UNIVERSIDADE FEDERAL DE ALFENAS

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Heat-killed *Pseudomonas aeruginosa* drives proinflammatory polarization on BCG-primed macrophages

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Dissertação apresentada como parte dos requisitos para obtenção do título de Mestre em Ciências Biológicas pela Universidade Federal de Alfenas. Área de concentração: Interação patógeno-hospedeiro.

Orientador: Prof. Dr. Leonardo Augusto de Almeida

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Ao professor Leonardo, pela oportunidade e parceria de fazer Ciência.

Aos meus amigos de laboratório, Thiago, Natália, Ana, Bianca e Karen.

RESUMO

A imunidade inata treinada é conceito recente que diz respeito ao treinamento de macrófagos, células *natural killer* (NK), células dendríticas e células epiteliais frente à estimulação heteróloga, levando ao treinamento ou à tolerância de resposta. Considerando os recentes indícios de treinamento em modelos de estudo de pneumonias e as possíveis implicações na prevenção e no tratamento dessas afecções, o presente trabalho se propôs a avaliar o perfil de resposta de macrófagos estimulados previamente pelo Bacilo de Calmatte-Guérin (BCG) e/ou *Pseudomonas aeruginosa* morta pelo calor (HKPa) seguido de estímulos homólogos ou heterólogos. Para isso, macrófagos derivados da medula óssea de camundongos C57BL/6 foram obtidos e estimulados com BCG e/ou HKPa. Houve ativação de macrófagos CD11b+ pelo aumento da imunofluorescência emitida por CD80/86, quando de estimulação homóloga ou heteróloga com BCG e/ou HKPa. As mesmas células demonstraram tendência pró-oxidante pelos testes da NADPH-oxidase, TBARS e TRAP, quando do estímulo com BCG e aumento da secreção de óxido nítrico quando da estimulação heteróloga iniciada com BCG. Ainda, houve aumento da secreção de IL-6 quando da estimulação heteróloga iniciada por BCG. Contudo, a estimulação homóloga com HKPa aumentou tanto a expressão quanto a secreção de IL-10. Portanto, o presente trabalho sugere o *priming* de macrófagos pelo BCG, quando da estimulação heteróloga com HKPa, e sugere tolerância induzida pela estimulação homóloga de HKPa.

Palavras-chave: Imunidade inata treinada; macrófagos; BCG; *Pseudomonas aeruginosa.*

ABSTRACT

Innate trained immunity is a recent concept regarding the training or tolerance acquired by macrophages, natural killer (NK) cells, dendritic cells and epithelial cells before heterologous stimulus. Given the novel data concerning macrophage training in pneumonia models and, thereby, the possibility of prevention and/or treatment of respiratory infections, this work aims to evaluate macrophage priming *in vitro* by BCG and/or HKPa. For this purpose, C57BL/6 mice bone-marrow-derived macrophages were plated and stimulated with BCG and/or HKPa. CD11b+ macrophage displayed activation through higher immunofluorescence of CD80/86, when homologous or heterologous stimulation occurred with BCG and/or HKPa. These cells also showed pro-oxidant status before BCG stimulus, through TBARS, TRAP and NADPH-oxidase tests. Nitric oxide concentration was augmented in BCG heterologous stimulation. Moreover, there was higher secretion of IL-6 when BCG heterologous stimulation occurred. However, homologous HKPa stimulation augmented IL-10 expression and secretion levels. Therefore, this study suggests that macrophages were primed by BCG, when there was heterologous stimulation with HKPa, and that homologous stimulation with HKPa induced immune tolerance.

Key-words: Innate trained immunity; macrophages; BCG; *Pseudomonas aeruginosa.*

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

ATP   Adenosina trifosfato

BMDM   Macrófagos derivados da medula óssea

HKPa   Heat-killed *Pseudomonas aeruginosa*

HIF-1α   Hypoxia-inducible factor 1-alpha

IL-1β   Interleucina 1 beta

IL-6   Interleucina 6

IL-10   Interleucina 10

iNOS   Óxido nítrico sintetase induzível

NADPH Hidrogênio de fosfato de nicotinamida adenina dinucleotídeo

NF-κB   Fator nuclear kappa B

NO   Óxido nítrico

ROS   Reactive oxygen species

RNS   Reactive nitrogen species

RT-PCR Transcriptase Reversa - Reação em Cadeia da Polimerase

TLR   Toll-like receptor

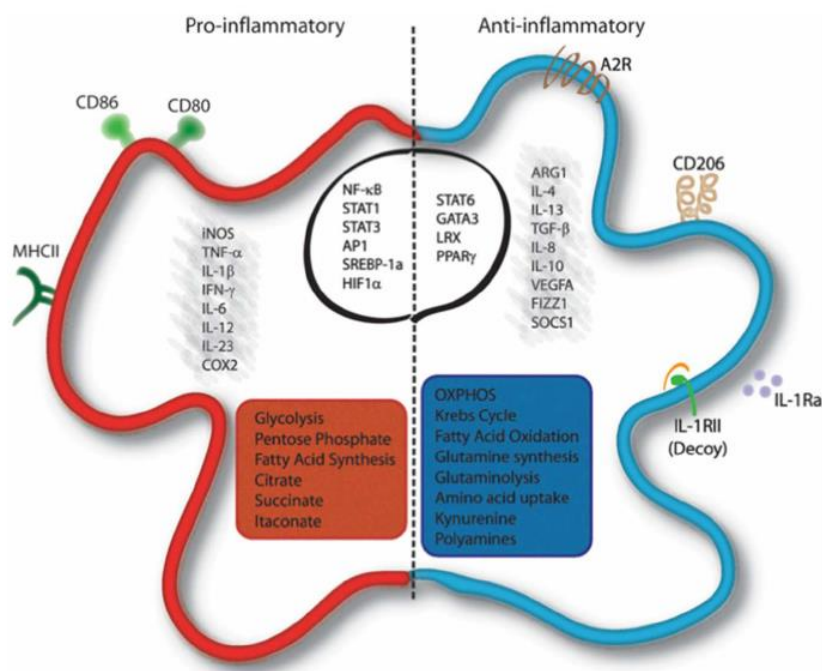TNF-α   Fator de necrose tumoral alpha

SUMÁRIO

1 INTRODUÇÃO

A imunidade inata treinada é um conceito corrente em imunologia de plantas e invertebrados, mas com aplicabilidade recente em vertebrados. Ela consiste nas alterações metabólicas e epigenéticas adquiridas após um segundo estímulo por células da imunidade inata, como as progenitoras da medula óssea, incluindo macrófagos, células assassinas naturais (NK- *natural killer)*, células dendríticas, e também células epiteliais, levando a um recrudescimento ou à tolerância de resposta (NETEA *et al*, 2020). Estas células podem ser treinadas de diversas formas, com o bacilo de Calmette-Guérin (BCG) (ARTS *et al*, 2018), β-glucanas (MOERINGS *et al*, 2021), lipopolissacarídeo (LPS) (FOSTER *et al*, 2007), adrenalina (VAN DER HEIJDEN *et al*, 2020), aldosterona (VAN DER HEIJDEN *et al,* 2020) e lipoproteínas de baixa densidade (BEKKERING *et al*, 2014).

Apesar de alguns dados já apontarem o potencial treino do sistema imune pelo BCG na metade do século XX (NAESLUND *et al*, 1931), este só ganhou notoriedade no fim do século XX e no século XXI (NIOBEY *et al*, 1992; GARLY *et al*, 2003). Estudos epidemiológicos apontam para a proteção cruzada do BCG contra infecções bacterianas e virais de crianças após a vacinação (COVIÁN *et al*, 2019; ARTS *et al*, 2016).

Os macrófagos podem ser treinados pelo BCG (ARTS *et al*, 2016). Eles são células derivadas de precursores mieloides da medula óssea. Estes precursores são diferenciados a monócitos que, quando da diapedese nos sítios de infecção, ou lesão, tornam-se macrófagos (GEISSMAN *et al*, 2010). Os macrófagos sofrem uma reprogramação imunológica e metabólica, o que os leva a polarizarem-se dentro do espectro de dois polos: os macrófagos pró-inflamatórios, M1, e os macrófagos anti-inflamatórios, M2. Os macrófagos pró-inflamatórios M1 expressam marcadores de reatividade CD80 e MHCII, ativam fatores de transcrição NF-κB e HIF-1α, o que os leva a expressar genes como *inos* e *il6*, além de ativar a via da glicólise, a via das pentoses, a síntese de ácidos graxos e a glutaminólise. Já os macrófagos anti-inflamatórios M2 expressam marcadores CD206, ativam fatores de transcrição STAT6 e GATA3, o que os leva a expressar de *il10* e *arg1*, além de induzir o ciclo de Krebs e a oxidação de ácidos graxos (VIOLA *et al*, 2019), conforme mostra a figura 1 (Fig. 1).

Figura 1- Representação gráfica da polarização de macrófagos.

Fonte: Retirado de VIOLA *et al*, 2019.

Nota: À esquerda, marcadores metabólicos e proteicos de macrófagos pró-inflamatórios M1. À direita, marcadores metabólicos e sinalizadores anti-inflamatórios do polo M2.

O treinamento de macrófagos *in vitro* ou *ex vivo* por BCG leva ao aumento da secreção de citocinas pró-inflamatórias, como TNF-α e IL-6, conduzindo à ativação pró-inflamatória de macrófagos. Isso ocorre tanto por alterações epigenéticas, quanto por reprogramação metabólica (ARTS *et al*, 2016).

A readaptação genética se dá por metilação de lisinas ou argininas e acetilação de lisinas, presentes nas caudas livres da cromatina de histonas. No geral, a metilação de lisinas, como a H3K4me3 leva a abertura de regiões de DNA a fatores de transcrição, assim como a acetilação (VAN DER HEIJDEN *et al*, 2018), processo que perdura por algum tempo, o que permite a rápida resposta imunológica inata, quando há reexposição frente a um segundo estímulo. Recentemente, ainda foi verificada a transmissibilidade das alterações epigenéticas à prole em modelo animal (KATZMARSKI *et al*, 2021).

A reprogramação metabólica se dá mediante a ativação da via Akt-mTor-HIF1α (CHENG *et al*, 2014) que leva ao desvio metabólico a favor da glicólise mesmo com níveis adequados de oxigênio (RIKSEN; NETEA, 2021), caracterizando o efeito Warburg (WARBURG, 1956). Apesar de acidificar o meio através da produção de lactato, o treinamento de macrófagos com BCG não diminui a taxa de consumo de oxigênio (ARTS *et al*, 2016). A glicólise permite, então, a síntese rápida de ATP, enquanto a glicose-6-fosfato

pode ser usada pela via das pentoses na produção de NADPH (O'NEILL *et al*, 2016). A inibição da oxidação fosforilativa permite a utilização do citrato na conversão de acetil-CoA, que poderia ser substrato para a acetilação epigenética (RIKSEN; NETEA, 2021); o aumento de fumarato, o que permite a metilação de histonas (ARTS *et al*, 2016); e o uso de succinato na estabilização de HIF-1α (RYAN; O'NEILL, 2020). O aumento do NADPH aumenta a produção citosólica de ROS mediada por NADPH-oxidase (CANTON *et al*, 2021). HIF-1α, ainda, promove a ativação do fator de transcrição NF-κB, o que aumenta a transcrição não somente de citocinas pró-inflamatórias (BONELLO *et al*, 2007), como também de *inos*, aumentando a síntese de óxido nítrico (NO), espécie reativa de nitrogênio (BRUNE *et al*, 2013). Todas estas mudanças permitem o aumento de substâncias que permitem a morte de patógenos e a amplificação da resposta inflamatória. É possível verificar as vias de sinalização intracelular que reconhecem estímulos que levam ao treinamento e que conduzem à reprogramação metabólica em macrófagos na figura 2 (Fig. 2).

Figura 2- Ligantes, metabolismo e reprogramação epigenética durante o treinamento de células imunes

Fonte: Retirado de RIKSEN;NETEA, 2021.
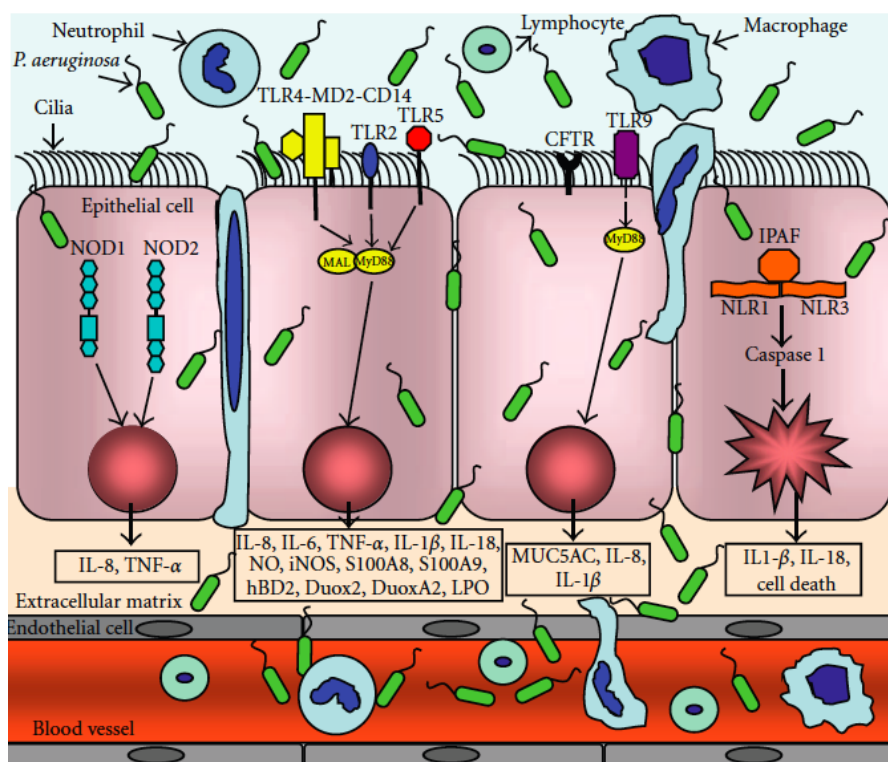
Nota: Estímulos como o BCG ativam a via Akt-mTOR, o que permite a transcrição de HIF-1α, o que direciona as vias metabólicas até a glicólise. Isso possbilita a conversão de citrato a acetil-Coa, propiciando a acetilação de histonas por enzimas como HAT; além disso, o fumarato pode ser substrato de KDM5 para acetilação de histonas, permitindo a reprogramação epigenética.

Um modelo de pneumonia induzida por *Streptococcus pneumoniae*, recentemente, evidenciou a menor susceptibilidade de camundongos após o treinamento de macrófagos alveolares com probióticos (RAYA *et al*, 2021). Ademais, a sensibilização de macrófagos com BCG e o posterior desafio com *Mycobacterium tuberculosis* levou ao treinamento destas células inatas (MATA *et al*, 2021). Considerando mo mesmo princípio, BCG poderia levar à imunomodulação de macrófagos frente à exposição com *Pseudomonas aeruginosa*.

P. aeruginosa é uma bactéria Gram-negativa, anaeróbia facultativa. É um importante patógeno oportunista causador de infecções nosocomiais, como pneumonia associada à ventilação mecânica, infecção de pele de indivíduos com grande extensão de queimadura, infecção do trato urinário de pacientes sondados, além da pneumonia em pacientes com fibrose cística (CIGANA *et al*, 2012). Apresenta vários fatores de virulência importantes na relação entre patógeno e hospedeiro, dentre os quais fímbrias, pilli, sistemas de secreção dos tipos 2 e 3 e exotoxinas (JURADO-MARTÍN *et al*, 2021).

No momento da infecção pulmonar aguda por *P. aeruginosa*, foco do presente trabalho, células epiteliais e macrófagos alveolares reconhecem LPS e flagelina por meio de receptores TLR 4 e TLR 5, respectivamente. Isso leva à ativação da via NF-κB, à síntese de citocinas pró-inflamatórias, como TNF-α, o que leva tanto ao recrutamento de neutrófilos para o sítio de infecção, quanto à amplificação da resposta inflamatória. O reconhecimento do sistema de secreção do tipo 3 leva ao acoplamento de inflamassomos, catalisando a secreção de IL-1β (LAVOIE e *et al*, 2011). Esse microambiente imunológico caracteriza, assim, a resposta imune inata contra *P. aeruginosa*, conforme demonstrado na figura 3 (Fig. 3).

Figura 3- Representação gráfica dos mecanismos da imunidade inata em resposta a *P. aeruginosa*.

Fonte: Retirado de CIGANA *et al,* 2012.

Nota: TLR4 e TLR5 de membrana plasmática em macrófagos reconhecem LPS e flagelo da bactéria, respectivamente, levando à ativação da via NF-κB e, consequentemente à transcrição de citocinas pró-inflamatórias.

Dada a importância de *P. aeruginosa* como bactéria causadora de pneumonia e de outras doenças graves, além da possibilidade de o BCG ser utilizado como indutor de resposta imune inata treinada, o presente trabalho objetivou analisar o papel da estimulação de macrófagos com BCG e/ou HKPa e avaliar qual a repercussão na ativação ou não deste tipo celular em uma segunda estimulação homóloga ou heteróloga.

CAPÍTULO 1: Heat-killed *Pseudomonas aeruginosa* drives proinflammatory polarization on BCG-primed macrophages

1 **Heat-killed *Pseudomonas aeruginosa* drives proinflammatory polarization on BCG-**
2 **primed macrophages**

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11

12 **Abstract**

13 Innate-trained immunity is a recent concept regarding the training or tolerance acquired by
14 macrophages, natural killer (NK) cells, dendritic cells, myeloid progenitors, and epithelial
15 cells before the heterologous stimulus, leading toward a stronger or weaker innate immune
16 response. Given the novel data concerning macrophage training in pneumonia models and,
17 thereby, the possibility of prevention and/or treatment of respiratory infections, this work
18 aims to evaluate macrophage priming *in vitro* by BCG and/or HKPa. For this purpose,
19 C57BL/6 mice bone-marrow-derived macrophages were plated and stimulated with BCG
20 and/or HKPa. CD11b+ macrophage displayed reactivity through higher
21 immunofluorescence of CD80/86, when homologous or heterologous stimulation occurred
22 with BCG and/or HKPa. These cells also showed pro-oxidant status before BCG stimulus,
23 through TBARS, TRAP and NADPH-oxidase tests. Nitric oxide concentration were
24 augmented in BCG heterologous stimulation. Moreover, there was higher expression of IL-
25 6 and higher secretion of IL-6 when BCG heterologous stimulation occurred. However,
26 homologous HKPa stimulation augmented IL-10 expression and secretion. Therefore, this
27 study suggests that macrophages were primed by BCG, when there was heterologous
28 stimulation with HKPa, and that homologous stimulation with HKPa induced immune
29 tolerance. These results may lead to novel respiratory infections approaches, either through
30 training by BCG or tolerance by HKPa.

31 Key-words: Innate-trained immunity; macrophages; BCG; *Pseudomonas aeruginosa.*

1. Introduction

32 Innate-trained immunity is a recent issue in vertebrate immunology, despite being common

33 knowledge to invertebrate and plant defense systems. It is characterized by an enhanced or

34 tolerized response after being exposed to a second stimulus, orchestrated through epigenetic

35 and metabolic reprogramming (Netea et al. 2020). Macrophages, natural killer cells,

36 dendritic cells, myeloid progenitor cells, and epithelial cells may be trained by Bacille

37 Calmette-Guérin (BCG) (Arts et al. 2018), β-glucans (Moerings et al. 2021),

38 lypopolissacaryde (LPS) (Foster, Hargreaves and Medzhitov 2007), adrenalin (Van Der

39 Heijden et al. 2020a), aldosterone (Van Der Heijden et al. 2020b) and low-density

40

41 lipoproteins (Bekkering et al. 2014).

42 BCG-trained macrophages display a proinflammatory profile, enhanced TNF-α and IL-6

43 secretion, augmented glycolytic rate, and H3K4me3 epigenetic methylation (Arts et al.

44 2018). Since macrophages were trained by BCG in a mycobacterium-pneumonia model, it

45 may be used to face *Pseudomonas aeruginosa* acute pneumonia (Mata et al. 2021).

46 Acute pneumonia is among the most common nosocomial infections, being *P. aeruginosa* a

47 great bacterial burden in this setting (Zaragoza et al. 2020). *P. aeruginosa* is an opportunist

48 Gram-negative bacillus pathogen. It presents fimbriae, pili, exotoxins, and secretion system

49 types 2 and 3 as virulence factors, which promote infection (Jurado-Martín, Sainz-Mejías

50 and McClean 2021). This bacterium imposes a great burden on humans through skin

51 infection, urinary tract infection, ventilation-associated pneumonia, and cystic fibrosis

52 pneumonia (Cigana et al. 2011).

53 Therefore, our work aimed to evaluate if BCG and heat-killed *Pseudomonas aeruginosa*

54 (HKPa) would induce differential profiles on macrophage response during homologous or

55 heterologous stimulation by both bacteria. Thereby, it may lead toward novel respiratory

56 infection approaches, either inducing training by BCG or tolerance by HKPa.

2. Materials and Methods

58 2.1 Animal Model and Bacteria

59 This study was carried out in strict accordance with Brazilian laws 6638 and 9605 in Animal

60 Experimentation and the protocol was approved by the Committee on the Ethics of Animal

61 Experiments of the Federal University of Alfenas (CEUA 23/2021). Female C57BL/6 mice

62 between 6 and 8 weeks old received water and food *ad libitum* and their boxes were cleaned

63   regularly. The PA14 virulent strain of *P. aeruginosa* was obtained from the bacterial culture

64   collection of the Laboratory of Molecular Microbiology of Microorganisms of the Federal

65   University of Alfenas. Heat-killed *P. aeruginosa* was made through 15-minute water

66   incubation at 80ºC after the exponential growth of *P. aeruginosa*. Bacterial killing was

67   confirmed by plating heat-killed bacteria on Luria Broth (LB) agar.

68   2.2 Bone-marrow-derived macrophages and cell stimulation

69   Bone-marrow-derived cells from C57BL/6 mice were stimulated and differentiated until

70   macrophages (BMDM), as previously described by our group (de Almeida *et al.* 2011). On

71   the tenth day, BMDMs were stimulated with 1:0.1 multiplicity of infection (MOI) of BCG

72   or with 1:10 MOI of HKPa. The stimulation was removed 24 hours after and then, fresh

73   medium was added without any differentiation stimulus. On the sixteenth day, cells were

74   again stimulated with a 1:0.1 MOI of BCG or with a 1:10 MOI of HKPa and supernatant

75   was collected 24 hours later to be evaluated to secretion molecules, and the cells were

76   evaluated to surface markers.

77   2.3 Analysis of CD11b, CD80 and CD86 surface markers by fluorescence microscopy

78   BMDMs under stimulation were evaluated to surface markers by fluorescence microscopy.

79   BMDMs (5×10^5 cells per well) were plated on imaging slides (μ-Slide 12-well, glass-bottom,

80   Ibidi GmbH, Munich, Germany). The cells were then incubated with anti-CD80/86 and anti-

81   CD11b, followed by staining with FITC-conjugated and PE-conjugated (BD Biosciences),

82   respectively, overnight at 4°C. The slides were washed with PBS and the nuclei were stained

83   with 150 ng/mL 40,6-diamino-2-phenylindole (DAPI; Thermo Scientific) for 1 h. All images

84   were captured using a Nikon Eclipse 80i fluorescence microscope (Melville, New York,

85   U.S.A). Finally, photos were taken from laminae, and adjustments were made through

86   ImageJ software, in order to analyze the nucleus (blue fluorescence), CD11b+ cells (green

87   fluorescence) and CD80+/86+ cells (red fluorescence).

88   2.4 Measurement of ROS produced by NADPH-oxidase, aldehydes generated from the lipid

89   oxidation, and total peroxyl radical-trapping antioxidant parameter

90   ROS synthesized by NADPH-oxidase was done as previously described (Amaral *et al.*

91   2015). Aldehydes generated from the lipid oxidation process were measured by the

92   thiobarbituric acid-reactive substance assay. Lipid samples (100 lL) were mixed with 0.67%

93   TBA (1000 lL) and 20% trichloroacetic acid (500 lL) followed by incubation at 100°C for

94   20min. After cooling, the reaction mixture was centrifuged for 5 min and the absorbance of

95   the supernatant was read at 532 nm. Triplicate experiments were carried out and the average

96   values were used(Yin and Porter 2003). TRAP (total peroxyl radical-trapping antioxidant

97   parameter) analysis. The luminol-enhanced CL assay for TRAP is based on the measurement

98   of peroxyl radicals The TRAP value was determined from the duration of the period during

99   which the CL signal was diminished by samples or standard antioxidants. Briefly, trolox, a

100   water-soluble analog of a-tocopherol, was used as a reference inhibitor. PBS, luminol and

101   PUFAs sample or trolox were added to 96-well plates and incubated at 37º C for 10 min.

102   After incubation, AMPA was added, and the CL immediately measured (Ambrozova,

103   Pekarova and Lojek 2010).

104   2.5 Nitric oxide quantification

105   Supernatant from BMDM groups, after stimulation with BCG or HKPa, was submitted to

106   Griess method (Green *et al.* 1982). The final reaction was measured using a microplate

107   reader (Bio-Tropsch Tek Instruments, Winooski, Vt., USA) and read at 405 nm with

108   wavelength correction set at 650 nm.

109   2.6 Real time RT-PCR (qPCR)

110   RNA from BMDM was isolated with trizol. Reverse-transcription of 1 μg total RNA was

111   performed using Illustra™ Ready-To-Go RT-PCR Beads (GE Healthcare,

112   Buckinghamshire, UK). Real-time RT-PCR was conducted in a final volume of 10 μL

113   containing the following: SYBR® Green PCR Master Mix (Applied Biosystems, Foster

114   City, CA, USA), with cDNA as the PCR template and primers to amplify specific fragments

115   corresponding to specific target genes: IL-6 F: 5'-CCAGGTAGCTATGGTACTCCAGAA-

116   3', IL-6 R: 5'- GATGGATGCTACCAAACTGGA-3'; IL-10 F: 5'-

117   GGTTGCCAAGCCTTATCGGA-3', IL-10 R: 5'-ACCTGCTCCACTGCCTTGCT-3';

118   iNOS F: 5'-CAGCTGGGCTGTACAAACCTT-3', iNOS R: 5'-

119   CATTGGAAGTGAAGCGTTTCG-3'. The PCR reaction was performed with ABI 7500

120   Real-Time PCR System (Applied Biosystems, Foster City, CA), using the following cycling

121   parameters: 60°C for 10 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec, and 60°C for 1

122   min, and a dissociation stage of 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec, 60°C for

123   15 sec. All data are presented as relative expression units after normalization to the β-actin

124   gene F: 5'-AGGTGTGCACTTTTTATTGGTCTCAA-3', R: 5'-

125   TGTATGAAGGTTTGGTCTCCCT3'. PCR measurements were conducted in triplicate.

126 The differences in the relative expression were analyzed by analysis of variance (ANOVA)

127 followed by Tukey's test ($p < 0.05$ denotes statistical significance).

128 2.7 Enzyme-Linked Immunosorbent Assay

129 Enzyme-Linked Immunosorbent Assay was made to IL-6 and IL-10 cytokines, according to

130 Murine IL-6/IL-10 Mini ABTS ELISA Development kit (PeproTech, Cranbury, NJ, USA).

131 Final cytokine concentrations were calculated using the standard curve for IL-6 or IL-10.

132 The final reaction was measured using a microplate reader (Bio-Tropsch Tek Instruments,

133 Winooski, Vt., USA) and read at 405 nm with wavelength correction set at 650 nm.

134 2.8 Statistical analysis

135 All experiments were repeated, at least, three times. Considering similar results among them,

136 figures display data from one representative experiment. Data were analyzed through

137 GraphPad Prism 8.0 software, using ANOVA-oneway variance test followed by Tukey's

138 test ($p < 0.05$ denotes statistical significance).

139 **3. Results**

140 3.1 Heterologous stimulation induced macrophage reactivity

141 Macrophage reactivity was evident only after homologous stimulation with HKPA or BCG

142 and heterologous stimulation with BCG and/or HKPA, as CD80/86 shows (Fig. 1a).

143 No macrophage unstimulated displayed reactivity. Those stimulated once with HKPA or

144 BCG displayed less than 50%, while homologous or heterologous stimulation presented

145 more than 60% of reactivity (Fig. 1b).

146 This highlights that both stimuli, BCG and HKPa, can induce proinflammatory markers on

147 the macrophage surface.

148 3.2 BCG and HKPA stimulation enhanced antioxidant capacity and increased production of

149 ROS

150 Total antioxidant capacity was measured through the TRAP test. First stimulus with BCG

151 followed by medium exposure and homologous stimulation presented a decrease in total

152 anti-oxidant capacity in medium (Fig. 2a). It suggests that BCG may result in depletion of

153 antioxidant in cell. Interestingly, heterologous stimulation followed by BCG and HKPA, in

154 this order, displayed an oxidative state not different from medium.

155 To verify the NADPH-oxidase-derived oxygen species, these species were measured by

156 luminescence. Only HKPa followed by no stimulus and BCG stimulus presented increased

157 reactive oxygen species levels. Notably, both augmentations were significant compared to

158 homologous stimulation (Fig. 2b).

159 Since ROS may react with plasmatic membrane lipids, TBARS was performed to quantify

160 lipid peroxidation. Second stimulation with HKPa had greater peroxidation compared to the

161 medium. Besides, heterologous stimulus of BCG followed by HKPa showed not only greater

162 reactions than medium, but also from homologous stimulation (Fig. 2c).

163 Overall, our results show that heterologous stimulation initiated by BCG has the tendency

164 of greater oxidative stress.

165 3.3 Heterologous stimulation led to higher nitric oxide synthesis

166 Nitric oxide was quantified through the Griess test. Reactive nitrogen species had significant

167 increase compared to medium. Moreover, heterologous stimulation initiated with BCG

168 presented with the greatest augmentation compared to heterologous stimulation started with

169 HKPa and homologous signals, as demonstrated in figure 3. Overall, BCG showed to be able

170 to prime macrophage to produce higher NO when boosted with other stimuli.

171 3.4 Differential gene expression profiles during homologous and heterologous stimulation

172 showed a tendency toward proinflammatory polarization

173 We investigated *il6*, *il10*, and *inos* gene expression. The highest IL-6 expression levels were

174 found among the second stimulus with HKPa and homologous stimulation with HKPa (Fig.

175 4a). Heterologous stimulation, no matter the stimulus order, has diminished levels of *il6*

176 expression compared to homologous stimulation (Fig. 4a). The highest *il10* expression was

177 found during homologous stimulation with HKPa (Fig. 4b). Meanwhile, there was higher

178 *inos* expression during heterologous stimulation started with BCG and when there was one

179 stimulus with HKPa (Fig.4c).

180 This indicates that not only BCG but also HKPa can induce both proinflammatory and anti-

181 inflammatory cytokines. Interestingly, BCG-primed macrophages improve *inos* expression

182 impressively.

183 3.5 Heterologous stimulation induced higher proinflammatory cytokine secretion

184   Since it was observed differential expression of proinflammatory cytokine IL-6 and anti-
185   inflammatory cytokine IL-10, both secreted cytokine levels were assessed through ELISA.
186   One stimulation with HKPA or heterologous stimulation initiated by BCG displayed the
187   highest levels of IL-6 when compared to the medium (Fig. 5a). On the other hand, anti-
188   inflammatory cytokine IL-10 displayed higher levels only when heterologous stimulation
189   started with BCG or homologous HKPa stimulation (Fig. 5b).

190   It shows that BCG-primed macrophages had more proinflammatory and anti-inflammatory
191   cytokine secretion, while homologous HKPa stimulation tended towards high levels of anti-
192   inflammatory secretion.

193   **4. Discussion**

194   Our work has demonstrated that both BCG and HKPa can induce proinflammatory markers
195   and proinflammatory cytokine expression. However, only BCG seems capable of inducing
196   a pro-oxidant status and BCG-primed macrophages secrete more NO and IL-6. Meanwhile,
197   HKPa homologous stimulation tends towards an anti-inflammatory profile, inducing
198   significant expression and secretion of IL-10.

199   First, we verified if BCG and HKPa could augment proinflammatory markers on cell surface.
200   We depicted macrophage reactivity through CD80/86 staining in immunofluorescence after
201   homologous or heterologous stimulation. CD80/86 were also augmented in THP-1
202   macrophages after LPS stimulation, with a positive correlation with IL-6 synthesis (Jiménez-
203   Uribe *et al.* 2019). Both CD86 and MHC-II markers were higher in BCG-trained lung
204   macrophages derived from mice and had an increase after the *Mycobacterium tuberculosis*
205   challenge (Mata *et al.* 2021). Differently, Koeken and colleagues found reduced expression
206   of HLA-DR, another reactivity marker, in human alveolar macrophages after BCG
207   vaccination (Koeken *et al.* 2020).

208   Since both stimuli induced proinflammatory macrophage reactivity markers, we
209   hypothesized that oxidative status may be influenced by BCG and/or HKPa. NADPH-
210   derived ROS concentration tended to be higher among heterologous stimulation. NADPH-
211   oxidase 2 also had an important role in proinflammatory macrophages in the context of fatty
212   liver disease, being responsible for ROS production (Kim *et al.* 2017). Moreover, human
213   BCG-trained macrophages displayed higher levels of ROS after zymosan stimulation
214   (Bekkering *et al.* 2016) and ROS depicted through flow cytometry was augmented among

215 BCG-trained macrophages, which was sustained for six days (Ferreira *et al.* 2021). Overall,
216 oxidative burst is increased among BCG-trained macrophages after restimulation.

217 Given the oxidative status change, we verified if RNS species would also be achieved.
218 Heterologous stimulation revealed greater amounts of NO and *inos* expression. Mata *et al*
219 also showed great intensity of iNOS levels of intra-nasally BCG-primed lung macrophages
220 at flow cytometry before and after *M. tuberculosis* defiance in a time-dependent manner
221 (Mata *et al.* 2021).

222 Overall, given the proinflammatory macrophage markers depiction, oxidative status
223 changes, and levels of RNS, we checked out for cytokine expression and secretion. Our
224 results showed higher levels of IL-6 secretion from heterologous stimulated group trained
225 with BCG. Differently, BCG-trained macrophages displayed IL-6 and TNF-α strong
226 transcriptomic correlations after LPS stimulation, although only BCG training had an anti-
227 inflammatory tendency (Kong *et al.* 2021). Moreover, neonatal BCG-trained macrophages
228 tended towards lower inflammatory profile than immune cells recovered from adults
229 (Angelidou *et al.* 2021). Notably, monocytes derived from BCG vaccinated humans secrete
230 higher amounts of proinflammatory cytokines after *Candida albicans* challenge (Cirovic *et*
231 *al.* 2020).

232 We used the BMDM model to prime cells with BCG. Kauffmann *et al* (2018) used the same
233 model and defied these cells with *M. tuberculosis*, recovering fewer *in vitro* colony forming
234 units. Furthermore, they depicted epigenetic priming in H3K27 ac and H3K4me3
235 (Kaufmann *et al.* 2018), two histone modifications responsible for BCG training (Van Der
236 Heijden *et al.* 2018). Another model challenged mice with *Streptococcus pneumoniae* after
237 probiotic exposure, suggesting that alveolar macrophages were trained and released more
238 proinflammatory cytokines (Raya Tonetti *et al.* 2021). Human alveolar macrophages were
239 also target of study, secreting higher amounts of IL-1β and IL-6 after *M. tuberculosis*
240 challenge (Koeken *et al.* 2020), as did our study.

241 We found huge amounts of IL-10 in HKPa homologous stimulation. It may suggest
242 immunotolerance induced by LPS present in HKPa membrane, as depicted by Foster *et al*
243 (2007) after homologous stimulation with LPS (Foster, Hargreaves and Medzhitov 2007).

244

245

246   **5.   Conclusion**

247   Our study shows that BMDM macrophages were primed by BCG after heterologous

248   stimulation with HKPa, polarizing towards an oxidative state characterized by NO and ROS

249   derived from NADPH-oxidase. Moreover, these cells transcript high amounts of *inos*, as

250   well as secreted huge amounts of proinflammatory cytokine IL-6, corroborating BCG

251   priming.

252   On the other hand, homologous stimulation with HKPa displayed not only higher expression

253   of *il10*, but also secreted it, suggesting an immune tolerance process.

254   As indicated by our findings, it is important to explore the potential of a well-known vaccine,

255   such as BCG, or a ubiquitous bacterium, such as *P. aeruginosa*, like possible inducers of

256   innate immune training, using the former as a response enhancer and the last as a response

257   tolerized, in order to boost bacterial clearance with BCG or to minimize cytokine storm with

258   HKPa.

259

269   **8.   References**

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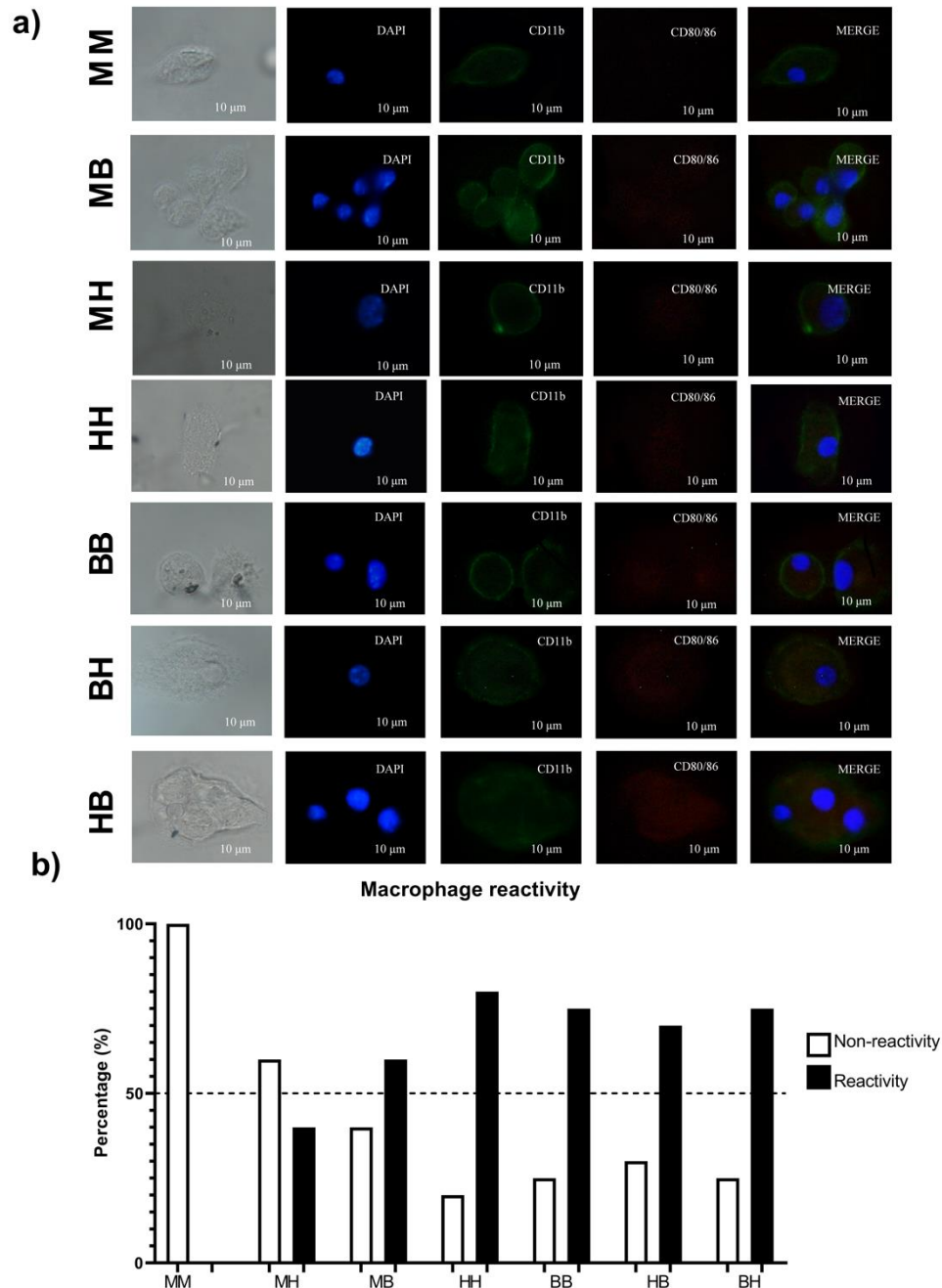306   *Antioxidants Redox Signal* 2018;**29**:1023–40.

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340

341 **9. Figure and Figure Legends**

342

343 **Figure 1. BMDM immunofluorescence and macrophage counting.** A)
344 Immunofluorescence staining with DAPI, CD11b and CD80/86 of BMDM after stimulation
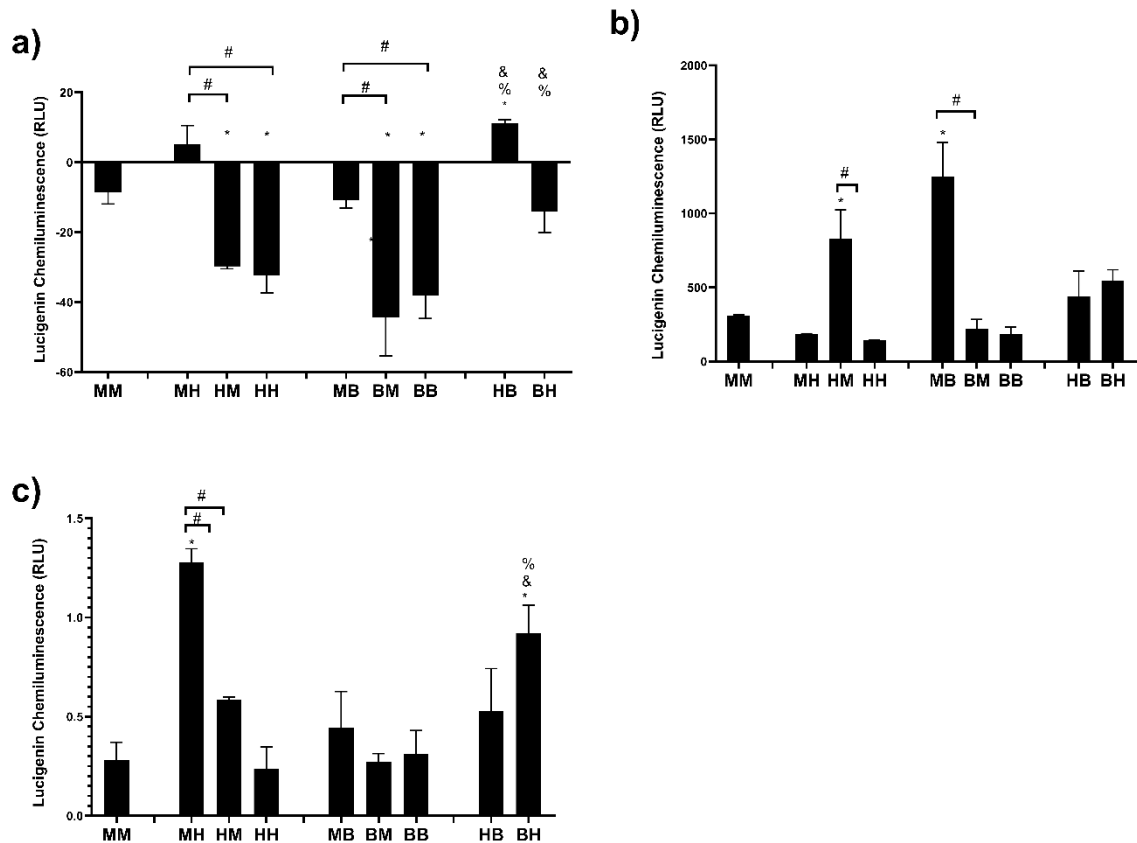345 with BCG and/or HKPa. B) Percentage of how much reactive macrophages were found out
346 of 20 cells. Experimental groups: MM - Medium, MH- medium followed by HKPa, HM -
347 HKPa followed by medium, HH - HKPa followed by HKPa, MB - medium followed by
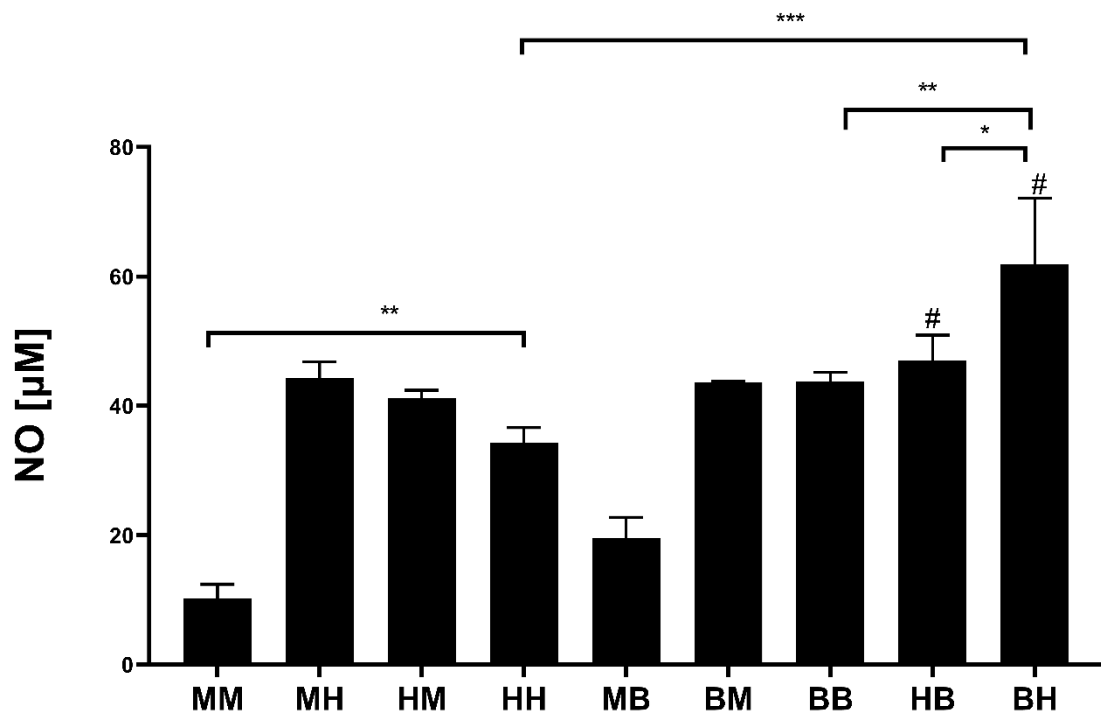348 BCG, BM - BCG followed by medium, BB – BCG followed by BCG HB - HKPa followed
349 by BCG, BH - BCG followed by HKPa.

Figure 2. Oxidant assays on BMDM stimulated with BCG and/or HKPa. A) Oxidative status given by TRAP test. B) ROS levels given by NADPH-oxidase. C) Lipid peroxidation levels given by TBARS. Groups: MM - Medium, MH- medium followed by HKPa, HM - HKPa followed by medium, HH - HKPa followed by HKPa, MB - medium followed by BCG, BM - BCG followed by medium, HB - HKPa followed by BCG, BH - BCG followed by HKPa. #p<0,05; * p<0,05 compared to MM; % P<0.05 compared to HH; & p<0,05 compared to BB. Experimental groups: MM - Medium, MH- medium folloewd by HKPa, HM - HKPa followed by medium, HH - HKPa followed by HKPa, MB - medium followed by BCG, BM - BCG followed by medium, BB – BCG followed by BCG HB - HKPa followed by BCG, BH - BCG followed by HKPa.

Figure 3. Nitric oxide levels after BCG and/or HKPa stimulation. Griess test carried out after homologous and/or heterologous stimulation. Experimental groups: MM - Medium, MH- medium followed by HKPa, HM - HKPa followed by medium, HH - HKPa followed by HKPa, MB - medium followed by BCG, BM - BCG followed by medium, BB – BCG followed by BCG HB - HKPa followed by BCG, BH - BCG followed by HKPa. *p<0,05; ** p< 0,005; *** p<0.0001; # p<0,05 compared to MM.
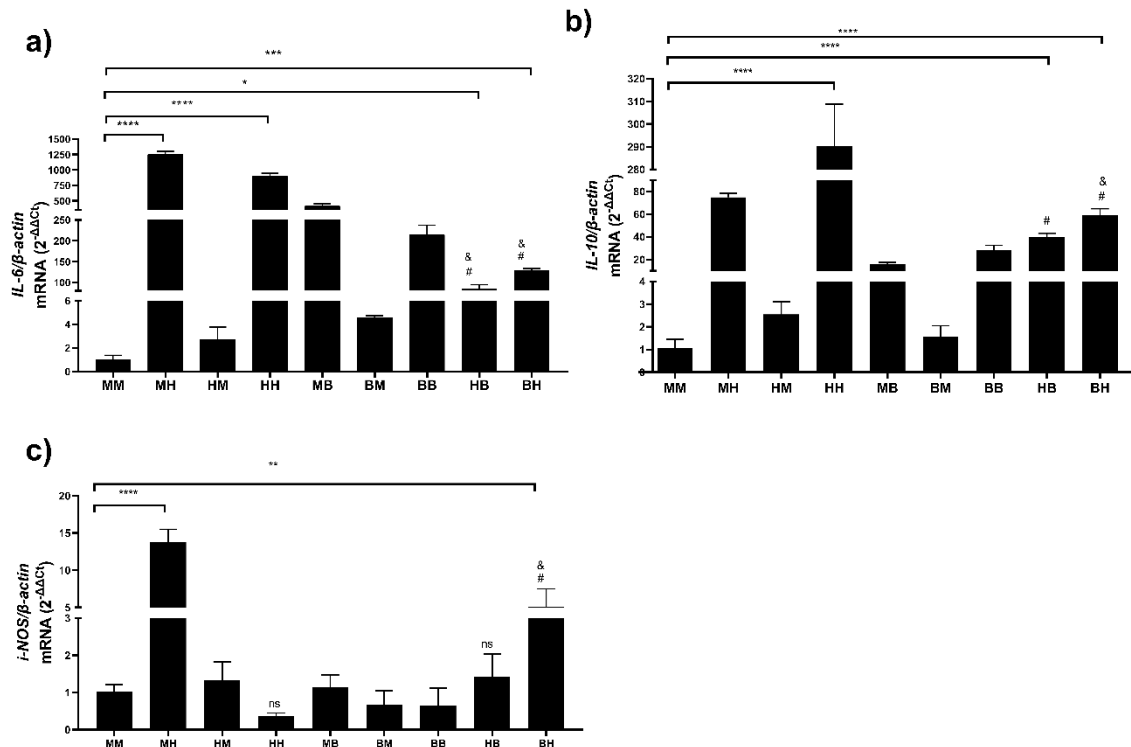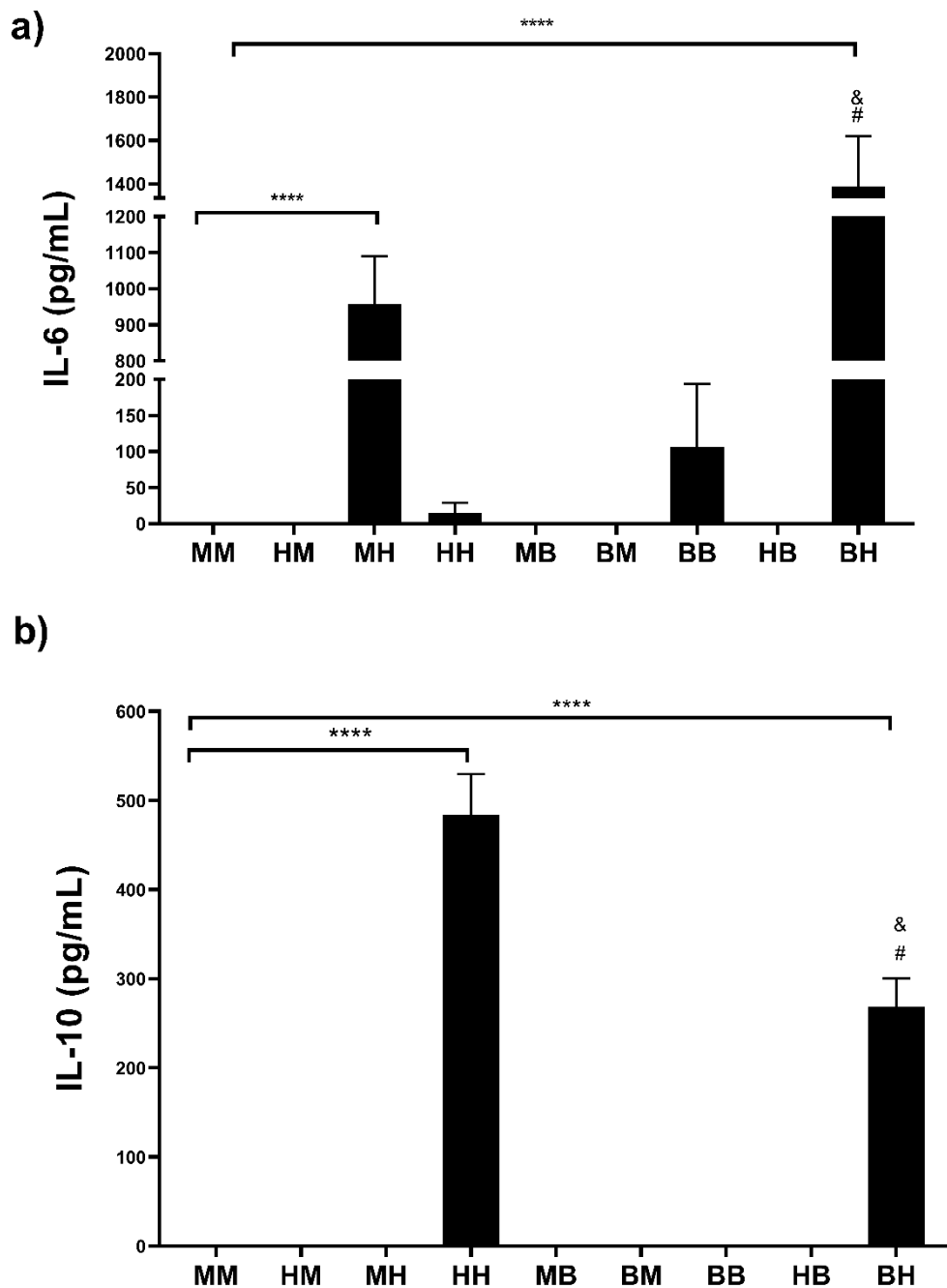
Figure 4. Expression levels of *il6, il10, inos*. PCR analysis of gene expression after BCG and/or HKPa stimulation. A) IL-6. B) IL-10. C) iNOS. Experimental groups: MM - Medium, MH- medium followed by HKPa, HM - HKPa followed by medium, HH - HKPa followed by HKPa, MB - medium followed by BCG, BM - BCG followed by medium, BB – BCG followed by BCG HB - HKPa followed by BCG, BH - BCG followed by HKPa.. *p<0,05; ** p< 0,001; *** p<0.0001; **** p<0,00001; # p<0,05 compared to HH; & p<0,05 compared to BB.

Figure 5. Secretion levels of IL-6 and IL-10 after stimulation with BCG an/or HKPa.
ELISA analysis of cytokine secretion. A) IL-6. B) IL-10. Experimental groups: MM - Medium, MH- medium followed by HKPa, HM - HKPa followed by medium, HH - HKPa followed by HKPa, MB - medium followed by BCG, BM - BCG followed by medium, BB – BCG followed by BCG HB - HKPa followed by BCG, BH - BCG followed by HKPa..
**** $p<0,00001$; # $p<0,05$ compared to HH; & $p<0,05$ compared to BB

CONCLUSÃO

O presente trabalho sugere a polarização M1 de macrófagos pelo BCG, sugere o processo de *priming* dessas células imunes pelo BCG e sugere a tolerância causada por HKPa.

Isso se demonstra pela ativação de macrófagos na estimulação homóloga ou heteróloga com BCG e/ou com HKPa, indicando certa polarização; pela tendência oxidante dos meios com BCG e no aumento da secreção de NO em estimulação heteróloga iniciada por BCG; e pela expressão aumentada de citocinas pró-inflamatórias e secreção de IL-6, quando do estímulo heteróloga também iniciado pelo BCG.

Diferentemente, a estimulação homóloga com HKPa não foi capaz de aumentar a resposta pró-inflamatória, outrossim, aumentou a expressão e secreção de IL-10, sugerindo um processo de tolerância imunológica.

Estudos *in vivo* tornam-se essenciais, para avaliar a aplicabilidade do potencial treinamento e tolerância imunológicos encontrados nos resultados frente a infecções do trato respiratório inferior.

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ANEXO A - CEUA-UNIFAL

MINISTÉRIO DA EDUCAÇÃO
Universidade Federal de Alfenas - UNIFAL-MG
Lei nº 11.154, de 29 de julho de 2005

Comissão de Ética no Uso de Animais - CEUA/UNIFAL-MG

Certificado

Certificamos que a proposta intitulada **ALTERAÇÕES DA MICROBIOTA BACTERIANA INTESTINAL PELA AÇÃO DE FÁRMACOS DE INTERESSE MÉDICO E VETERINÁRIO E SUAS REPERCUSSÕES SISTÊMICAS NO CONTROLE DE INFECÇÕES OPORTUNISTAS**, registrada com o nº **0023/2021**, sob a responsabilidade de **Leonardo Augusto de Almeida**, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de **pesquisa científica**, com vigência de **01/07/2021 a 28/06/2024**, encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA-UNIFAL) DA UNIVERSIDADE FEDERAL DE ALFENAS.

Espécie/linhagem/raça	Total de animais	Total de machos	Total de fêmeas	Origem
Roedor / C57bl/6	180	90	90	Biotério central

Alfenas, 18 de Janeiro de 2023

Prof(a). Dr(a). Pollyanna Francielli de Oliveira
Coordenador(a) do CEUA/UNIFAL - MG

Para verificar autenticidade acesse: http://sistemas.unifal-mg.edu.br/app/ceua/autenticidadecertificado/ e digite a chave: 1c427e8ed6a35e743491a8d7185ea30d