current investigation.

CONCLUSION

In contrast to the initial hypothesis, regular moderate-intensity exercise did not reduce the gene expression and protein content of TLR4 but improved cardiac function and performance. The TLR4 deletion attenuated the apoptosis and ER stress pathways at the basal condition. Also, the lack of improvement in heart function and performance markers in this condition, even after a chronic exercise protocol, suggests the TLR4 plays a crucial role in the training-induced beneficial adaptations.

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AUTHORS' CONTRIBUTIONS

LGV, VRM, APP, and ASRS designed the paper. LGV and ASRS wrote the paper. LGV, VRM, APP, RLR, ALR, BBM, MEAT, and GDF performed the data collection, application of techniques, and/or statistical analysis. LGV, VRM, RLR, and ASRS designed the figures of the manuscript. GRT, LCA, FGF, FMS, ERR, LPM, DEC, JRP, and ASRS contributed to data analysis, discussion, and/or provided laboratory support. All authors have read and approved this manuscript.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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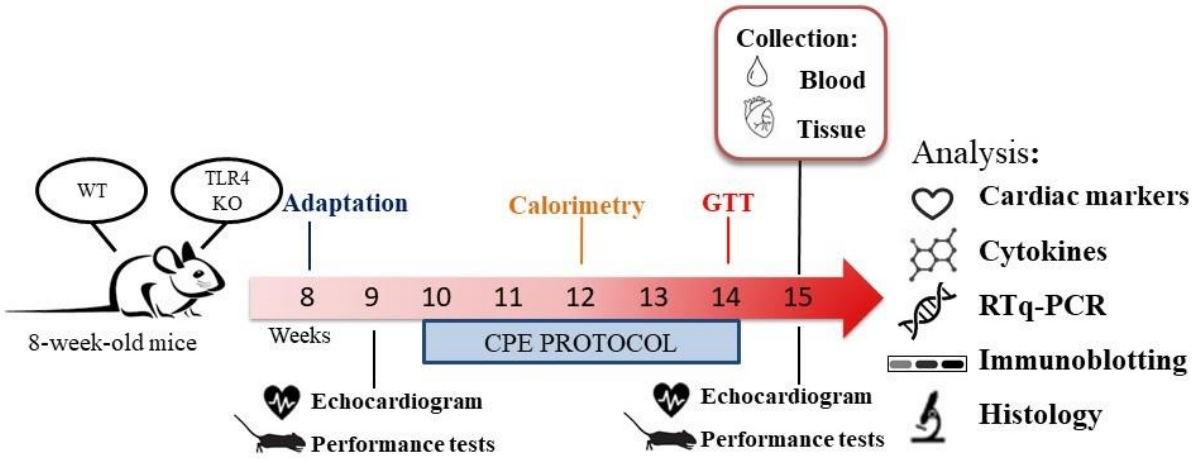

Figure 1. Schematic diagram of the experimental procedures. CPE: chronic physical exercise; GTT: glucose tolerance test; WT: wild-type mice; KO: TLR4 knockout mice.

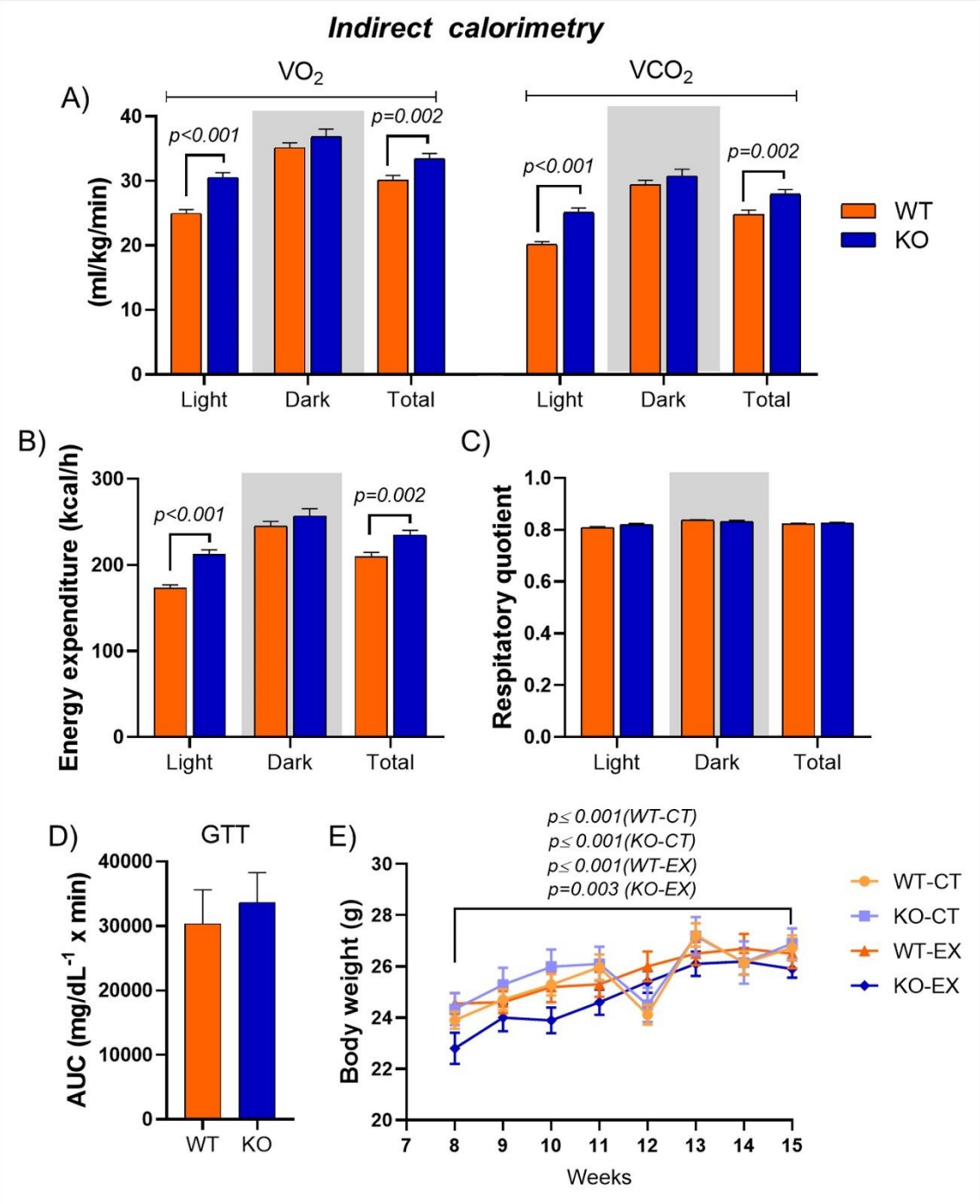

Figure 2. Oxygen uptake/VO2 and Carbon dioxide output/VCO2 **(A)**; Energy expenditure **(B)**; Respiratory quotient **(C)**; Area under the curve (AUC) for glucose tolerance test **(D)**; Body weight **(E)**. Data correspond to the mean ± SEM of n = 5 per group for A, B, and C, n=6 per group for D, and n=9-10 per group for E. dL: deciliter; g: grams; Kcal/24h: kilocalories in 24 hours; mg: milligram; ml/kg/min: milliliters per kilogram minute; WT: wild-type mice; KO: TLR4 knockout mice.

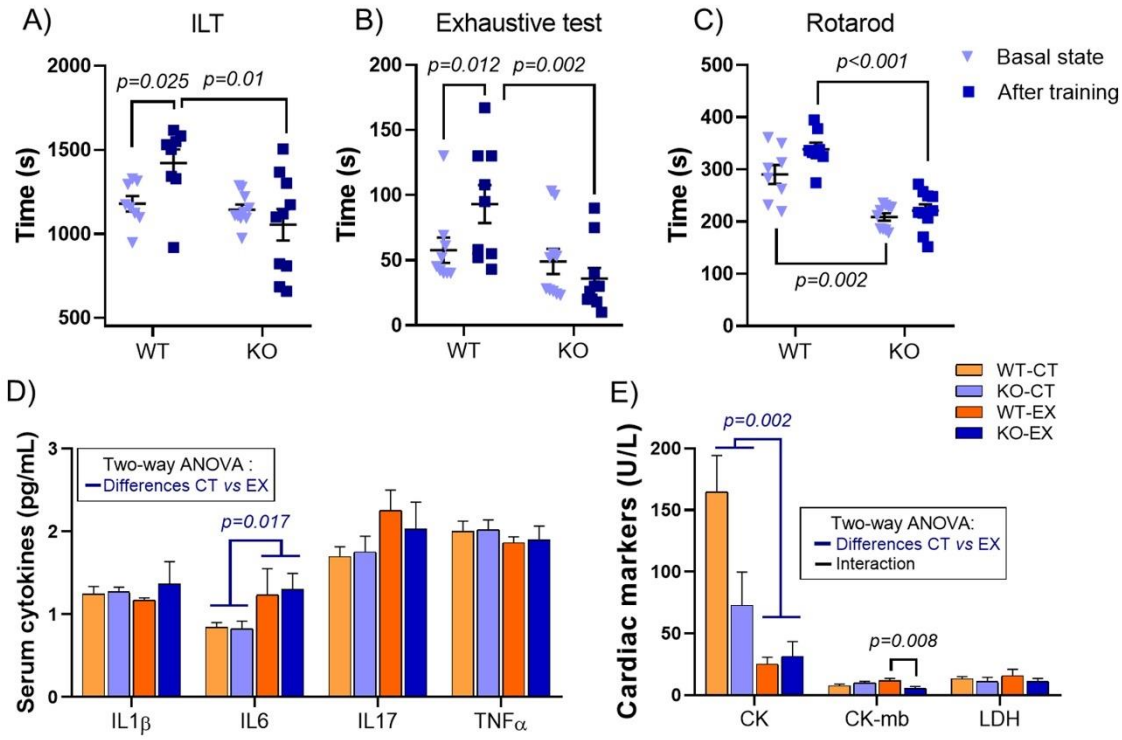

Figure 3. Incremental load test **(A)**; Exhaustive test **(B)**; Rotarod test **(C)**; Serum levels of the interleukin (IL) 1β, IL-6, IL-17, and tumor necrosis factor-alpha (TNFα) **(D)**; Serum levels of cardiac markers creatine kinase (CK), CK-mb, and lactate dehydrogenase (LDH) **(E)**. Data correspond to the mean ± SEM of n=5-10 mice per group. Pg/ml: picogram per milliliter; U/L: units per liter; s: seconds; WT-CT: wild-type control group; KO-CT: TLR4 knockout control group; WT-EX: wild-type exercise group; KO-EX: TLR4 knockout exercise group.

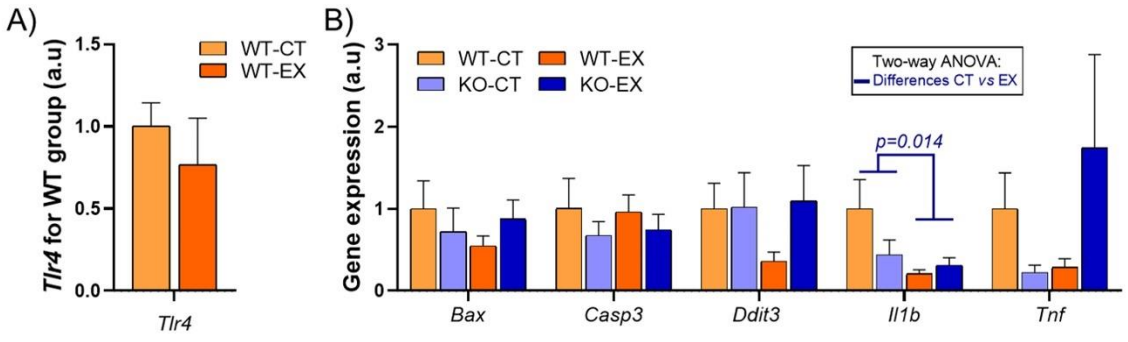

Figure 4. Levels of *Tlr4* expression in the heart for WT mice **(A)**; Levels of *Bax*, *Casp3*, *Ddit3*, *Il1b*, and *Tnf* expressions in the heart **(B)**. Data correspond to the mean ± SEM of n=3 (*Il1b* only for the WT-CT)-5 mice per group. a.u: arbitrary units; WT-CT: wild-type control group; KO-CT: TLR4 knockout control group; WT-EX: wild-type exercise group; KO-EX: TLR4 knockout exercise group.

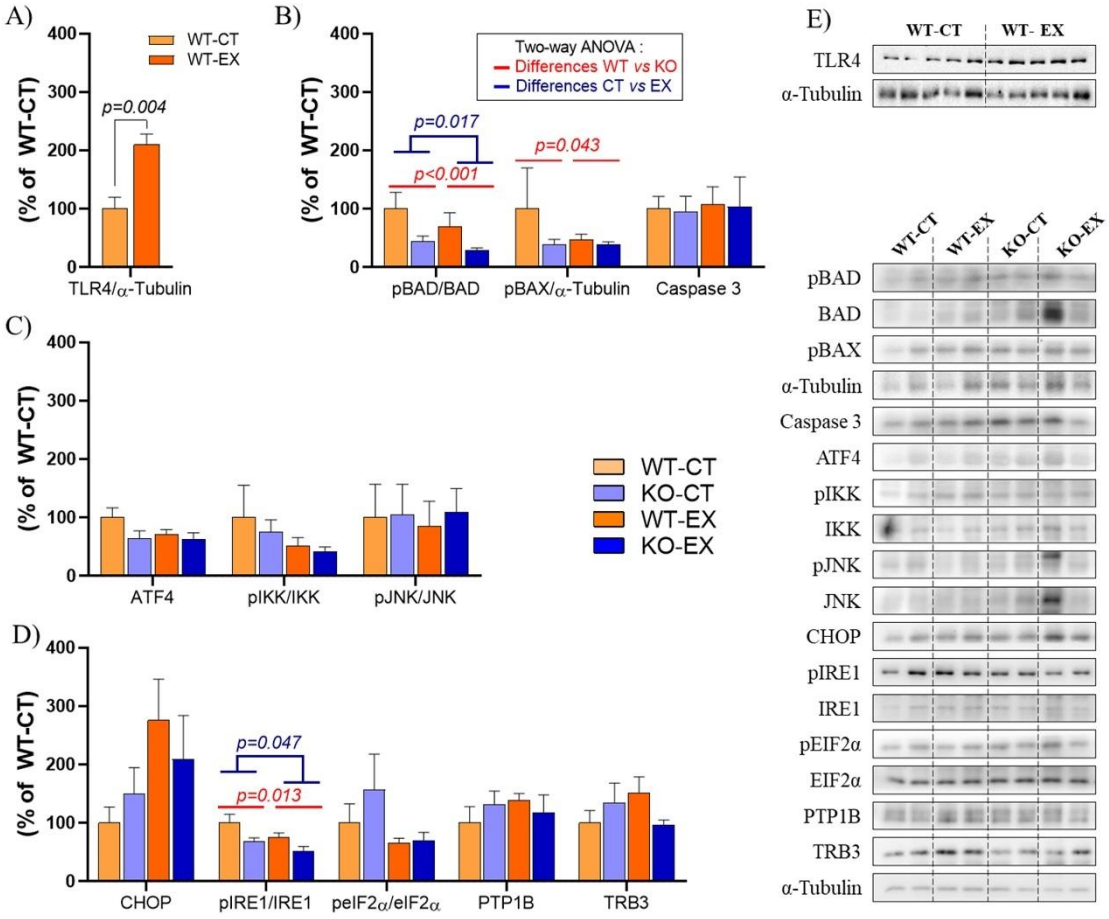

Figure 5. TLR4 protein content in the heart for WT mice **(A)**; Content of proteins related to apoptosis pathway **(B)**; Content of proteins related to inflammation pathway **(C)**; Content of proteins related to endoplasmic reticulum stress pathway **(D)**; Representative blots **(E)**. All immunoblotting experiments are available in supplementary file 1. Data correspond to the mean ± SEM of n=5 mice per group. a.u: arbitrary units; WT-CT: wild-type control group; KO-CT: TLR4 knockout control group; WT-EX: wild-type exercise group; KO-EX: TLR4 knockout exercise group.

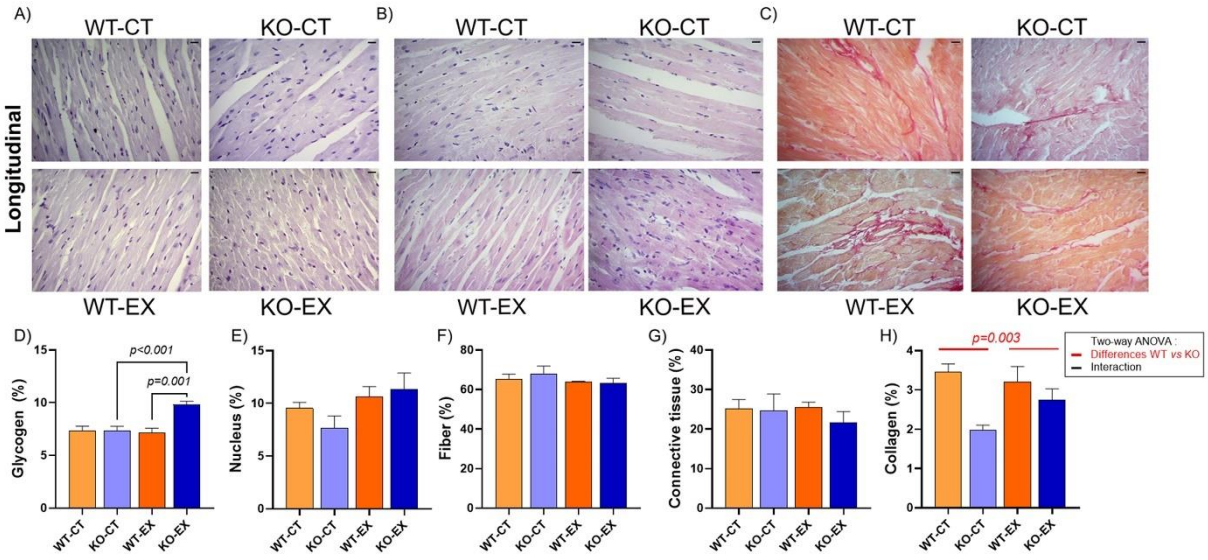

Figure 6: Histological characterization (x400) of longitudinal fibers of cardiac tissue by Periodic Acid Schiff (PAS) **(A)**; Hematoxylin-Eosin **(B)**; Picrosirius Red **(C)**; Graph representative of glycogen **(D)**; Graph representative of the nucleus **(E)**; Graph representative of fiber **(F)**; Graph representative of connective tissue **(G)**; Graph representative of collagen **(H)**. Bar=20µm. WT-CT: wild-type control group; KO-CT: TLR4 knockout control group; WT-EX: wild-type exercise group; KO-EX: TLR4 knockout exercise group.

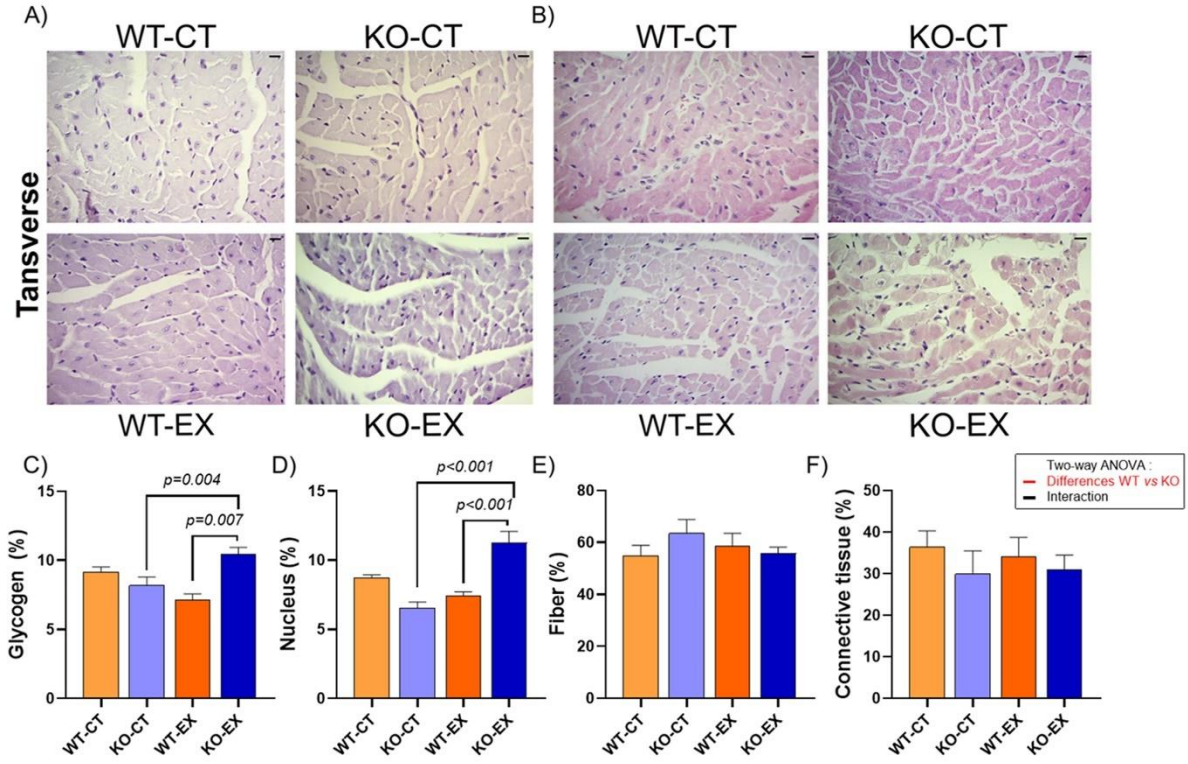

Figure 7. Histological characterization (x400) of transverse fibers of cardiac tissue by Periodic Acid Schiff (PAS) **(A)**; Hematoxylin-Eosin **(B)**; Graph representative glycogen **(C)**; Graph representative of the nucleus **(D)**; Graph representative of fiber **(E)**; Graph representative of connective tissue by stereology analyzes **(F)**. Bar=20µm. WT-CT: wild-type control group; KO-CT: TLR4 knockout control group; WT-EX: wild-type exercise group; KO-EX: TLR4 knockout exercise group.

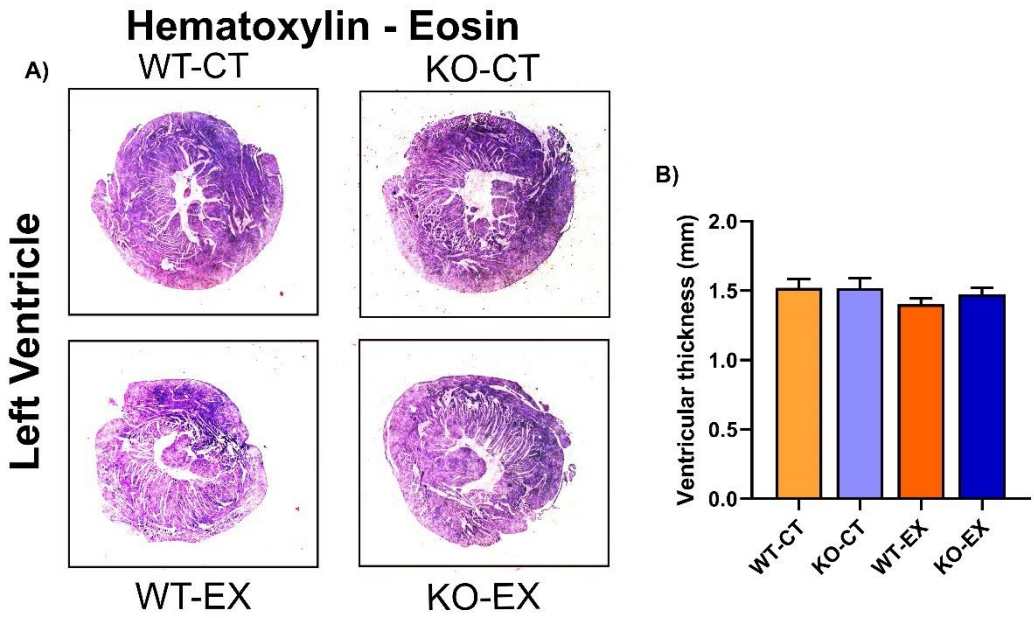

Figure 8. Histological characterization (x12) of the left ventricle by hematoxylin-eosin **(A)**; Graph representative of left ventricle thickness **(B)**. WT-CT: wild-type control group; KO-CT: TLR4 knockout control group; WT-EX: wild-type exercise group; KO-EX: TLR4 knockout exercise group.

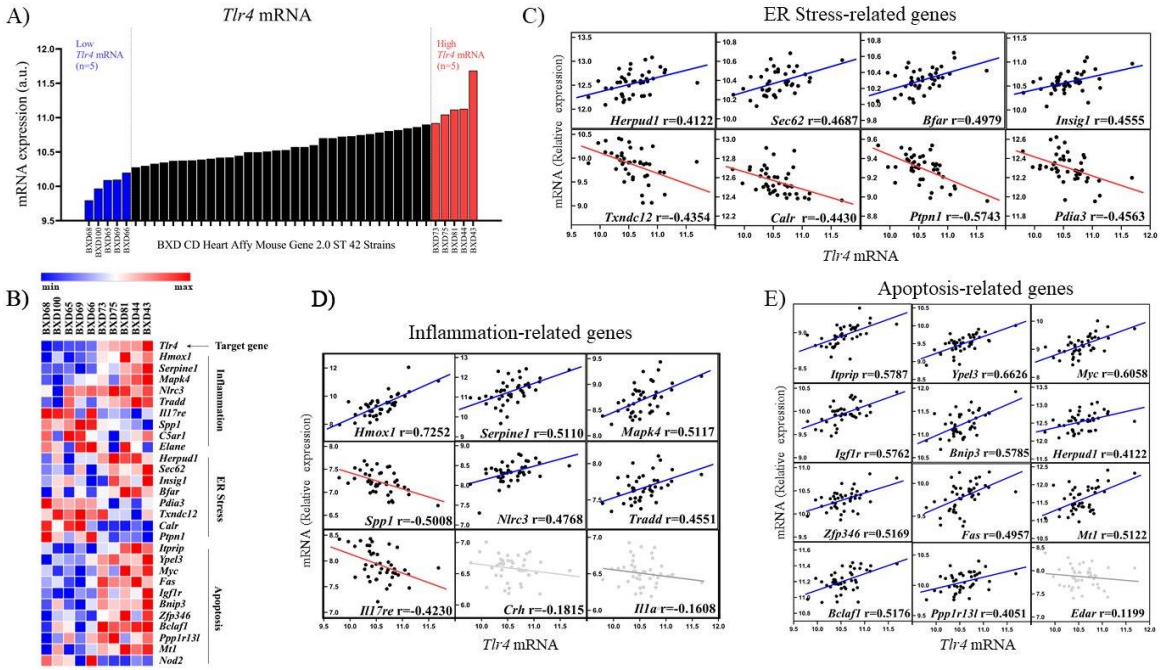

Figure 9. Distribution of *Tlr4* mRNA in the heart (n =42) of isogenic BXD mice, highlighting five strains with lower (blue) and five strains with higher (red) levels of *Tlr4* mRNA in the heart **(A)**; Heatmap showing the relationship of heart *Tlr4* mRNA levels with inflammation, ER stress, and apoptosis-related genes in the heart in 10 BXD mice strains **(B)**; The main correlations of heart *Tlr4* mRNA levels with ER stress-related genes in the heart **(C)**; The main correlations of heart *Tlr4* mRNA levels with inflammation-related genes in the heart **(D)**; The main correlations of heart *Tlr4* mRNA levels with apoptosis-related genes in the heart **(E)**. Blue ($p \leq 0.05$), red ($p \leq 0.05$), and grey lines represent significant positive, significant negative and, no significant correlations.

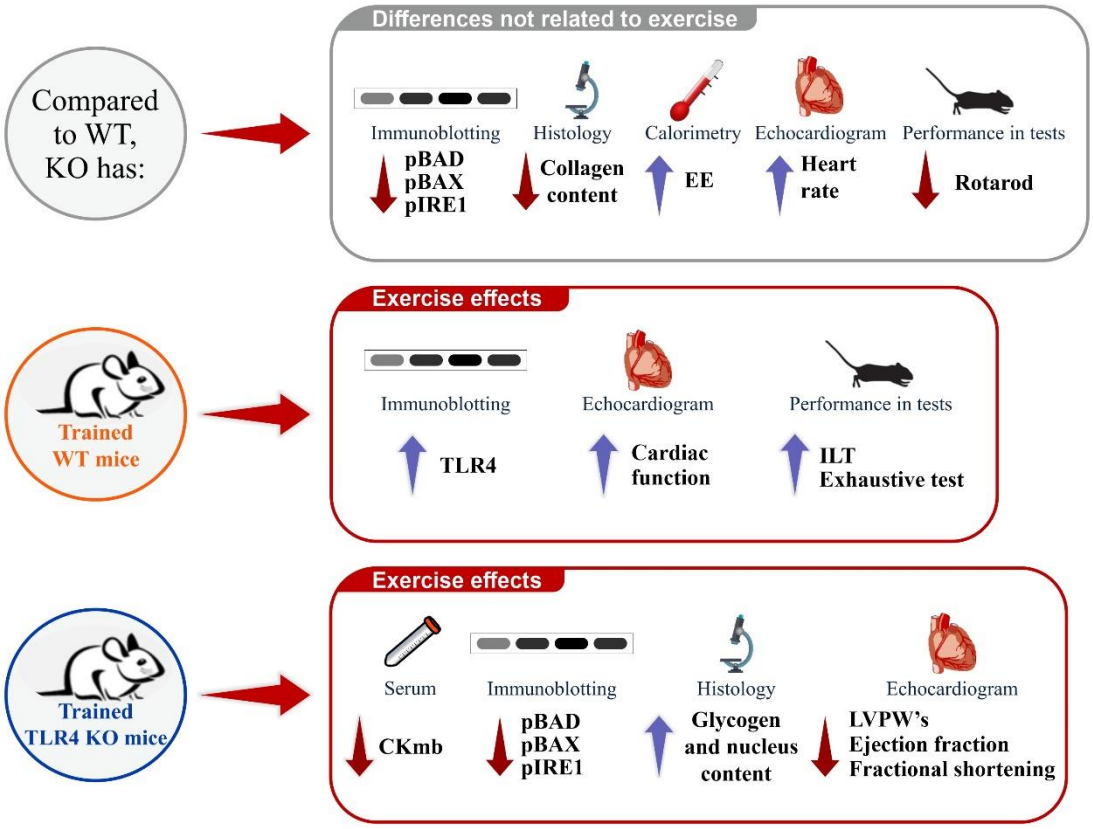

Figure 10. Schematic representation summarizing the main results of the present study.

Anexo 1:

DE VICENTE, L.G. PINTO, A.P; MUÑOZ, V.R; ROVINA, R.L; DA ROCHA, A.L; GASPAR, R.C; DA SILVA, L.E.C.M.; SIMABUCO, F.M; FRANTZ, F.G; PAULI, J.R; DE MOURA, L.P; CINTRA, D.E, ROPELLE, E.R; DA SILVA, A.S.R. Tlr4 participates in the responses of markers of apoptosis, inflammation, and ER stress to different acute exercise intensities in mice hearts. Life sciences, v. 240, p. 117107, 2020.

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Tlr4 participates in the responses of markers of apoptosis, inflammation, andER stress to different acute exercise intensities in mice hearts

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ABSTRACT

Background: Toll-like receptor 4 (Tlr4) is recognized due to its role in the immune response. Also, this protein can participate in the signaling pathway of events triggered by physical exercise such as apoptosis, inflamma- tion, and endoplasmic reticulum (ER) stress. The main objective of this study was to evaluate the role of Tlr4 in the markers of these events in the myocardium of mice submitted to acute physical exercise (APE) protocols at different intensities.

Methods: Echocardiogram, RT-qPCR, and immunoblotting technique were used to evaluate the left ventricle of wild-type (WT) and *Tlr4* knockout (*Tlr4* KO) submitted to APE protocols at 45, 60, and 75% of their maximal velocity. Also, we performed the bioinformatics analysis to establish the connection of heart mRNA levels of *Tlr4* with heart genes of inflammation and ER stress of several isogenic strains of BXD mice.

Results: Under basal conditions, the *Tlr4* deletion diminished the performance, and expression of inflammation and ER stress genes in the left ventricle, but increased the serum levels of CK, Il-17, and Tnf-alpha. Under the same exercise conditions, the *Tlr4* deletion reduced the glycemia, serum levels of CK, Il-17, and Tnf-alpha, as well as genes and/or proteins related to apoptosis, inflammation and ER stress in the left ventricle, but increased the levels of CK-mb and LDH, as well as other genes related to apoptosis, inflammation, and ER stress in the left ventricle.

Conclusion: Altogether, the current findings highlighted the effects of different acute exercise intensities were attenuated in the heart of *Tlr4* KO mice.

1. Introduction

Toll-like receptor 4 (Tlr4) is a transmembrane protein with an important role in the immune response that can be found in several types of human body cells, including cardiomyocytes [1–3]. Some studies have described the role of Tlr4 in inducing apoptosis [4,5], inflammation [1], and endoplasmic reticulum (ER) stress [6,7], a phenomenon that can also be initiated in response to an extreme aerobic exercise [6]. Acute physical exercise can activate Tlr4 [1], which may induce deleterious changes in the heart like cardiac fatigue [8] and myocardial injury [9]. Also, the increased levels of creatine kinase (CK) and lactate dehydrogenase (LDH) were linked to Tlr4 activation in a rat chronic stress model [10]. However, the effects of different exercise intensities on Tlr4 activation have not been explored until the present moment.

When associated with a specific ligand, Tlr4 is activated, initiating its signaling cascade [11]. The Tlr4 activation leads to the translocation

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of the nuclear factor kappa B (NF-κB) to the cell nucleus, which subsequently induces the translation of proinflammatory cytokines [12] (i.e., interleukin 1 beta/Il-1beta, interleukin 6/Il-6, and tumor necrosis factor-alpha/Tnf-alpha) [11], causing myocardium inflammation. Also, this inflammation can lead to ER stress [13], caused by the accumulation of unfolded or misfolded proteins [14–17].

Another pathway independent of the inflammation by which Tlr4 can trigger ER stress is the protein kinase RNA-like ER kinase (Perk) autophosphorylation. When autophosphorylates, Perk disconnects from chaperone Binding Protein (Bip) [18] and interacts with C/EBP-Homologous Protein (Chop), generating the ER stress response [13]. Also, the Perk/Chop axis seems to contribute to apoptosis generation [13], once an increase in the ER stress-induced Chop expression may induce the Bcl-2 associated protein X (Bax) translocation from cytosol to mitochondria [19]. Finally, Tlr4 can promote apoptosis by the p38 mitogen-activated protein kinase (p38Mapk) phosphorylation [5], which causes the localization of Bax to the mitochondria and increases the mitochondria-dependent apoptosis leading to the cleavage of Caspases 3 and 9 [4].

Based on the previous information, the main objective of the present investigation was to evaluate the role of Tlr4 in the markers of apoptosis, inflammation, and ER stress in the myocardium of wild-type (WT) and *Tlr4* knockout (*Tlr4* KO) mice submitted to acute physical exercise protocols at different intensities. Our hypothesis is the higher the acute exercise intensity, the higher the *Tlr4* expression leading to apoptosis, inflammation, and ER stress.

2. Methods

2.1. Experimental animals

Eight-week-old C57BL/6 $Tlr4^{wt/wt}$ (n = 20) and $Tlr4^{-/-}$ (n = 20) mice from the Central Animal Facility of the Ribeirão Preto campus from the University of São Paulo (USP) were used for the WT and KO groups. The rodents were accommodated in sterile micro-insulators (three animals per cage) in a ventilated rack (INSIGHT™, Ribeirão Preto, São Paulo, Brazil) with controlled temperature (22 ± 2 °C) on a 12:12-h light-dark inverted cycle (light: 6 PM to 6 AM, dark: 6 AM to 6 PM), food (Nuvilab® CR1; Sogorb Indústria e Comércio Ltda, São Paulo, Brazil) and water were provided ad libitum. The diet macronutrient composition is 63% of carbohydrates, 26% of proteins, and 11% of lipids. All experimental procedures were according to the Brazilian College of Animal Experimentation (COBEA) and were approved by the Ethics Committee of the University of São Paulo (I.D 2016.5.82.90.7).

2.2. Echocardiogram

Forty-eight hours before the acute physical exercise protocols, the exam was performed using a Vevo 2100® ultrasound system (VisualSonics, Toronto, ON) with a 30Mhz transducer on a heated platform for handling mice with electrocardiogram detectors and monitoring of vital signs. The animals were anesthetized with isoflurane inhalation anesthetic at 3% of the chamber volume, and the loss of the foot reflex was the parameter to evaluate the efficacy of the anesthesia. After anesthetized, the animals were positioned with the legs fixed to the side of the body on the heated platform and inhaled isoflurane enriched with a flow of 5 L/min of oxygen. Two-dimensional (B-Mode) and M-mode (M-Mode) images were recorded on the parasternal long axis, with the center of the left ventricle (LV) like a reference. The variables measured in the M-mode were: LV mass, left ventricular internal dimension at the end of the diastole and systole, LV ejection fraction, interventricular septum thickness at the end of the diastole and systole, fractional shortening and left ventricular posterior wall thickness at the end of the diastole and systole. In the B-Mode, the analyzed variables were: heart rate, cardiac output, systolic and diastolic volume, and stroke volume.

2.3. Incremental load test

The animals were adapted to exercise on a treadmill (INSIGHT™, Ribeirão Preto, São Paulo, Brazil) for five days, 10 min/day at a speed of 6 m/min. After one day of rest as standardized [20], the test started at a speed of 6 m/min, with 0% of inclination and 3 m/min of increments every 3 min until the mice voluntary exhaustion, which occurred when they touched five times at the end of the treadmill in the interval of 1 min. When the mice became exhausted before finishing the stage, the exhaustion velocity (EV) was corrected as proposed by Kuipers et al. [21] and used for prescribing intensities of the acute physical exercise protocols.

2.4. Acute physical exercise (APE) protocols

The maximal lactate steady state (MLSS) is defined as the highest exercise intensity in which balance between the production and removal of blood lactate occurs, and is used as the gold standard to determine exercise intensity [20,22]. Thus, the physical exercise below the MLSS intensity corresponds to the moderate domain [22], and exercise above the MLSS intensity corresponds to the intense domain. Ferreira et al. [20] verified the intensity corresponding to 60% of EV obtained in the incremental load test was similar to the MLSS intensity. Here, three different physical exercise intensities were used: 1) moderate intensity, below the MLSS (45% of the EV during 30 min; APE 45%); 2) MLSS intensity (60% of the EV during 22 min and 30 s; APE 60%); 3) high intensity, above the MLSS (75% of the EV until exhaustion; APE 75%). Also, the training loads (i.e., the product between exercise intensity and volume) of the APE 45% and APE 60% were equalized to isolate the exercise intensity effect. Fig. 1A illustrates the schematic representation of the experimental procedures.

2.5. Glucose levels

The mice have fasted for 2 h, and the blood from the tail tip was collected, and glucose levels were measured before and immediately after the APE protocols using a glycemic monitoring system (Accu-Chek™ Active model, Roche, Santo André, São Paulo, Brazil).

2.6. Blood collection, heart extraction, and left ventricle isolation

Two hours after the APE protocols and in the fasted state, mice were anesthetized by an intraperitoneal administration of xylazine (10 mg/kg body weight) and ketamine (100 mg/kg body weight). As soon as the loss of pedal reflexes confirmed the effect of anesthesia, blood was collected by cardiac puncture and serum was separated by centrifugation (1100 ×*g*) for 15 min at 4 °C and stored at −80 °C. Immediately after blood collection, each mouse heart was removed, the left ventricle (LV) was isolated, washed with saline, and divided into two samples, which were designated for reverse transcription-quantitative polymerase chain reaction RTq-PCR (n = 5 mice for each experimental group) and immunoblotting (n = 5 mice for each experimental group) techniques.

2.7. Serum analysis

According to the instructions of the manufacturers, the serum samples were used to determine the concentrations of creatine kinase (CK), creatine kinase MB isoenzyme (CK-mb), and lactate dehydrogenase (LDH) using conventional assay kits (Analisa, Belo Horizonte, Minas Gerais, Brazil), as well as interleukins 1beta, 6, 17 (Il-1beta, Il-6, and Il-17) and tumor necrosis factor-alpha (Tnf-alpha) using Luminex ™ multiplex method (Millipore, St Charles, MO, USA). For the measurement of cytokines, samples were pipetted on the Luminex

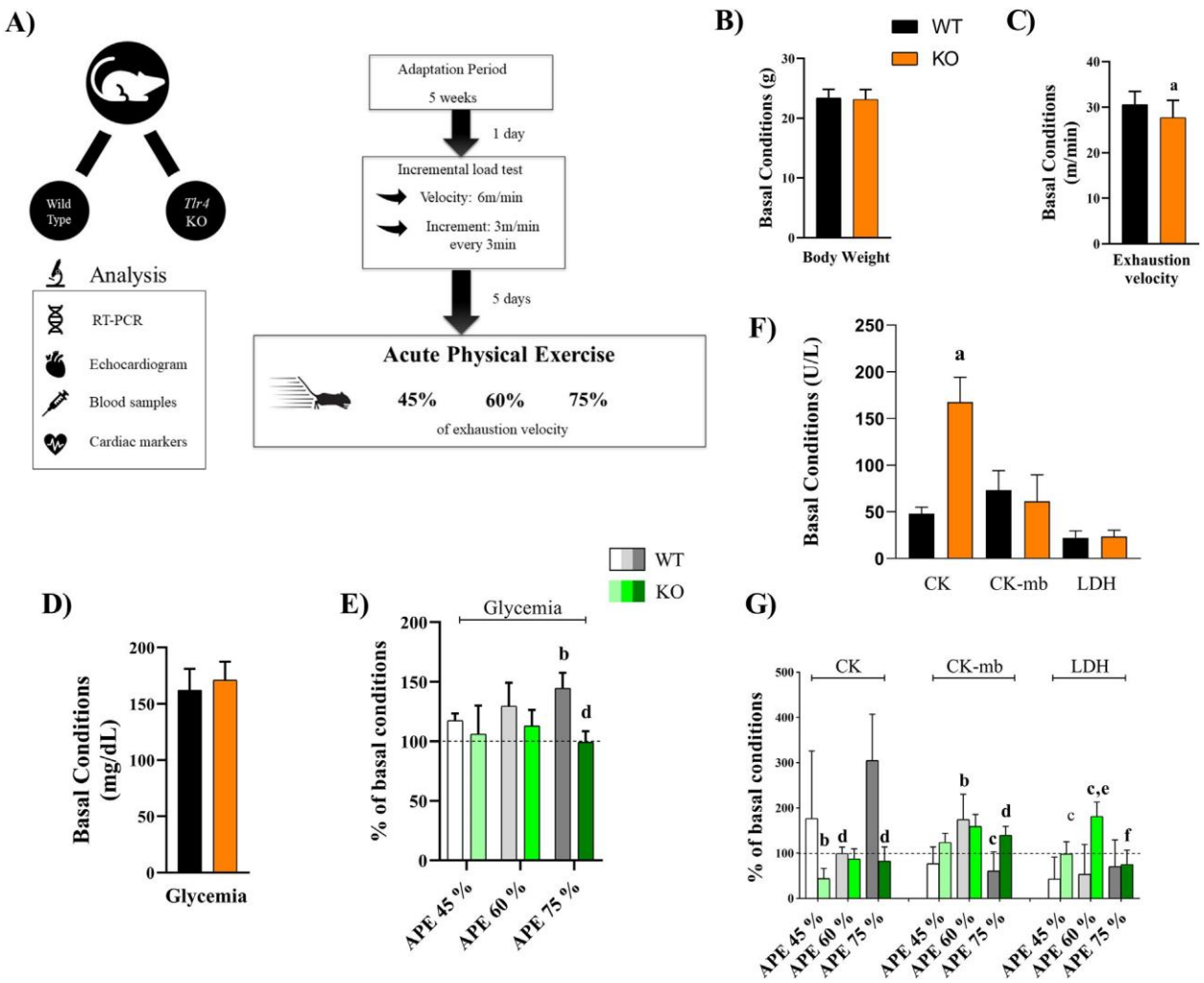

Fig. 1. Schematic representation of the experimental procedures (A), Body weight under basal conditions (B), Exhaustion velocity under basal conditions (C), Glycemia under basal conditions (D), Responses of glycemia to the different exercise intensities (E), Serum levels of CK, CK-mb, and LDH under basal conditions (F), Responses of CK, CK-mb, and LDH to the different exercise intensities. Data correspond to the mean ± SEM of n = 20 mice per group for B, C, and D, n = 5 per group for E, F, and G. WT: wild-type mice; KO: *Tlr4* knockout mice; $^{a}p \leq 0.05$ vs. WT/Basal; $^{b}p \leq 0.05$ vs. WT/APE 45%; $^{c}p \leq 0.05$ vs. WT/APE 60%; $^{d}p \leq 0.05$ vs. WT/APE 75%; $^{e}p \leq 0.05$ vs. KO/APE 45%; $^{f}p \leq 0.05$ vs. KO/APE 60%.

Table 1

The primers design.

Gene	Forward	Reverse
Actb	GACTCATCGTACTCCTGCTTG	GATTACTGCTCTGGCTCCTAG
Bax	TTTGCTACAGGGTTTCATCCAG	GTCCAGTTCATCTCCAATTCGC
Casp3	AGCTTGGAACGGTACGCTAA	CCAGAGTCCACTGACTTGC
Casp9	ATATTCAGCAGGCAGGATCTGG	TGTCCTCTAAGCAGGAGATGA
Ddit3	ATCTTGAGCCTAACACGTCGAT	GACCAGGTTCTCTCTCCTCAG
Eif2ak3	AAAGCAGTGGGATTTGGACG	AAGTTTTGTGGGTGCCCTCT
Gapdh	AAGAGGGATGCTGCCCTTAC	CGGGACGAGGAAACACTCTC
Hspa5	GTGTGTGAGACCAGAACCGT	GCAGTCAGGCAGGAGTCTTA
Il1b	TGCCACCTTTTGACAGTGATG	GCTCTTGTTGATGTGCTGCT
Nfkb1	GATTCCGGGCAGTGACG	GATGAGGGGAAACAGATCGTCC
Mapk14	GGACTGTGAGCTGAAGATCCTA	CGCATCCAATTCAAGATGACCT
Tlr4	GTTCTCTCATGGCCTCCACT	GGAACTACCTCTATGCAGGGAT
Tnf	CCACCACGCTCTTCTGTCTA	CCACTTGGTGGTTTGTGAGT

MAP200 instrument and were analyzed using the 3.1 xPONENT System.

2.8. Reverse transcription-quantitative polymerase chain reaction (RTq-PCR)

Total RNA from the left ventricle was extracted using Trizol (Invitrogen, Carlsbad, CA, USA), and the cDNA was synthesized with 600 ng of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer instructions. Quantitative real-time PCR was performed on the ViiA7 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) to analyze the relative mRNA expression of the following genes associated with the proteins in parentheses: *Il1b* (Il-1beta), *Tnf* (Tnf-alpha), *Nfkb1* (Nf-κb), *Eif2ak3* (Perk), *Hspa5* (Bip), *Ddit3* (Chop), *Casp3* (Caspase 3), *Casp9* (Caspase 9), *Bax* (Bax), *Mapk14* (p38Mapk) and *Tlr4* (Tlr4).

The amplification reactions in a final volume of 10 μL were performed in triplicate with the following reagents: 5 μL 2× Power Sybr Master Mix (Thermo Fisher Scientific, Wilmington, DE, USA), 1 μL primer forward, 1 μL primer reverse, 1 μL cDNA diluted in 1:6 and 2 μL of H_2O. *Gapdh* and *Actb* were used as reference genes for the normalization of the data. Each amplification reaction occurred in the standard cycling in the following cycles: 10 min at 95 °C and a further 40 cycles with 15 s at 95 °C and 1 min at 60 °C. Relative quantification was calculated by the 2 −ΔΔCT method using the Thermo Fisher Cloud Software, RQ version 3.7 (Life Technologies Corporation, Carlsbad, CA, USA). The primers' designs are available in Table 1.

2.9. Immunoblotting technique

The immunoblotting technique was performed as previously described by our research group [17,23]. The antibodies used were Perk (SC13073), phospho(p)-Perk (Thr981; SC32577), Bip (SC33757), and Bax (SC7480) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); p-Bax (Ser184; orb4658) from Biorbyt Ltd. (Cambridge, Cambridgeshire, England); p-p38Mapk (T180/Y182; #9211s), Caspase 9 (#9508s) and beta-actin (#4967s) from Cell Signaling Technology (Cell Signaling Technology, MA, USA); p38Mapk (OPA1-10080) from Thermo Fisher Scientific (Waltham, MA USA); Tnf-alpha (60291-1-Ig) from Proteintech (Rosemont, IL, USA). All the primary antibodies were utilized at a dilution of 1:1000, and the secondary antibodies (#7074s and #7076s) from Cell Signaling Technology (Cell Signaling Technology, MA, USA) were utilized at a dilution between 1:10,000 and 1:20,000. Images were acquired by the C-Digit Blot Scanner (LI-COR, Lincoln, Nebraska, USA) and quantified using the software Image Studio for C-DiGitTM Blot Scanner.

2.10. Bioinformatics analysis

Correlation analyses were performed using a data set from heart *Tlr4* (EPFL/LISP BXD CD Heart Affy Mouse Gene 2.0 ST Gene Level [Jan 14] RMA), and heart genes from inflammation and ER stress of genetically diverse BXD mice as previously published [24]. The four strains with the highest *Tlr4* values (BXD75, BXD81, BXD44, and BXD43) and the four strains with the lowest *Tlr4* values (BXD68, BXD100, BXD65, and BXD69) were selected to perform the correlation with the other genes. All data are accessible on Genetwork (http://www.genenetwork.org). The heatmap graph was obtained using the Gene-E software.

2.11. Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). While the normality was checked using the Shapiro–Wilks *W*-test, the homogeneity was checked using the Levene's test. The unpaired Student's *t*-test analyzed the basal differences between WT and *Tlr4* KO mice. The one-way analysis of variance (ANOVA) analyzed the effects of the acute physical exercise protocols on the *Tlr4* gene expression for the WT mice. The two-way analysis of variance (ANOVA) was used for the comparison of the other parameters between exercise protocols and genetic groups. When the one or two-way ANOVA indicated significance, Bonferroni's post hoc test was performed. All analyses were bilateral, and the level of significance was fixed at $p \leq 0.05$.

3. Results

The echocardiogram parameters, body weight, glycemia, CK-mb, and LDH were not different between the experimental groups (Table 2; Fig. 1B, D, and F) under basal conditions, but the exhaustion velocity of the *Tlr4* KO group was lower than WT group (Fig. 1C). Otherwise, the CK of the *Tlr4* KO group was higher than the WT group (Fig. 1F). The glycemia of the *Tlr4* KO group was lower than the WT group at the APE 75%. For the WT group, the glycemia at the APE 75% was higher than APE 45% (Fig. 1E).

Fig. 1G demonstrates the CK values of the *Tlr4* KO group were lower than the WT group at the APE 45% and 75%. For the WT group, the CK values at the APE 75% were higher than APE 60%. Compared to the WT group, the *Tlr4* KO group presented higher values of CK-mb at the APE 75% and LDH at APE 60%. The CK-mb values of the WT group and the LDH values of the *Tlr4* KO group at the APE 60% were higher than APE 45% and 75%.

Fig. 2A and B shows the Il-1beta and Il-6 serum levels were not different between the experimental groups under basal and same exercise conditions. Otherwise, the Il-17 and Tnf-alpha serum levels of the *Tlr4* KO group were higher than the WT group (Fig. 2A). According to Fig. 2B, compared to the WT group, the *Tlr4* KO group presented lower serum values of Il-17 at the APE 75% and Tnf-alpha at APE 45%, 60%, and 75%. For the WT and *Tlr4* KO groups, the serum levels of cytokines were not sensitive to the different acute physical exercise intensities.

While the *Bax* mRNA levels of the *Tlr4* KO group were lower than the WT group, the *Casp3* and *Casp9* mRNA levels were not different between the experimental groups in the left ventricle (Fig. 2C). According to Fig. 2D, the *Bax* and *Casp9* mRNA levels of the *Tlr4* KO group were higher than the WT group at the APE 60% and 75%. The *Casp3* mRNA levels of the *Tlr4* KO group were higher than the WT group at the APE 75%. The *Bax* mRNA levels of the WT and KO groups, and *Casp3* and *Casp9* mRNA levels of the *Tlr4* KO group were not sensitive to the different acute physical exercise intensities. For the WT group, the *Casp3* and *Casp9* mRNA levels at the APE 75% and 60% were lower than APE 45%.

The *Il1b* and *Nfkb1* mRNA levels were not different in the left ventricle between the experimental groups, but the *Mapk14* and *Tnf* mRNA levels of the *Tlr4* KO group were lower than the WT group (Fig. 2E). Fig. 2F shows the mRNA levels of *Il1b* at the APE 60% and 75%, *Mapk14* at APE 75%, and *Nfkb1* at APE 60% of the *Tlr4* KO group were higher than WT group. Otherwise, the *Mapk14*, *Nfkb1*, and *Tnf* mRNA levels at the APE 45% of the *Tlr4* KO group were lower than the WT group. For the WT group, the mRNA levels of *Il1b*, *Mapk14*, *Nfkb1*, and *Tnf* at the APE 75% and 60% were lower than APE 45%. For the *Tlr4* KO group, the *Il1b*, *Nfkb1*, and *Tnf* mRNA levels were not sensitive to the different acute physical exercise intensities, but the *Mapk14* mRNA levels at the APE 75% was higher than APE 60% and 45%.

While the *Hspa5* mRNA levels were not different between the experimental groups under basal and same exercise conditions (Fig. 2G and H), the *Ddit3* and *Eif2ak3* mRNA levels of the *Tlr4* KO group were lower than WT group (Fig. 2G). Fig. 2H shows the *Ddit3*, and *Eif2ak3* mRNA levels of the *Tlr4* KO group were lower than the WT group at the APE 75%, but the *Eif2ak3* mRNA levels of the *Tlr4* KO group was higher than WT group at APE 60%. The *Ddit3* mRNA levels for the WT and *Tlr4* KO groups and the *Eif2ak3* and *Hspa5* mRNA levels for the *Tlr4* KO group were not sensitive to the different acute physical exercise

Table 2

M and B-mode echocardiogram variables between the wild-type and *Tlr4* knockout mice.

Variables	WT	*Tlr4* KO
M-Mode		
IVS;d (mm)	0.95 ± 0.03	0.89 ± 0.03
IVS;s (mm)	1.40 ± 0.04	1.31 ± 0.04
LVID;d (mm)	3.81 ± 0.09	3.74 ± 0.09
LVID;s (mm)	2.61 ± 0.09	2.55 ± 0.10
LVPW;d (mm)	0.82 ± 0.03	0.80 ± 0.04
LVPW:s (mm)	1.15 ± 0.05	1.10 ± 0.04
Ejection fraction (%)	59.23 ± 2.28	59.96 ± 2.07
Fractional shortening (%)	31.20 ± 1.56	31.56 ± 1.38
LV Mass (mg)	100.80 ± 4.55	91.53 ± 4.52
B-Mode		
Heart Rate (bpm)	422.52 ± 15.60	417.38 ± 14.23
Cardiac output (mL/min)	14.46 ± 0.86	13.98 ± 1.27
Stroke volume (uL)	34.72 ± 1.27	31.57 ± 2.07
Diastolic volume (uL)	62.88 ± 1.66	54.35 ± 4.29
Systolic volume (uL)	27.20 ± 1.13	22.91 ± 2.02

Data correspond to the mean ± SEM of n = 20 mice per group. IVS;d - Interventricular septum thickness at end-diastole; IVS;s - Interventricular septum thickness at end-systole; LV - Left ventricle; LVID;d - Left ventricular internal dimension at end-diastole; LVID;s - Left ventricular internal dimension at end-systole; LVPW;d - Left ventricular posterior wall thickness at end-dia- stole; LVPW;s - Left ventricular posterior wall thickness at end-systole; Tlr4 - Toll-like receptor 4.

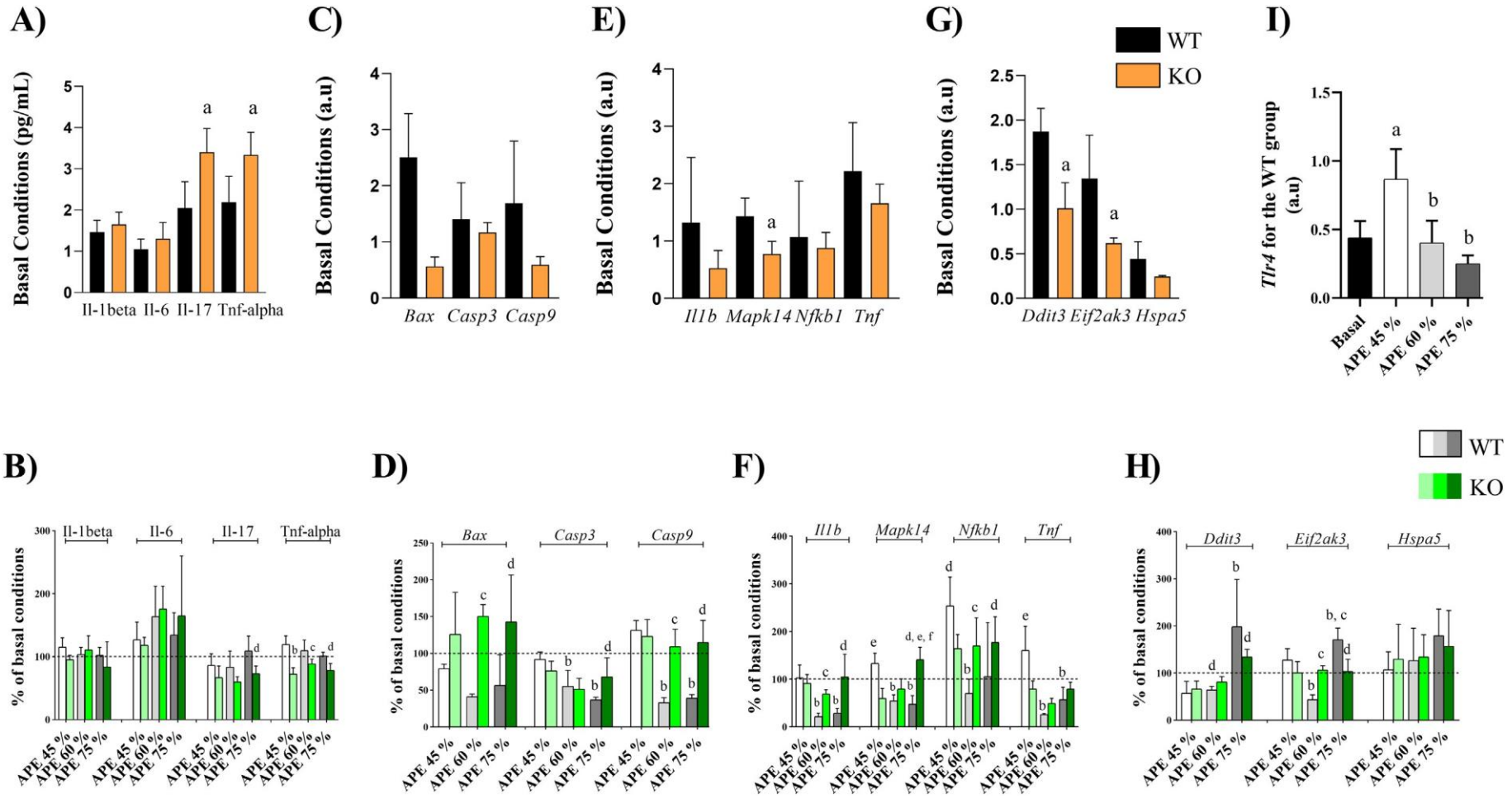

Fig. 2. Serum levels of Il-1beta, Il-6, Il-17, and Tnf-alpha under basal conditions (A), Responses of Il-1beta, Il-6, Il-17, and Tnf-alpha to the different exercise intensities (B), Gene expressions of *Bax*, *Casp3*, and *Casp9* under basal conditions (C), Responses of *Bax*, *Casp3*, and *Casp9* to the different exercise intensities (D), Gene expressions of *Il1b*, *Mapk14*, *Nfkb1*, and *Tnf* under basal conditions (E), Responses of *Il1b*, *Mapk14*, *Nfkb1*, and *Tnf* to the different exercise intensities (F), Gene expressions of *Hspa5*, *Ddit3* and *Eif2ak3* under basal conditions (G), Responses of *Hspa5*, *Ddit3* and *Eif2ak3* to the different exercise intensities (H), Gene expression of *Tlr4* for the WT group (I). Data correspond to the mean ± SEM of n = 5 mice per group. WT: wild-type mice; KO: *Tlr4* knockout mice; $^{a}p \leq 0.05$ vs. WT/Basal; $^{b}p \leq 0.05$ vs. WT/APE 45%; $^{c}p \leq 0.05$ vs. WT/APE 60%; $^{d}p \leq 0.05$ vs. WT/APE 75%; $^{e}p \leq 0.05$ vs. KO/APE 45%; $^{f}p \leq 0.05$ vs. KO/APE 60%.

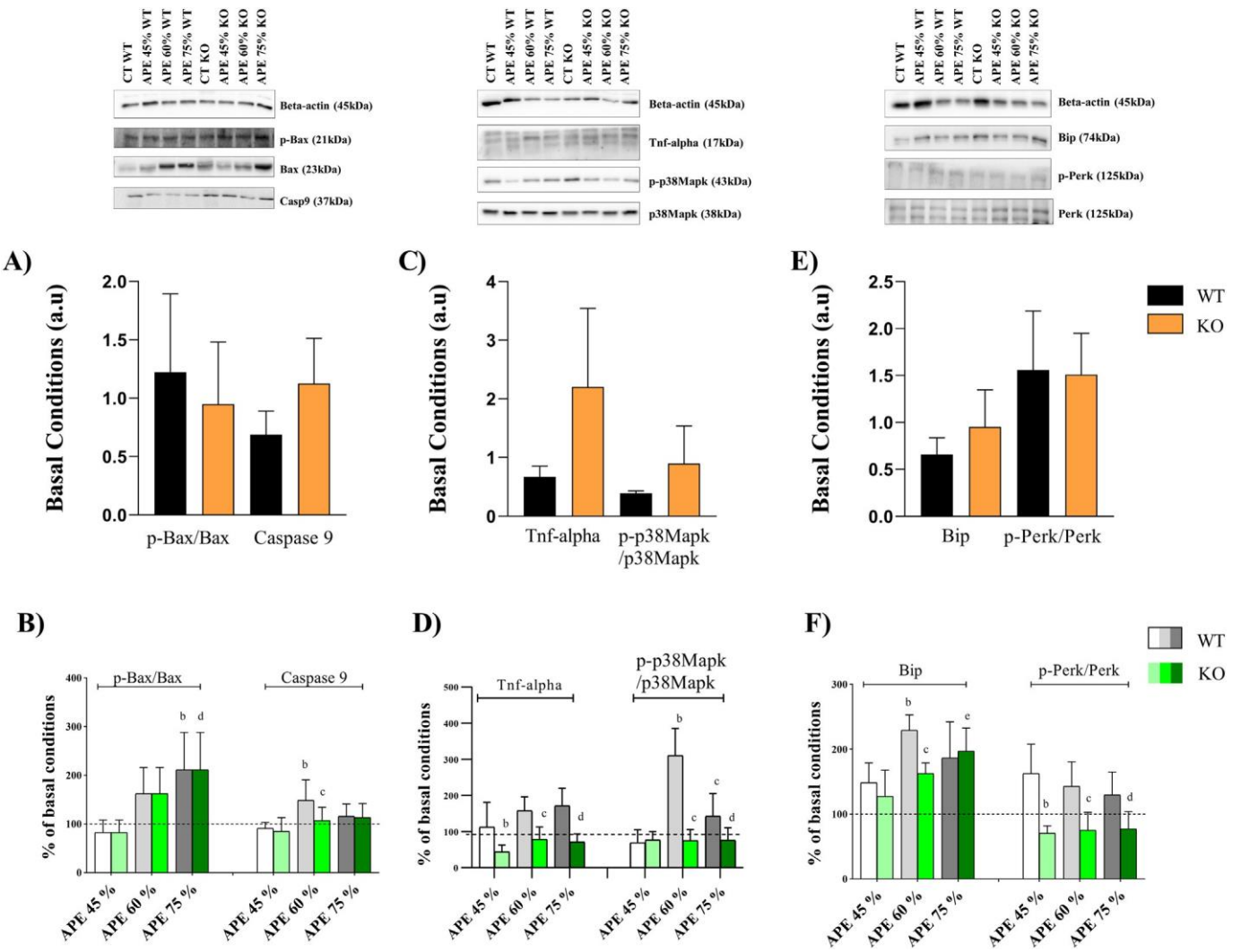

Fig. 3. Protein contents of p-Bax/Bax, Caspase 9, and Beta-actin under basal conditions (A), Responses of p-Bax/Bax, Caspase 9, and Beta-actin to the different exercise intensities (B), Protein contents of Tnf-alpha, p-p38Mapk/p38Mapk, and Beta-actin under basal conditions (C), Responses of Tnf-alpha, p-p38Mapk/p38Mapk, and Beta-actin to the different exercise intensities (D), Protein contents of Bip, p-Perk/Perk, and Beta-actin under basal conditions (E), Responses of Bip, p- Perk/Perk, and Beta-actin to the different exercise intensities (F). Data correspond to the mean ± SEM of n = 5 mice per group. WT: wild-type mice; KO: *Tlr4* knockout mice; $^{b}p \leq 0.05$ vs. WT/APE 45%; $^{c}p \leq 0.05$ vs. WT/APE 60%; $^{d}p \leq 0.05$ vs. WT/APE 75%; $^{e}p \leq 0.05$ vs. KO/APE 45%.

intensities. For the WT group, the *Ddit3* and *Eif2ak3* mRNA levels at the APE 75% were higher than APE 60% and 45%. Also, the *Eif2ak3* mRNA levels at the APE 60% was lower than APE 45%. Finally, the *Tlr4* mRNA levels of the WT group were higher at the APE 45% than basal, APE 60%, and 75% (Fig. 2I).

Fig. 3A, C, and E shows the protein contents were not different between the experimental groups under basal conditions. Compared to the WT group, the *Tlr4* KO group presented lower protein contents of p-Bax/Bax at the APE 75%, Caspase 9 and Bip at APE-60%, p-p38Mapk/p38Mapk at APE 60% and 75%, as well as Tnf-alpha and p-Perk/Perk at APE 45%, 60%, and 75% (Fig. 3B, D, and F).

For the WT group, the protein contents of p-Bax/Bax at the APE 75%, as well as Caspase 9, p-p38Mapk/p38Mapk, and Bip at APE 60% were higher than APE 45%. Also, the protein contents of p-p38Mapk/p38Mapk at the APE 75% was lower than APE 60%. The Tnf-alpha and p-Perk/Perk protein contents for the WT and *Tlr4* KO groups, as well as p-Bax/Bax, Caspase 9, p-p38Mapk/p38Mapk, Tnf-alpha and p-Perk/Perk for *Tlr4* KO group, were not sensitive to the different acute physical exercise intensities (Fig. 3B, D, and F). Finally, the Bip at the APE 75% was higher than APE 45% (Fig. 3F).

To extend our experimental findings, we performed a bioinformatics analysis using the BXD database. The BXD database is among the largest and best-categorized family of isogenic strains, providing a broad set of data appropriate for studies involving metabolic disorders [24]. In the bioinformatics analysis, we first observed the distribution of heart *Tlr4* mRNA levels in 42 strains of isogenic BXD mice, highlighting four strains with lower (BXD68, BXD100, BXD65, and BXD69) and four strains with higher (BXD75, BXD81, BXD44, and BXD43) levels of *Tlr4* mRNA in the heart (Fig. 4A). The transcriptomic analysis demonstrated that several inflammatory genes were upregulated in the heart of the strains with lower *Tlr4*, while several ER stress genes were down-regulated in the heart of the strains with lower *Tlr4* (Fig. 4B).

4. Discussion

The main findings of the present study were: 1) under basal conditions, the KO of *Tlr4* decreased the exhaustion velocity, and expressions of *Mapk14*, *Ddit3*, and *Eif2ak3*, but increased the serum levels of CK, Il-17, and Tnf-alpha; 2) under the same exercise conditions, the *Tlr4* KO attenuated the glycemia, serum levels of CK, Il-17, and Tnf-alpha, expressions of *Mapk14*, *Tnf*, *Ddit3*, and *Eif2ak3*, as well as protein levels of Caspase 9, Tnf-alpha, p-p38Mapk/p38Mapk, Bip, and p-Perk/Perk,

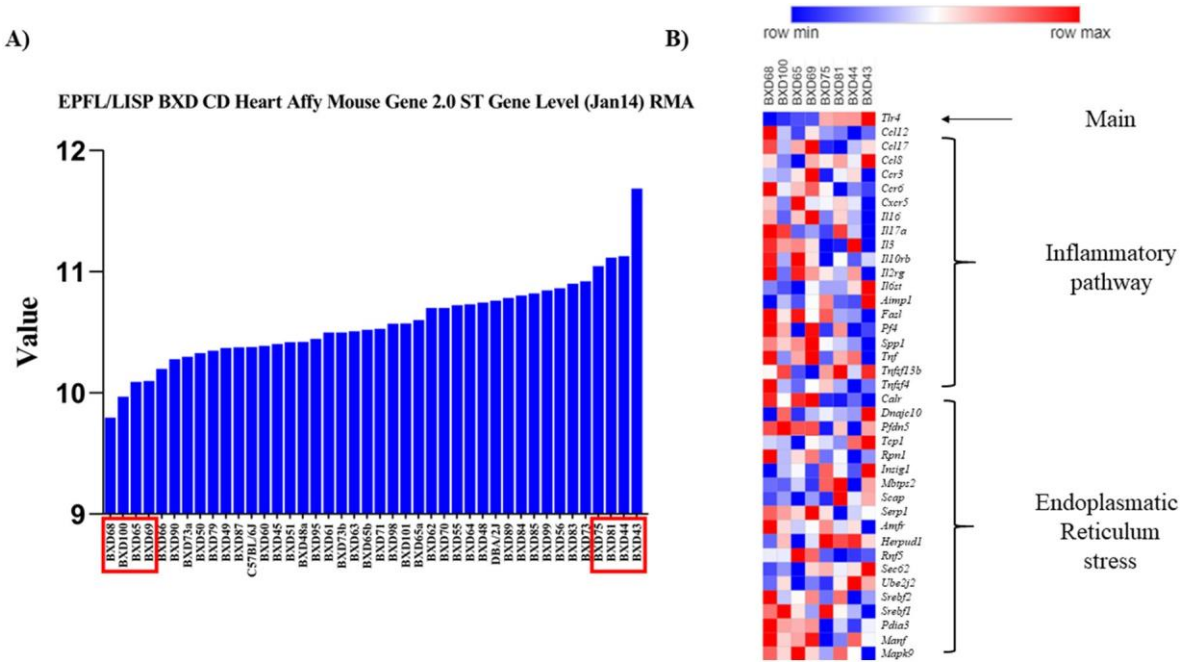

Fig. 4. Distribution of heart *Tlr4* mRNA levels in 42 strains of isogenic BXD mice, highlighting four strains with lower (BXD68, BXD100, BXD65, and BXD69) and four strains with higher (BXD75, BXD81, BXD44, and BXD43) levels of *Tlr4* mRNA in the heart (A), Heatmap showing the inflammation and ER stress gene expressions in the heart and their relationship with heart *Tlr4* mRNA levels (B).

but amplified the serum levels of CK-mb and LDH, and expressions of *Bax*, *Casp*3, *Casp*9, *Il1b*, *Mapk14*, *Nfkb1*, and *Eif2ak3*; 3) for the WT group, the different exercise intensities influenced the glycemia, serum levels of CK and CK-mb, expressions of *Casp*3, *Casp*9, *Il1b*, *Mapk14*, *Nfkb1*, *Tnf*, *Ddit3*, *Eif2ak3*, and *Tlr4*, as well as protein levels of p-Bax/Bax, Caspase 9, p38Mapk/p38Mapk, and Bip; 4) for the *Tlr4* KO group, the different exercise intensities influenced the serum levels of LDH, expression of *Mapk14*, and protein levels of Bip.

4.1. Comparisons between the WT and Tlr4 KO mice under basal conditions

In agreement with our results, Camandola and Simonetta [25], as well as Pierre et al. [26], did not observe significant differences for the body weight between the WT and *Tlr4* KO mice. Here, the *Tlr4* KO mice presented a lower exhaustion velocity than the WT mice, which is probably not related to the cardiovascular system since the WT and *Tlr4* KO mice display no significant differences for the echocardiogram variables, corroborating the previous data of Hu and Zhang [27]. On the other hand, Zbinden-Foncea et al. [28] did not find significant differences for the maximal velocity between the WT and *Tlr4* KO mice, which may be explained by their small sample size (i.e., six animals) since the values of the *Tlr4* KO mice were numerically lower than the WT pairs (i.e., 16.3 ± 0.60 versus 18.0 ± 1.15 $m \cdot min^{-1}$, respectively).

Interestingly, our *Tlr4* KO mice displayed higher serum CK levels than the WT mice under basal conditions. The elevated serum levels of CK and decreased values of muscle performance are physiological hallmarks of animal models of Duchenne muscular dystrophy [29]. Thus, the higher CK values for the *Tlr4* KO mice may have influenced their lower locomotor performance. The increased serum levels of Il-17 and Tnf-alpha for the *Tlr4* KO mice were not observed by Tang et al. [30] and Zhang et al. [31], respectively. These differences may be related to different cytokine analysis methods. To the best of our knowledge, this is the first time the decrease of the inflammation (*Mapk14*) and ER stress genes (*Ddit3* and *Eif2ak3*) is described in the *Tlr4* KO mice hearts. This result may be explained by the relationship between Tlr4 activation and inflammation and ER stress [1,6,7]. Moreover, our transcriptomic analysis highlighted that other ER stress genes were downregulated in the heart of the strains with lower *Tlr4*.

4.2. Comparisons between the WT and Tlr4 KO mice under the same exercise conditions

This is the first investigation analyzing the *Tlr4* KO mice hearts in response to exercise. The global deletion of *Tlr4* decreased the pro-inflammatory cytokines (Il-17 and Tnf-alpha), cardiac gene expressions of inflammation (*Mapk14* and *Tnf*) and ER stress (*Ddit3* and *Eif2ak3*), as well as heart protein contents related to apoptosis (Caspase 9), inflammation (Tnf-alpha and p-p38Mapk/p38Mapk), and ER stress (Bip and p-Perk/Perk). In accordance, Zbinden-Fonseca et al. [28] verified the activations of inflammatory proteins (p-p38Mapk, p-Sapk/Jnk, and p-c-Jun) were completely blunted in the *Tlr4* KO mice skeletal muscle in response to two bouts of 60-min running at 70% of maximal velocity. The downregulation of Il-17 and Tnf-alpha may be linked to the anti-inflammatory effects of physical exercise [32].

On the other hand, we also verified an upregulation of expression of cardiac genes related to apoptosis (*Bax*, *Casp3*, and *Casp9*), inflammation (*Il1b*, *Mapk14*, and *Nkkb1*), and ER stress (*Eif2ak3*) in the *Tlr4* KO mice. Based on the relationship between Tlr4 activation, apoptosis, inflammation, and ER stress [1,4–7], the reader may be wondering how these increases occurred without the presence of Tlr4. Because mice hearts can express at least the other five receptors (Tlr2, Tlr3, Tlr5, Tlr7, and Tlr9) related to the Tlr signaling [33,34], these increases may be explained by the activation of one or more of these Tlrs. For instance, Cavalcante et al. [35] showed that different types (aerobic and resistance) of acute and chronic exercise models can modulate both Tlr2 and 4 in several samples of humans and rodents.

4.3. Effects of different acute exercise intensities on the WT and Tlr4 KO mice

For the first time, the effects of different acute exercise intensities on the heart Tlr4 signaling were investigated. Corroborating the findings of Cristi-Montero et al. [1], we also demonstrated this receptor was

sensitive to an acute exercise session. In contrast to our hypothesis, we did not observe a cause and effect relationship between exercise intensity and *Tlr4* expression for the WT group. Indeed, the *Tlr4* expression was raised after the APE 45% compared to the basal, APE 60%, and 75%. These findings suggest the *Tlr4* expression is more influenced by the volume (APE 45%, volume = 30 min; APE 60%, volume = 22.5 min; APE 75%, volume = 13.9 ± 2.9 min for the WT group) than exercise intensity. Zbinden-Fonseca et al. [28] linked the skeletal muscle Tlr4 activation with the plasma elevation of extracellular nonesterified fatty acids (NEFA), which are more responsive to the low than high-intensity exercise [36].

For the WT mice, the apoptosis (*Casp3* and *Casp9*) and inflammation (*Il1b*, *Mapk14*, *Nfkb1*, and *Tnf*) gene expressions were downregulated in response to the acute exercise protocols with higher intensities, corroborating the *Tlr4* behavior. On the other hand, the ER stress gene expressions (*Ddit3* and *Eif2ak3*), as well as the protein contents related to apoptosis (p-Bax/Bax and Caspase 9), inflammation (p-p38Mapk/p38Mapk), and ER stress (Bip) were upregulated with the exercise intensity increase. These data suggest higher exercise intensities can also activate the other receptors (Tlr2, Tlr3, Tlr5, Tlr7, and Tlr9) related to the Tlr signaling in the heart of mice [33,34]. Therefore, further investigations should evaluate these Tlrs and their relationship to apoptosis, inflammation, and ER stress after different acute exercise intensities.

Interestingly, most of the responses to the different exercise intensities were blunted for the *Tlr4* KO group, highlighting its partial function in the acute exercise-induced apoptosis, inflammation, and ER stress modulations. In summary, the global deletion of *Tlr4* led to significant alterations of mRNA and protein levels of genes related to apoptosis, inflammation, and ER stress in mice hearts under basal and same exercise conditions. Also, the effects of different acute exercise intensities were attenuated in the heart of *Tlr4* KO mice. Fig. 5 summarizes the findings of the present study. Future studies are needed to evaluate the role of Tlr4 in the cardiac morphological and functional adaptations to chronic physical exercise and their relationships with the markers of apoptosis, inflammation, and ER stress.

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Authors' contributions

LGV, APP, RLR, ALR, and ASRS designed the paper. LGV and ASRS wrote the paper. VRM and RCG performed the immunoblotting technique experiments. LGV, APP, RLR, and ASRS designed the figures of the manuscript. LGV, APP, RLR, ALR, VRM, RCG, LECMS, FMS, and FGF performed other experiments, data collection, and/or statistical analysis. LGV, LECMS, FMS, FGF, JRP, LPM, DEC, ERR, and ASRS contributed to data analysis, discussion and/or supported financial costs. All authors have read and approved this manuscript.

Declaration of competing interest

The authors declare that they have no competing interests.

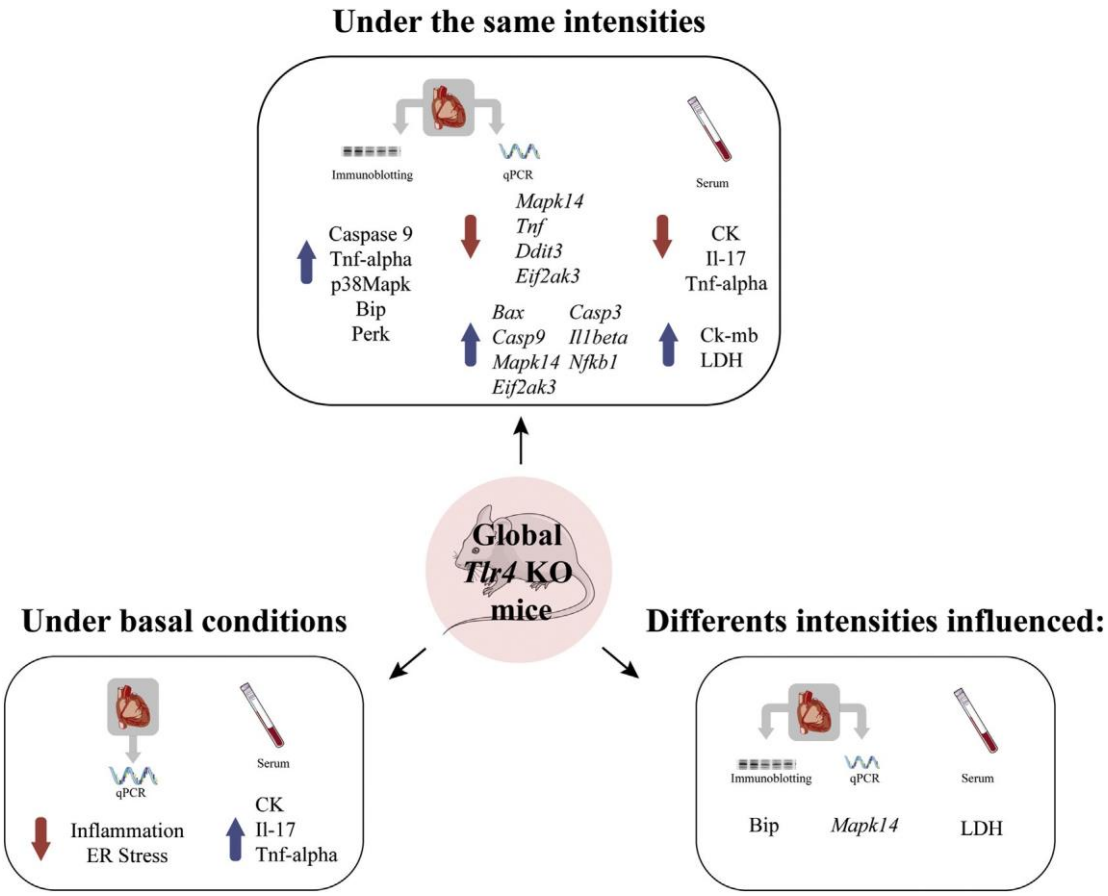

Fig. 5. Schematic representation summarizing the main findings of the present study.

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