

PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL
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PAPEL IMUNORREGULADOR DA HSP70 DE *Mycobacterium*
tuberculosis, MURINA E HUMANA

Porto Alegre

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Dissertação apresentada como requisito para a obtenção do grau de Mestre pelo Programa de Pós-Graduação em Biologia Celular e Molecular da Pontifícia Universidade Católica do Rio Grande do Sul.

Orientador: Cristina Bonorino

Porto Alegre

2016

Dedico esta dissertação aos meus familiares, colegas e amigos que me apoiaram de alguma forma para que eu atingisse meus objetivos

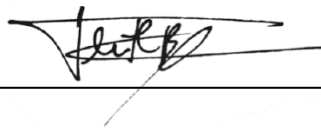
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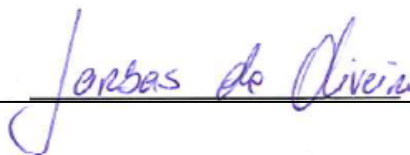
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RESUMO

A Hsp70 (*Heat shock protein 70 kDa*) é uma proteína de choque térmico de 70 kDa que possui funções intra e extracelulares. Nos estudos iniciais, somente as funções intracelulares eram exploradas, como o auxílio no dobramento, impedir a agregação e o redobramento protéico. Há décadas mostrou-se que essa proteína pode ser exportada para o ambiente extracelular e exercer efeitos sobre outras células, como as do sistema imune. O nosso grupo vêm explorando o papel dessa proteína em células chave da resposta imune, como macrófagos e células dendríticas. O presente trabalho observou que a Hsp70 de *Mycobacterium tuberculosis* pode polarizar macrófagos a um fenótipo M2 e que essa resposta é dependente de IL-10. Macrófagos peritoneais e derivados de medula óssea foram tratados com DnaK (homólogo procariótico da Hsp70) de *Mycobacterium tuberculosis* por 24h. Após esse período observou-se o aumento de marcadores clássicos de fenótipo M2, como: FIZZ1, YM1, CD206, IL-10, Arg1. Por outro lado, houve uma diminuição na expressão de IL-6, MCP-1, TNF- α , NO, MHC de classe II e CD86, além de essas células serem capazes de promover o crescimento tumoral em modelo alogênico. Observou-se também, com células KO ou bloqueando o IL-10R que essa modulação é dependente de IL-10. Além disso, essa modulação celular não é restrita aos macrófagos, podendo essa proteína agir sobre células dendríticas, modulando-as a um fenótipo imaturo, como já mostrado em trabalhos anteriores do grupo (1,2). Porém, não se sabe ainda qual região específica da proteína é essencial para esse efeito e se ele é restrito a esse homólogo, DnaK de *M. tuberculosis*. Portanto, outro objetivo do trabalho foi expressar e purificar no mesmo sistema os seus homólogos Hspa1a (Hsp70 murina) e HSPA1A (Hsp70 humana) e posteriormente, testá-las em paralelo em modelos experimentais imunológicos. O trabalho está em andamento atualmente em relação a purificação da Hspa1a e os primeiros testes de estado de ativação de células dendríticas foram realizados. Acredita-se que se há um efeito dos homólogos de DnaK, esse efeito é menos evidente, segundo resultados prévios.

Palavras-chave: Hsp70. Macrófago. célula dendrítica. modulação.

ABSTRACT

Hsp70 (Heat shock protein 70 kDa) is a chaperone protein which has intra- and extracellular functions. In the early studies, only intracellular functions were operated, as to aid in folding, prevent aggregation and protein refolding. In 80's a study has shown that this protein can be exported into the extracellular environment exerting effects on other cells, such as the immune system. Our group are exploring the role of this protein in key immune response cells such as macrophages and dendritic cells. Present study noted that the Hsp70 of *Mycobacterium tuberculosis* can polarize macrophages to a M2 phenotype and this response is dependent on IL-10. Peritoneal and bone marrow derived macrophages were treated with DnaK (Hsp70 prokaryotic homolog) from *M. tuberculosis* for 24 hours. After this period there was an increase of M2 classic phenotype markers, such as FIZZ1, YM1, CD206, IL-10 and Arg1. On the other hand, there was a decrease in expression of IL-6, MCP-1, TNF- α , NO, MHC class II and CD86 (M1 markers), and these cells are able to promote tumor growth in allogeneic model. It was also observed with KO cells or blocking IL-10R that this modulation is dependent on IL-10. In addition, this cellular modulation is not restricted to macrophages; such protein acting on dendritic cells by modulating them with an immature phenotype, as has been shown in prior works of our group (Borges et al, 2010;. Motta et al., 2007). However, it is not yet known what specific region of the protein is essential for that purpose and whether it is restricted to that homologues, DnaK from *M. tuberculosis*. Therefore, another objective was to express and purify in the same system their counterparts Hspa1a (murine Hsp70) and HSPA1A (human Hsp70) and subsequently testing them in parallel in immunological experimental models. Work is currently in progress with regard to purification of Hspa1a and initial dendritic cells activation state tests were conducted. It is believed that whether there is an effect with DnaK homologues, this effect is less obvious, according to previous results.

Keywords: Hsp70. macrophage. dendritic cell. modulation.

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LISTA DE SIGLAS

BMDC – *Bone marrow dendritic cell*; Célula dendrítica diferenciada da medula óssea

CD – *Cluster of differentiation*; Grupo de diferenciação

DC – *Dendritic cell*; Célula dendrítica

DNA - *Deoxyribonucleic acid*; Ácido desoxirribonucleico

Foxp3 – *Factor forkhead Box P3*

GM-CSF - *Granulocyte–macrophage colony-stimulating factor*; Fator estimulador de colônia de granulócitos e macrófagos

Hsp - *Heat shock protein*; Proteína de choque térmico ou Proteína de choque de calor

IFN - Interferon

LPS - Lipopolissacarídeo

MHC – *Major histocompatibility complex*; Complexo principal de histocompatibilidade

OVA - Ovalbumina

PBS – *Phosphate buffered saline*; Tampão fosfato-salino

TGF – *Transforming growth factor*; Fator de crescimento transformador

TNF - *Tumor necrosis factor*; Fator de necrose tumoral

TLR- *Toll like receptor*; Receptor do tipo Toll

UV – Radiação ultravioleta

Hsp70 – *Heat shock protein 70 kDa* – Proteína de choque de calor de 70 kDa

PBMC – *Peripheral blood mononuclear cells* – Células mononucleares periféricas do sangue

IL-1, IL-6, IL-10 e IL-12 – Interleucina-1,6,10 e 12

EU – *Endotoxin units* – unidades de endotoxina

EDTA – Ácido etileno diamino tetra-acético

SBF – Soro bovino fetal

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Capítulo I

1 INTRODUÇÃO

1.2 Proteínas de choque térmico (HSPs – *Heat shock proteins*)

As proteínas de choque térmico são proteínas induzidas nas células por um variado conjunto de estressores, como: calor, radiação UV, infecções virais e bacterianas, câncer, entre outros. Essas proteínas possuem um papel de extrema importância no ambiente intracelular frente a situações de estresse. Dentre suas funções intracelulares estão: dobramento adequado de proteínas, impedir a agregação de proteínas, desfazer agregados, endereçamento correto de proteínas para suas respectivas organelas, marcação para a degradação. As HSPs se encontram em diversos compartimentos celulares, como: citosol, retículo endoplasmático, núcleo e mitocôndria (3–5).

Sua descoberta foi de forma acidental, quando um colega de Ferruccio Ritossa, o descobridor, aumentou a temperatura da estufa onde o pesquisador analisava larvas de *Drosophilla*. Após observá-las ao microscópio viu que apresentavam formação de *puffs* cromossômicos. Posteriormente, repetiu o experimento, porém agora, com os controles adequados e observou o mesmo comportamento. Somente anos depois, em 1974, Tissieres, associou esse fenômeno a resposta de choque térmico, quando então “nasceram” as HSPs (6,7).

As Hsps são subdivididas em famílias, de acordo com seu peso molecular aproximado, sendo denominadas, como segue no exemplo: família Hsp27, Hsp40, Hsp70, Hsp100. A denominação Hsp não implica que cada família possua funções semelhantes ou homologia de sequência entre si. Isso porque cada família possui diversos genes que codificam suas proteínas e os mesmos são regulados por promotores diferentes (5). Além disso, são proteínas que possuem uma alta conservação evolutiva mesmo se compararmos desde os procariotos até os humanos. Isso também salienta sua importância nos sistemas vivos (8,9).

1.2.1 Proteína de choque térmico de 70 kDa

A estrutura molecular da HSP70 é formada por dois domínios, um de ligação ao nucleotídeo e outro de ligação ao substrato (SBD – *substrate binding domain*). O sítio de ligação ao nucleotídeo ao receber o ligante, transmite o sinal até o sítio que liga ao substrato. A região de ligação ao substrato tem um formato de fenda com uma tampa que proporciona uma região hidrofóbica de ligação ao substrato (**Figura 1**). Para auxiliar no dobramento das proteínas há uma cooperação de outras chaperonas, como Hsp40, Hsp100 e fatores de troca de nucleotídeos (4,10).

Dentre as características importantes dessa proteína está sua conservação evolutiva, sendo a mais conservada proteína documentada, desde arqueobactérias e plantas até os humanos. A conservação evolutiva não se limita apenas a sequência protéica, mas também a função. Hsp70 de *Drosophilla* quando expressada em células de mamíferos é capaz de prevenir contra o estresse térmico (11), Hsp70 humana previne células de fibroblasto de rato contra estresse térmico induzido (12,13).

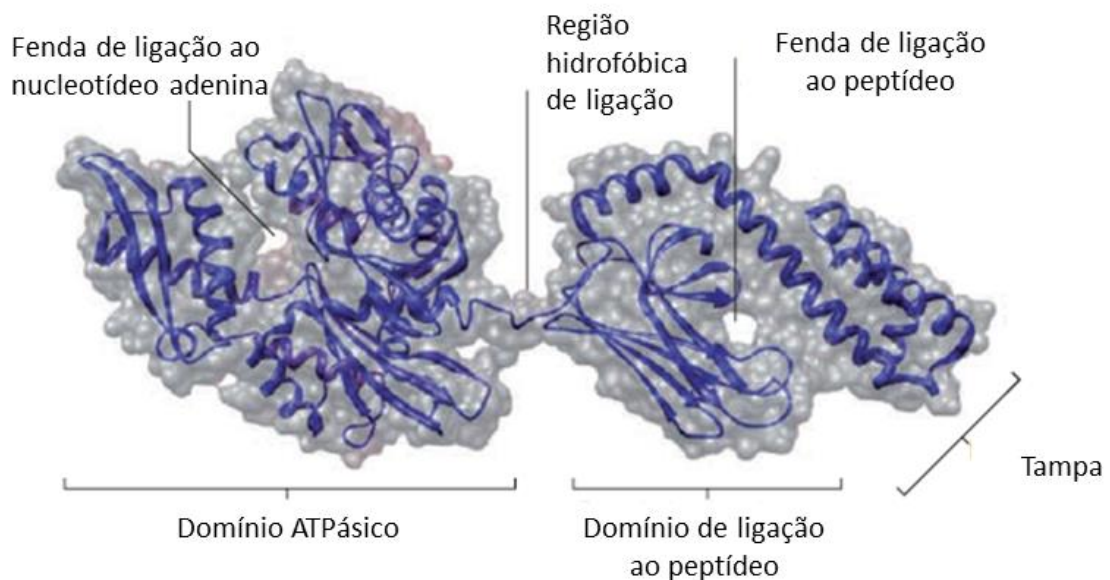


Figura 1 Estrutura da Hsp70

Essa é a estrutura da Hsp70 de *E. coli* resolvida por Bertelsen e colegas (14). A sua estrutura é formada por dois domínios, um de ligação ao nucleotídeo e o outro de ligação ao substrato, sendo ambos ligados por uma fina região hidrofóbica (15).

Abaixo, uma tabela que mostra os nomes atuais e alguns sinônimos utilizados antigamente para as Hsp70 de diferentes espécies discutidas neste trabalho.

Tabela 1. Nomenclatura dos ortólogos de Hsp70

Nome do gene	Nome antigo da proteína (sinônimos)	Organismo
HSPA1A	Hsp70-1, Hsp72, HSPA1A	<i>H. sapiens</i>
DnaK	Hsp70	<i>M. tuberculosis</i>
Hspa1a	Hsp70-3, Hsp70.3	<i>Mus musculus</i>

(16,17)

1.3 Macrófagos

Macrófagos são células que possuem mais de uma origem. Uma de suas origens provém de células tronco hematopoiéticas, podendo se originar também a partir do saco vitelino na embriogênese ou do fígado fetal a medida que a medula óssea vai desenvolvendo-se em camundongos (18,19).

Os macrófagos são células fagocíticas que desempenham papéis importantes na resposta imune inata, além de interligar-se à resposta imune adaptativa. (20). Essas células exercem sua função ajudando a eliminar patógenos, desde a fagocitose de patógenos, passando pela apresentação de antígenos até a citotoxicidade mediada por ROS (*Reactive oxygen species*-espécies reativas de oxigênio). Tais células auxiliam na resposta inflamatória, liberando citocinas pró-inflamatórias, como IL-1, IL-12, TNF- α (21).

A ativação ocorre via componentes microbianos, chamados de PAMPs (*Pathogen-associated molecular pattern* - Padrões moleculares associados a patógenos), como o LPS (*Lipopolysaccharide* - Lipopolissacarídeo), PGN (*Peptidoglycan* - Peptideoglicano), através do engajamento de TLRs, receptores de manose, integrinas (ex.: CD11b/CD18), além de poderem ser ativados por citocinas, como IFN- γ , IL-6, via receptores de citocina . Quando ativados, aumentam sua capacidade de fagocitose, aumenta a secreção de mediadores inflamatórios, o que culmina em um maior poder de combate aos patógenos invasores do organismo (22).

Sua ativação leva a uma expressão aumentada da enzima *iNOS* (*inducible nitric oxide synthase* - Óxido nítrico sintase induzida), levando a produção e consequente liberação de óxido nítrico por essas células, o qual possui uma alta atividade anti-microbiana (21,23,24). Outra forma de eliminação de patógenos ocorre via complexos formados por componentes do sistema complemento e complexos antígeno-anticorpo ou célula-anticorpo. A ligação de um componente do sistema complemento ao macrófago leva a fagocitose da célula ou do complexo formado. Além disso, há também a apresentação de antígenos via molécula de superfície MHC de classe II, em que o macrófago fagocita patógenos opsonizados ou não, digere-o, leva ao seu processamento e apresenta os antígenos em sua superfície, que levará a interações com células T auxiliares, auxiliando na resposta humoral e celular (25).

1.3.1 Heterogeneidade de macrófagos

As primeiras observações feitas a respeito da modulação de fenótipo dos macrófagos ocorreram por volta da década de 90, quando constatou-se que macrófagos sob o estímulo de IL-4 inibiam a produção de espécies reativas de oxigênio (ROS) a produção de IL-8 e IL-1 β (26). Até que em 1992, Michael Stein et al. publicaram o trabalho em que mostraram que a IL-4 induz macrófagos inflamatórios a adquirirem um fenótipo ativado alternativamente, distinto daquele clássico ativado por IFN- γ . Esse fenótipo era caracterizado por alta capacidade de depuração de ligantes de manose, reduzida secreção de citocinas pró-inflamatórias, além da presença de mudanças morfológicas nas células (27).

Após anos de estudos, a ativação alternativa dos macrófagos está mais definida. De acordo como conceito atual, macrófagos ativados classicamente (CaM ϕ – *Classical activated macrophages* – macrófagos ativados classicamente) expressam altos níveis de IL-1 β , IL-6, IL-12, IL-23, TNF- α e baixos níveis de IL-10, além da produção de ROS; são células presentes em respostas Th1. Esse fenótipo é ativado por IFN- γ e TNF- α e/ou produtos bacterianos (ex.: LPS) e também é denominado como M1. Macrófagos M1 possuem um importante papel contra patógenos intracelulares e mecanismos antitumorais. Em uma resposta inflamatória contra um patógeno as células NK (*Natural killer*) reagem produzindo IFN- γ transientemente, citocina que em uma resposta adaptativa é produzida por células Th1, o que pode levar os macrófagos a secreção de citocinas pró-inflamatórias, radicais de

nitrogênio e oxigênio, levando a uma maior capacidade de eliminação do micro-organismo invasor (18)

Já os macrófagos ativados alternativamente expressam altos níveis de IL-10 e TGF- β ; da enzima arginase, altos níveis dos genes *Chi3l3* (YM1) e *Retnla* (FIZZ1), além da expressão aumentada de receptores tipo galactose, manose (CD206) e *scavengers*; essas células participam de respostas Th2 (28–30).

O gene *Chi3l3* é membro da família das quitinases, originalmente descrito como um fator quimiotático de eosinófilo produzido por células T CD8. É um gene altamente expresso nos pulmões e baço de camundongos, porém sua função nos macrófagos ainda não está bem elucidada (33,34). Já o gene *Retnla* foi primeiramente identificado em fluido de lavagem de camundongo com asma experimentalmente induzida e é um membro da família das moléculas secretadas ricas em cisteína, sua função nos macrófagos ainda não é clara também (33,34).

O fenótipo ativado alternativamente (aaM ϕ – *Alternative activated macrophages* – macrófagos ativados alternativamente) pode ser induzido por IL-4 e/ou IL-13 e é também conhecido como M2 (**figura 2**) (35). Em muitas doenças parasitárias os macrófagos sofrem polarização a um fenótipo M2. Em infecções causadas por *Taeniasolium* e *Taenia crassiceps* em humanos, observa-se a polarização de macrófagos

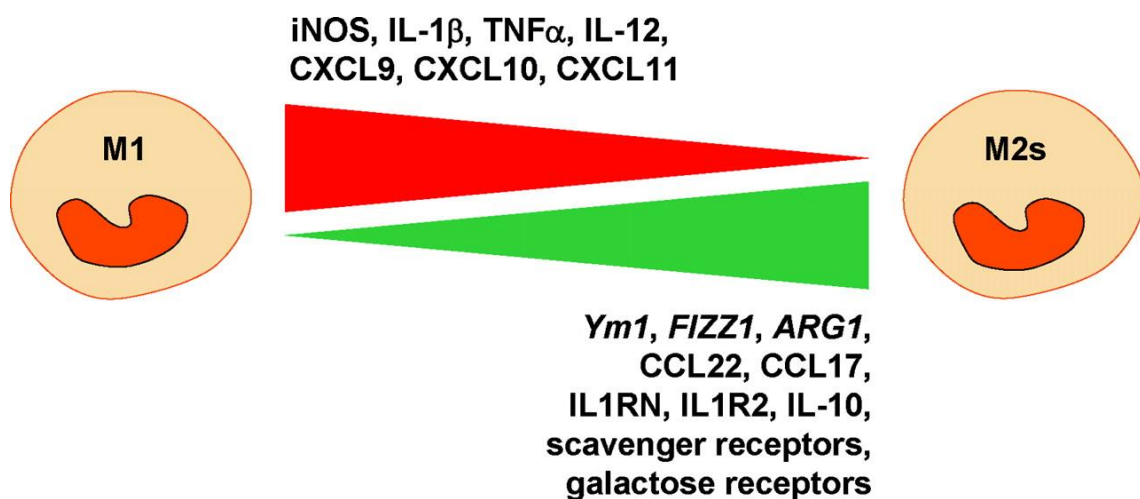


Figura 2 Ativação e polarização de macrófagos.

Acima em vermelho estão ilustradas algumas moléculas com aumentada expressão em macrófagos M1. Já em macrófagos M2 (em verde) são mostradas as moléculas mais expressas em macrófagos M2. IL1RN, antagonista do receptor de IL-1; IL1R2, receptor de IL-1 do tipo 2. (31,32)

a um fenótipo M2 após a fase aguda da doença (>4 semanas). Em infecções por *Schistosoma* os carboidratos dos ovos são os principais produtos que levam a uma resposta Th2, que pode formar um microambiente favorável ao desenvolvimento de macrófagos M2 (33). Macrófagos

ativados alternativamente estão presentes também em alergias, visto que essas propiciam um ambiente Th2, que é propício a polarização de macrófagos. A asma está associada a remodelação tecidual, incluindo a deposição de colágeno e hiperplasia de células caliciformes e os macrófagos M2 estão intimamente envolvidos neste processo (30).

1.3.2 Macrófagos e câncer

Em 1863, foi quando pela primeira vez se viu a presença de leucócitos em um tecido canceroso por Rudolph Virchow (36). Séculos se passaram e os estudos relacionando o papel e as características das células imunes associadas ao câncer continuam incessantes.

Os macrófagos são os leucócitos presentes em maior número na maioria dos tumores (37). Essas células, denominadas TAMs (*Tumor associated macrophages* - macrófagos associados a tumores) são recrutadas aos tumores através de quimiocinas. Funcionalmente, os TAMs possuem duas vias as quais podem atuar, uma, matando as células tumorais, na outra, podem promover a angiogênese, o crescimento tumoral e a metástase (36).

Nos estudos iniciais pensava-se que era necessária a presença de células T e NK para o recrutamento de TAMs. Após experimentos de transplante tumoral em hospedeiros com defeitos na imunidade mediada por células T e células NK, mostrou-se que a imunidade específica não é determinante para a infiltração de macrófagos, mas fatores liberados pelas próprias células tumorais são os que fazem o recrutamento celular (37).

Ao serem recrutados para os sítios tumorais, os macrófagos recebem estímulos que os levam a adquirir um fenótipo *M2-like*, visto a ausência de IFN- γ , ligantes de TLR, a presença de IL-10 e TGF- β secretados por células neoplásicas, fibroblastos e células T reguladoras (38). Estudos mostram a relação entre fenótipos *M2-like* e o aumento do grau e largura do tumor em pacientes com câncer de mama (39), *M2-like* e fibroblastos associados ao tumor com a piora de prognóstico em câncer colorretal (40) e com aumento da progressão tumoral em câncer de próstata (41).

1.4 Células dendríticas

As células dendríticas (DCs – “*Dendritic cells*”) são células que fazem uma ponte entre o sistema imune inato e adaptativo. Elas são ditas apresentadoras de antígeno profissionais e dentre esse grupo são as que apresentam com maior eficiência. São células essenciais na defesa do organismo contra uma série de patógenos. Essas células estão distribuídas por quase todo o corpo fazendo a vigilância do organismo e impedindo a disseminação silenciosa dos patógenos. Estão presentes principalmente em regiões de ligação com o ambiente, como mucosas e pele compondo 1 a 2% do total de células (42).

As células dendríticas possuem estágios de ativação e em cada um ela está voltada para funções específicas (**figura 3**). Uma DC imatura, é uma célula com alta capacidade de endocitose localizada nos tecidos periféricos. Essa célula expressa níveis baixos de moléculas co-estimulatórias e de MHC de classe II, dessa forma não possui a capacidade de ativar linfócitos T, levando-os a anergia. A DC também pode virar uma célula migratória, quando entra em contato com alguns estímulos, como: células apoptóticas, lactobacilos do intestino, TNF- α . Isso leva a uma menor expressão de algumas integrinas, que são moléculas que mantêm a adesão dessa célula ao tecido adjacente, maior expressão de metaloproteinase de matriz (MMPs – “*Matrix metalloproteinasis*”), além de uma maior expressão de receptores de quimiocinas, como CCR7, necessário para a entrada da DC no linfonodo. Porém, isso não é o suficiente para uma DC tornar-se madura, sendo necessário a estimulação por citocinas pró-inflamatórias ou PAMPs (*pathogen associated molecular patterns* – padrões moleculares associados aos patógenos) em PRRs (*pattern recognition receptors* – receptores de reconhecimento de padrões), como: TLRs, FcR (Receptores de porção Fc de anticorpos), receptores de moléculas do sistema complemento. Após esses estímulos, a DC está com sua maturação completa e isso leva a produção e liberação de citocinas como IL-1 β , IL-6, IL-12, TNF- α , que é o último sinal necessário para a ativação da célula T. Uma vez ativados, os linfócitos T promovem um verdadeiro diálogo com outras células do sistema imune, como linfócitos B e macrófagos, com o intuito de neutralizar as ações nocivas do patógeno (43–45)

Uma DC imatura é incapaz de apresentar antígenos via MHC de classe II, devido a alguns fatores importantes: (1) a degradação dos antígenos é ineficiente, podendo eles ser internalizados e permanecerem intactos nos lisossomos devido a falta de proteases; (2) o MHC pode ser exposto na superfície celular vazio ou ligado a molécula CLIP, o que impede a

ligação a qualquer peptídeo. Posteriormente, esse MHC é internalizado e pode retornar a superfície ou é movido para a degradação (43,46).

Em uma DC madura a situação se inverte, havendo muitos peptídeos para serem processados, muitas moléculas de MHC disponíveis, as enzimas proteolíticas aumentam sua atividade, degradação do MHC de classe II é diminuída, permitindo mantê-lo na superfície celular (43,45).

O estado de ativação das células dendríticas é muito importante em transplantes de órgãos, por exemplo. Visto que, após o órgão ser transplantado, DCs do enxerto e do receptor são ativadas, migram e apresentam antígenos do órgão transplantado no linfonodo drenante a células T. Dessa forma, torna-se importante, em situações como essa, criar-se um ambiente tolerogênico. Com isso, visto o papel essencial das DCs nessa resposta, modular essas células e conseqüentemente outras células, como os linfócitos T, torna-se um desafio essencial para obtermos avanços promissores nesse campo (47,48).

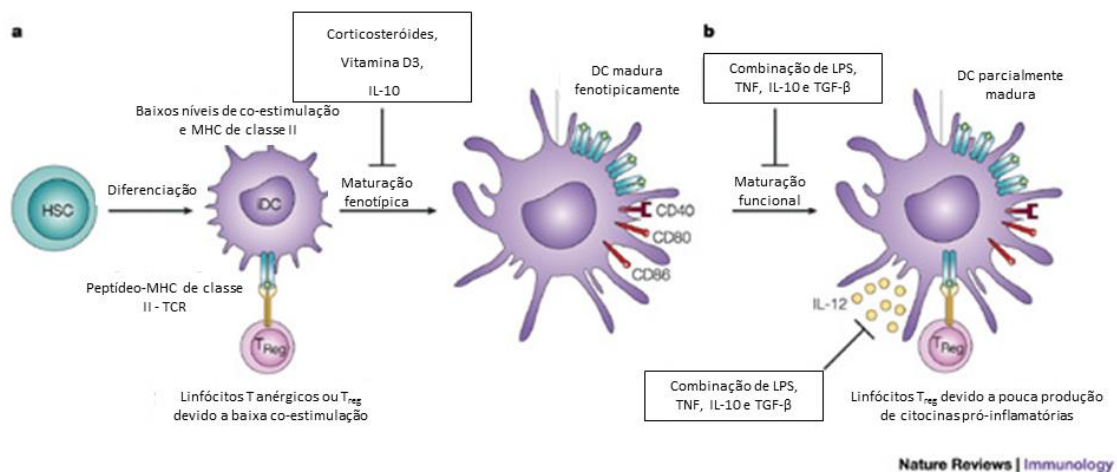


Figura 3 Estágios da maturação de DCs.

(a) A figura mostra que DCs imaturas apresentam baixos níveis de moléculas co-estimulatórias (ex.: CD40, CD80, CD86) e MHC de classe II. Dessa forma, essa célula é incapaz de ativar células T e leva-las a proliferação. Os estímulos que podem levar a inibição da maturação estão citados na figura, como: corticosteróides, vitamina D3, IL-10. Atualmente, essa figura teria uma lista maior de estímulos que bloqueiam a maturação das DCs e interessante a **DnaK de *M. tuberculosis*** está nessa lista (1). (b) Mostra as características da DC tolerogênica, que apresenta altos níveis de MHC de classe II, pouca produção de IL-12. Esse estado pode ser gerado pela combinação de agentes que promovem e suprimem a maturação (ex.: LPS, TNF, IL-10, TGF). (Figura traduzida de 49)

1.5 HSP70 e sistema imune

O primeiro estudo que mostrou que uma proteína de choque térmico pode ser liberada para o ambiente extracelular foi realizado em axônio gigante de lula (50,51). Além disso, foi observado que células tumorais submetidas a choque térmico e um período de recuperação apresentavam Hsp70 na superfície celular (52). A liberação de Hsp70 para o ambiente extracelular foi observada também em células de glioma submetidas a choque térmico ou condições normais (53). Também foi observado a liberação de Hsp70 via exossomos por uma via não-clássica em células brancas de sangue periférico (PBMCs – *Peripheral blood mononuclear cells*) (54). Após amplas evidências de sua liberação para o ambiente extracelular, muitos trabalhos começaram a relatar sua ação sobre outras células, como as do sistema imune (55–59).

Porém, grande parte dos estudos mostra que a Hsp70 é capaz de ativar células do sistema imune, mostrando até mesmo que podem se ligar a TLRs. Acredita-se que em sua maioria, esses efeitos se devem a endotoxinas contaminantes provenientes da purificação de proteínas recombinantes que não passaram por processos apropriados de limpeza ou com controles de limpeza inadequados. Outra possível causa é a presença de mais de um homólogo de Hsp70 presente na amostra (60), além de peptídeos que podem estar ligados à proteína (61), influenciando seu efeito sobre as células. Alguns métodos utilizam a purificação através de órgãos ou tecidos, como fígado e até mesmo sarcoma murino (60). Uma vez que há uma ampla variedade de genes que codificam as Hsps da mesma família, purificando a proteína de um tecido, estará se mascarando o efeito de um homólogo, para se avaliar o efeito de praticamente uma família inteira de proteínas.

Diversos estudos, mantendo controles adequados e cuidados com a purificação da Hsp70 mostraram efeitos reguladores sobre células do sistema imune inato, adaptativo e em algumas doenças (62) (**Figura 4**). Um estudo de nosso grupo em PBMCs de pacientes com artrite, observou um aumento na produção de IL-10 e uma concomitante diminuição na produção de IFN- γ após um tratamento *in vitro* de 48h com Hsp70 (DnaK) proveniente de *Mycobacterium tuberculosis* (63). U outro grupo observou que a Hsp70 humana (HSPA1A) levou a uma diminuição na produção de IL-6, IL-8 e MCP-1 induzidos por TNF- α em sinoviócitos de pacientes com Artrite reumatoide (64).

Esse efeito também é observado em células dendríticas, monócitos e macrófagos. Um estudo de nosso grupo observou que a Hsp70 de *Mycobacterium tuberculosis* impede a maturação de células dendríticas derivadas de medula óssea (BMDCs – *Bone marrow dendritic cells*), induzindo uma população de células com baixa expressão de MHC-II e CD86, além de uma aumentada produção de IL-10 (1). Observamos ainda em células dendríticas uma diminuição, quando tratado com DnaK de *Mycobacterium tuberculosis*, na expressão de dois fatores de transcrição característicos de citocinas pró-inflamatórias, C/EBP β e C/EBP δ , sendo um processo dependente de IL-10 e TLR2. Outro estudo de nosso grupo, mostrado em detalhes a seguir, observou que macrófagos derivados de medula óssea (BMM – *Bone marrow macrophages*) e peritoneais quando

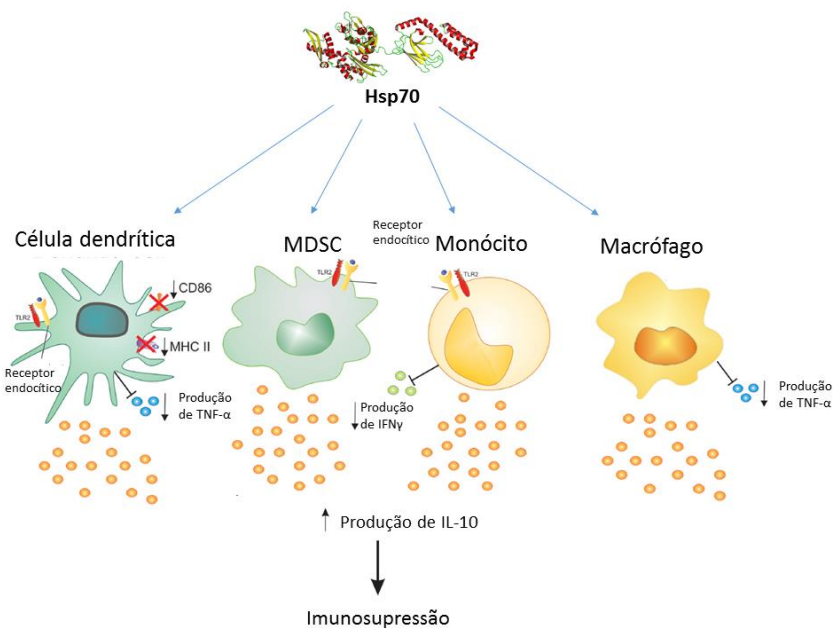


Figura 4 Hsp70 modulando células do sistema imune.

Uma ilustração dos diversos achados nos quais a Hsp70 modula células do sistema imune a um estado imunossupressor. A proteína é capaz de levar ao aumento de IL-10 em todas as células citadas acima e a uma diminuição na produção de citocinas pró-inflamatórias, como TNF- α e IFN- γ , além de baixar os níveis de co-estimulação. (Modificado e traduzido de 62)

tratados com DnaK por 24h são polarizados ao fenótipo *M2-like*. Isso ocorre devido ao aumento CD206 na superfície celular, produção aumentada de IL-10, aumento da expressão dos genes Fizz1 e Ym1 e a diminuição de TNF- α , IL-6, MCP-1 e de CD86 na superfície celular (65).

Ainda não se sabe o porquê dos efeitos desses efeitos celulares e em doenças ocorrerem com essa proteína e o porquê disso estar mais restrito a DnaK de *Mycobacterium tuberculosis*. Uma vez que essa é a proteína mais conservada evolutivamente, podia-se esperar que alguns

de seus homólogos possuísem efeitos semelhantes. Pensando nisso, um estudo avaliou o efeito da imunização com proteínas microbianas altamente conservadas, como: superóxido dismutase, aldolase, GAPDH e Hsp70 sobre ratos induzidos a desenvolver artrite reumatoide. Apesar de todas proteínas apresentarem-se imunogênicas, a única que apresentou efeito protetor foi a Hsp70, levando a uma resposta Th2 e uma produção aumentada de IL-10 e IL-4 (66). Além disso, outros estudos avaliaram o efeito de Hsps da mesma família, como a Hsp60.1 e a Hsp60.2 de *Mycobacterium tuberculosis* em PBMCs, tendo 76% de similaridade de sequência entre ambas. Observou-se que a Hsp60.1 levou a uma produção de 10 a 100 vezes superior de TNF- α , IL-1 β , IL-6, IL-8, IL-10 em relação a Hsp60.2 (67). Outro estudo comparando duas Hsp60 de *Rhizobium leguminosarum* mostrou que somente monócitos tratados com Hsp60.3 tiveram um aumento na produção de IL-1 β , TNF- α , IL-6, IL-8, IL-10 e IL-12 em comparação com Hsp60.1, sendo que ambas tem 74% de identidade de sequência (68). Apesar de não serem Hsp70, esses estudos ilustram bem que mesmo uma alta similaridade de sequência não garante funções semelhantes (69).

Por a Hsp70 ser uma proteína relativamente grande, pode ser que diferentes epítomos possam interagir com a célula e levar a mais de um tipo de resposta por essas proteínas. Foi observado que a DnaK possui um epítomo estimulador da maturação de células dendríticas, epítomo esse localizado na região de fenda de ligação ao peptídeo da Hsp70. Além desse epítomo, outro foi localizado, porém inibidor da produção de citocinas e da maturação de células dendríticas (70).

Além desses fatores que podem influenciar nas diferenças de efeitos das Hsp70, nosso grupo questiona-se também que diferentes modificações pós-traducionais podem estar influenciando no efeito da proteína. Uma vez que os estudos nesse campo não possuem uma padronização quanto a expressão da proteína em laboratório, isso pode levar a diversos vieses que podem influenciar os resultados da pesquisa. Pois, como comentado anteriormente, alguns laboratórios purificam a proteína de órgãos, outros com coluna de ADP ou ATP, expressam-na em levedura ou *E. coli*. Todos esses tipos de produções podem levar a pequenas modificações na estrutura da proteína que podem ser suficientes para influenciar no efeito sobre as células do sistema imune.

2 OBJETIVOS

2.1 OBJETIVO PRINCIPAL

Comparar o efeito imunomodulador da DnaK, Hspa1a murina e HSPA1A humana em macrófagos e células dendríticas.

2.2 OBJETIVOS ESPECÍFICOS

- 2.2.1 Validar o bioensaio para indicar o grau de contaminação com LPS das amostras protéicas;
- 2.2.2 Avaliar os marcadores fenotípicos de macrófagos tratados com DnaK *in vitro*;
- 2.2.3 Verificar a funcionalidade dos macrófagos tratados com DnaK *in vivo*;
- 2.2.4 Avaliar o mecanismo pelo qual ocorre a modulação fenotípica dos macrófagos pela DnaK;
- 2.2.5 Expressar e purificar a DnaK e Hspa1a no mesmo sistema de expressão;
- 2.2.6 Avaliar comparativamente o efeito imunomodulador da DnaK e HSPA1A em células imunes.

3 JUSTIFICATIVA

Os estudos no campo do efeito da Hsp70 extracelular em células imunes têm às vezes resultados controversos. Provavelmente devido a variáveis metodológicas (como sistema de produção, grau de contaminação e método de purificação) os resultados acabam variando muito entre os grupos de pesquisa focados nesse tema. O presente estudo visa avaliar o efeito da DnaK e alguns de seus ortólogos sobre células do sistema imune usando o mesmo sistema de purificação. Dessa forma, em um futuro, tentar padronizar a forma com que os experimentos são conduzidos nesse campo e contribuirá para o entendimento dos mecanismos moleculares pelos quais os ortólogos de Hsp70 exercem suas funções imunes.

Capítulo II

4 Bioensaio

Antes de todas amostras protéicas serem utilizadas para experimentos *in vitro* e *in vivo*, elas passaram por um bioensaio para determinar contaminação por endotoxinas.

Controle de qualidade de endotoxinas na amostra protéica (Bioensaio)

Esse procedimento foi descrito por (71) e tem o intuito de avaliar a presença da endotoxina LPS de uma maneira indireta, através do estado de ativação de células dendríticas do baço. Uma injeção intravenosa na veia caudal é dada ao camundongo da proteína ou controles que se pretende dar. Proteínas e PBS passam previamente por um protocolo de Triton X-114 para retirar o LPS das amostras e posteriormente são incubados com *Biobeads* (*Biorad*) para a remoção do Triton das amostras. Após 6h, o baço é extraído, é feito um protocolo de digestão enzimática com colagenase (72) e essas células são marcada com anticorpos contra proteínas de superfície celular de DCs. Dessa forma, caso a DC aumente a expressão de CD86 e MHC de classe II em sua superfície, isso indica que ela é uma DC madura e a amostra está contaminada com LPS. Esse teste é realizado, pois segundo observações de laboratório, embora não haja publicações a respeito, o teste *Limulus* (ensaio que visa realizar a detecção e quantificação de LPS em amostras) apresenta-se com baixa sensibilidade.

injeção i.v.:

- LPS
- DnaK antes do triton
- DnaK e HSPA1A depois do triton
- PBS

Análise de CD86 e MHC II em esplenócitos CD11c+



Figura 5 Esquema do bioensaio

Injeção intravenosa na veia caudal de PBS, LPS, DnaK ou HSPA1A por 6h. Após 6h o animal é eutanasiado, seu baço é extraído e o tecido é digerido com a enzima colagenase. Uma marcação é realizada com os anticorpos α -CD11c, α -B220, α -CD86, α -IA^b. As amostras são lidas em citômetro de fluxo e analisadas no programa flowjo (Imagem presente em manuscrito recém submetido presente nesta dissertação)

Abaixo está representada a estratégia de gates utilizada para analisar a expressão de CD86 e MHC de classe II na superfície celular das DCs.

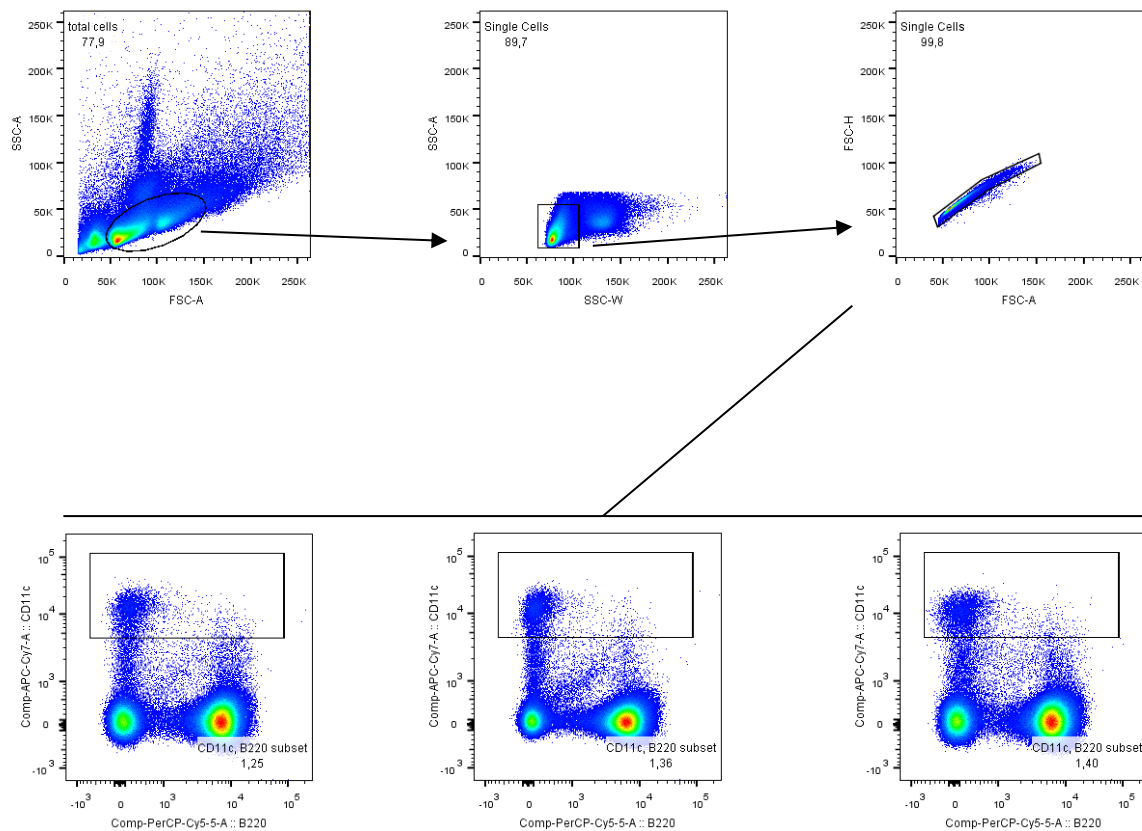


Figura 6 Estratégia de gates.

À direita em cima é feito o primeiro gate que leva em conta o tamanho e granulosidade nas DCs e linfócitos. Os próximos dois ao lado são os gates nas *single cells*. Dessa forma, há a exclusão de células que poderiam passar juntamente pela detecção do laser no citômetro. Abaixo nos três *dot plots* são feitos *gates* em células dendríticas de acordo com a exclusão de células CD11c⁻.

Na **figura 7a** abaixo uma representação da expressão de MHCII e CD86 na forma de histograma em relação a HSPA1A. Pode-se observar que o camundongo injetado com HSPA1A apresentou uma expressão de CD86 e IA^b na superfície das DCs semelhante ao camundongo PBS, assim como pode ser observado no gráfico de barras da MFI (*mediam fluorescence intensity* = intensidade de fluorescência média) na **figura 7b**. Na **figura 7c-d** também pode ser observado o mesmo padrão em relação a DnaK, apresentando uma expressão de CD86 e IA^b até mesmo inferior ao grupo PBS. É importante observar que a DnaK que não passou pelo processo de limpeza do LPS apresentou uma expressão de ambos marcadores muito alta, sendo muito semelhante ao grupo LPS.

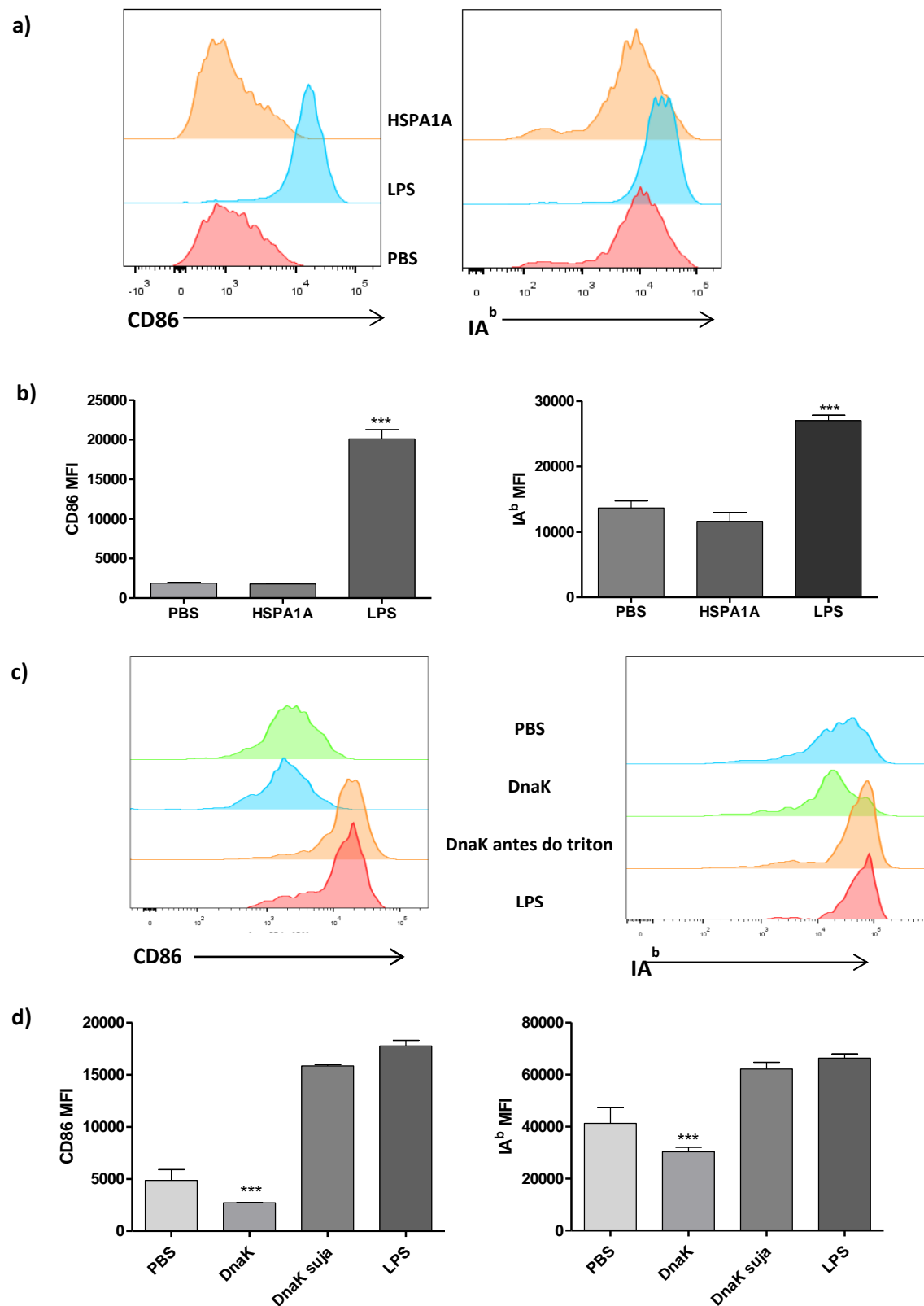


Figura 7 Expressão de CD86 e MHC de classe II nas DCs do baço.

Tratamentos com PBS, DnaK suja (antes do triton), DnaK, HSPA1A e LPS por 6h através da veia caudal e posterior extração do baço e preparação celular (**a e b**) Histogramas e gráficos em barra da MFI da expressão de CD86 e MHCII em células tratadas com HSPA1a, PBS ou LPS; (**c e d**) Histogramas e gráficos em barra da MFI de CD86 e MHCII em células tratadas com DnaK, PBS ou LPS.

Capítulo III

5 Artigo publicado na revista PLOS ONE, intitulado:

“Extracellular Mycobacterial DnaK Polarizes Macrophages to the M2-Like Phenotype”
em 24 de novembro de 2014

RESEARCH ARTICLE

Extracellular Mycobacterial DnaK Polarizes Macrophages to the M2-Like Phenotype

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Abstract

Macrophages are myeloid cells that play an essential role in inflammation and host defense, regulating immune responses and maintaining tissue homeostasis. Depending on the microenvironment, macrophages can polarize to two distinct phenotypes. The M1 phenotype is activated by IFN- γ and bacterial products, and displays an inflammatory profile, while M2 macrophages are activated by IL-4 and tend to be anti-inflammatory or immunosuppressive. It was observed that DnaK from *Mycobacterium tuberculosis* has immunosuppressive properties, inducing a tolerogenic phenotype in dendritic cells and MDSCs, contributing to graft acceptance and tumor growth. However, its role in macrophage polarization remains to be elucidated. We asked whether DnaK was able to modulate macrophage phenotype. Murine macrophages, derived from bone marrow, or from the peritoneum, were incubated with DnaK and their phenotype compared to M1 or M2 polarized macrophages. Treatment with DnaK leads macrophages to present higher arginase I activity, IL-10 production and FIZZ1 and Ym1 expression. Furthermore, DnaK increased surface levels of CD206. Importantly, DnaK-treated macrophages were able to promote tumor growth in an allogeneic melanoma model. Our results suggest that DnaK polarizes macrophages to the M2-like phenotype and could constitute a virulence factor and is an important immunomodulator of macrophage responses.



OPEN ACCESS

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Introduction

Macrophages are myeloid cells which have an important role during inflammation, infection resolution, tissue repair and cancer [1]. These cells have a marked phenotypic heterogeneity, which is dependent on the microenvironment conditions. T helper 1 (Th1) or T helper 2 (Th2) cytokines stimulate macrophage to differentiate into two opposed phenotypes. Classically activated macrophages (M1) are induced by Th1 cytokines (IFN- γ), or by bacterial products (e.g LPS). They are able to control infections, have a tumoricidal activity and secrete high levels of pro-inflammatory cytokines. Alternatively activated macrophages (M2) are induced by Th2 cytokines (IL-4 and/or IL-13) and have important roles in allergy, parasitic infections and tissue repair [2]. Both phenotypes can be differentiated by surface receptors, gene expression and cytokines profile produced. M1 macrophages express CD80, CD86, produce NO and secrete the pro-inflammatory cytokines TNF- α , IL-12, IL-6 and IL-1 β . M2 macrophages express CD206 and CD163. They can produce IL-10, TGF- β and show an increased arginase I activity [3]. In addition, M2 macrophage polarization can be defined based on a specific genetic signature characterized by the upregulation of Ym1 (also known as *Chil3l3*) and FIZZ1 (also known as *Retnla*) genes [4, 5].

Both in infections and tumors, a switch from Th1 (or M1) to Th2 (or M2) immunity can occur, leading to the generation of a suppressive environment that abrogates effector immunity [6]. During mycobacterial infections, the generation of suppressive macrophage populations coincides with a switch from a Th1 to a Th2 response, and such macrophages are important for the persistence of the pathogen [7]. *Mycobacterium tuberculosis* can reprogram macrophages to M2 via secretion of IL-10 [8], a major immunosuppressant that counteracts IFN- γ and TNF- α , the two major cytokines that drive the effective response that clears the infection. In tumors, macrophages infiltrate the microenvironment, and modulate T-cell and stroma activity, either promoting or inhibiting tumor progression [9]. In established tumors, macrophages are biased toward the M2-like phenotype which has tumor-promoting functions [10–13] correlating with poor prognosis [14, 15].

The chaperone DnaK is the major bacterial counterpart of heat shock protein 70 (HSP70) [16]. Extracellular HSP70 from different sources has been demonstrated to have protective and regulatory roles in different inflammatory disease models as arthritis [17, 18], colitis [19], transplants [20] and brain ischemia [21]. These effects were reported to be due to modulation of dendritic cells (DCs) [22–24] and monocytes [25] to a tolerogenic state, inducing IL-10 production and downregulating MHC class II.

Recently, DnaK was found in vesicles released by *Mycobacterium tuberculosis* [26]. Administration of these vesicles to mice before infection accelerated the pathogenesis of the disease, suggesting that it could have a role in mycobacterial infection. Nevertheless, the immune effects of prokaryotic Hsp70 in macrophages have never been addressed.

In the present study we investigated whether mycobacterial DnaK polarizes murine macrophages. Macrophages treated with DnaK behaved like M2 macrophages. Furthermore, these cells presented M2 function *in vivo* in an allogeneic murine melanoma model, enhancing tumor growth. Our results indicate that macrophages treated with DnaK become functional M2-like cells, with tumor promoting potential.

Results

Extracellular DnaK induces the expression of M2 markers in bone marrow-derived macrophages

To verify the effect of DnaK treatment on macrophages polarization, we treated macrophages differentiated from bone-marrow cells (BMMs) of B6 mice with different DnaK concentrations and compared iNOS and arginase activities between cells stimulated with LPS (M1), IL-4 (M2) or untreated cells. iNOS activity was induced in M1 macrophages but not in cells treated with DnaK (30 or 60 $\mu\text{g}/\text{mL}$) or in M2 (Fig. 1A). However, DnaK treatment increased the activity of arginase in both concentrations tested when compared with control or M1 macrophages (Fig. 1B). The increase of arginase activity by DnaK was similar to the one in macrophages treated with IL-4 (Fig. 1B). Because there were no differences between both DnaK concentrations that were tested, we used 30 $\mu\text{g}/\text{mL}$ in all of the following experiments.

Murine polarized macrophages exhibit a distinct gene signature which can be used as polarization-associated markers [27]. The M2 phenotype is associated with the expression of Ym1 and FIZZ1 [4, 5]. To evaluate whether DnaK can induce these M2 gene markers, we treated BMMs with LPS, IL-4 or DnaK for 24 h and then assessed both Ym1 and FIZZ1 mRNA levels by real time PCR. DnaK induced Ym1 (Fig. 1C) and FIZZ1 (Fig. 1D) mRNA expression by macrophages to levels superior to the ones in IL-4 polarized M2 macrophages. LPS treated BMMs (M1) did not express any of the two markers. Altogether, these data demonstrate that the treatment of BMMs with DnaK induces the expression of well-characterized markers associated with M2 phenotype.

DnaK induces release of M2-like cytokines by macrophages

To investigate the profile of cytokines released by the BMMs, we analyzed the production of TNF- α , MCP-1, IL-6, IL-10 and TGF- β upon stimulation with LPS, DnaK or IL-4 for 24 h. The treatment of BMMs with LPS led to an increased production of TNF- α when compared to control, IL-4 and DnaK treatments (Fig. 2A). Production of IL-6 was lower in the DnaK group when compared to LPS, and similar to M2 macrophages (Fig. 2B). Likewise, MCP-1 production was lower in DnaK-treated macrophages when compared to M1 and M2 phenotype and similar to the control (Fig. 2C). In contrast, DnaK or M2 macrophages produced higher levels of IL-10 when compared with LPS or control (Fig. 2D). We also evaluated the TGF- β production by treated BMMs. DnaK treatment did

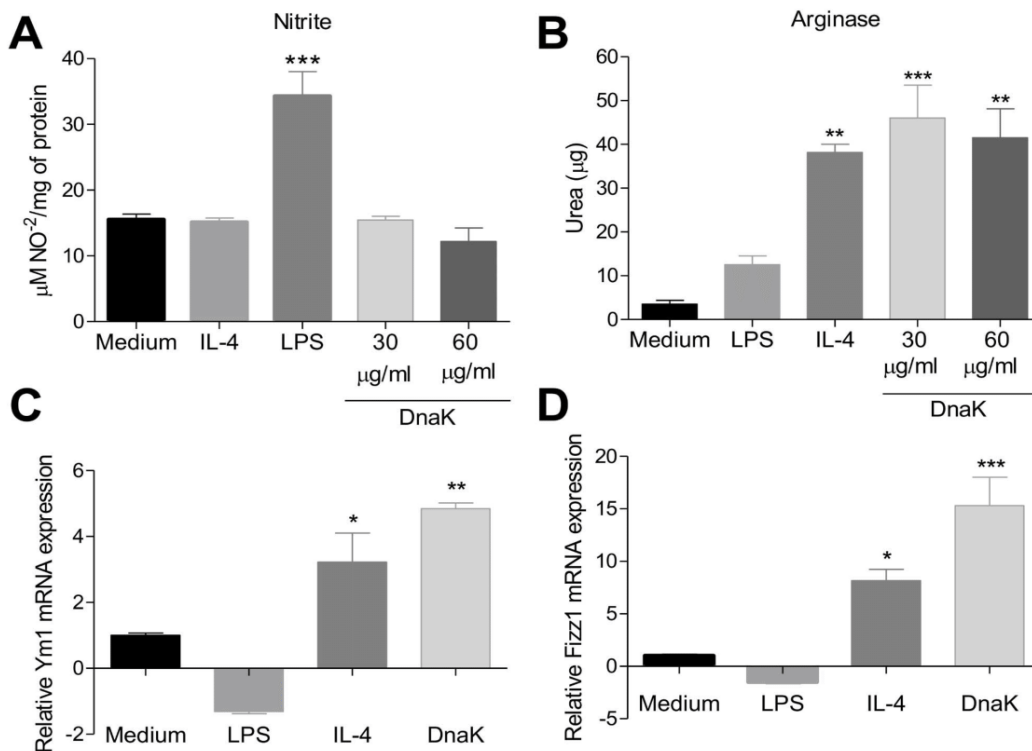


Figure 1. Extracellular DnaK induces the expression of M2 markers in bone marrow-derived macrophages. BMMs were treated with LPS (30 ng/ml), IL-4 (40 ng/mL), DnaK (30 µg/mL or 60 µg/mL), or left unstimulated for 24 h. (A) iNOS activity was determined by nitrite (NO²⁻) accumulation in the supernatant of macrophages. Data are the mean ± S.D. from triplicates. Data representative of three independent experiments. (***) p<0.001 indicates difference between LPS and other treatment groups. (B) Arginase activity was assessed by measuring the formation of urea from arginine. Data are the mean ± S.D. from triplicates. (***) p<0.001 and (**) p<0.01 indicate difference between treated groups and the medium group. Effect of DnaK on Ym1 (C) and FIZZ1 (D) expression in macrophages were quantified by real time PCR. The total amount of Ym1 and FIZZ1 mRNA were normalized to β-microglobulin signals and expressed as 2^{-ΔΔCT}. The values represent means ± SEM from triplicates. Data representative of three independent experiments. All data were analyzed by one-way ANOVA with Tukey post hoc test.

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not induce the production of TGF-β (data not shown). Thus, murine macrophages treated with DnaK are similar to M2 macrophages, producing low levels of M1 cytokines but high levels of IL-10.

DnaK induces M2 phenotype surface markers

Classically activated macrophages (M1) express CD80 and CD86 on surface. CD206 - the mannose receptor - is a specific marker of alternatively activated macrophages (M2). To analyze the expression of these surface molecules, we stimulated BMMs with DnaK, LPS or IL-4 and analyzed them for the expression of CD206 and CD80 by flow cytometry. [Figure 3A](#) shows representative dot plots of the cell surface stainings analyzed. DnaK treatment decreased the percentage of CD80+ macrophages ([Fig. 3A and C](#)) as well as CD80 MFI ([Fig. 3E](#)) in cells. In

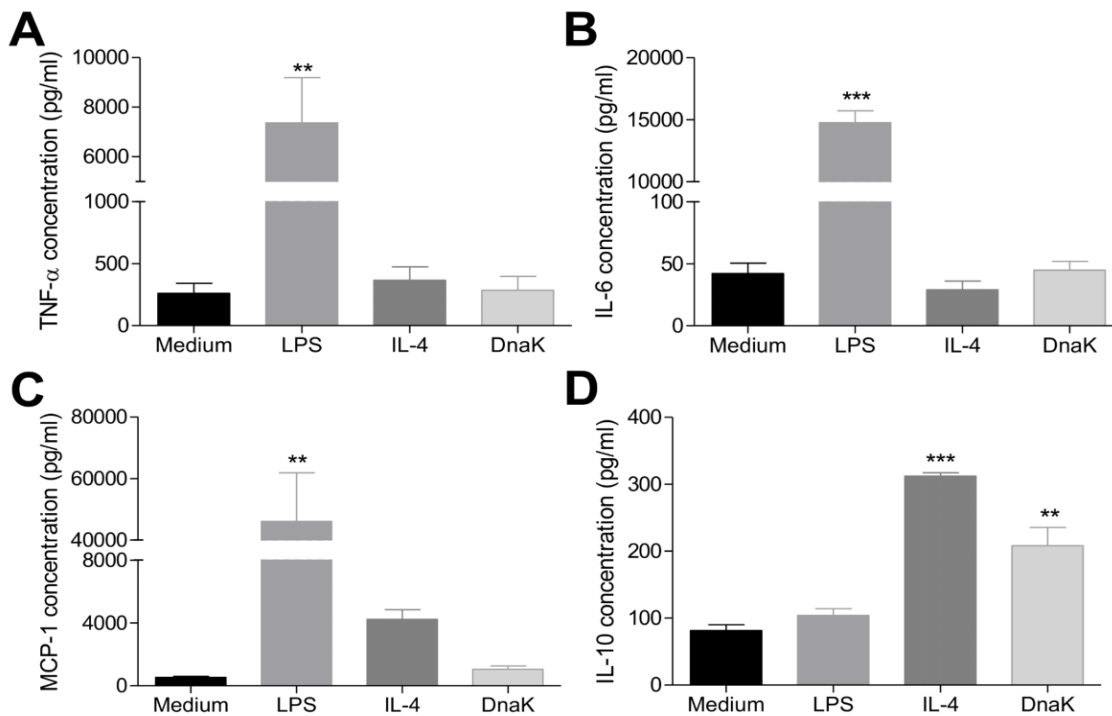


Figure 2. Macrophages release an M2-like cytokine profile upon stimulation with DnaK. BMMs were treated with 30 ng/mL of LPS, 40 ng/mL of IL-4, 30 μ g/mL or 60 μ g/mL of DnaK or left unstimulated for 24 h. (A) TNF- α , (B) IL-6, (C) MCP-1 and (D) IL-10 were measured from culture supernatants by flow cytometry. The values represent means \pm SEM in pg/ml from triplicates. (**) $p < 0.01$ and (***) $p < 0.001$ indicate significant difference between treated groups and medium group. All data has been by one-way ANOVA with Tukey post hoc test. Data representative of four independent experiments.

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contrast, treatment with DnaK increased the percentage of CD206+ cells (Fig. 3B and D) as well as CD206 MFI (Fig. 3F) in comparison to other treatments. These results indicated that macrophages treated with DnaK presented a profile of surface molecules consistent with that is observed in alternative activated (M2) macrophages.

We also asked if DnaK could have modulatory effect in another macrophage population. To answer that, we treated peritoneal macrophages with DnaK and compared them with M1 and M2 macrophages. DnaK-treated peritoneal macrophages presented a lower percentage of CD80+ cells when compared control and M1 macrophages (Fig. 4A and B). Also, macrophages expressing CD80 were similar in the M2 and DnaK-treated cells (Fig. 4A and B). In contrast, macrophages treated with extracellular DnaK showed a higher expression of CD206 than other treatments, including M2 macrophages (Fig. 4C). Thus, extracellular DnaK has immune modulatory effects in both bone marrow-derived and peritoneal macrophages.

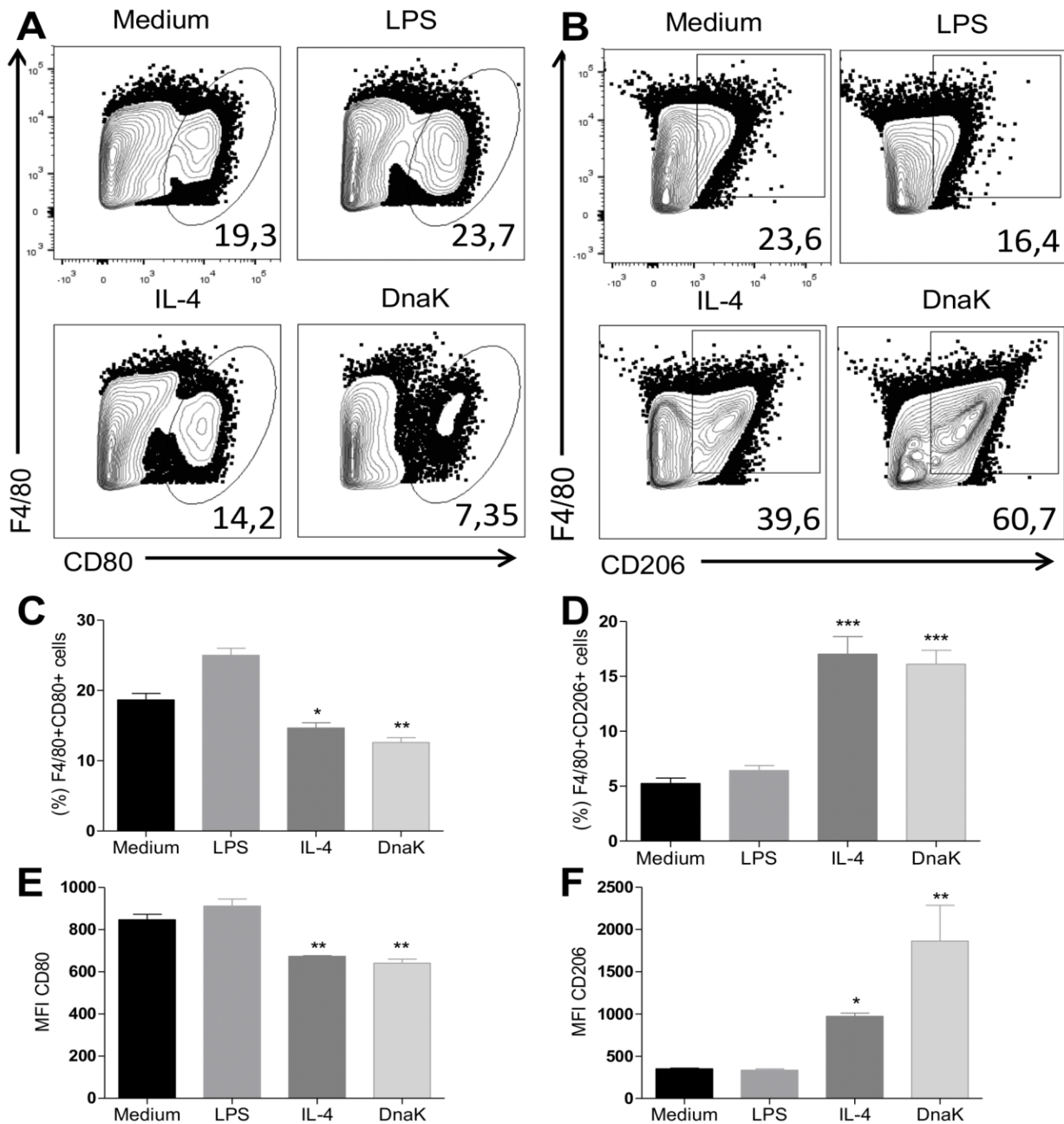


Figure 3. Induction of M2 surface marker CD206 by DnaK treatment. Representative dot plots of surface (A) CD80 and (B) CD206 expression in BMMS treated with 30 ng/mL of LPS, 40 ng/mL of IL-4, 30 μ g/mL or 60 μ g/mL of DnaK, or left unstimulated for 24 h. The percentage values of (C) F4/80⁺CD80⁺ and (D) F4/80⁺CD206⁺ cells represent means \pm SEM from triplicates. (E) and (F) show respective values for MFI analyses. (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ indicate significant difference treated groups in relation to medium group. All data were analyzed by one-way ANOVA with Tukey post hoc test. Data are representative of three independent experiments.

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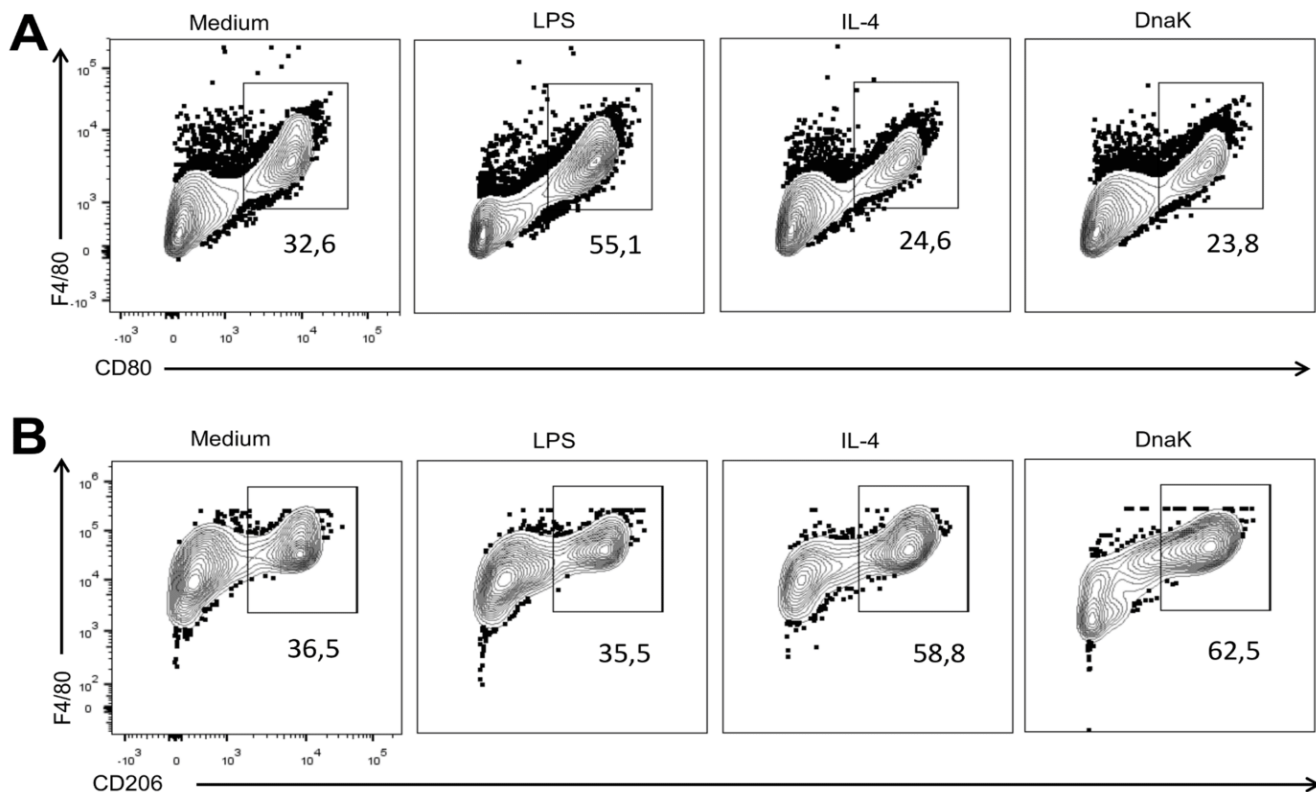


Figure 4. Extracellular DnaK induces the expression of CD206 in peritoneal macrophages. Peritoneal macrophages were isolated from B6 mice and treated with 30 ng/mL of LPS, 40 ng/mL of IL-4, 30 μ g/mL or 60 μ g/mL of DnaK, or left unstimulated for 24 h. After that, cells were analyzed by flow cytometry and data presented as representative dot plots of (A) CD80, (B) CD206 expression. Data representative of three independent experiments.

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DnaK-treated macrophages promote melanoma growth in mice

To test whether the M2-like macrophages generated by DnaK treatment were functional M2s, we tested their ability to promote tumor growth in an allogeneic murine melanoma model. We co-injected BMMs previously treated with DnaK or M1 macrophages, or untreated BMMs, with B16F10 (B16) cells (I-A^b) in BALB/c mice (I-A^d) and followed tumor growth over several days.

On the 16th day after co-injection of B16 tumors and BMMs, the mice were euthanized and their tumors removed and dissected. Tumors were first digested with collagenase D and the cells in a single cell suspension were stained with antibodies for flow cytometry analysis. The results of this analysis are shown in [Figure 5](#). Sixteen days after co-injection of tumors and treated allogeneic BMMs, these macrophages can no longer be found alive inside the tumors. In fact, most of the macrophages inside the tumors are not viable ([Figure 5, A and B](#)). Of the viable macrophages found infiltrating the tumors, none are IA^b+ ([Figure 5C](#)).

[Figure 6](#) shows the results for tumor growth upon injection with polarized BMMs. Tumors co-injected with M1 macrophages, untreated macrophages, or alone could not develop in BALB/c hosts ([Fig. 6](#)). Nevertheless, when B16F10 cells

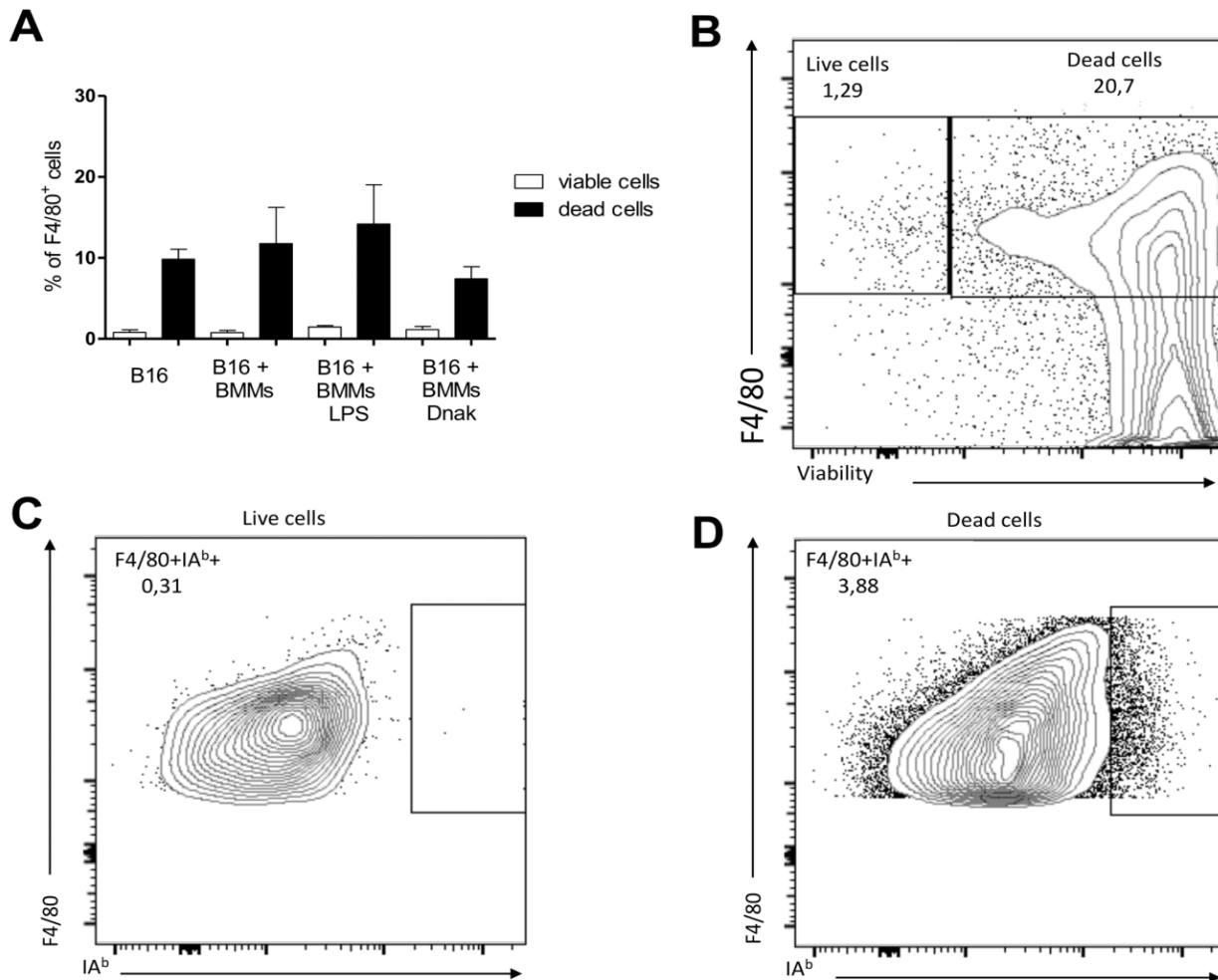


Figure 5. Viability of tumor infiltrating F4/80⁺IA^{b+} cells 16 days after co-injection. All mice were euthanized and tumors dissected and digested with collagenase D. The single cell suspension obtained was stained for flow cytometry, with antibodies against MHC class II allotype (I-A^{b+}), F4/80 as well as viability stain. (A) Bar graph with the percentages of tumor infiltrating F4/80⁺ cells, dead or alive; (B) Dot plot representative of the difference in viability of these two populations; (C) representation of F4/80⁺IA^{b+} in the viable population; (D) representation of F4/80⁺IA^{b+} in the non-viable population.

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were injected together with DnaK-polarized macrophages, tumors were capable of growth in the allogeneic host (Fig. 6B). This difference in tumor growth could be observed macroscopically (Fig. 6C). In addition, tumors that were co-injected with DnaK-treated macrophages were bigger when compared to other groups (Fig. 6D). Altogether, these findings showed that extracellular DnaK induces M2-like macrophages with tumor-promoting potential.

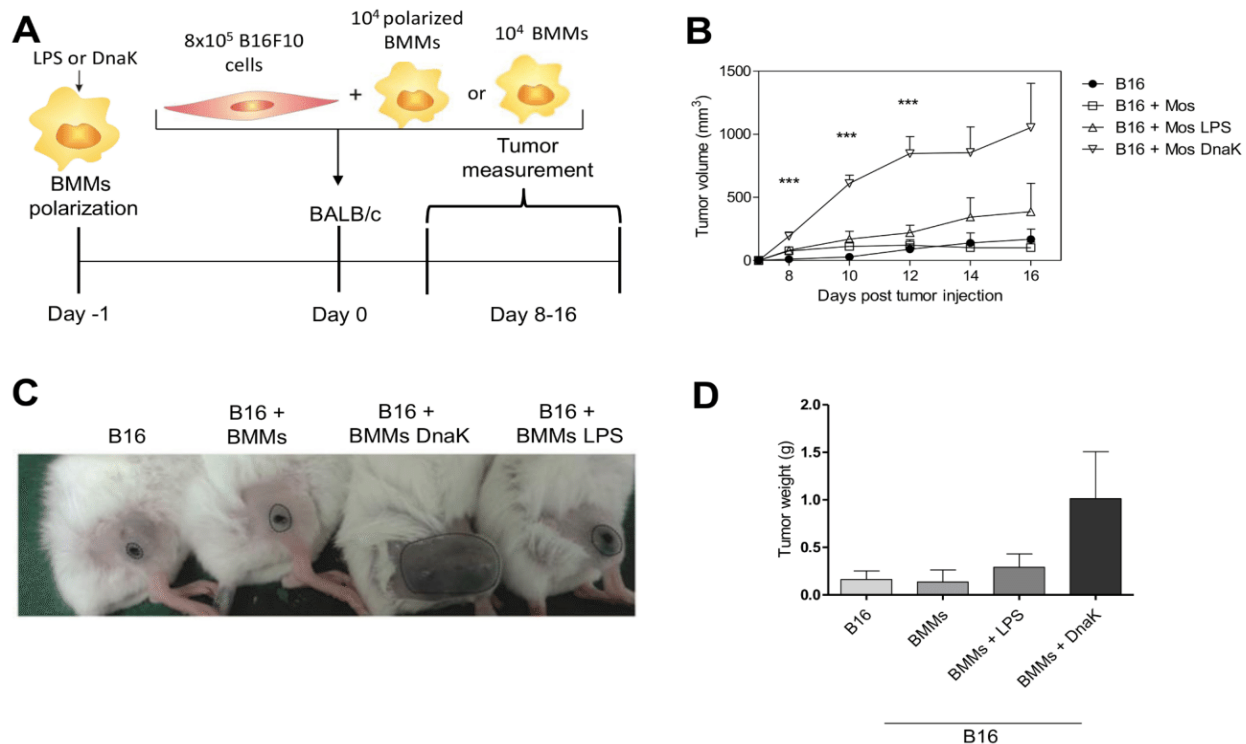


Figure 6. DnaK-treated macrophages enhance tumor growth in murine allogeneic melanoma model. (A) The murine melanoma cell line B16F10 was co-injected with macrophages exposed to 30 μ g/mL DnaK, 30 ng/ml of LPS, untreated macrophages, or no other cells for 24 h as illustrated in experimental design. (B) Cells were subcutaneously injected into BALB/c mice (4 mice per group) and the tumor volume was measured 8 days later as indicated. The values represent means \pm SEM. (*) $p < 0.05$ and (***) $p < 0.001$ indicate significant difference between the macrophages exposed to DnaK in relation to B16 group. The data were analyzed by one-way ANOVA with Tuckey post hoc test in each time point. (C) Macroscopically view of tumor size. (D) Tumor weight on day 16 after tumor injection. The values represent means \pm SEM. All data representative of three independent experiments.

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Discussion

In this study we provide functional evidence that DnaK from *Mycobacterium tuberculosis* skews macrophages towards the M2 phenotype. This may, at least in part, explain the role of DnaK in mycobacterial virulence.

Some of the strategies used by mycobacteria to polarize macrophages to M2 have been described. *M. tuberculosis* can secrete lipoarabinomannan which inhibits IFN- γ induced macrophage activation [8]. In addition, mycobacteria can shift macrophages to M2 by inducing IL-10 [28, 29]. Indeed, high levels of macrophage-derived IL-10 are correlated with active TB in humans [30, 31]. After efficient treatment of TB patients, this anti-inflammatory cytokine profile shifts to inflammatory [31, 32]. Also, in human macrophages infected with *M. tuberculosis*, the repression of IL-10 leads to an enhancement of phagosome protease activity, leading to a higher eradication of the pathogen [33].

In vivo, macrophage polarization induced by IL-10 is associated with the production of this cytokine in infected organs [30, 34] and tumor microenvir-

onment [35, 36]. To assess the role of IL-10 specifically produced by macrophages, Schreiber *et al.* infected macrophages from mice in which IL-10 was upregulated in these cells with *Mycobacterium tuberculosis* [28]. These animals were more susceptible to infection, died early and exhibited a higher bacterial load in the lungs. In the same study, *M. tuberculosis* infected macrophages had a M2 phenotype. In *Toxoplasma gondii* infected RAW 264.7 macrophages, the parasite Hsp70 inhibited production of NO and NF- κ B activation, resulting in increased parasite load [37].

In both cases, macrophage modulation is a major strategy to evade effector immune responses, avoiding tumor destruction and pathogen eradication. We show that DnaK induce the production of IL-10 by macrophages and polarization to M2 phenotype. Because most of the co-injected allogeneic macrophages are found dead inside the tumors on day 16, we believe that the effect of the DnaK polarized BMMs is very robust and occurs very early, allowing the implantation of the tumor in the host. It is possible that DnaK acts as an immunomodulator with a putative virulence role in bacterial infections, polarizing macrophages to M2, with production of IL-10.

DnaK was found within vesicle membranes released by mycobacteria which can modulate macrophages in a pathway dependent on TLR2 [26]. Other molecules from *Mycobacterium tuberculosis*, like the PPE18 protein, induce IL-10 production through TLR2 in order to evade effector immune responses mediated by CD4⁺ T cells [38, 39]. Chalmin *et al.* demonstrated that both murine and human HSP70 present in exosome membranes released from tumor cells enhances immunosuppressive functions of MDSCs [40], leading to tumor growth, in a TLR-2, IL-10 dependent mechanism. It is thus possible to hypothesize that other members of the Hsp70 family, when released from infected or tumor cells in vesicles, can engage a TLR2 pathway, leading to IL-10 production in myeloid cells, and polarizing macrophages to an M2 phenotype. This pathway would be activated both in infections and tumors, and further experiments are necessary to test this hypothesis.

Materials and Methods

Mice

6 to 10-weeks-old female C57BL/6 and BALB/c mice were purchased from FEPPS (Rio Grande do Sul, BRA). All mice were housed in individual and standard mini-isolators (Techniplast) in an SPF facility (Institute of Biomedical Research – PUCRS) with free access to water and food. Mice used in experiments have a range of weight between 18–22 g. The method of euthanasia used was a carbon dioxide (CO₂) chamber (Beirmar). All procedures were performed in accordance with the guidelines of the Federation of Brazilian Societies for Experimental Biology and approved by the Ethics Committee for the Use of Animals of Pontifícia Universidade Católica do Rio Grande do Sul (CEUA-PUCRS) under protocol ID CEUA 12/00316.

Protein purification and LPS extraction

Recombinant DnaK of *Mycobacterium tuberculosis* was produced with the construct pET23a(+)/MtbDnaK in XL1-blue *Escherichia coli* and purified according to Mehlert [41]. To remove LPS, Triton X-114 was used according to the method described in Aida et al. [42]. Contaminating Triton X-114 was removed by incubating overnight with Bio-Beads (Bio-Rad) at 4°C with agitation, as described in [22]. Protein concentration was determined using Qubit Protein Assay Kit (Invitrogen) and the Qubit Fluorometer (Invitrogen).

Macrophages cultures and polarization

Macrophages were derived from bone marrow of C57BL/6 WT mice. Cells (10^6) were cultured in 24-well plates in serum-free medium AIM-V (Gibco) with 10 ng/mL of GM-CSF (Peprotech). At day 3, medium was collected and cells were cultured for a further 3 days in the presence of fresh AIM-V with 10 ng/mL of GM-CSF. On the seventh day of culture, the non-adherent cells were separated from adherent cells (macrophages) and stimulated as described below. The purity in BMMs cultures was higher than 90% as assessed by staining with anti-F4/80 antibodies (data not shown).

Peritoneal macrophages were collected by peritoneal cavity wash with 5 mL of sterile serum-free AIM-V medium (Gibco). The cells were washed twice with sterile PBS and suspended in AIM-V, transferred to a 24 multi-well plates and allowed to attach for 30 min. Unattached cells were washed out with medium. The adherent cells, mainly peritoneal macrophages, were used for the experiments thereafter. Macrophages were evaluated by microscopic examination with May-Grunwald and Giemsa stains, indicating macrophage purity higher than 80%. Purity was confirmed by flow cytometry, using the F4/80 Ab (data not shown).

The obtained macrophages (from bone marrow or peritoneum) were stimulated for 24 h in serum-free AIM-V with 30 or 60 μ g/mL of DnaK or left unstimulated. For the generation of classically or alternatively activated macrophages, cells were stimulated with LPS (30 ng/mL) or IL-4 (40 ng/mL) (both purchased from Peprotech) for 24 h, respectively.

Arginase Assay

Arginase activity in cell lysates was measured based on the conversion of L-arginine to L-ornithine and urea according to the technique described by Corraliza and collaborators [43] with minor modifications. Briefly, cells were lysed for 30 min with 40 μ L of PBS containing 0.1% Triton-X-100. 30 μ L of 25 mM Tris-HCl, pH 7.4 and 10 μ L of 10 mM MnCl₂ were added and the enzyme was heat-activated for 10 min at 56°C. Similar amounts of samples (40 μ L) and 0.5 M L-arginine (pH 9.7) were mixed and incubated for 1 h at 37°C. The reaction was stopped by adding 400 μ L of H₂SO₄ (96%), H₃PO₄ (85%), H₂O (1/3/7, v/v/v). The urea concentration was measured at 540 nm after the addition of 8 μ L of

α -isonitropropiofenone 6%, followed by heating at 95°C for 30 min. Values were compared with a standard curve of urea concentration.

Nitrite Assay

Nitrite concentrations were measured using the Greiss reaction [44]. We used the Greiss Reagent Kit for Nitrite Determination (Molecular Probes), according to manufacturer's instructions. Samples were quantified by spectrophotometry at 540 nm using sodium nitrite as standard.

Flow cytometry

The Fc receptors of macrophages were blocked with 24G2 supernatant containing 10% mouse serum and 10% rat serum, and later stained for F4/80 (BM8) from eBioscience; CD80 (16-10A1) from BD Biosciences; and CD206 (MR5D3) from AbDSerotec. Cells were analyzed using FACSCanto II (BD Biosciences) and BD FACSDiva software (BD Biosciences). Data obtained were analyzed using Flowjo software (version 7.6.5, Tree Star).

On Day 16 after the subcutaneous co-injection of polarized BMMs and B16 tumor cells, the mice were euthanized and tumors were excised and digested with collagenase D (Roche). The single cell suspension obtained was filtered to eliminate debris and stained for flow cytometry, using antibodies to F4/80, IA^b, CD86 and viability (Fixable Viability Dye from eBioscience).

Total RNA isolation and cDNA synthesis

Total RNA was isolated from murine macrophage cultures using RNAeasy kit (Qiagen) according to manufacturer's instructions. The concentration of the purified total RNA samples was measured using a Qubit RNA Assay Kit (Invitrogen) and the Qubit Fluorometer (Invitrogen). We added 50 ng of RNA each cDNA synthesis reaction using the SuperScript-III RT pre-amplification system (Invitrogen, Carlsbad, CA, USA). cDNA concentrations were measured using Qubit dsDNA HS Assay Kit (Invitrogen) and the Qubit Fluorometer (Invitrogen).

Real time PCR

Real time PCR was carried out StepOne Real-Time PCR System (Applied Biosystems) using Platinum SYBR Green qPCRSuperMix-UDG (Invitrogen) following the manufacturer's instructions. The thermal cycling conditions included an initial denaturation for 2 min at 95°C and 40 cycles consisting of a denaturation step at 95°C for 15 s, an annealing step at 60°C for 30 s and an extension step for 1 min at 70°C. Samples was analyzed in triplicates. The relative mRNA levels were calculated using the comparative Ct method [45], using the

house keeping gene β -microglobulin as a normalizer. Non-treated macrophages served as a reference for treated macrophages.

Primers sequences we used for β -microglobulin were F: TCCTGGCTCACACTGAATTC and R: CTGCGTGCATAAATTGTATAGCA; for Fizz1 (Retnla) F: TCCCAGTGAATACTGATGAGA and R: CACTCTGGATCTCCCAAGA; and for Ym1 (Chi3l3) F: GGCATACCTTTATCCTGAG and R: CCACTGAAGTCATCCATGTC

Cytokines release measurement

Supernatants of cell cultures were analyzed for the presence of TNF, IL-10, MCP-1, IL-6, IL-12p70 and IFN- γ with the CBA Mouse Inflammation kit (BD Biosciences), according to manufacturer's instructions. Samples were analyzed using FACSCanto II (BD Biosciences) and BD FACSDiva software (BD Biosciences). Data obtained were analyzed using FCAP Array software (version 3.0, Soft Flow, Inc.) and expressed in pg/ml. TGF- β measurements were made using a human/mouse TGF- β 1 (2nd Gen) ELISA Ready-SET-Go! kit (eBioscience), according to the manufacturer's instructions.

Tumor and BMMs co-injection

The murine melanoma cell line B16F10 (ATTC CRL-6475) was cultured with DMEM media (Cultilab) supplemented with 10% of fetal calf serum (FCS) (Cultilab), 1 \times essentials amino acids (Gibco), 1 \times vitamins (Gibco) and 55 μ M of β -mercaptoethanol at 37°C with 5% of CO₂ atmosphere.

B16F10 cells (8×10^5) were co-injected with 10^4 of BMMs treated with LPS or DnaK as previously described in 100 μ L of serum-free RPMI. Injections were performed subcutaneously in the thigh of male BALB/c or C57BL/6 mice, after anesthesia with 83 mg/kg of ketamine and 17 mg/kg of xylazine. Mice were photographed and tumor growth was evaluated using a digital caliper (Mitutoyo) in days 8, 10, 12, 14 and 16 post tumor injections. We used a modified ellipsoid formula $0.52 (\text{Length} \times \text{Width}^2)$ to access the tumor volume [46]. On 16th day after tumor injection, mice were euthanized; the primary tumor was removed and weighted. All procedures were performed in the afternoon between 1–6 p.m. Tumor borders were drawn in photographs (Figure 5) using CorelDRAW (version 12.0).

Statistical analysis

Statistical analysis was performed using the Prism software (version 5.00, Graphpad Software Inc.). The one-way ANOVA test was used to determine differences between groups. Multiple comparisons among levels were checked with Tukey post hoc test. Differences between specific points were determined by a t test. The level of significance was set at $p < 0.05$.

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Author Contributions

Conceived and designed the experiments: RLL TJB RFZ CB. Performed the experiments: RLL TJB JFA NGP LSB SPM RFZ. Analyzed the data: RLL TJB JFA NGP LSB AOB SPM ADS RFZ CB. Contributed reagents/materials/analysis tools: LSB AOB SPM ADS. Wrote the paper: RLL TJB RFZ CB.

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Capítulo IV

6 Manuscrito submetido à revista “*Cytokine*”, intitulado:

“IL-10 is required for polarization of macrophages to M2-like phenotype by DnaK”

IL-10 is required for polarization of macrophages to M2-like phenotype by mycobacterial DnaK (Heat shock protein 70)

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Abstract (150-250 words)

Macrophages are key cells in the innate immune system. They phagocytose pathogens and cellular debris, promote inflammation, and have important roles in tumor immunity. Depending on the microenvironment, macrophages can polarize to M1 (inflammatory) or M2 (anti-inflammatory) phenotypes. Extracellular DnaK (the bacterial ortholog of the mammalian Hsp70) from *Mycobacterium tuberculosis* (*Mtb*) was described to exert immune modulatory roles in an IL-10 dependent manner. We have previously observed that endotoxin-free DnaK can polarize macrophages to an M2-like phenotype. However, the mechanisms that underlie this polarization need to be further investigated. IL-10 has been described to promote macrophage polarization, so we investigated the involvement of this cytokine in macrophages stimulated with extracellular DnaK. IL-10 was required to induce the expression of M2 markers - Ym1 and Fizz, when macrophages were treated with DnaK. Blockade of IL-10R also impaired DnaK induced polarization, demonstrating the requirement of the IL-10/IL-10R signaling pathway in this polarization. DnaK was able to induce TGF- β mRNA in treated macrophages in an IL-10 dependent manner. However, protein TGF- β could not be detected in culture supernatants. Finally, using an *in vivo* allogeneic melanoma model, we observed that DnaK-treated macrophages can promote tumor growth in an IL-10-dependent manner. Our results indicate that the IL-10/IL-10R axis is required for DnaK-induced M2-like polarization in murine macrophages.

Key words (4-6): macrophage; anti-inflammatory; DnaK; M2-phenotype

1. Introduction

Macrophages are major players in innate immunity and can dynamically alter phenotypes, depending on the microenvironment [1]. The two main macrophage phenotypes described are M1 and M2. M1 macrophages produce pro-inflammatory cytokines and NO. They are present at sites of viral and bacterial infections and can have tumoricidal activity [2]. M2 macrophages have important roles in extracellular pathogen infections, wound-healing, and pro-tumoral growth [3]. They produce IL-10, TGF- β [4] and express the markers Fizz1 and Ym1 [5]. These environments harbor different molecules involved in the activation and polarization of macrophages, and some molecules are essential for cells to develop a specific phenotype.

Skewing towards M2 phenotype occurs both in infections and in tumors, contributing to create a suppressive environment that blunts pro-inflammatory responses [6, 7]. M1 macrophages are also denominated as classically activated macrophages and are induced by IFN- γ and/or by bacterial products, like LPS. M2 macrophages are induced by Th2 cytokines - IL-4 and/or IL-13 – and they are denominated M2a. When macrophages are polarized by the ligation of Fc γ Rs and toll-like receptors (TLRs), they are described as belonging to the M2b category [8]. Macrophages stimulation with IL-10/TGF- β or glucocorticoid hormones leads to a more suppressive phenotype, called M2c. This phenotype has been used to treat inflammatory disorders in experimental models [9].

We recently demonstrated that *Mycobacterium tuberculosis* (*Mtb*) extracellular DnaK (the bacterial ortholog of the mammalian Hsp70) can polarize murine macrophages to M2-like macrophages [10]. These macrophages expressed Fizz and Ym1, produced IL-10, and presented a higher arginase I activity. Additionally, they were able to sustain tumor growth of allogeneic melanoma cells [10]. However, the mechanisms that underlie this polarization need to be further investigated. Extracellular DnaK was described to exert immune suppressive roles in an IL-10-dependent manner [11]. So, using cells from IL-10 KO mice and blockade of IL-10R with specific antibody, we evaluated the phenotypic and functional roles of IL-10 and IL-10R in macrophages treated with DnaK.

2. *Materials and Methods*

2.1 *Animals*

Female C57BL/6 (B6) and BALB/c mice were purchased from CeMBE-PUCRS (Rio Grande do Sul, Brazil). Il10^{-/-} (IL-10 KO) mice were provided by Ana Faria (Federal University of Minas Gerais, Belo Horizonte, Brazil). All animals used were between 6 and 10 weeks old with a range of weight between 18-22 g. Mice were housed in mini-isolator cages (Techniplast) in the CeMBE/FABIO – PUCRS SPF facility. A carbon dioxide (CO₂) chamber (Beiramar) was used to euthanize the animals. All procedures were approved by the Ethics Committee for the Use of Animals of PUCRS (CEUA-PUCRS) under protocol ID CEUA 15/00466, following guidelines of the Federation of Brazilian Societies for Experimental Biology.

2.2 *Recombinant protein expression and purification*

Mycobacterium tuberculosis DnaK gene were cloned into the plasmid pET23a(+) (GenScript, USA). BL21 *Escherichia coli* were transformed with our construct and protein purified as described in Mehlert [12]. To remove endotoxin contaminants such as LPS, we used Triton X-114 (Sigma) in a method described by Aida et al [13]. Residual Triton X-114 was removed by incubating overnight the protein preparation with Bio-Beads (Bio-Rad) with agitation at 4°C [14]. Protein concentrations were measured using the Qubit Protein Assay Kit (Invitrogen) and samples were read by the Qubit Fluorometer (Invitrogen). To verify the purity of DnaK preparations, we performed in vivo assay as described in Fig 1.

2.3 *Flow cytometry*

Spleens from B6 treated mice were excised, disrupted against a nylon screen and treated with Collagenase D (Roche) for 30 minutes at 37°C. Cells then had their Fc receptor blocked and were stained with the following antibodies: CD11c (clone HL3), B220 (clone RA3-6B2), MHC II (I-A^b, clone AF6-120.1) and CD86 (clone GL1), all purchased from BD Biosciences. Cells were analyzed in a FACSCanto II flow cytometer (BD Biosciences) using the FACSDiva software (BD Biosciences).

2.4 *Macrophages differentiation and polarization*

Macrophages were differentiated from bone marrow (BMMs) of C57BL/6 WT or IL-10 KO mice as described in [10]. Briefly, 10⁶ cells were cultured serum-free medium AIM-V (Gibco) in the presence of 10 ng/mL of GM-CSF (Peprotech). After seven days of culture, the non-adherents cells were stimulated. Culture purity was assessed by flow cytometry (staining for

F4/80) and only cultures with purity higher than 90% were used. For IL-10R blockade, cells were treated with 10µg/mL of anti-mouse IL-10R (clone 1B1.3a, Biolegend) prior to the stimulation. BMMs were treated with 30µg/mL of mycobacterial DnaK.

2.5 Total RNA isolation and cDNA synthesis

To extract total RNA from macrophages, we used a Rnaeasy kit (Qiagen). To measure the concentration of total RNA, we used a Qubit RNA Assay Kit (Invitrogen). To cDNA synthesis, we used the SuperScript-III RT pre-amplification system (Invitrogen) with 50 ng of RNA in each reaction. We used a Qubit dsDNA HS Assay Kit (Invitrogen) to measure cDNA concentrations. RNA and cDNA samples were read using the Qubit Fluorometer (Invitrogen).

2.6 Gene expression quantification

To measure gene expression we used the real-time polymerase chain reaction (PCR) using the StepOne™ Real-Time PCR System (Applied Biosystems), along with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) following the manufacturer's instructions. Cycling conditions used were an initial denaturation for 2 min at 95 °C followed by 40 cycles consisting of a denaturation step during 15 s at 95 °C, an annealing step during 30 s at 60 °C and finally an extension step for 1 min at 70 °C. To amplify the β -microglobulin gene, we used the following primers sequences F: TCCTGGCTCACACTGAATTC and R: CTGCGTGCATAAATTGTATAGCA. For Retnla (Fizz1) amplification, we used F: TCCCAGTGAATACTGATGAGA and R: CACTCTGGATCTCCCAAGA; and for Chi3l3 (Ym1) F: GGGCATAACCTTTATCCTGAG and R: CCACTGAAGTCATCCATGTC primers were used.

Tgfb1 mRNA levels were analyzed using the *Mm01178820_m1* Taqman Gene Expression assay (Applied Biosystems). The house keeping gene β -microglobulin was used as a normalizer. We analyzed all samples in triplicates and we calculated the relative mRNA levels using the comparative Ct method [15] and non-treated cells served as a reference for treated macrophages.

2.7 Cytokines release measurement

TGF- β concentrations were assessed using the kit human/mouse TGF- β 1 (2nd Gen) ELISA Ready-SET-Go from eBioscience, following manufacturer's instructions.

2.8 Melanoma cell line and macrophages co-injection

B16F10 cells (murine melanoma - ATTC CRL-6475) were cultured with DMEM media (Cultilab) supplemented with 10% of fetal calf serum (Cultilab), 1x vitamins and 1x essential amino acids (Gibco) and 55 μ M of β -mercaptoethanol (Sigma) at 37°C with 5% of CO₂ atmosphere.

For allograft tumor inoculation, we co-injected 8×10^5 B16F10 cells with 10^4 of B6 WT or IL-10 KO macrophages treated with DnaK diluted in 100 μ l of media (serum-free). Mice were anaesthetized with ketamine (83 mg/kg) and xylazine (17 mg/kg) and cells were injected into the right thigh of BALB/c mice. Tumor growth was measured using digital caliper (Mitutoyo) every 2 days from day 8 to day 16 after tumor inoculation. Sixteen days after tumor inoculation, mice were sacrificed and tumors were weighed. Tumor volume was assessed by the formula $0.52 (\text{Length} \times \text{Width}^2)$, according to [16].

2.9 Statistical analysis

Prism software (version 6.00, Graphpad Software Inc.) was used for statistical analysis. For differences between groups, we used one-way ANOVA test. We used Tukey post hoc test for analysis of multiple comparisons between levels. A t test was made to verify differences between specific points. Significance points were set at $p < 0.05$.

3. Results

3.1 Characterization of LPS-free *M. tuberculosis* DnaK

Members of the extracellular Hsp70 family can interact with immune system cells. However, some of the described effects of such interactions were shown to be due to the presence of endotoxins in recombinant protein preparations [17, 18]. To exclude the possibility that our results were influenced by undesirable endotoxin contamination, we performed a “Bio-assay” to test the quality of our preparations. Khoruts and collaborators [19] demonstrated that 6h after intravenous injection of LPS, splenic dendritic cells (DCs) upregulated their CD86 levels. Recombinant DnaK was produced in *Escherichia coli* and endotoxins were removed using Triton X-114 (see *Methods* for further details). After that, B6 mice were intravenously injected with 25µg of LPS or 30µg of DnaK, treated or not with Triton. Six hours post injection, CD86 expression was analyzed in splenic DCs by flow cytometry (Fig. 1a,b). We observed that LPS and untreated DnaK upregulated CD86 expression in DCs (Fig. 1c). In contrast, Triton-treated DnaK as well as PBS did not (Fig. 1c). MHC II is also upregulated upon LPS stimulation [20]. In accordance with our CD86 results, only LPS and DnaK that did not undergo Triton treatment were able to increase MHC II levels, whereas PBS or DnaK treated with Triton were not able to alter or increase MHC II levels (Fig. 1d). Interestingly, DnaK before Triton treatment increased DCs MHC II levels in greater extension than LPS (Fig 1d).

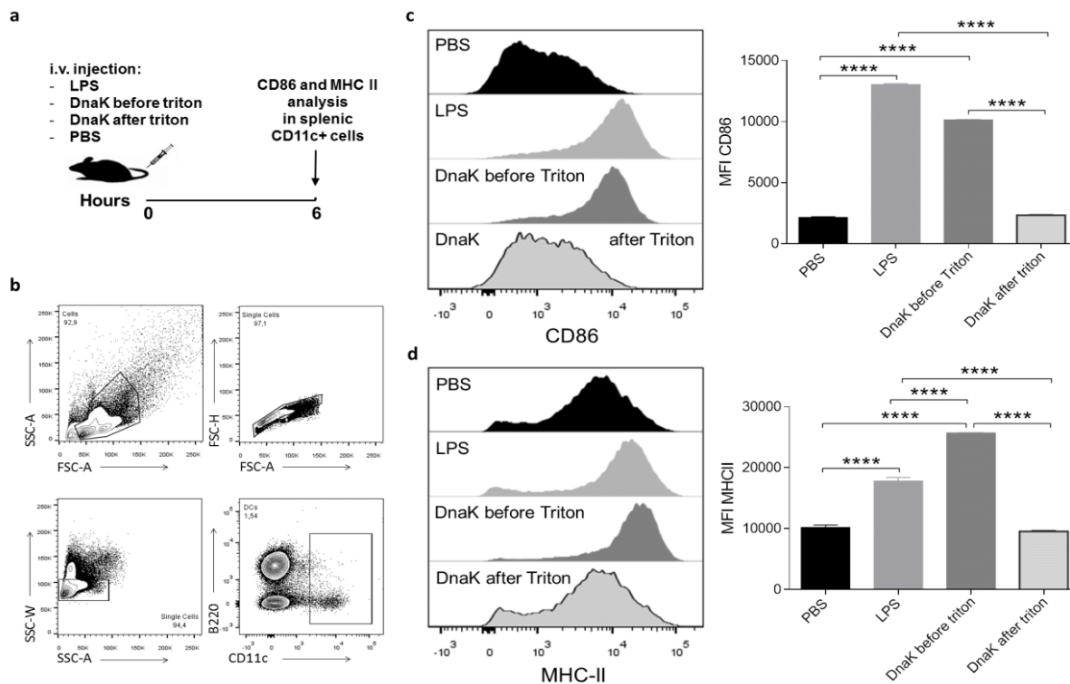


Fig. 1 Bio-assay to exclude DnaK contamination by endotoxin. a) Naïve B6 mice were intravenously injected with 25µg of LPS, 30µg of DnaK treated or not with Triton or PBS. Six hours after the injection, CD86 expression in splenic dendritic cells (CD11c⁺) was analyzed by flow cytometry. **b)** Gating strategy for splenic dendritic cells. **c)** Representative histograms and CD86 mean fluorescence intensity (MFI) in

splenic dendritic cells gated as in b). **d**) Representative histograms and MHC II MFI in splenic dendritic cells gated as in b). ****p<0.0001 by one-way ANOVA with Tukey post hoc test.

3.2 Absence of IL-10 or IL-10R blockade decreases the expression of M2 markers in DnaK-treated macrophages

In order to investigate the role of the IL-10 signaling pathway in the DnaK-induced polarization, we pre-treated macrophages with an anti-IL-10 receptor (IL-10R) or isotype control and then stimulated them with LPS, IL-4 or clean DnaK. As expected, IL-4 and DnaK treatment increased the mRNA levels of Fizz1 (Fig. 2a) and Ym1 (Fig. 2b) when compared to control or LPS. Blocking IL-10R in BMM prior to DnaK stimulus decreased the expression of Fizz1 and Ym1 in comparison to DnaK + Isotype control and IL-4 treatment (Fig. 2a,b). Accordingly, when we treated IL-10 KO macrophages with DnaK, the expression of Fizz1 (Fig. 2c) and Ym1 (Fig. 2d) was decreased when compared to WT BMM treated either with DnaK or IL-4.

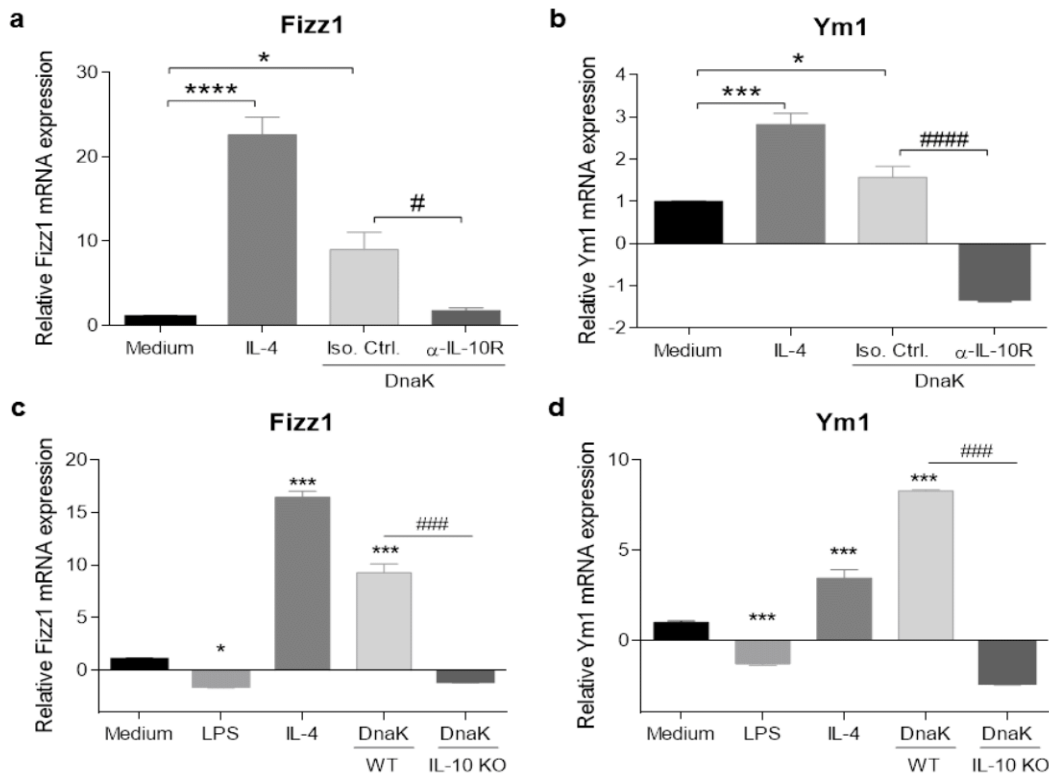


Fig. 2 Fizz1 and Ym1 mRNA expression induced by DnaK depends on IL-10 signaling pathway. WT Bone marrow macrophages were previously blocked with an antibody anti-IL-10R or isotype control and then treated with DnaK (30 μ g/mL), IL-4 (40 ng/mL) or medium. After that, **a**) Fizz1 or **b**) Ym1 gene expression was assessed by real-time PCR. **c**) Bone marrow macrophages from WT or IL-10 KO mice were treated with DnaK (30 μ g/mL), IL-4 (40 ng/mL), LPS (100 ng/mL) or medium. After that, **c**) Fizz1 or **d**) Ym1 gene expression was assessed by real-time PCR. For all experiments total amounts of Ym1 and Fizz1 mRNA were normalized to β -microglobulin levels and expressed as fold change compared to untreated cells. Data are the mean \pm SD from triplicates. (*) p<0.05, (***) p<0.001 and (****) p<0.0001 indicate difference between treated groups and the medium group. (#) p<0.05, (###) p<0.001 and (####) p<0.0001.

p<0.0001 when WT groups were compared to IL-10 KO groups. All data were analyzed by one-way ANOVA with Tukey post hoc test. Data are representative of three independent experiments.

3.3 DnaK upregulates TGF- β mRNA, but not protein levels, via an IL-10 dependent pathway

TGF- β production was associated with M2 macrophages polarization [21]. Also, macrophage stimulation with IL-10 was described to induce the expression of the *Tgfb1* gene [22]. We questioned whether DnaK-induced IL-10 could influence TGF- β expression in the polarized macrophages. To answer this, WT or IL-10 KO BMMs were stimulated for 24h with LPS, IL-4, DnaK or medium. Subsequently, TGF- β production was analyzed in culture supernatant. We observed that only BMM stimulated with IL-4, could produce TGF- β (Fig. 3a). We next investigated *Tgfb1* gene expression by real-time PCR. IL-4 treatment led to a ~20 fold increase in *Tgfb1* mRNA levels in WT macrophages (Fig. 3b), whereas DnaK increased ~100 fold. Surprisingly, while IL-4 treatment was still able to increase *Tgfb1* mRNA levels in IL-10 KO BMMs, the effect observed for DnaK in WT cells was abolished in the IL-10 KO BMMs (Fig. 3b).

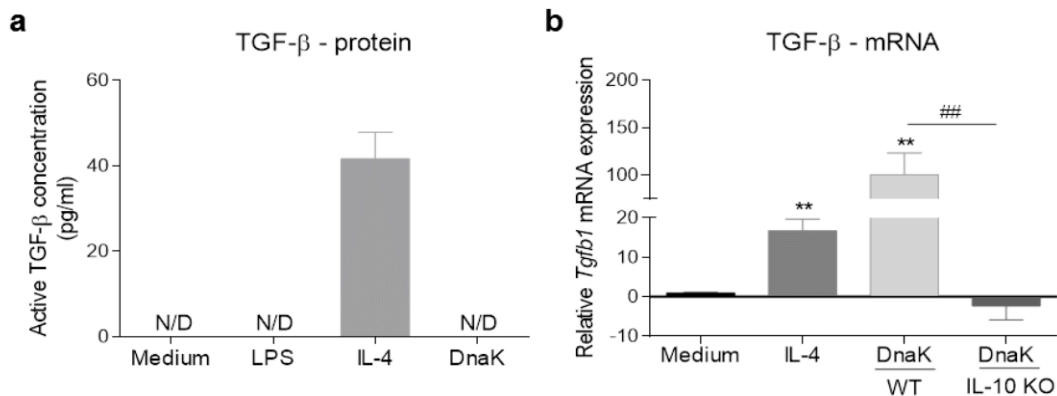


Fig. 3 DnaK-induced *Tgfb1* gene expression is IL-10 dependent. Bone marrow macrophages were treated with DnaK (30 μ g/mL), IL-4 (40 ng/mL) or medium for 24h. **a**) TGF- β protein production was measured by ELISA in culture supernatants. The values represent means \pm SD in pg/ml from triplicates. **b**) TGF- β mRNA levels were assessed by real-time PCR. Total amounts of mRNA were normalized to β -microglobulin signals and expressed as fold change. The values represent means \pm SD from triplicates. (***) p<0.01 indicate a significant difference between treated groups and medium group. (##) p<0.01 when WT groups were compared to IL-10 KO groups. All data were analyzed by one-way ANOVA with Tukey post hoc test. Data are representative of three independent experiments.

3.4 DnaK-induced M2-like macrophages promotes melanoma growth in mice via IL-10

We had previously shown that DnaK polarized macrophages to M2-like that were able to promote allogeneic tumor growth in a murine melanoma model [10]. To test whether IL-10 was essential to this phenomenon, we used the same allograft melanoma model [10, 23]. We co-

injected WT or IL-10 KO BMMs previously treated with DnaK, together with B16F10 (B16) cells (H-2^b) in BALB/c mice (H-2^d). Tumor growth was observed between days 8 and 16 post-injection (Fig. 4a). As expected, tumors could not grow in mice that received only melanoma cells (B16 group - Fig. 4b), as the tumors were rejected by an alloresponse. Mice that received co-injections of B16 and DnaK-treated WT BMM presented significantly increased tumor growth (Fig. 4b), according to our previously published results [10]. However, when DnaK-treated BMM from IL-10 KO mice were co-injected with B16 tumor cells, no tumor growth was observed (Fig. 4b). These results indicate that DnaK-polarized macrophages promote murine melanoma growth through a mechanism dependent on IL-10. Moreover, we have tested DnaK effects in a syngeneic model. We co-injected WT BMMs previously treated with DnaK (M2) or LPS (M1) along with B16 cells (H-2^b) in C57Bl/6 hosts (H-2^b) (Fig. 4c). Tumors could grow in a more potent manner in syngeneic hosts (Fig. 4d) when compared to allogeneic ones (Fig. 4b). Hosts that had the melanoma inoculated together with M1 (treated with LPS) macrophages presented decreased tumor growth compared with the ones that received only the B16 cells (Fig. 4d). Interestingly, mice that received co-injections of melanoma cells and DnaK-treated BMMs presented similar tumor growth compared to control (Fig. 4d). Therefore, DnaK-treated macrophages can support tumor growth in an allogeneic, but not in a syngeneic melanoma model.

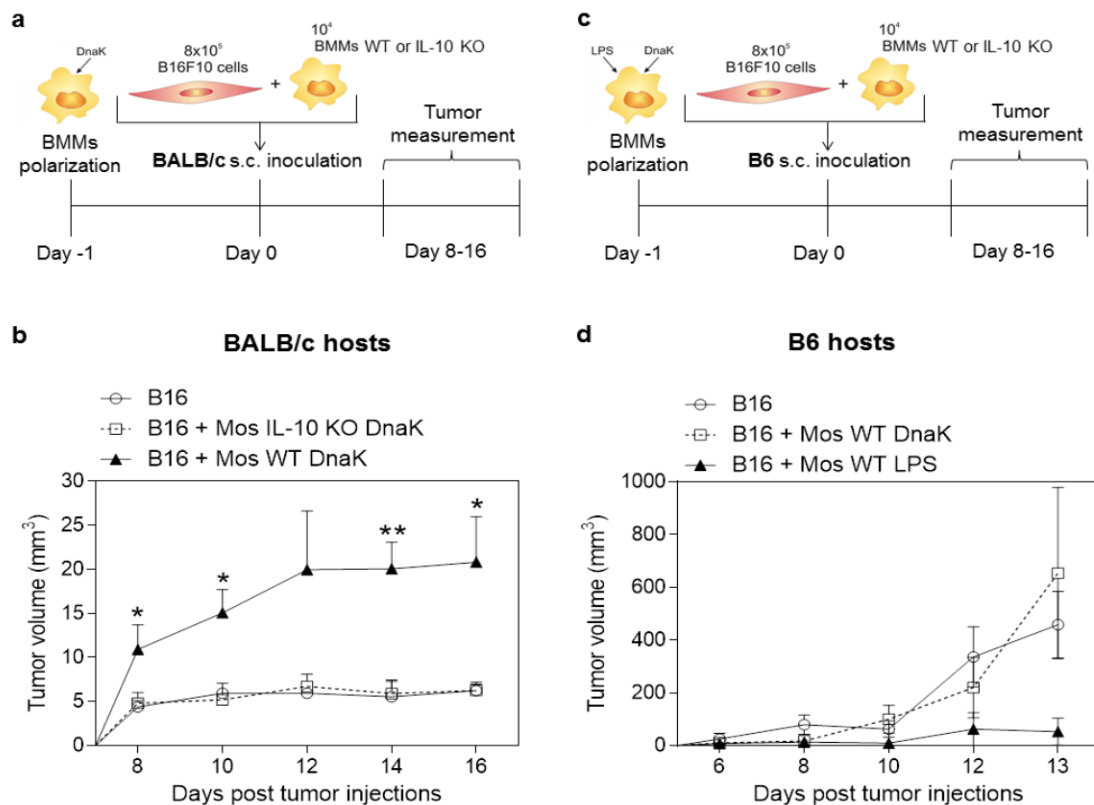


Fig. 4 DnaK-polarized M2-like macrophages promote allogeneic tumor growth in an IL-10-dependent manner. a, b) Bone marrow macrophages (BMMs) were differentiated from WT or IL-10 KO and treated with DnaK for 24 h. Following this, those cells were co-injected with B16F10 in allogeneic BALB/c mice hosts. Tumor volume was measured every 2 days from Day 8 (when tumors were established) to 16, post-tumor inoculation. The values represent means \pm SEM of each group. Statistics were generated using two-way ANOVA, * $p < 0.05$, ** $p < 0.01$ when comparing the Mos WT DnaK group to other groups. **c, d) WT** BMMs were treated with DnaK or LPS for 24 h. Following this, those cells were co-injected with B16F10 in syngeneic C57Bl/6 mice hosts. Tumor volume was measured every 2 days from Day 8 (when tumors were established) to 13, post-tumor inoculation. The values represent means \pm SEM of each group. Data are representative of two independent experiments.

4. Discussion

Hsp70 family members have been proposed to act as virulence factors, contributing to the induction of a non-protective immunity. Mice infected with a *Cryptococcus neoformans* strain lacking Ssa1 – Hsp70 ortholog – presented decreased lung fungal burden compared to controls [24]. It was also proposed that Ssa1 directly promoted the polarization of macrophages to an M2 phenotype in order to evade host protective immune responses [24]. Ssa1-lacking *Candida albicans* strain showed a reduced virulence in murine model of candidiasis due to a reduced capacity to be endocytosed by endothelial and oral epithelial cells [25]. Additionally, human macrophages exposed to *Toxoplasma gondii* strains which had a reduced Hsp70 expression presented a reduced production of NO and iNOS mRNA levels when compared to control strains [26]. Hsp70 orthologs present in fungi and parasites are linked to inhibition of NO production and an induction of M2 markers in host's macrophages during infection. It is possible that similar mechanisms are triggered by some forms of extracellular mammalian Hsp70, as in the tumor microenvironment, demonstrating a tumor's strategy to avoid immune responses. Hsp70 found in membranes of tumor exosomes binds to myeloid-derived suppressor cells (MDSCs) and increase their suppressive capacity, promoting tumor growth [27]. In any case, our results reinforce that studies that employ recombinant Hsps to analyze immunomodulatory effects of these proteins need to have bacterial contaminants thoroughly removed. This is in order to avoid possible confusion over their true immune modulatory effects, as first demonstrated by Gao and collaborators [17] and further discussed in [the following article](#) [28]. A detailed comparison of the immune effects by the very same Hsp isoform isolated from the pathogen with one produced in a recombinant way has not yet been performed. It will undoubtedly shed light on this complex phenomenon.

IL-10 appears to be a central hub mediating extracellular mycobacterial DnaK (Hsp70) effects. DnaK has been demonstrated to have anti-inflammatory and protective roles in different inflammatory murine models such as arthritis [29, 30] and transplant rejection [23]. Also, these effects were either dependent on IL-10 [31] or have been associated with this cytokine [11, 32]. DnaK can induce the production of IL-10 in dendritic cells [14], macrophages [10], and synovial cells from arthritis patients [33]. Moreover, in *Mycobacterium tuberculosis* (*Mtb*) DnaK can modulate the production of pro-inflammatory cytokines via IL-10 in murine dendritic cells [34].

Previous studies have emphasized the role of IL-10 in macrophage polarization by *Mtb* infection [6]. IL-10 production by macrophages triggered by *Mtb* infection is linked with the ability of the pathogen to evade immune responses [35]. Mice lacking IL-10, or that had had IL-10R blocked, showed an increased clearance of certain pathogens [36-39]. IL-10 is also associated with long-term *Mtb* lung infections in humans [35]. This cytokine can act on dendritic cells and

macrophages by modulating the levels of MHC [40] and co-stimulatory molecules. It can also block IFN- γ and TNF- α production, impairing inflammatory effector immune responses [41]. Additionally, IL-10 can block CXCL10 production and limit Th1 cells migration to lungs infected with *Mtb* [42]. In macrophages, IL-10 can limit the production of reactive oxygen species (ROS) inhibiting phagocytosis and microbial killing [43]. Thus, IL-10 induction by mycobacteria compounds – such as DnaK – could constitute a mechanism to establish chronic infection and perpetuate the microorganism inside host cells. In fact, DnaK has been found inside vesicles released by *Mtb*, modulating macrophages to an anti-inflammatory phenotype [44].

Macrophages polarized with IL-10 are presently included in the M2 group. However, according to current knowledge, these cells show few different functional aspects from those polarized with IL-4, being denominated M2c macrophages by Martinez and Gordon [8]. IL-4-polarized macrophages were described to produce TGF- β [1]. Moreover, *in vivo*, or upon IL-4 stimulation, the TGF- β signaling pathway is required for M2 polarization [45]. Interestingly, we found that DnaK was able to induce TGF- β mRNA, but not protein (Fig. 3), and that was dependent on IL-10. It is remarkable that the induced TGF- β mRNA is not translated in this situation. TGF- β transcription involves *v-src* gene products and is mediated by AP-1 complex [46]. A protein called P311 was recently described as stimulator of TGF- β translation [47]. P311 needs to interact with eIF3b, and the disruption of the P311-eIF3b complex inhibits translation of TGF- β 1 [47]. It is possible that after DnaK stimulation, *v-src* genes' products are induced, or the AP-1 complex is activated, leading to TGF- β mRNA transcription. However, for translation to occur, an additional step – probably involving co-stimulation or co-receptor engagement, which is absent in our *in vitro* system or is not induced by DnaK treatment, would be required for the induction/activation of the P311- eIF3b complex, and translation of the subsequent induced TGF- β mRNA.

In this study, we have also determined that TGF- β mRNA induction by DnaK is dependent on IL-10. IL-10 commonly signals through the IL-10 receptor (IL-10R), which is a dimer of IL-10R1 and IL-10R2 [41], however the molecular pathways by which IL-10 induces TGF- β are still unclear. In the case of DnaK-induced polarization of macrophages, as well as their effects on allogeneic tumor progression, a role for TGF- β cannot yet be discarded, and further testing of this hypothesis is needed. For example, it is possible that *in vivo* other signals are provided which would allow for the translation of the TGF- β mRNA, thus contributing to tumor growth. We will address this possibility in future research

Finally, we observed *in vivo* that DnaK-treated macrophages could not enhance tumor growth in syngeneic hosts. We interpreted this result as evidence that the mechanisms through which DnaK prevents rejection of allogeneic tumor cells are not the same as those used for syngeneic

tumors to grow *in vivo*. To explore the tumor microenvironment and to attempt to clarify the mechanisms of tumor rejection, we have previously demonstrated that most macrophages that we treated *in vitro* and co-injected are already dead after 15 days [10]. We hypothesize that intra-tumoral cell death may be a major mechanism in preventing rejection. Indeed, there are several reports showing that transfer of allogeneic tolerized cells can be used to prolong allograft survival in mice [48]. However, it was shown those transferred donor cells are short-lived and hosts' dendritic cells in fact mediate the effect [49, 50]. Hosts' DCs can acquire donor-derived antigens from dead transferred cells and impair alloreactive effector T cells in a tolerogenic context [49, 50]. In our model, we would predict that the transfer of DnaK-treated macrophages along with allogeneic melanoma cell creates a tolerogenic microenvironment in which recipients' APCs acquired donor antigens and impaired alloreactive T cell function, allowing tumor establishment and growth.

Alternatively, it is possible that the type of cell death that occurs *in situ* modulates the response. For example, Chaurio and collaborators induced *in vitro* death of B16F10 melanoma cells by four different pathways: overexpression of active caspase-3 (casp-3), BH3-interacting domain death agonist (Bid), a necrosis-inducing toxin (CpnT_{CTD}) and by irradiation with UVB [51]. Importantly, each pathway activated a different type of cell death. Dead cells were then transferred to allogeneic BALB/c hosts. Following this immunization, mice were challenged with viable cells and tumor growth was assessed. They showed that melanoma cell death induced through UVB irradiation or Bid overexpression was more tolerogenic and allowed tumor growth. However, cells overexpressing casp-3 or CpnT_{CTD} induced immunogenic cell death and reduced tumor growth [51]. Consequently, different types of cell death can modulate the interaction between immune cells and dead cells, impacting on tumor growth and persistence. In our system, the IL-10 produced by DnaK-treated macrophages could change or interfere with the type of cell death induced *in vivo*, changing the outcome of tumor growth. Indeed, IL-10 has been shown to inhibit apoptotic cell death in some cases [52, 53]. Future studies should address this possibility in order to further understand the molecular mechanisms involved in this phenomenon.

Conclusion

We find that M2-like polarization of macrophages by extracellular mycobacterial DnaK depends not only on IL-10, but also on IL-10R signaling, suggesting an autocrine feedback loop. Altogether, our findings highlight the overall complexity of macrophages phenotype polarization, which is probably more dependent on the microenvironment where these cells will be inserted than other factors.

Conflict of Interest Disclosure

The authors report no conflicts of interest.

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Capítulo V

7 Expressão e purificação de Hspa1a

7.1 Expressão de Hspa1a

O plasmídeo pM70.1 foi gentilmente cedido pelo professor Clayton Hunt. Esse plasmídeo consiste na sequência da Hspa1a (Hsp70 murina) em um plasmídeo de clonagem pUC 9, que possui um gene de resistência a ampicilina. A sequência que codifica a Hspa1a está entre os sítios de restrição HindIII e EcoRI.

Primeiramente, descongelou-se do ultrafreezer -80°C as bactérias BL21 que estavam transformadas com o plasmídeo pM70.1. Essas células foram colocadas em meio de cultura LB com ampicilina ($50\ \mu\text{g}/\text{mL}$) e estimuladas a crescer por 18h a 37°C sob agitação de 180 RPM.

Para purificação do plasmídeo em bactérias transformadas foi utilizado o *PureLink® Quick Plasmid Miniprep Kit* (Thermo Fisher Scientific).

Posteriormente, foi feito um gel de agarose 10% para observar se o plasmídeo foi extraído com sucesso (fig. 8).

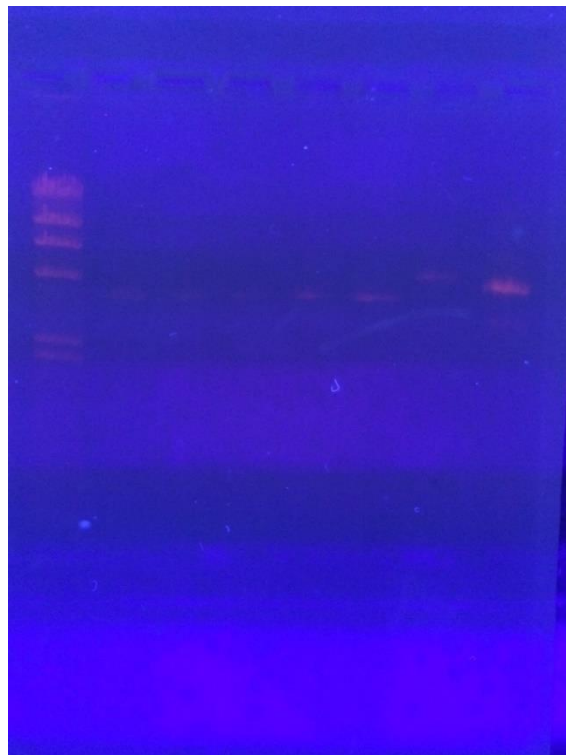


Figura 8 Migração do plasmídeo pM70.1 em gel de agarose.

O plasmídeo pM70.1 foi extraído e migrado em um gel de agarose 10%. A canaleta 1 (à esquerda) representa o marcador de peso molecular, a canaleta 8 (última à direita) representa o plasmídeo pM70.1 na forma fechada.

Visto que o plasmídeo apresentou uma expressão muito fraca no gel, optamos pela transformação em uma bactéria que permita a sua propagação de forma eficiente. O plasmídeo pM70.1 então foi transformado em bactérias *One shot*[®] *TOP10 Chemically Competent E. coli* (Invitrogen) para que pudéssemos ter o plasmídeo propagado em grande quantidade.

O DNA plasmidial foi incubado com a cepa de bactérias TOP10 para propagação do plasmídeo (vetor de clonagem) ou BL21 para expressar a proteína (vetor de expressão). O tubo é deixado por 20 minutos no gelo e depois 90 segundos a 42°C. Novamente é colocado no gelo, agora por 2 minutos. As células são transferidas para o meio SOC (triptona 20g, extrato de levedo 5g, NaCl 0,5g, KCl 10 mL de solução a 250 mM) e deixadas na estufa a 37°C por 1 hora. Após o tempo, o tubo é centrifugado a 12.000 RPM por 2 minutos a 4°C. Despreza-se o sobrenadante até ficar no mesmo nível do *pellet*. Então, ressuspende-se o *pellet* e semeia na placa de *Petri* com meio LB sólido e kanamicina (50 µg/mL). A placa é deixada em estufa a 37°C *overnight*. A observação de pequenas colônias espalhadas pela placa, indica um possível sucesso na transformação.

Após a transformação, essas bactérias foram semeadas em placa de LB sólido com ampicilina (50 µg/mL) *overnight*. No outro dia, foi feito um inóculo dessas bactérias em meio líquido com ampicilina na mesma concentração. Uma nova extração plasmidial foi realizada no dia posterior. Agora, há um evidente aumento na concentração plasmidial após a transformação nas bactérias TOP10, como segue na figura abaixo. (Fig. 9).

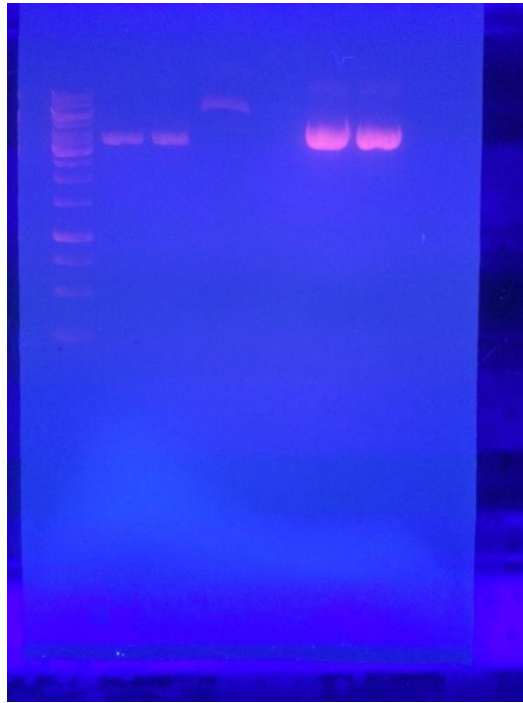


Figura 9 Migração do plasmídeo pM70.1 em gel de agarose transformado em bactérias TOP10.

O plasmídeo pM70.1 foi isolado e colocado para migrar em gel de agarose 10%. Na canaleta 1 (à esquerda) está o marcador de peso molecular. Nas canaletas 6 e 7 (à direita) estão o plasmídeo pM70.1 agora extraído de bactérias TOP10.

O próximo passo foi a clivagem do plasmídeo no sítio HindIII (Promega) para que ele ficasse linearizado e isso pudesse ser observado em gel de agarose, como segue na **figura 10** logo abaixo. O plasmídeo purificado é misturado a enzima de restrição EcoRI (Promega). Para a enzima HindIII foi utilizado o tampão *10x REACT 2* e o restante do volume completado com H₂O mili-Q (*Millipore*). A reação de restrição foi realizada a 37°C por 1h.

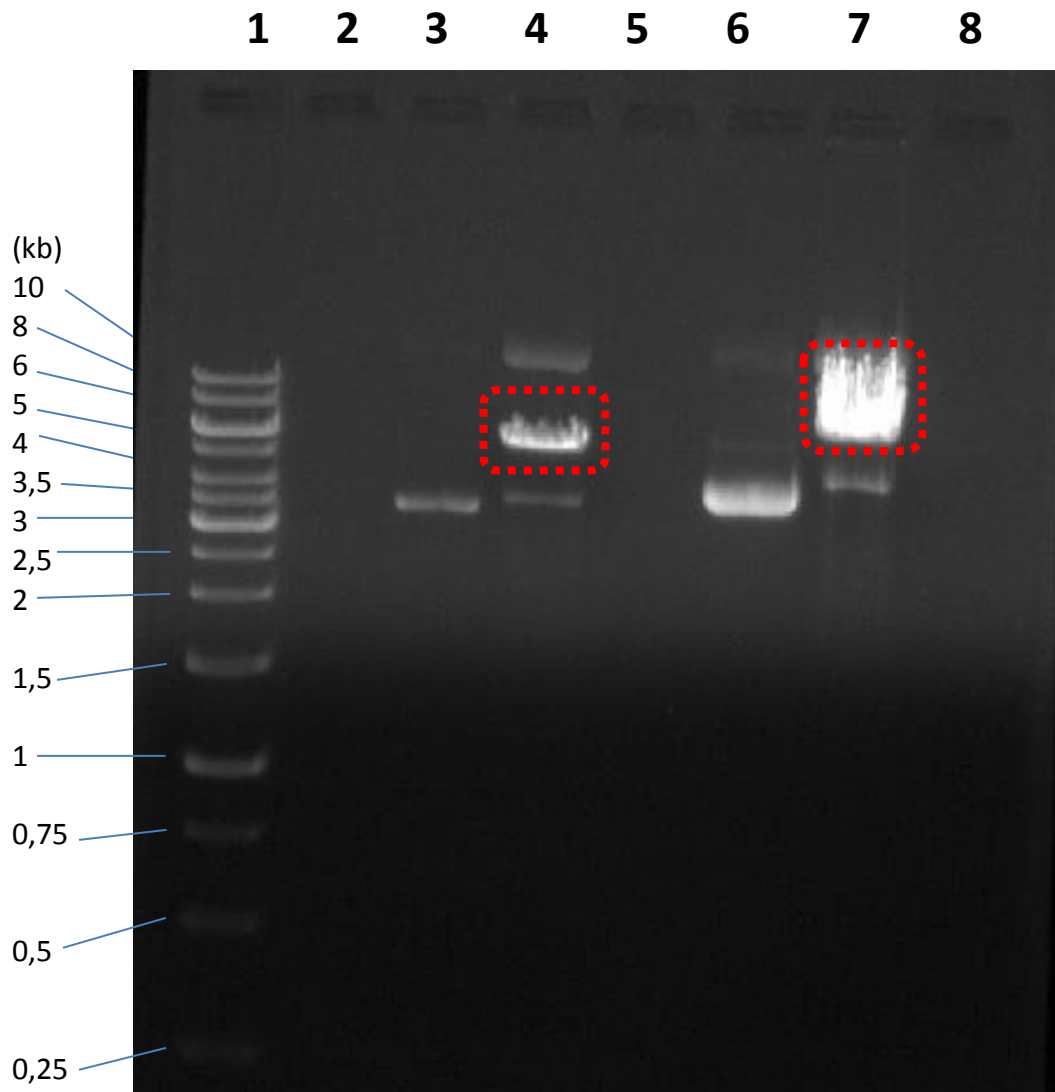


Figura 10 Migração do plasmídeo pM70.1 e do pET30a(+).

(1) Marcador de peso molecular Scada 1 kb DNA ladder. (3) pET30a+ circular; (4) Círculo pontilhado representa pET30a+ linearizado (clivado com HindIII); (6) pM70.1 circular; (7) Círculo pontilhado pM70.1 linearizado (clivado com HindIII). O plasmídeo pET30a(+) possui 5422 pb.

A banda logo acima na canaleta 4 pode ser a forma supertorcida do pET30a(+) e essas formas migram com mais dificuldade no gel de agarose, ficando mais acima. As bandas em destaque com o envoltório em vermelho foram recortadas e seu DNA purificado (com o kit *Illustra GFX PCR DNA and Gel band purification kit*) para a clivagem com a outra enzima (EcoRI). Já para a purificação de plasmídeo em gel de agarose foi utilizado o *illustra GFX PCR DNA and Gel Band Purification Kit* (GE Healthcare).

Após a purificação do DNA do fragmento do gel recortado, houve a clivagem com a enzima EcoRI e um novo gel foi feito (**Fig. 11**). Para a enzima EcoRI foi utilizado o *IX buffer EcoRI*, adicionado BSA e o volume foi completado com H₂O milli-Q. A restrição foi realizada a 37°C por 1h.

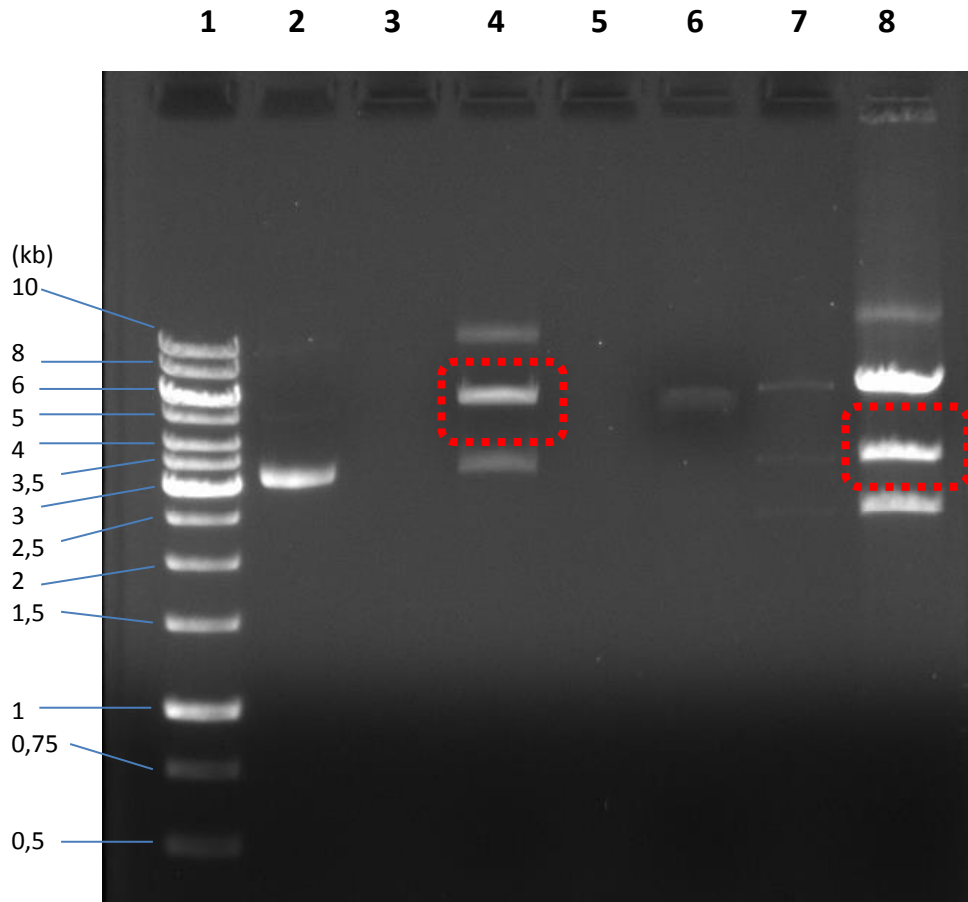


Figura 11 Clivagem dos plasmídeos pM70.1 e pET30a(+) com enzima de restrição EcoRI.

(1) Marcador de peso molecular Scada 1 kb DNA ladder; (2) pET30a+ circular. (4) Círculo pontilhado representa pET30a+ linearizado (clivado com HindIII e EcoRI); (6) pM70.1 circular; (8) Círculo pontilhado representa pM70.1 com sequência da Hsp1a separada (clivado com HindIII e EcoRI).

As bandas destacadas nas canaletas 4 e 8 foram recortadas e o DNA foi purificado. Para a purificação plasmidial a partir da banda do gel foi utilizado *Illustra GFX PCR DNA and Gel Band Purification Kit* (GE Healthcare). A banda recortada na canaleta 4 representa o vetor pET30a(+) linearizado em que o inserto irá se ligar. A banda destacada na canaleta 8 representa a sequência da Hsp1a segundo o peso observado no gel comparando-o com o banco de dados online (NCBI). A banda logo acima não se sabe a que sequência representa.

7.2 Ligação do vetor (pET30a(+)) ao inserto (gene da Hsp1a)

Foi realizada uma reação de ligação com o vetor pET30a(+) e o inserto Hsp1a. Essa reação ocorreu durante 16h a 37°C. Os volumes e concentrações a serem utilizados foram ajustados de acordo com a página <http://nebiocalculator.neb.com> (NEB®). Após a reação de clivagem,

com o conteúdo do tubo foi realizada uma clivagem com EcoRI para verificar se o plasmídeo com a sequência linearizaria e se ficaria na posição correta no gel.

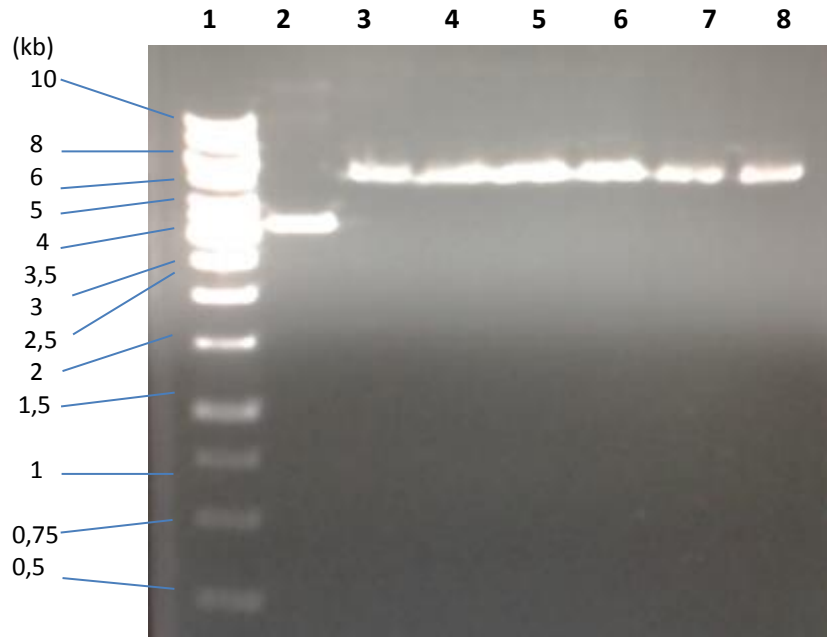


Figura 12 Clivagem do plasmídeo pET30a(+):Hspa1a com enzima EcoRI.

(1) Marcador de peso molecular Scada 1 kb DNA ladder; (2) Plasmídeo pET30a(+):Hspa1a na forma circular (3-8) Amostras do plasmídeo clivadas com EcoRI.

No gel é possível ver que a clivagem com EcoRI foi capaz de linearizar o plasmídeo. O mesmo ficou em uma altura que condiz com o peso aproximado do plasmídeo ligado ao inserto. Porém, para certificar-se de que a ligação ocorreu com eficiência, realizamos a clivagem do plasmídeo com a outra enzima de restrição, HindIII.

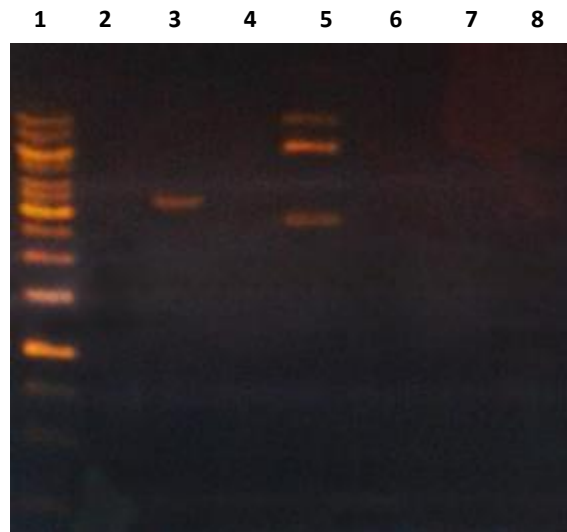


Figura 13 Clivagem do plasmídeo pET30a(+):Hspa1a com EcoRI e HindIII.

(1) Marcador de peso molecular Scada 1 kb DNA ladder; (3) Plasmídeo circular; (5) Banda inferior = inserto, banda superior = plasmídeo aberto.

Quando clivado com ambas enzimas de restrição, a reação foi capaz de liberar o inserto com sucesso, como mostrado na canaleta 5 da imagem 13 acima.

7.3 Transformação e padronização da expressão

Após obtermos no plasmídeo pET30a(+) a sequência da Hspa1a inserida com sucesso entre os sítios EcoRI e HindIII, foi dado início ao processo de transformação e posterior padronização da expressão da proteína.

A transformação foi realizada na cepa BL21 de *E. coli*. Após o protocolo de transformação, as bactérias cresceram em meio LB sólido com kanamicina (50 µg/mL) *overnight*. Foi feito um pré-inóculo (5 mL) com kanamicina na mesma concentração e após o crescimento *overnight* esse pré-inóculo foi dividido em pequenos Erlen-meyers com capacidade para até 250 mL com as seguintes condições:

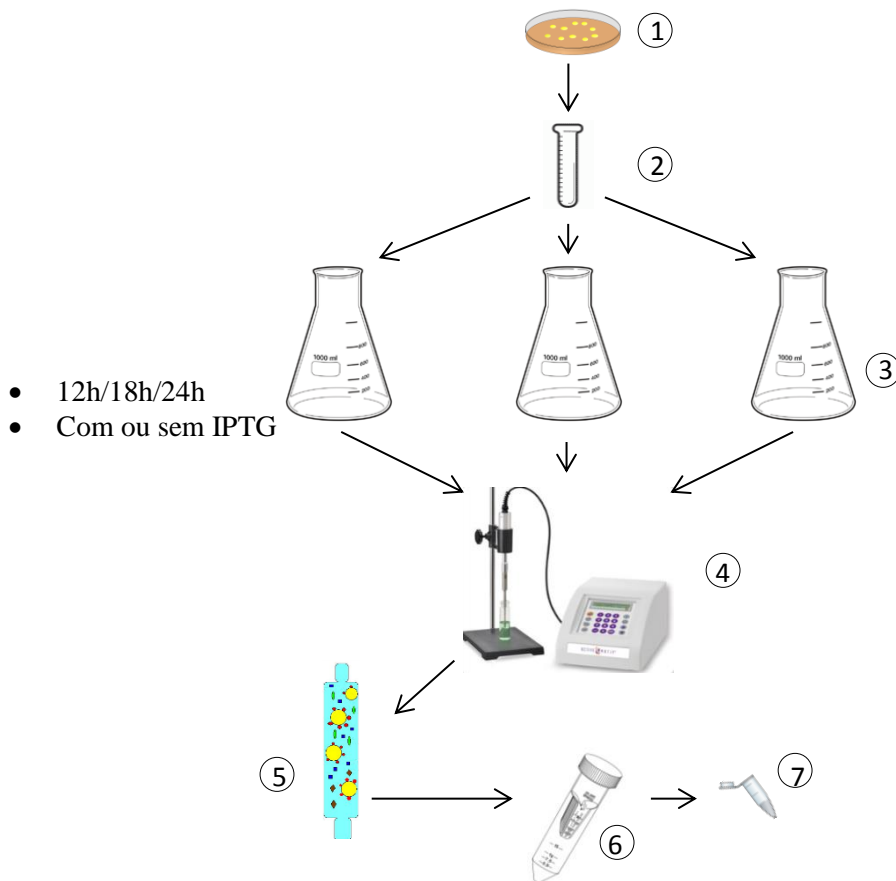


Figura 14 Esquema da padronização da expressão da Hsp1a.

(1) Placa de Petri com colônias isoladas de bactérias BL21 contendo o plasmídeo pET30a(+) com a sequência da Hsp1a ligada nele, sendo que uma colônia é pescada e inoculada; (2) O pré-inóculo é crescido *overnight* sob agitação; (3) Há uma divisão em tubos Erlenmeyer em que cada tubo cresce sob uma condição, sendo: 12h, 18h, 24h, 48h, sendo que cada tempo tem um tubo com e outro sem IPTG. O tempo só começa a contar, após o inóculo atingir a OD_{600} , medida em espectrofotômetro; (4) As bactérias são sonicadas e centrifugadas; (5) o sobrenadante da centrifugação é filtrado e passado na coluna. Após uma série de tampões a proteína é eluída; (6) um filtro amicon é utilizado para concentrar a amostra e diminuir a molaridade de ATP presente na amostra; (7) quando finalmente o LPS é extraído da amostra com o detergente triton X-114, que sequestra o LPS da proteína. Para se livrar do triton na amostra, são adicionadas *Biobeads*® que retiram o triton

Até o presente momento a padronização chegou ao ponto de testar alguns tempos, porém não todos. O protocolo de sonicação é o mesmo utilizado para o homólogo micobacteriano que é descrito no último capítulo . Abaixo segue a imagem de um gel de poli-acrilamida a 12%.

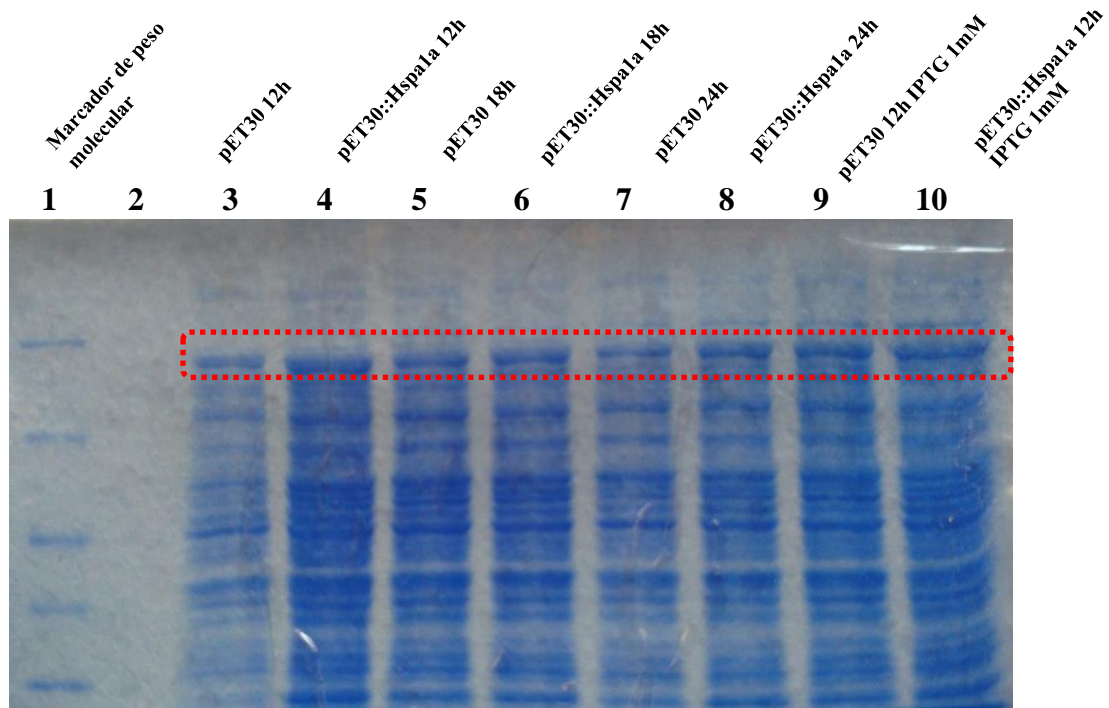


Figura 15 Gel de poliacrilamida com diferentes condições de crescimento bacteriano.

Bactérias BL21 transformadas com o plasmídeo pET30a(+):Hspa1a foram crescidas em diferentes condições. O tempo passa a ser contado a partir do momento em que as bactérias atingem a OD_{600} . As condições de crescimento foram 12, 18 e 24 horas. Após esse crescimento as bactérias foram sonicadas e uma amostra de cada tubo foi separada para correr em gel de poliacrilamida 12%.

O passo atual em que se encontra esse processo é o de teste de mais condições para tentar otimizar mais o crescimento. A ideia é testar o crescimento bacteriano com meios mais ricos, como SB e TB, além de temperaturas diferentes de crescimento.

Capítulo VI

8 Efeito comparado da DnaK e HSPA1A sobre DCs

8.1 Produção da DnaK de *M. tuberculosis* e HSPA1A

Bactérias estão transformadas com o plasmídeo pET23a que contém a sequência da DnaK de *Mycobacterium tuberculosis* e um gen de resistência a ampicilina. Essas bactérias crescem em meio LB sólido em placa de Petri com ampicilina (50 µg/mL) *overnight*. Essas bactérias são pescadas com palito de madeira e inoculadas em 5 mL de meio LB líquido com ampicilina (50 µg/mL). As bactérias crescem *overnight* novamente, porém agora com agitação de 180 RPM a 37°C. Esse pré-inóculo é adicionado a um Erlenmeyer com 495 mL de LB líquido e ampicilina na mesma concentração e colocado para crescer sob agitação de 180 RPM a 37°C. Essas bactérias foram centrifugadas a 13.000 RPM em centrífuga.

O *pellet* de bactérias obtido após a última centrifugação é ressuspendido em PBS e isso é colocado em um sonicador a uma amplitude de 80% (4 pulsos de 30 segundos com intervalo de 2 minutos entre pulsos). Esses tubos são centrifugados novamente a 13.000 RPM e o sobrenadante, onde encontram-se as proteínas é filtrado com papel filtro e filtro acrodisk de 22 µm. Esse filtrado é colocado em uma coluna de ATP-agarose, visto que a proteína DnaK possui um sítio de ligação a nucleotídeo e liga-se à coluna. O filtrado é eluído com três tampões, sendo que o último possui uma molaridade de ATP superior a da coluna, fazendo com que a proteína se ligue a ele e seja eluída. Após esse passo, o eluído é passado por um filtro amicon que concentra o eluído e com adições seriadas de PBS, diminui-se a concentração de ATP na amostra proteica, o que seria um interferente nos experimentos (73).

À proteína concentrada presente em um tubo eppendorf é adicionado 1% de triton X-114, um detergente que fará o sequestro do LPS ligado a DnaK. É dado *vortex* nesse tubo e é colocado no gelo por 5 minutos. Um novo *vortex* é dado e colocado em estufa por 5 minutos. O tubo é centrifugado por 5 minutos a 13.000 RPM e duas fases se formam, sendo a superior, a fase proteica que passará pelo processo novamente por mais 4 vezes. Após o fim desses processos, a proteína é colocada em contato com Biobeads® (Biorad) *overnight* (74). Após isso, a proteína estará pronta para o bioensaio, que testará de forma indireta e *in vivo* a presença de LPS na amostra, como explicado no **capítulo II**.

A proteína HSPA1A foi expressada em levedura *Pichia pastoris* e purificada em coluna de ATP por uma empresa contratada.

8.2 Efeito comparado da DnaK e HSPA1A sob células dendríticas de linfonodo de camundongo

Nesse experimento, visamos avaliar o estado de ativação de células dendríticas de linfonodo comparativamente entre DnaK de *Mycobacterium tuberculosis* e HSPA1A.

Os animais são eutanasiados e os linfonodos isolados com auxílio de coluna magnética (*Miltenyi Biotec*). Após a eutanásia, é seguido o protocolo de colagenase (75). Utilizando o tampão 1 (2% SBF, 1:10 colagenase D em RPMI zero) em contato com os linfonodos macerados a 37°C com 5% de CO₂ por 25 minutos. Depois disso, é adicionado o tampão 2 (0,1M de EDTA em PBS 1x, pH 7,2) por 5 minutos a 37°C com 5% de CO₂ por 5 minutos. Finalmente, é adicionado o tampão 3 (5mM de EDTA em PBS 1% SBF) e as células são filtradas, centrifugadas e ressuspensas. Posteriormente, essas células são submetidas a separação por coluna magnética, que separa somente as células que possuem as *beads* magnéticas de CD11c ligadas a coluna. Após esse processo, essas células separadas são contadas, plaqueadas e estimuladas para o experimento.

As células foram marcadas com anticorpo α -CD11c ligado a *beads* magnéticas para que possam ser separadas em coluna magnética. Após isso, as células foram colocadas em cultura e tratadas com OVA, DnaK, HSPA1A ou sem tratamento por 24h.

Foram utilizados os seguintes anticorpos para a marcação de citometria: α -CD86, α -IA^b, α -CD11c, α -B220 (todos da BD Biosciences) e corante de viabilidade (eBioscience). Os protocolos de marcação estão no *Data sheet* de cada anticorpo, disponíveis também, no site do fabricante (BD Biosciences).

Como podemos ver na **figura 16** DnaK e a HSPA1A foram capazes de levar a uma menor expressão de CD86, porém, em relação ao MHC de classe II somente a DnaK foi capaz de baixar a expressão. Ainda são necessários mais repetições e outros experimentos quanto a liberação de citocinas e expressão gênica para se ter uma ideia melhor de como a HSPA1A (Hsp70 humana) e a Hspa1a (Hpsp70 murina) agem sobre células dendríticas.

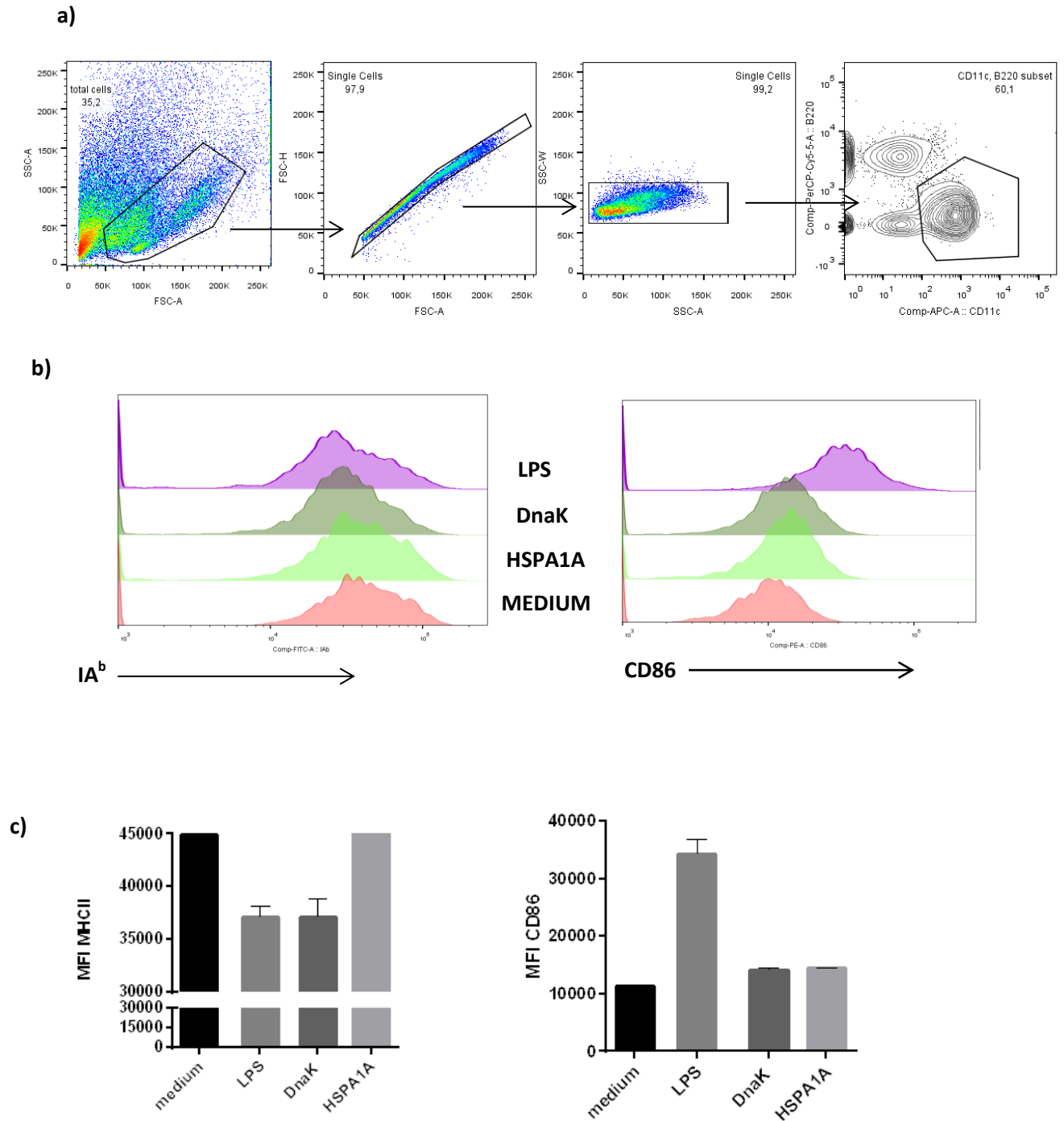


Figura 16 Modulação de células dendríticas por DnaK e HSPA1A.

Células de linfonodos, marcadas com anticorpo α -CD11c e separadas em coluna magnética. Foram tratadas com LPS, DnaK, HSPA1A ou sem tratamento por 24h. Após isso, foram marcadas com α -CD11c, α -B220, α -CD86, α -IA^b e corante de viabilidade. **a)** Estratégia de gates; **b)** Histogramas da expressão das moléculas CD86 e MHC de classe II **c)** Gráfico de barras da MFI de MHC de classe II e CD86.

Capítulo VII

9 CONCLUSÃO

Com esse trabalho podemos observar a ampla esfera em que atua a DnaK de *M. tuberculosis* no sistema imune. Visamos explorar um tipo celular ainda não muito bem documentado quanto sua relação na modulação pela DnaK. Interessantemente, observamos que essa proteína é capaz de polarizar macrófagos a um fenótipo M2. Além disso, baseando-se em estudos prévios, mostrando a relação de IL-10 e imunomodulação por DnaK (1,63,64) nós procuramos investigar a relação da citocina com a polarização de macrófagos. Observamos com uma série de ensaios, *in vitro* e *in vivo*, que a IL-10 é uma citocina crucial na polarização de macrófagos a M2 pela DnaK de *M. tuberculosis*. Através da dependência de IL-10 para o aumento de FIZZ1, YM1, TGF- β e para o crescimento tumoral, obtivemos dados suficientes para nos certificar da importância da citocina nessa sinalização.

Em relação ao homólogo de DnaK purificado em laboratório, Hspa1a (Hsp70 murina), não obtivemos o sucesso pretendido. No momento, estamos intensificando a busca por uma melhor expressão da proteína, testando outros tempos de crescimento bacteriano, outras concentrações de IPTG, meios mais ricos em nutrientes e temperaturas de crescimento diferentes. Além disso, para nos certificarmos de todo o processo de clivagem e ligação da sequência no plasmídeo pET30a(+), amostras foram mandadas para sequenciamento durante o desenvolvimento dessa dissertação. Caso o resultado seja condizente com a sequência inserida no plasmídeo corretamente, o próximo passo será testar todas condições citadas acima.

O teste inicial realizado com HSPA1A (Hsp70 humana) mostrou que ela levou a uma modulação parcial de células dendríticas em relação a DnaK de *M. tuberculosis*. Acreditamos que o efeito de ambos homólogos de mamíferos não seja semelhante ao da DnaK, sendo capaz de polarizar macrófagos (65), impedir a maturação de células dendríticas (1) ou prolongar a aceitação do aloenxerto cutâneo (2). Isso vai ao encontro do que já foi citado anteriormente que diz que não necessariamente uma proteína mesmo com quase 80% de similaridade tenha um efeito semelhante no mesmo sistema experimental.

O interessante do atual trabalho e futuro experimentos é a diminuição de variáveis que acabam confundindo muito os estudos nessa área. Purificando as proteínas no mesmo sistema, com os devidos controles de limpeza para a retirada de LPS e testando-as em paralelo esses interferentes deixam de mascarar o efeito dessas proteínas sobre células imunes.

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ANEXO A - Parecer de aprovação da Comissão de Experimentação e Uso de Animais –
CEUA



Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA, INOVAÇÃO E DESENVOLVIMENTO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Ofício 89/2015 - CEUA

Porto Alegre, 19 de novembro de 2015.

Prezado Sr(a). Pesquisador(a),

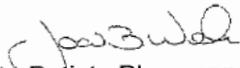
A Comissão de Ética no Uso de Animais da PUCRS apreciou e aprovou seu Protocolo de Pesquisa, registro CEUA 15/00466 intitulado **“Papel imunoregulador da HSP70 extracelular de M. tuberculosis, murina e humana”**.

Sua investigação, respeitando com detalhe as descrições contidas no projeto e formulários avaliados pela CEUA, está **autorizada** a partir da presente data.

Informamos que é necessário o encaminhamento de relatório final quando finalizar esta investigação. Adicionalmente, ressaltamos que conforme previsto na Lei no. 11.794, de 08 de outubro de 2008 (Lei Arouca), que regulamenta os procedimentos para o uso científico de animais, é função da CEUA zelar pelo cumprimento dos procedimentos informados, realizando inspeções periódicas nos locais de pesquisa.

Nº de Animais	Espécie	Duração do Projeto
126	Camundongos C57BL/6 e BALB/c	12/2015 – 02/2016

Atenciosamente,


Prof. Dr. João Batista Blessmann Weber
Coordenador da CEUA/PUCRS

Ilma. Sra.

Profa. Dra. Cristiana Beatriz C. Bonorino

FABIO

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ANEXO B – Carta de submissão do Manuscrito

Date: June 15, 2016 at 11:03:19 AM GMT-3

Ms. No.: CYTO-16-60

Title: IL-10 is required for polarization of macrophages to M2-like phenotype by mycobacterial DnaK (Heat shock protein 70)

Corresponding Author: Dr. Cristina Bonorino

Authors: Rafael L Lopes, MSc; Thiago J Borges, Ph.D.; Rafael F Zanin, Ph.D.;

Dear Dr. Bonorino,

Your submission, referenced above, has been assigned the following manuscript number: CYTO-16-60

You will be able to check on the progress of your paper by logging on to the Elsevier Editorial System as an author:

<http://ees.elsevier.com/ycyto/>

Your username is: cbonorino@pucri.br

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http://ees.elsevier.com/ycyto/automail_query.asp

Thank you for submitting your work to Cytokine.

Kind regards,

Cytokine

ANEXO C – Carta de aceite para publicação do Manuscrito

Date: Monday, February 1, 2016 at 10:37

Ms. No.: CYTO-16-60R1

Title: IL-10 is required for polarization of macrophages to M2-like phenotype by mycobacterial DnaK (Heat shock protein 70)

Corresponding Author: Dr. Cristina Bonorino

Authors: Rafael L Lopes, MSc; Thiago J Borges, Ph.D.; Rafael F Zanin, Ph.D.;

Dear Dr. Bonorino,

I am pleased to inform you that your manuscript referenced above has been accepted for publication in Cytokine.

Your accepted manuscript will now be transferred to our production department and work will begin on creation of the proof. If we need any additional information to create the proof, we will let you know. If not, you will be contacted again in the next few days with a request to approve the proof and to complete a number of online forms that are required for publication.

Cytokine is the official journal of the International Cytokine Society whose principal function is to hold a major conference annually at different sites around the world. If you are not already a member of the International Cytokine Society and wish to know more about its activities, please visit <http://www.weizmann.ac.il/cytokine/>.

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Thank you for sending the results of your interesting work to Cytokine. I look forward to receiving additional papers from you in the future.

With kind regards,

Cytokine