PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL
FACULDADE DE BIOCIÊNCIAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

THIAGO DE JESUS BORGES

VIAS EFETORAS PELAS QUAIS A Hsp70 DE *Mycobacterium tuberculosis* INIBE A REJEIÇÃO AGUDA EM UM MODELO DE ALOENXERTO CUTÂNEO

Porto Alegre
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DISSERTAÇÃO DE MESTRADO

VIAS EFETORAS PELAS QUAIS A Hsp70 DE *Mycobacterium tuberculosis* INIBE A REJEIÇÃO AGUDA EM UM MODELO DE ALOENXERTO CUTÂNEO

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

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Dedico este trabalho aos meus pais e seus companheiros, às minhas irmãs, à minha namorada e aos meus familiares que me ampararam e auxiliaram nos momentos de dificuldades. E que foram meu suporte e inspiração para continuar nesta caminhada profissional.

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RESUMO

O transplante de órgãos sólidos emergiu como uma terapia viável para o tratamento de uma variedade de patologias. A rejeição dos enxertos é resultado de uma série complexa e coordenada de interações envolvendo o sistema imune inato e adaptativo. Com isso, o maior desafio no transplante de órgãos sólidos é induzir um estado irresponsivo e específico ao doador em um sistema imune maduro sem que haja uma imunossupressão sistêmica e de longo prazo, tudo isso livre de rejeição crônica. As limitações no estabelecimento de estratégias imunossupressoras nos levaram a buscar novos métodos para a modulação dos mecanismos homeostáticos que previnem e limitam as respostas inflamatórias nos tecidos enxertados. A proteína de choque térmico (*Heat shock protein* – Hsp) 70 tem um papel antiinflamatório e protetor em modelos animais experimentais como artrite, colite, fibrose pulmonar e danos cerebrais. Essa proteína pode modular tanto o sistema imune inato quanto o adaptativo. Nosso grupo demonstrou que a Hsp70 de *Mycobacterium tuberculosis* (Mt Hsp70) pode inibir a maturação de células dendríticas diferenciadas da medula óssea (*bone marrow dendritic cells* – BMDCs), porém o mecanismo envolvido nesse processo ainda não foi totalmente esclarecido. Nesse trabalho, demonstramos que a Mt Hsp70 foi capaz de aumentar a sobrevida do enxerto em dois modelos murinos de transplantes (um modelo tumoral e um modelo de aloenxerto cutâneo). Em ambos os modelos, observamos o envolvimento de Tregs. Demonstramos que a administração s.c. da Mt Hsp70 levou a um aumento dessas células nos linfonodos drenantes, além de um aumento na produção de IL-10. Também observamos que a inibição da rejeição aguda induzida pela Mt Hsp70 no modelo de aloenxerto cutâneo é dependente da presença do receptor do tipo toll (*toll like receptor* – TLR) 2 no enxerto, e não no receptor. Nas BMDCs, vimos que a indução da IL-10 induzida pela Mt Hsp70 é dependente de TLR2. Ainda nessas células, analisamos a fosforilação de moléculas como a ERK, p38 e Akt após o estímulo com a Mt Hsp70. Observamos um aumento na expressão de p-ERK e nenhuma alteração nos níveis de p-p38 e p-Akt. A inibição da ERK aboliu a produção de IL-10 induzida pela Mt Hsp70. Propomos que esse efeito da Mt Hsp70 sobre as DCs pode servir como intervenção terapêutica em modelos de transplantes.

Palavras-chaves: Hsp70, transplante, imunossupressão, Tregs, células dendríticas

ABSTRACT

Transplantation of solid organs has emerged as a viable therapeutic modality for the treatment of a variety of disorders. Rejection of solid organ allografts is the result of a complex range of interactions involving coordination between both the innate and adaptive immune system. Therewith, a major goal of clinical organ transplantation is to induce a donor-specific unresponsive state in a mature immune system that is free from long-term immunosuppression and chronic rejection. The limitations in the establishment of immunossupressive stratagies led us to search new methods to the modulation of the homeostatic mechanisms that limit and prevent inflammatory responses in allograft tissue. The heat shock protein 70 (Hsp70) has a protective and antiinflamatory role in several animals models like arthritis, colitis, pulmonary fibrosis and brain injury. This protein can modulates both the innate and adaptative immune system. Our group demonstrated that *Mycobacterium tuberculosis* Hsp70 (Mt Hsp70) can inhibit bone marrow dendritic cells (BMDCs) maturation; however the mechanisms involved in this process has not been completely elucidated. In the present work, we demonstrated that Mt Hsp70 inhibited the acute rejection in two allograft models (a tumor model and a skin allograft model). In both models, we observed an involvement of Tregs. In addition, s.c. Mt Hsp70 injection leds to an increase in Treg population and IL-10 production in the draining lymph node. We also observed that the inhibition of acute rejection induced by Mt Hsp70 was dependent on the presence of toll-like receptor (TLR) 2 in the allograft, and not in the host. In BMDCs, we demonstrated that IL-10 production induced by Mt Hsp70 is dependent on TLR2. Also, we analyzed the phosphorylation of ERK, p38 and Akt after Mt Hsp70 stimulus. We observed an increase in p-ERK expression, but no difference in p-38 and p-Akt levels. The inhibition of ERK abolished the IL-10 production induced by Mt Hsp70. We propose that Mt Hsp70 effect on DCs can be used as a therapeutic approach in transplantation models.

Key- words: Hsp70, transplantation, immunosuppression, Tregs, dendritic cells

LISTA DE FIGURAS

Capítulo 1 - Figura 1: Dinâmica célular da rejeição em um modelo de aloenxerto cutâneo murino.

Capítulo 1 - Figura 2: Modulação do sistema imune inato e adaptativo pela Hsp70.

Capítulo 2 - Figura 1: Hsp70 can interact directly with innate immune cells.

Capítulo 2 - Figura 2: Hsp-specific immunoregulation in the healthy and aged immune system.

Capítulo 3 - Figura 1: B16F10 tumor allograft in BALB/c mice.

Capítulo 3 - Figura 2: Treg cells are observed at the tumor allograft site.

Capítulo 3 - Figura 3: Local injection of TBHsp70 induces Tregs, IL-10 and leads to suppression in the draining lymph nodes.

Capítulo 3 - Figura 4: TBHsp70 delays skin graft rejection.

Capítulo 3 - Figura 5: CD4+CD25+ regulatory T cells are crucial for prolonged survival induced by TBHsp70.

Capítulo 4 - Figura 1: A inibição da rejeição aguda induzida pela Mt Hsp70 não depende da presença do TLR2 nos receptores.

Capítulo 4 - Figura 2: A inibição da rejeição aguda induzida pela Mt Hsp70 depende da presença do TLR2 nos enxertos.

Capítulo 4 - Figura 3: A Mt Hsp70 induz a produção de IL-10 nas BMDCs em um mecanismo dependente do TLR2.

Capítulo 4 - Figura 4: A Mt Hsp70 induz a expressão de p-ERK 1/2 nas BMDCs via TLR2.

Capítulo 4 - Figura 5: A Mt Hsp70 não induz a expressão de p-p38 nas BMDCs.

Capítulo 4 - Figura 6: A Mt Hsp70 não induz a expressão de p-Akt nas BMDCs.

Capítulo 4 - Figura 7: Nas BMDCs, a produção de IL-10 induzida pela Mt Hsp70 é dependente de ERK.

LISTA DE SIGLAS

APC – *Antigen-presenting* cell*;* Célula apresentadora de antígenos
ASGPR – *Asialoglycoprotein receptor*
BM – *Bone marrow*; Medula óssea
BMDC – *Bone marrow dendritic cell*; Célula dendrítica diferenciada da medula óssea
CCR5 – *C-C chemokine receptor*; Receptor de quimiocina C-C
CD – *Cluster of diferentiation;* Grupo de diferenciação
CTLA-4 - *Cytotoxic T-lymphocyte antigen-4*
DC – *Dendritic cell;* Célula dendrítica
DC-SIGN - *Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin*
DEXA - Dexametasona
DNA - *Deoxyribonucleic acid*; Ácido desoxirribonucleico
Foxp3 – *Factor forkhead Box P3*
GM-CSF - *Granulocyte–macrophage colony-stimulating*
Hsp - *Heat shock protein;* Proteína de choque térmico
IFN - Interferon
Ig - Imunoglobulina
IL - Interleucina
LLR – *Lectin-like receptors*
LOX-1 - *Lectin-like Oxidized Low-density Lipoprotein Receptor 1*
LPS - Lipopolissacarídeo
MFI - *Mean Fluorescence Intensity;* Média da intensidade fluorescente
MHC – *Major histocompatibility complex;* Complexo principal de histocompatibilidade
Mt – *Mycobacterium tuberculosis*
OVA - Ovalbumina
PBS – *Phosphate buffered saline;* Tampão fostato-salino
PGN - Peptidoglicano
TGF – *Transforming growth factor;* Fator de crescimento transformador
TNF - *Tumor necrosis factor;* Fator de necrose tumoral
TLR- *Toll like receptor*; Receptor do tipo Toll

SUMÁRIO

CAPÍTULO 1 ..... **10**

INTRODUÇÃO **11**

1.1 PROBLEMA DOS TRANSPLANTES 11

1.1.1 Contexto histórico *11*

1.1.2 Base celular da rejeição *11*

1.1.3 Tolerância ao enxerto e as células imunes *13*

1.1.4 Desafios *14*

1.2 PROTEÍNAS DE CHOQUE TÉRMICO (HSPS) 15

1.2.1 Hsp70 e seus efeitos imunoreguladores *17*

2. OBJETIVOS **20**

2.1. OBJETIVO GERAL 20

2.2. OBJETIVOS ESPECÍFICOS 20

3. JUSTIFICATIVA **21**

CAPÍTULO 2 ..... **22**

ARTIGO DE REVISÃO PUBLICADO EM *FRONTIERS IN IMMUNOLOGY*. 2012 MAY 4; 3:95. 22

CAPÍTULO 3 ..... **35**

ARTIGO CIENTÍFICO PUBLICADO EM *PLOS ONE*. 2010 DEC 8;5(12):E14264. 35

CAPÍTULO 4 ..... **44**

RESULTADOS COMPLEMENTARES 44

CAPÍTULO 5 ..... **57**

CONSIDERAÇÕES FINAIS 57

REFERÊNCIAS **62**

Capítulo 1

INTRODUÇÃO

1.1 Problema dos Transplantes

1.1.1 Contexto histórico

Queimaduras sofridas por vítimas de ataques aéros na 2ª Guerra Mundial motivaram o biólogo Sir Peter Medawar (ganhador do prêmio Nobel de Fisiologia e Medicina de 1960) em seus famosos estudos sobre a imunologia de transplantes. Buscando entender os mecanismos da rejeição a transplantes cutâneos, ele observou que coelhos rejeitavam enxertos cutâneos de outros indivíduos mais rapidamente do que se os animais fossem re-transplantados com um enxerto do mesmo doador (1).

Em 1953, Billingham, Brent e Medawar descreveram, em camundongos, a tolerância imunológica adquirida a aloantígenos (2). Esse artigo é considerado um marco na história da imunologia dos transplantes. No ano seguinte, a equipe lidera por Joseph Murray realizou com sucesso, no Hospital *Brigham and Women*, o primeiro transplante renal entre gêmos idênticos (3).

1.1.2 Base celular da rejeição

O transplante de órgãos sólidos emergiu como uma terapia viável para o tratamento de uma variedade de patologias. A rejeição dos enxertos é resultado de uma série complexa e coordenada de interações envolvendo o sistema imune inato e adaptativo (4). A base molecular da rejeição está na habilidade das células T reconhecerem versões polimórficas de uma gama de proteínas, nesse caso, aloantígenos provenientes de um organismo geneticamente diferente do receptor. No momento o qual as células T específicas reconhecerem esses aloantígenos, elas irão proliferar, se diferenciar em células efetoras e ocorrerá a migração para o local do enxerto onde irão promover a destruição do tecido (rejeição) (5).

A dinâmica celular envolvida no processo de rejeição a enxertos cutâneos é complexa e está associada com uma resposta imune potente levando à destruição das células do doador. Em um modelo de transplante cutâneo murino, no primeiro dia após o transplante, monócitos do

receptor começam a entrar no tecido transplantado (6) e células dendríticas (DCs) da pele migram, através de vasos linfáticos, e infiltram o linfonodo drenante do receptor. No linfonodo drenante, as DCs da pele apresentam antígenos do doador através de dois mecanismos: (a) a via direta, na qual células T reconhecem antígenos intactos do MHC nas DCs do doador; (b) via indireta, que envolve o reconhecimento pelas células T de peptídeos do doador sendo apresentados em molécular de MHC na superfície das DCs do receptor (7). Foi visto recemente que além das DCs, um grande número de monócitos do receptor, carregados com peptídeos do doador, entram no linfonodo drenante e ativam um grupo de células T (8). As células NK, que são ativadas pela ausência da expressão de moléculas de MHC classe I nas células do doador, também estão envolvidas no processo de rejeição a enxertos (5). Elas contribuem matando DCs dermais do doador que vieram do enxerto (8, 9) e produzem citocinas proinflamatórias como o INF-γ e TNF-α (10).

No dia 6 após o transplante, é observado um grande infiltrado de monócitos no tecido da pele, e células T efetoras (CD4+ e CD8+) começam a migrar lateralmente do tecido adjacente para o enxerto (8). Finalmente, no dia 10 após o transplante, células T CD8+ destroem as células estranhas do doador, levando a necrose e aos estágios finais da rejeição (Figura 1).

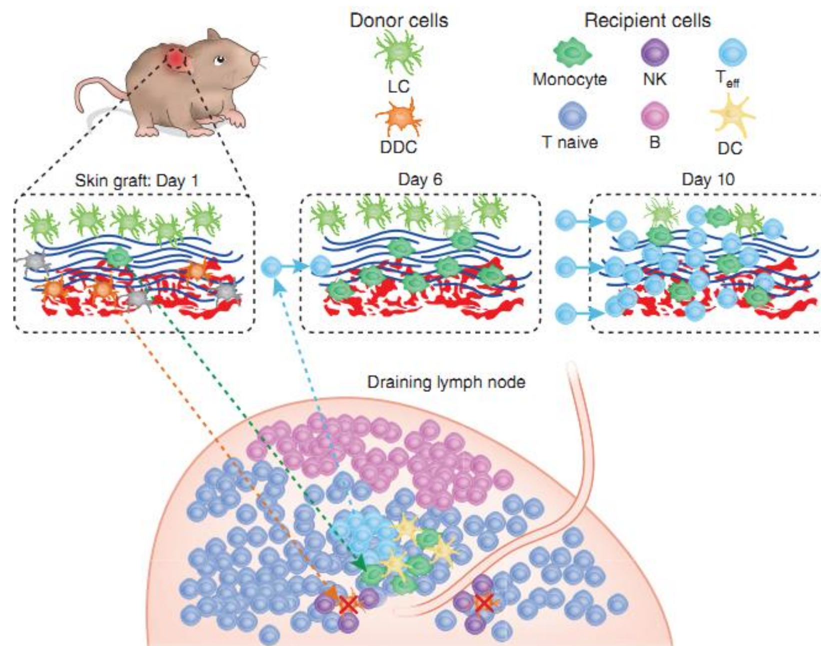

Figura 1 – Dinâmica célular da rejeição em um modelo de aloenxerto cutâneo murino. Retirado de Celli *et al.* (2011).

1.1.3 Tolerância ao enxerto e as células imunes

A tolerância a enxertos ideal seria um estado no qual o órgão do doador é aceito sem uma terapia imunossupressora crônica, enquanto o restante do sistema imune é mantido intacto. Deste modo, a falta de uma resposta patogênica aos aloantígenos seria específica, e o receptor seria capaz de responder a microorganismos patogênicos e danos. A tolerância não implica na falta de respostas imunes. Na verdade, há evidencias de mecanismos imunoreguladores ativos, os quais podem operar para manter a tolerância aos enxertos (11).

Um desses mecanismos são as células T reguladoras (Tregs). Elas possuem um papel fundamental na supressão de respostas imunes efetoras que são excessivas e que podem causar dano ao tecido (12). Essas células podem ser divididas em dois subtipos: (a) as Tregs naturais (nTregs) que são desenvolvidas no timo, expressam o fator de transcrição Foxp3 (13) e são HELIOS+ (14); (b) as Tregs induzidas (iTregs), as quais podem se desenvolver nos tecidos periféricos quando forem expostas a sinais específicos provenientes das células apresentadoras de antígenos (APCs) (15). Além disso, as iTregs podem expressar ou não o Foxp3 e são HELIOS- (14). Ambos os subtipos são essenciais para a manutenção da tolerância à autoantígenos, e na sua ausência, há o desenvolvimento de uma síndrome autoimune específica ao tecido (16).

As células Tregs são CD25+. Essas células possuem uma habilidade em inibir a proliferação de células T efetoras nos linfonodos drenantes e também são responsáveis por controlar sua homeostase (13, 17, 18). Essas células utilizam alguns mecanismos supressivos de acordo com seu modo de ação: i) a produção de citocinas anti-inflamatórias como a interleucina (IL)-10, IL-35 e o fator de crescimento transformante-β (TGF-β); ii) citólise por mecanismos de morte celular dependentes de perforinas, granzima A e granzima B; iii) comprometimento metabólico e iv) conversão de APCs a um estado tolerogênico como, por exemplo, através da ligação da molécula CTLA-4 presente nas Tregs com os receptores CD80 ou CD86 presentes nas APCs (19).

As Tregs podem ser usadas para controlar as respostas contra antígenos não próprios que são introduzidos no hospedeiro, como no caso dos transplantes, e promovem a sobrevivência indefinida de aloenxertos em vários modelos experimentais (20, 21). Além disso, evidências de estudos experimentais e clínicos indicam que um balanço entre a regulação e a deleção de células T responsivas ao tecido do doador é uma estratégia eficaz para controlar a resposta imune após o

transplante de órgãos ou células (22, 23). Isso torna as Tregs atrativas para a aplicação na tolerância a transplantes.

Outro mecanismo que pode contribuir para a tolerância aos enxertos são as DCs tolerizadas. Essas células são caracterizadas pela baixa produção de citocinas proinflamatórias e por uma alta produção de citocinas anti-inflamatórias, como a IL-10 e o TGF-β. Foi demonstrado que essas células expressam níveis baixos do MHC II e de moléculas co-estimulatórias, como o CD80 e o CD86 (24-26). Mesmo com baixos níveis de MHC II, essas células conseguem apresentar antígenos a células T específicas. Porém, com a falta dos sinais co-estimulatórios (segundo sinal) não há a ativação e proliferação de células T efetoras. Os baixos níveis da apresentação de antígeno juntamente com a falta de co-estimulação tem sido associado à diferenciação das células em Tregs (27-29).

Com isso, as DCs tolerizadas podem regular respostas imunes inatas e adaptativas (26), incluindo a resposta de células T CD4+ e CD8+ de memória (30, 31). Foi visto que essas DCs sozinhas podem promover a tolerância a transplantes em modelos exprimentais, ou em conjunto com terapias imunossupressivas convencionais ou experimentais (32). Assim, os transplantes oferecem uma oportunidade para manipular as DCs antes ou depois do início da resposta imune. Além disso, as vias direta e indireta de reconhecimento de aloantígenos proporcionam possíveis alvos para a tolerização das DCs, provomendo assim, uma melhor sobrevida do enxerto (26).

Portanto, as células Tregs e as DCs tolerizadas são consideradas como terapias promissoras para a rejeição a enxertos e para a promoção de uma tolerância específica para o doador. O uso de moléculas que estimulem a interação entre essas duas populações de células reguladoras podem ser de grande importância para a manutenção da tolerância e o sucesso de protocolos que visam uma melhora em longo prazo na sobrevida de enxertos.

1.1.4 Desafios

Desde a introdução do transplante de órgãos na prática médica, o conhecimento deste campo se mostrou rápido e otimista. O aperfeiçoamento das drogas imunossupressoras e o auxílio com os transplantados aumentaram as taxas de sobrevida em curto prazo dos pacientes e dos enxertos entre um e três anos (11). Porém, esse avanço é mascarado por graves problemas, como a baixa taxa de sobrevida do enxerto em longo prazo (menor que cinco anos). Com isso, o maior desafio no transplante de órgãos sólidos é induzir um estado irresponsivo e específico ao doador

em um sistema imune maduro sem que haja uma imunossupressão sistêmica e de longo prazo, tudo isso livre de rejeição crônica. Podemos citar três problemas que nos levam a não alcançar esse objetivo: (a) a alta incidênciade rejeição crônica após o quinto ano do transplante; (b) a necessidade de uso contínuo de imunossupressores que levam a maior suscetibilidade a infecções oportunistas e tumores, além de inúmeros efeitos colaterais; (c) discrepância entre a demanda e a disponibilidade de órgãos (33).

Essas limitações no estabelecimento de estratégias imunossupressoras nos leva a buscar novos métodos para a modulação dos mecanismos homeostáticos que previnem e limitam as respostas inflamatórias nos tecidos enxertados.

1.2 Proteínas de choque térmico (Hsps)

As proteínas de choque térmico ou estresse (Hsps) foram primeiramente descritas em 1962 (34) e formam um grupo de proteínas induzidas por estresses celulares como o calor e radiação ionizante, sendo distribuídas de forma ubíqua entre organismos procarióticos e eucarióticos. As Hsps de mamíferos podem ser classificadas em cinco principais famílias de acordo com seu peso molecular: Hsp100, Hsp90, Hsp70, Hsp60 e sHsp (*small heat shock proteins*) e estão presentes no citosol, membrana, núcleo, retículo endoplasmático e mitocôndria da célula (35).

Cada família é composta por membros expressos constitutivamente e outros induzidos. Funcionam principalmente como chaperonas moleculares, transportando proteínas entre compartimentos celulares, ajudando no dobramento de proteínas que estão sendo formadas ou no redobramento de proteínas que sofreram danos, protegendo a agregação de outras proteínas, além de direcionar proteínas às rotas de degradação e auxiliar na dissolução de complexos protéicos (36).

Em condições fisiológicas, as Hsps distribuídas ubiquamente mantém a integridade da função de outras proteínas celulares quando expostas a um estímulo de estresse. Elas são as proteínas mais conservadas e imunogênicas compartilhadas entre mamíferos e microorganismos (37). Nos mamíferos, durante infecções por bactérias, membros bacterianos das famílias da

Hsp60 e da Hsp70 (GroEL e DnaK, respectivamente) são alvos comuns da resposta humoral e da resposta imune mediada por células (38-40).

A análise das respostas imunes às Hsps em modelos experimentais e pacientes tem indicado a capacidade das Hsps de induzir respostas de células T reguladoras, sugerindo que essas proteínas possuem características únicas que permite a elas terem essa capacidade de induzir uma imunoregulação (41).

Historicamente, tem sido demonstrado que as Hsps possuem algumas características que fazem delas alvos importantes do sistema imune (42, 43). Isso parece refletir no fato de que as Hsps são proteínas imunodominantes. Em alguns estudos, entre 10-20% das células T reconheceram a Hsp60 de *Mycobacterium tuberculosis* após a imunização com a micobactéria (44). Foi demonstrado que a Hsp70 de *Mycobacterium leprae* é um antígeno relevante em humanos infectados com *M. leprae* (44, 45). Essas respostas de células T específicas a Hsps micobacterianas também foram observadas em indivíduos saudáveis que não foram expostos a infecções micobacerianas (46). A imunodominância das Hsps micobacterianas foi inesperada dada pelo grande nível de conservação e, portanto uma forte homologia com Hsps próprias (47-49).

Interessantemente, uma análise detalhada dos peptídeos que são reconhecidos pelas células T, tanto de indivíduos saudavéis quanto de infectados, revelou que alguns desses peptídeos eram altamente conservados (50, 51). Com a observação de que as Hsps procarióticas e eucarióticas possuem um alto grau de homologia, foi proposta a hipótese na qual as Hsps são canditadas potenciais para o mimetismo molecular e podem agir, potencialmente, como autoantígenos (52). Com isso, pode haver uma resposta de reatividade cruzada entre Hsps microbianas exógenas e Hsps próprias (53). Parece que o efeito protetor das Hsps microbacterianas está relacionado, pelo menos em parte, a capacidade delas induzirem respostas de mediadas por células T as quais tem uma reatividade cruzada com Hsps próprias.

Em 1991, Cohen e Young propuseram o *immunological homunculus* que consistem em: na tentativa de se evitar respostas imunes excessivas contra antígenos próprios e estrangeiros, algumas proteínas imunodominantes poderiam ser usadas pelo organismo para balancear e monitorar o sistema imune (54, 55). As Hsps parecem fazer parte dessas proteínas e podem ser fundamentais para inibir uma resposta inflamatória exacerbada *in vivo*, como no caso da rejeição a enxertos.

1.2.1 Hsp70 e seus efeitos imunoreguladores

A família Hsp70 é a mais conservada e a melhor estudada entre as outras famílias de proteínas de choque de calor (56, 57). Sua expressão é induzida nas células expostas ao calor e a uma variedade de outros estímulos estressantes, como espécies reativas de oxigênio, infecção, inflamação, hipóxia e drogas antitumorais (58). A Hsp70 utiliza dois domínios funcionais para realizar sua atividade de chaperona, o domínio C-terminal de 18 kDa que se liga ao substrato protéico e o domínio N-terminal de 44 kDa que possui atividade ATPásica e controla a abertura e o fechamento do domínio C-terminal (59, 60). A ligação e liberação do substrato protéico são moduladas pela afinidade intrínseca do peptídeo com a Hsp70 em ciclos de ligação e hidrólise de ATP (61, 62). Para adaptar este mecanismo de ação para específicas funções uma variedade de proteínas acessórias, chamadas de co-chaperonas, interagem com a Hsp70 e regulam sua atividade ATPásica.

Alguns grupos verificaram que membros da família da Hsp70 eram capazes de induzir respostas antiinflamatórias e imunossupressoras em modelos inflamatórios animais, como por exemplo, a proteção contra a artrite pode ser proporcionada através de pré-imunizações com a Hsp70 de *Mycobacterium tuberculosis* (63-65). Além disso, a Hsp70 murina endógena foi capaz de proteger os animais contra dois modelos de colite experimental (66) e contra um modelo de fibrose pulmonar (67). Também foi observado que a superexpressão dessa proteína levou a uma proteção em diferentes modelos de injuria no sitema nervoso (68).

Mas como a Hsp70 estaria modulando a resposta imune? A primeira linha de pensamento se foca na modulação de células do sistema imune inato (69). Esses estudos analisaram a interação da Hsp70, disponível no meio extracelular ou presente na porção extracelular da membrana plasmática, com receptores presentes nessas células. Por exemplo, para os receptores do tipo *toll* (TLR) 2 e 4 (70), CD14 (71), CD91 (72), LOX-1 (73), CD40 (74) e o CCR5 (75) foi relatado que poderiam se ligar a Hsp70 e induzir resposta proinflamatórias. Porém, esses efeitos foram associados com a contaminação da proteína por LPS ou outros compostos microbianos (76, 77). Isso porque os efeitos inflamatórios da Hsp70 sumiram quando o LPS foi cuidadosamente removido da proteína (78, 79).

Em contrapartida, em estudos nos quais a Hsp70 está livre dos contaminantes, ela apresentou uma capacidade de modular células do sistema imune inato em um perfil

imunossupressor. Esses estudos mostraram esse perfil em células dendríticas (80), células supressoras mielóides (*myeloid-derived supressor cells* – MDSCs) (81) e monócitos (82). Um estudo do nosso grupo demonstrou que a Hsp70 de *Mycobacterium tuberculosis* (Mt Hsp70) bloqueia a diferenciação de DCs a partir de células precurssoras da medula óssea (*bone marrow* - BM). BMDCs foram tratadas com Hsp70 e foi observada uma inibição da maturação dessas células, a qual foi caracterizada pela baixa expressão de MHC II e CD86. Além disso, essas células apresentaram uma grande produção de IL-10, principal citocina antiinflamatória (80). Isso nos indica que DCs tratadas com Hsp70 se apresentam um fenótipo tolerizado e, *in vivo*, podem favorecer a criação de um ambiente supressor propicio ao surgimento de células Tregs.

Em outro estudo realizado pelo nosso grupo, células do líquido sinovial provenientes de pacientes com artrite foram incubadas com Mt Hsp70 por 48 horas. Após esse período, essas células apresentaram uma reversão do perfil inflamatório, uma alta produção de IL-10 e uma diminuição da produção de TNF-α e IFN-γ (82). Chalmin e colaboradores demonstaram, em um modelo murino, que células tumorais liberam moléculas de Hsp72 associadas à exossomos, resultando na ativação das funções supressoras das MDSCs. Os autores viram que esse efeito foi dependente de TLR2 e MyD88 e indepente de TLR4 e Trif (81). Essa dependência de TLR2 é corroborada pelo fato de que vem sendo demonstrado que esse receptor é ótimo em induzir a produção de IL-10 (83-86), além de ser importantíssimo na indução de respostas inflamatórias contra patógenos (87, 88). O fato da Hsp70 induzir IL-10 em diferentes sistemas, hipotetizamos que ela pode ser um ligante do TLR2.

A outra linha de pensamento se foca na modulação do sistema imune adaptativo pela Hsp70. O mecanismo antiinflamatório proposto é a indução de células T reguladoras específicas a Hsp70 em condições fisiológicas (53). Esse mesmo efeito foi associado a outras Hsps, como a Hsp60 (89). O papel da Hsp70 mediando à imunossupressão na imunidade adaptativa através das Tregs pode ser relacionado à apresentação de peptídeos conservados dessa proteína (43, 64) ou, como discutido acima, pela modulação do ambiente por células do sistema imune. Por exemplo, quando administrados de forma intranasal, peptídeos conservados entre a Hsp70 de *M. tuberculosis* e ratos podem suprimir artrite adjuvante. Esse efeito era mediado em ratos pela indução de Tregs produtoras de IL-10 e TGF-β. Também foi visto que essas Tregs eram específicas para a Hsp70 própria, indicando que houve uma reatividade cruzada (90). A apresentação de peptídeos da Hsp70 nas moléculas de MHC pode ser resultado de uma

superexpressão da Hsp70 endógena em situações fisiológicas de estresse ou a partir da endocitose de moléculas de Hsp70 extracelulares (91, 92).

Portanto, parece a Hsp70 pode modular tanto a imunidade inata quanto a adaptativa (Figura 2). Porém, as bases moleculares e em quais situações isso ocorre precisa ser esclarecido melhor. A possibilidade de essa proteína estar modulando simultaneamente ambas as vias celulares não pode ser excluída. A intenção desse trabalho foi tentar elucidar um pouco mais do mecanismo envolvido na imunossupressão induzida pela Hsp70 em um modelo de aloenxerto cutâneo murino.

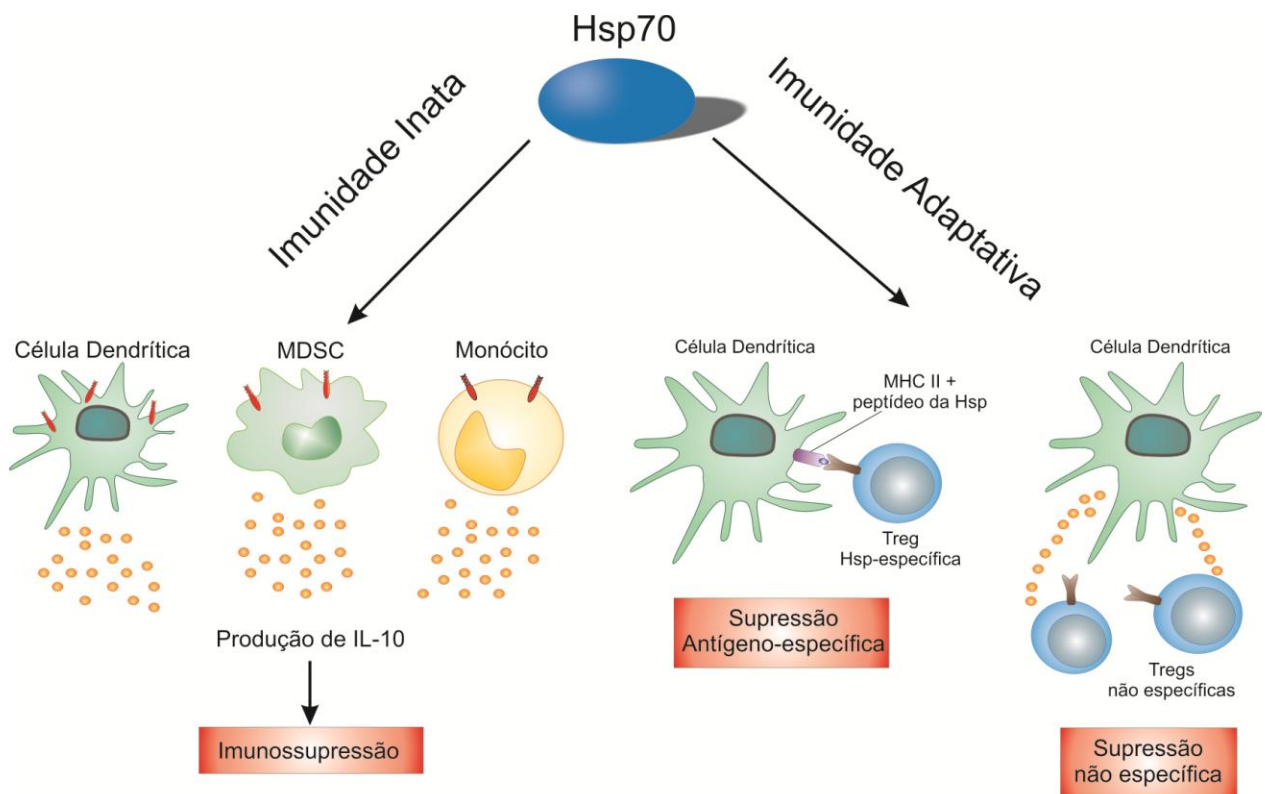

Figura 2 – Modulação do sistema imune inato e adaptativo pela Hsp70.

2. OBJETIVOS

2.1. Objetivo Geral

Analisar os mecanismos pelos quais a Hsp70 de *Mycobacterium tuberculosis* desempenha seu potencial efeito imunossupressor em um modelo de aloenxerto cutâneo.

2.2. Objetivos Específicos

2.2.1. Verificar o papel da Mt Hsp70 na inibição da rejeição aguda em um modelo de aloenxerto cutâneo;

2.2.2. Analisar se a Mt Hsp70 induz a geração de células T reguladoras;

2.2.3. Testar o papel das células T reguladoras na inibição da rejeição aguda de enxerto mediado pela Mt Hsp70;

2.2.4. Verificar os níveis de TGF-β, IL-10, IL-2, IL-4, IL-6, IL-17A, IFN-γ, TNF-α e IL-12 após administração de Mt Hsp70;

2.2.5. Testar o papel do TLR2 na imunossupressão mediada pela Mt Hsp70.

3. JUSTIFICATIVA

A necessidade do aprofundamento nos testes sobre o efeito imunomodulador da Mt Hsp70 e a identificação de peptídeos conservados que tenham o mesmo efeito em um modelo de transplante é essencial para a confirmação da geração de Tregs e do papel imunossupressor exercido por essa proteína. O modelo de aloenxerto cutâneo é bem estabelecido, estudado e caracterizado na literatura. Este modelo se torna uma excelente ferramenta para verificarmos se a Mt Hsp70 possui as mesmas propriedades vistas anteriormente pelo nosso grupo. Adicionalmente, poderemos verificar se os achados de que a Hsp70, neste caso a micobacteriana e não a endógena, como descrito, estaria se ligando no TLR2 e causando uma imunossupressão local se aplicaria, também, em nosso modelo de transplante. A confirmação desta atividade torna a Hsp70 uma forte candidata a se tornar uma terapia inovadora no tratamento da rejeição aguda a enxertos. Uma vez que provavelmente seu efeito é local evitando a contra-regulação de células T reguladoras, o receptor do transplante não entraria em um quadro de imunossupressão sistêmica causada pelos fármacos administrados atualmente.

Capítulo 2

The Anti-inflammatory mechanisms of Hsp70

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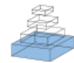

The anti-inflammatory mechanisms of Hsp70

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Immune responses to heat shock proteins (Hsp) develop in virtually all inflammatory diseases; however, the significance of such responses is only now becoming clear. In experimental disease models, Hsp administration can prevent or arrest inflammatory damage, and in initial clinical trials in patients with chronic inflammatory diseases, Hsp peptides have been shown to promote the production of anti-inflammatory cytokines, indicating immunoregulatory potential of Hsp. Therefore, the presence of immune responses to Hsp in inflammatory diseases can be seen as an attempt of the immune system to correct the inflammatory condition. Hsp70 can modulate inflammatory responses in models of arthritis, colitis and graft rejection, and the mechanisms underlying this effect are now being elucidated. Incubation with microbial Hsp70 was seen to induce tolerogenic dendritic cells (DCs) and to promote a suppressive phenotype in myeloid-derived suppressor cells and monocytes. These DC could induce regulatory T cells (Tregs), independently of the antigens they presented. Some Hsp70 family members are associated with autophagy, leading to a preferential uploading of Hsp70 peptides in MHC class II molecules of stressed cells. Henceforth, conserved Hsp70 peptides may be presented in these situations and constitute targets of Tregs, contributing to downregulation of inflammation. Finally, an interfering effect in multiple intracellular inflammatory signaling pathways is also known for Hsp70. Altogether it seems attractive to use Hsp70, or its derivative peptides, for modulation of inflammation. This is a physiological immunotherapy approach, without the immediate necessity of defining disease-specific auto-antigens. In this article, we present the evidence on anti-inflammatory effects of Hsp70 and discuss the need for experiments that will be crucial for the further exploration of the immunosuppressive potential of this protein.

Keywords: Hsp70, stress proteins, immunomodulation, adaptive immunity, innate immunity

Hsps ARE IMMUNODOMINANT PROTEINS

Heat shock proteins (Hsp) are highly conserved proteins, from microbes through mammals. They are preferentially induced in response to cell stresses including heat shock, oxidative stress, ultraviolet radiation, ischemia-reperfusion injury, viral infections, nutrient deprivation, and chemicals (Lindquist, 1986), protecting cells from injury and promoting refolding of denatured proteins. Hsp are grouped in families according to their molecular weight, and constitutive members of each family can be found in different cell compartments under non-stress conditions, performing chaperone functions (Lindquist and Craig, 1988).

Hsp70 is the most highly conserved protein known to date (Lindquist and Craig, 1988; Ellis, 1990; Feder and Hofmann, 1999). It was therefore surprising when Hsp, including Hsp70, were found to be immunodominant antigens. Early studies demonstrated that 10–20% of the T cells recognized Hsp60 of *Mycobacterium tuberculosis* after experimental mycobacterial immunization (Kaufmann et al., 1987). Hsp70 of *M. leprae* was shown to be a prominent antigen in humans infected with *M. leprae* (Kaufmann et al., 1987; Janson et al., 1991). Such mycobacterial-Hsp-specific T cell responses have also been observed in healthy individuals, not previously exposed to mycobacterial infections (Munk et al., 1989) and in cord blood (Fischer et al., 1992; Aalberse et al., 2011). Immunization with Hsp70 of *M. tuberculosis* (TB-Hsp70) led to a strong IgG response in 7 days without evidence of IgM production (Bonorino et al., 1998), suggesting that antigen-specific T cells able to provide help were already available in naïve mice. Interestingly, a detailed analysis of the peptides recognized by T cells, both in healthy and infected individuals, revealed that some of them were highly conserved (Quayle et al., 1992; Anderton et al., 1995).

Hsp70 AS AN IMMUNOMODULATORY AGENT

It was then hypothesized that, because of their homology with self, bacterial-Hsp would provoke autoimmunity through molecular mimicry with self-proteins. This idea was refuted by the finding that pre-immunization with bacterial-Hsp protected Lewis rats from adjuvant-induced arthritis (van Eden et al., 1988). Subsequently, immunoregulatory features of Hsp were demonstrated in various inflammatory diseases. The literature on immunomodulatory properties of Hsp is vast. In this review, we will focus on Hsp70. Although it may be tempting to generalize observations on different Hsp, it is important to consider that the different families of Hsp show no homology of sequence or structure, and are encoded by different genes, transcribed under the control

of different transcription factors, that are not always activated in coordinate manner. Rather, Hsp are grouped under the same banner because they are commonly induced in similar situations of stress, cooperating to promote cell recovery and protection from injury.

Hsp70 was demonstrated to have a disease suppressive role in experimental models of autoimmunity. One study demonstrated that T cells reactive to peptide 234–252 of TB-Hsp70 suppressed inflammatory responses against *Listeria monocytogenes* via production of IL-10 (Kimura et al., 1998). The same group later showed that pretreatment with peptide 234–252 of TB-Hsp70 suppressed the development of adjuvant-induced arthritis in Lewis rats, generating T cells that were specific for this peptide, and produced high levels of IL-10, but not IFN-g (Tanaka et al., 1999). Also the treatment with anti-IL-10 antibody abrogated protection. This peptide showed 58% amino acid identity between rat and mycobacterial Hsp70. Another study revealed that a different peptide of Hsp70, conserved between rat and mycobacteria, protected Lewis rats from development of arthritis when given intra-nasally (Wendling et al., 2000), preventing disease development by the induction of IL-10 producing T cells. Endogenous Hsp70 presence in the mouse, guaranteed by the presence of heat shock factor 1 (HSF1), its transcription factor, was found to protect from induced colitis (Tanaka et al., 2007). More recently, treatment with whole endotoxin-free TB-Hsp70 inhibited acute rejection of skin and tumor allografts (Borges et al., 2010). Consequently, disease suppressive effects have been observed in the case of both microbial and self (mammalian) Hsp70, some studies using whole protein, some studies using just the peptide, and IL-10 was always important.

How could the conservation of Hsp be reconciled with this apparent predisposition for recognition by the immune system? One idea was that the protective effects of microbial Hsp were related, at least in part, to their capacity to induce T cell responses which were cross-reactive with self-Hsp. Cohen proposed that, to avoid excessive immune responses to both self- and foreign-antigens, the immune system would be selective in its responsiveness and focus on particular immunodominant proteins: the so-called immunological homunculus (Cohen and Young, 1991; Cohen, 2007). Hsp were thus postulated to be such proteins. However, the regulatory capacity of Hsp could not be completely explained by immunodominance and homology between bacterial- and self-Hsp. This was demonstrated in studies using the adjuvant-induced arthritis model, in which Hsps, but not other highly immunogenic and conserved proteins of bacterial origin, were found to suppress disease development (Prakken et al., 2001). So, which additional features of Hsp would endow them with the capacity to suppress inflammatory responses? Along the years, different groups have collected evidence on Hsp70 involvement in innate and adaptive immune responses.

INNATE IMMUNE CELL MODULATION BY Hsp70 – EXTRACELLULAR Hsp70

The idea that Hsp70 could modulate innate cell function comes from studies that analyzed the interaction of Hsp70, either delivered extracellularly or present in the outer cell membrane/exosomes, with receptors on cells such as monocytes, dendritic cells (DCs) and myeloid-derived suppressor cells (MDSCs). This notion was surprising initially, because Hsp70 was then believed to be an intracellular chaperone. However, studies by Hightower and Guidon Jr. (1989) revealed that Hsp70 could be released from cells, in a mechanism that was independent of blockage of secretory pathways. A series of studies followed, revealing that soluble Hsp70 could be measured in the serum of both healthy and diseased individuals (Pockley et al., 1998); and that this extracellular Hsp70 could be either actively secreted by a non-classical pathway, or released from dying cells, review in De Maio (2011).

Two new functions were then reported for extracellular Hsp70. One study demonstrated that (mammalian) Hsp70–peptide complexes purified from MethA sarcomas could lead to priming of cytotoxic T cell (CTL) responses against these tumors (Udono and Srivastava, 1993). That meant that Hsp70 could probably bind to a membrane receptor in antigen-presenting cells (APCs), and get access to the endogenous route of antigen processing and presentation in MHC class I – i.e., cross-priming. A different group later reported that human Hsp70 could bind to and activate human monocytes, promoting the secretion of inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 (Asea et al., 2000a). Different groups went on to corroborate the findings of the cross-priming abilities of Hsp70 (Delneste et al., 2002; Kammerer et al., 2002; Ueda et al., 2004). However, the findings on the induction of pro-inflammatory cytokines were disputed (Gao and Tsan, 2004) when the removal of contaminating endotoxin of the recombinant preparations of human Hsp70 abrogated the induction of TNF-α by this protein. Hsp70 is a molecule with high affinity for hydrophobic moieties (Tsan and Gao, 2009) and the efficient removal of LPS and lipid-like contaminants from preparations of Hsp70 proved to be a challenge for those working with this protein. It is thus very likely that the ability of Hsp70 to bind cell surface receptors (see below) and be internalized, activating antigen presentation, which has been verified by independent groups, is independent of the induction of inflammatory cytokines by this protein, which, to this date, is still disputed.

The removal of contaminating endotoxin and lipopeptides by treatment with Triton X-114, a detergent, revealed that soluble Hsp70 had, in fact, anti-inflammatory properties. It was demonstrated that TB-Hsp70 could modulate cytokine production in blood and synovial cells of arthritis patients. *In vitro* treatment with endotoxin-free TB-Hsp70 for 48 h induced IL-10 production in peripheral blood mononuclear cells (PBMCs) from rheumatoid arthritis (RA) and reactive arthritis (ReA) patients as well as in normal controls PBMCs (Detanico et al., 2004). Concomitantly, PBMCs from these patients downregulated IFN-γ production (900-fold for RA patients and 750-fold for ReA patients when compared with untreated cells) and up-regulated IL-10 production (900-fold for RA patients and 500-fold for ReA patients). In addition, synovial cells incubated with TB-Hsp70 for 48 h showed a reversal of the inflammatory profile, with an induction of IL-10 [a 4.9-fold increase when compared with cells treated with bovine serum albumin (BSA) and LPS], correlating with a decrease in TNF-α and IFN-γ production. Synovial monocytes from the arthritis patients were the major source of IL-10 induced by TB-Hsp70. In accordance with these findings,

Luo et al. (2008) demonstrated that human Hsp70 downregulated in a concentration-dependent manner the TNF-α-induced production of pro-inflammatory mediators IL-6, IL-8, and MCP-1 in RA fibroblast-like synoviocytes when compared with OVA-treated cells. Thus, Hsp70, both bacterial and human, were shown to be associated with a protective phenotype in arthritis, corroborating the initial findings in adjuvant arthritis.

TB-Hsp70 could also modulate cytokine production in DCs. These cells provide a link between innate and adaptive responses, by presenting antigen to T cells, activating them, and shaping their differentiation into effector phenotypes (Heath and Carbone, 2009; Watowich and Liu, 2010). Production of IL-12 by DCs leads to a Th1 program of differentiation for the antigen-specific $CD4^+$ T cells, while IL-4 production induces a Th2 phenotype. Tolerogenic DCs, however, are characterized by low production of pro-inflammatory cytokines and high production of anti-inflammatory cytokines. It has been shown that cells expressing low levels of both MHC class II and T cell co-stimulatory molecules – such as CD80 and CD86, and that do or do not produce IL-10 and TGF-β, can be tolerogenic (Steinman et al., 2003; Rutella et al., 2006; Morelli and Thomson, 2007).

LPS-free TB-Hsp70 blocked the *in vitro* differentiation of DCs from bone marrow precursors. When murine bone marrow DCs (BMDCs) were treated with TB-Hsp70 for 24 or 48 h, an inhibition of maturation characterized by a failure to acquire MHC class II and CD86 expression was observed. TB-Hsp70-treated BMDCs had an eightfold increase in IL-10 production when compared with dexamethasone treated cells and produced 1,200-fold less TNF-α than LPS stimulated cells after 48 h of culture (Motta et al., 2007), suggesting not all transcription was inhibited in the treated BMDCs. More recently, a different group demonstrated that soluble inducible human Hsp70 (now known as HSPA1A) can also induce a regulatory phenotype in monocyte-derived DCs (MoDCs; Stocki et al., 2012). They tested three preparations of Hsp70, two commercial ones, with high or medium endotoxin levels, and one other with very low endotoxin levels. Only the Hsp70 preparations with high and medium endotoxin levels induced maturation of MoDCs in culture. The very low endotoxin level Hsp70, however, inhibited the maturation of MoDCs and reduced the capacity of those cells of stimulating allogeneic T cell proliferation. Together, these results indicated that both TB-Hsp70 and human Hsp70 produced a tolerogenic phenotype in DCs, provided that LPS contamination was eliminated.

These findings in DC have an important implication for a regulatory role of soluble forms of Hsp70. Tolerogenic DCs are known to contribute to the creation of a "suppressive environment" facilitating the peripheral generation of peripheral Tregs. Tregs play a crucial role in suppressing the excessive effector immune response that is harmful to the host (Sakaguchi et al., 2008). These cells can be divided into two subphenotypes. The first one is the Foxp3-expressing Tregs that develop in the thymus (nTregs; Feuerer et al., 2009). The second are the cells that can be induced in peripheral sites when given appropriate signals by the APCs (iTregs; Shevach, 2006). Tregs produce IL-10 or TGF-β, sometimes both, and actively suppress non-Treg proliferation (Vignali et al., 2008). Low levels of antigen presentation coupled to low co-stimulation have been linked to the differentiation of induced Tregs (iTregs; Jenkins et al., 1990; Steinman et al., 2000; Long et al., 2011). Thus, it was possible that, by modulating the APCs, Hsp70 could lead to the induction of Tregs in the periphery.

Confirming this prediction, soluble TB-Hsp70 was demonstrated to inhibit acute allograft rejection (Borges et al., 2010). When C57Bl/6 tumor cells or skin sections were pre-incubated in a solution with endotoxin-free TB-Hsp70 and then grafted onto a BALB/c host, the tumor cells formed a solid tumor, and skin rejection was delayed for 7–10 days, compared to controls. This effect was abrogated by depletion of Tregs, which were shown to infiltrate the accepted grafts. Interestingly, when soluble TB-Hsp70 was injected subcutaneously, this led to an increase in $CD4^+CD25^+Foxp3^+$ cells in the draining lymph node, which correlated to a diminished proliferation of lymph node cells in response to a T cell mitogen. The conclusion was that one single pretreatment with TB-Hsp70 could inhibit a powerful *in vivo* inflammatory process, and this correlated with the presence of Tregs.

The possibility that Hsp70 and Tregs are intimately linked is discussed in detail in the second part of this article (adaptive immunity). In the meantime, we wish discuss one more evidence that Hsp70 can act as an immunosuppressant – and this is related to another discovery, namely that Hsp70 could localize in membranes.

It was shown that Hsp70 (Vega et al., 2008), similarly to Hsc70 (Arispe and De Maio, 2000) could integrate into an artificial lipid bilayer, opening cationic conductance channels, and this ability was associated with the presence of phosphatidylserine (PS; Arispe et al., 2004). Other sphingolipids, such as globotriaosylceramide, have also been reported to enhance Hsp70 insertion into membranes (Gehrmann et al., 2008). This supported previous reports that Hsp70 could be found in the membrane of tumors (Ferrarini et al., 1992; Multhoff et al., 1995). Hsp70 was not simply associated with a receptor in the membrane, but rather inserted, because it could not be eluted by acid washes, or Triton X-1000 (Vega et al., 2008) and because only one antibody, recognizing a part of the C-terminus, but not antibodies that would recognize the N-terminus, would detect it (Botzler et al., 1998). The presence of Hsp70 in membranes of cells or exosomes of tumors presented one more way of extracellular interactions of Hsp70.

Myeloid-derived suppressor cells are a different, heterogeneous population of cells that are expanded during cancer, inflammation, and infection, with a remarkable ability to suppress T cell responses (Gabrilovich and Nagaraj, 2009). Chalmin et al. (2010) demonstrated, in mice and humans, that membrane-associated Hsp70 found in tumor-derived exosomes (TDEs) restrained tumor immune surveillance by promoting MDSCs suppressive functions. It was demonstrated that TDEs, contained in the tumor cell supernatant of three tumor cell lines, could mediate T cell-dependent immunosuppressive functions of MDSCs. The authors identified that the factor present on the TDEs that induced MDSCs activation was the inducible Hsp70 (HSPA1A) expressed on TDE cell surface. Hsp70 was only present on exosomal fractions, not in other microparticles. These findings indicated that immunomodulatory effects of tumor cells include their potential of inducing functional MDSCs by releasing exosomes expressing Hsp70.

Hsp70 PUTATIVE RECEPTORS AND RESPECTIVE SIGNALING PATHWAYS

Many studies asked the question of how would cells perceive the presence of extracellular Hsp. CD14 (Asea et al., 2000b), and Toll-like receptors (TLRs) 2 and 4 (Asea et al., 2002) were first proposed to be receptors for soluble extracellular human Hsp70 – and this was, as discussed above, disputed due to the contamination issue. CD40 (Wang et al., 2001) was then proposed as a receptor for mammalian Hsp70, however a different study (Binder, 2009) refuted this idea, demonstrating that Hsp70 would still bind to cells in CD40 knockout mice. CD91 (Basu et al., 2001) and LOX-1 (Delneste et al., 2002), two scavenger receptors, were shown to bind Hsp70–antigen complexes, increasing cross-presentation and eliciting a protective immune response against antigen-expressing tumor cells *in vivo*. Floto et al. (2006) suggested that TB-Hsp70 promoted DC aggregation, immune synapse formation between DCs and T cells, and an effector immune response the signaling through the CCR5 chemokine receptor. All these different results generated great confusion. A consistent finding among studies was the ability of extracellular Hsp70 to be internalized and interact with antigen presentation routes, inducing T cell responses to the peptides that associated with this protein. TLRs and CD40 are signaling receptors, rather than endocytic receptors. Scavenger receptors and lectin-like receptors are endocytic receptors, and the signaling events downstream binding and internalization that follows binding are not fully characterized.

A thorough study transfected Chinese hamster ovary (CHO) cells with cDNAs expressing each of these putative receptors, as well as other scavenger receptors and lectins, and studied their interaction with mammalian extracellular Hsp70 (Theriault et al., 2005). The authors verified no binding or internalization of Hsp70 with cells expressing TLR2, TLR4, CD40, or CD91. In a follow-up study, they used the same approach focusing on scavenger receptors (Theriault et al., 2006). They demonstrated that LOX-1, SREC-1, and FEEL-1 bind and internalize Hsp70. However, different forms of Hsp70 (peptide bound or ATP bound) interacted with each of these receptors with different affinities. In summary, while binding to signaling receptors was refuted by more than one study, different groups provided evidence for scavenger receptors as the likely receptors for extracellular Hsp70.

SIGNALING ROUTES ACTIVATED BY Hsp70

If extracellular Hsp70 indeed interacts with membrane-bound receptors, will it activate signaling pathways associated with these receptors? Few studies approached this issue.

Mitogen-activated protein (MAP) kinase cascade is one of the most ancient and evolutionarily conserved signaling pathways, which is also important for many processes in immune responses (Dong et al., 2002). TDE-associated Hsp70 was found to mediate the suppressive activity of the MDSCs via activation of STAT3 and ERK (Chalmin et al., 2010). An ERK-dependent route for IL-10 production by different immune system cells upon TLR stimulation has been described (Saraiva and O'Garra, 2010). It has been suggested that some TLR2 agonists are good inducers of IL-10 production (Dillon et al., 2006; Manicassamy et al., 2009; Saraiva and O'Garra, 2010; Yamazaki et al., 2011). It is an interesting feature of TLR2 that, depending on the nature of the ligand and the population of target cells, it can mediate either inflammatory or anti-inflammatory responses to the same infectious organism (Dillon et al., 2006; Frodermann et al., 2011), and the anti-inflammatory response is mediated by IL-10.

IL-10 is the main anti-inflammatory and immunosuppressive cytokine (Moore et al., 2001). However, depending on the situation, it can exert a pro-inflammatory role like in lupus erythematosus (Bussolati et al., 2000; Sharif et al., 2004). It has been suggested that type I interferons regulate the balance between anti- and pro-inflammatory role of IL-10 (Sharif et al., 2004). In monocytes of patients with systemic lupus erythematosus (SLE), it was demonstrated that IL-10 can stimulate production of platelet-activating factor (PAF) and this production was correlated with disease severity (Bussolati et al., 2000).

IL-10 production of by DCs stimulated via TLRs is diminished in presence of selective ERK inhibitors (Yi et al., 2002; Dillon et al., 2004; Kaiser et al., 2009) or in ERK-deficient cells (Agrawal et al., 2006). Besides, differences in IL-10 production by macrophages, myeloid DCs, and plasmacytoid DCs are correlated with different levels of ERK activation in these cells (Saraiva and O'Garra, 2010). Borges et al. (in preparation) observed that BMDCs treated with TB-Hsp70 showed a higher expression of phospho-ERK when compared with unstimulated cells, and inhibition of ERK expression with the specific ERK inhibitor PD98059 blocked IL-10 production upon incubation with Hsp70.

STAT3 is associated with IL-10 production and tolerance (Barton, 2006; Dhingra et al., 2011). Also, IL-10R recruits and activates JNK1-STAT3 pathway (Murray, 2006). In contrast, STAT3 can be activated by pro-inflammatory cytokines like IL-6, through IL-6R (Murray, 2007) and Oncostatin M (Halfter et al., 1999). Despite this duality in STAT3 activation, this transcription factor may be activated after IL-10 release induced by TB-Hsp70.

Based on this, we propose a model in which extracellular Hsp70 could regulate innate immune cell function, binding to cell surface receptors (a scavenger or lectin-like receptor), signaling through TLR2 via ERK to induce IL-10 production, resulting in an anti-inflammatory response. This model is depicted in **Figure 1**.

Is it possible to reconcile this model with what has been observed for the cross-priming and pro-inflammatory roles described for this protein? We believe that the next studies should test the possibility that extracellular Hsp70, upon binding to lectin-like or scavenger receptors, uses associated receptors to signal. It is possible that depending on the form of Hsp70 (associated with peptide; with membranes; with nucleotides; peptide-free) it will associate with a different receptor. Another issue that has to be considered is that, while in bacteria, Hsp70 comes from one gene, in mammals, there may be at least eight genes that code for Hsp70 (Kampinga et al., 2009). Bulk preparations of mammalian Hsp70 from cells contain not only the inducible, HSPA1A, but products from other genes as well. And this may also influence the outcome of the experiment. Finally, binding and internalization, followed by antigen presentation, may lead to inflammatory as well as to regulatory responses, depending on which receptor is engaged, as demonstrated in a recent study (Li et al., 2012). The authors verified that targeting an antigen to LOX-1 or DC-ASGPR on the surface of DCs led to internalization and cross-presentation of

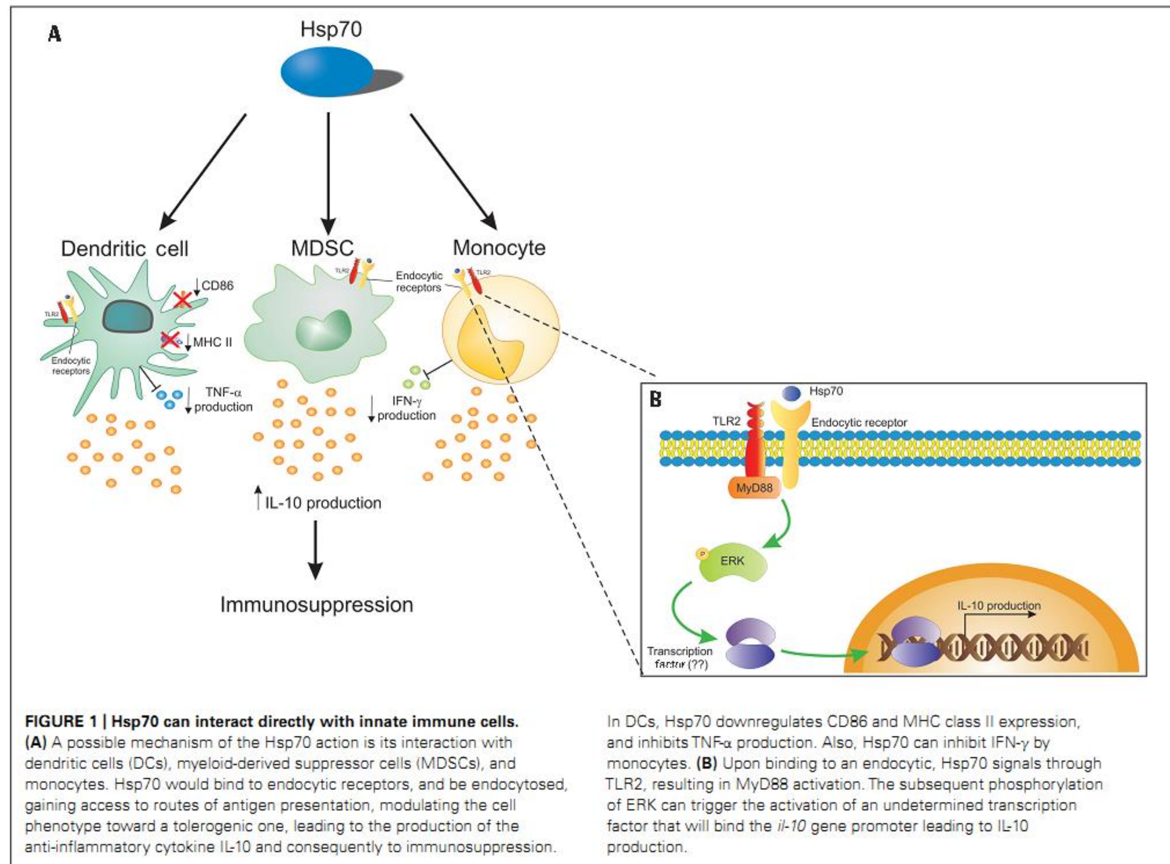

FIGURE 1 | Hsp70 can interact directly with innate immune cells. **(A)** A possible mechanism of the Hsp70 action is its interaction with dendritic cells (DCs), myeloid-derived suppressor cells (MDSCs), and monocytes. Hsp70 would bind to endocytic receptors, and be endocytosed, gaining access to routes of antigen presentation, modulating the cell phenotype toward a tolerogenic one, leading to the production of the anti-inflammatory cytokine IL-10 and consequently to immunosuppression. In DCs, Hsp70 downregulates CD86 and MHC class II expression, and inhibits TNF-α production. Also, Hsp70 can inhibit IFN-γ by monocytes. **(B)** Upon binding to an endocytic, Hsp70 signals through TLR2, resulting in MyD88 activation. The subsequent phosphorylation of ERK can trigger the activation of an undetermined transcription factor that will bind the *il-10* gene promoter leading to IL-10 production.

the antigen. However, while targeting to LOX-1 resulted in INF-gamma producing T cells, targeting to DC-ASGPR resulted in IL-10 producing CD4 T cells. Thus, it is possible that, depending on the form of extracellular Hsp70 and the target cell/tissue microenvironment, different outcomes may ensue. If this possibility is verified experimentally, that would in part explain some of the conflicting results previously discussed here. We are now left with the challenge to test these possibilities in order to elucidate the whole potential of Hsp70 as an immunomodulatory agent.

ADAPTIVE IMMUNITY REGULATION BY Hsp70

Besides the innate effects discussed above, several adaptive immunity associated mechanisms have been proposed for induction of Hsp-specific Tregs under physiological conditions.

The role of Hsp70 in adaptive immunity to mediate suppression through Tregs could be related to presentation of Hsp70 peptides, or to the modulation of the innate environment as described in the previous section, leading to the induction of Tregs.

The presentation of Hsp70 peptides in MHC molecules could result either from overexpression of endogenous Hsp70 in situations of physiological stress, or from endocytosis of extracellular Hsp; In response to physiological stress, intracellular levels of Hsp70 will rise in the stressed cells which can lead to presentation of Hsp peptides on MHC class I via the default MHC loading route for cytosolic proteins. This pathway includes degradation of the protein by the proteasome, transporter associated with antigen presentation (TAP) mediated translocation to the endoplasmic reticulum and subsequent loading of the peptides on MHC class I molecules (Neefjes et al., 2011). As will be discussed in more detail below, it is now becoming clear that via autophagy, intracellular Hsp can also be loaded on MHC class II molecules. Peptides derived from extracellular Hsp (pathogen-associated or secreted endogenous Hsp) can be presented via endocytic pathways by MHC class II molecules on APCs or on non-APCs upon stimulation with factors like IFNγ.

The mechanisms leading to production of Hsp-specific Treg can be manifold. Continuous encounter of bacterial-Hsp, in mucosal surfaces such as the gut can be a way to induce bacterial-Hsp-specific Treg, contributing to Hsp-specific mucosal tolerance (van Eden et al., 2005, 2007). Another possibility is the up-regulation of self-Hsp on non-professional APCs in response to various forms of stress in tissues. In the gut lamina propria of many species, MHC class II is also found to be present on non-professional APCs (Stokes et al., 1996). In addition, the inflammatory mediator IFN-γ is known to induce MHC class II in various cell types. Thus, MHC class II presentation of Hsp fragments in the absence

of proper co-stimulation may add to the production of tolerogenic or regulatory T cell responses. In addition, presentation of self-Hsp70 conserved peptides in presence of TGF-β (Sela et al., 2011) could lead to Treg induction and/or expansion (Rosenblum et al., 2011). Also, because some self-Hsp70 peptides are not completely identical to their bacterial homolog peptides, such presented self-peptides could function as altered peptide ligands for bacterial-Hsp-specific cells leading to induction of a partially agonistic and therefore downmodulated T cell response (Wauben et al., 1993). Finally, induction of Treg might be reinforced by the increased levels of the immunoregulatory cytokine IL-10, induced upon stress in multiple tissues (Stordeur and Goldman, 1998).

AUTOPHAGY, LOADING Hsp PEPTIDES ON MHC CLASS II

To activate $CD4^+$ T cells, peptides should be presented by MHC class II molecules. Cytosolic proteins, like Hsp70, are by default loaded on MHC class I molecules while extracellular proteins will be presented on MHC class II. Thus, another fundamental question can be raised; how do Hsp peptides end up to become presented by MHC class II? The distinct localization between MHC class I and MHC class II loading pathways has been proven incorrect because cytosolic proteins have been eluted from MHC class II and vice versa (Schmid et al., 2007). Autophagy has been initially found as a process to sustain metabolic fitness during food deprivation through bulk protein degradation (Kuma et al., 2004). The role of autophagy in the immune system is only now becoming clear (Schmid and Munz, 2007; Munz, 2009). Two pathways can result in loading of intracellular peptides on MHC class II. First, intracellular proteins can be incorporated in autophagosomes that subsequently fuse with lysosomes for degradation of their cargo (macroautophagy). In addition, cytosolic proteins can be transported via LAMP2a directly into the lysosome (chaperone mediated autophagy; Munz, 2006; Schmid et al., 2007; Strawbridge and Blum, 2007). Recently, the role of autophagy in loading Hsp70 peptides has been described; in human HLA-$DR4^+$ B cells a striking increase of especially Hsp70 peptides was eluted from HLA-DR4 upon induction of autophagy by amino acid deprivation (Dengjel et al., 2005). Autophagy induction coincided with elevated Hsp70 mRNA levels. In other words, especially under conditions of cell stress, fragments of Hsp70 will be presented on APCs to T cells, possibly initiating a regulatory T cell response.

PHENOTYPE OF Hsp-SPECIFIC Treg

The phenotype of Hsp-specific Treg has not been studied in detail. However, since Hsp-specific T cells have been observed in cord blood, some of them will probably be thymus derived $CD4^+CD25^+Foxp3^+$ natural Treg (Sakaguchi et al., 1995; Tang and Bluestone, 2008). Also, Hsp-specific Treg can be induced in the periphery, which potentially leads to induction of several induced Treg subsets. For example, $Foxp3^-$ Tr1 cells, which are induced by repetitive stimulation with antigen in the presence of IL-10 (Groux et al., 1997; Roncarolo and Battaglia, 2007). Alternatively, mucosal exposure of Hsp can produce iTregs, expressing a $CD4^+CD25^+Foxp3^+$ phenotype (Chen et al., 1994; Weiner, 2001). Or, conversion of naïve $CD4^+$CD25-$Foxp3^-$ cells into induced $CD4^+CD25^+Foxp3^+$ can occur in the presence of IL-2 and TGF-β at low levels of pro-inflammatory cytokines (Horwitz et al., 2008).

The phenotype of the Hsp-specific Treg may depend on the exposure route. Intraperitoneal (i.p.) immunization with endotoxin-free TB-Hsp70 or OVA as a control resulted in $CD4^+CD25^+$ T cells from Hsp70 immunized mice expressing slightly enhanced levels of regulatory cytokine IL-10, but not increasingly expression of Foxp3 (Wieten et al., 2009a). In contrast, in a study in a mouse atherosclerosis model, oral Hsp administration increased Foxp3 expression (van Puijvelde et al., 2007). Enhanced Foxp3 expression, both systemically in the spleen and locally in the inflamed joint, was also found upon up-regulation of endogenous Hsp70 in Peyer's patches of carvacrol (a co-inducer of Hsp70) fed mice (Wieten et al., 2010). The finding that Foxp3 levels were increased in cells obtained from joint synovial fluid suggested that induced Treg could have actually migrated to the site of inflammation.

In a recent study, after local injection of whole TB-Hsp70, a higher percentage of $CD4^+CD25^+Foxp3^+$ cells in draining lymph nodes compared with local injection with OVA was observed. Moreover, TB-Hsp70 inhibition of lymph node cell proliferation was superior to the inhibition induced by dexamethasone after PHA stimulation. The authors also observed that inhibition of acute rejection induced by TB-Hsp70 was dependent on $CD4^+CD25^+$ T cells in a skin allograft model (Borges et al., 2010).

To study the phenotype of Hsp-specific Treg in more detail, the expression of the transcription factor Helios in Tregs elicited by Hsp70 treatment, to verify if they are nTregs or iTregs (Thornton et al., 2010), since peripherally induced Tregs do not usually express this molecule. It will also be interesting to see if T cells found at the site of inflammation are Hsp70 specific, and if they indeed express special homing receptors. Future studies should tell us the relative proportions of nTregs and iTregs in Hsp70-specific Tregs, as well as what are the mechanisms by which they can mediate suppression in each of these models.

SUPPRESSIVE MECHANISM OF Hsp-SPECIFIC Treg

Hsp-specific Treg will probably use similar suppressive mechanisms as other antigen-specific Treg, like the production of anti-inflammatory cytokines, cell contact dependent suppression or killing of effector T cells and conversion of APC into a tolerogenic state (Vignali et al., 2008). Most Treg subsets use IL-10 for suppression (Bluestone, 2005). It has been recently demonstrated that Treg IL-10 is important for local responses, and not for the systemic suppression of inflammation (Rubtsov et al., 2008). In previous studies, we showed that cross-reactive Hsp-specific T cell responses coincided with the production of IL-10 (Anderton et al., 1995; Wendling et al., 2000; Prakken et al., 2001). Subcutaneous injection of soluble TB-Hsp70 increased IL-10 production and the number of Tregs in draining lymph nodes when compared with OVA injection (Borges et al., 2010). Moreover, while addressing the role of IL-10 in modulation of Proteoglycan-induced arthritis (PGIA) upon i.p. immunization with TB-Hsp70 and after nasal administration of Hsp70 peptides, it was observed that both treatment strategies enhanced Hsp70-specific T cell proliferation and IL-10 production. TB-Hsp70 immunization failed to rescue IL-10

deficient mice from PGIA development. In both wild type and IL-10 deficient mice, Hsp70-specific T cell responses were found, but only in wild type mice these responses suppressed arthritis (Wieten et al., 2009a). In addition, increased PG-specific T cell proliferation, IFN-γ and IL-10 production were found in wild type, but not in IL-10 deficient mice. This illustrates that Hsp70 immunization also modified the PG response to a more anti-inflammatory response. It is therefore possible that Hsp70-induced Tregs generated a tolerogenic micro-milieu by their cytokine production that enabled the outgrowth of new Tregs with antigen specificities beyond Hsp and that IL-10 was required for this effect.

These findings emphasize that Hsp-specific Tregs use mechanisms of infectious tolerance for modulation of inflammation. This has been shown before in transplantation (Qin et al., 1993; Borges et al., 2010), type-1 diabetes (Tarbell et al., 2007), and experimental autoimmune encephalomyelitis (EAE; Mekala et al., 2005) models. Besides IL-10, the role of other cytokines associated with Tregs, like IL-35 has not been addressed but might be relevant.

HOW IMPORTANT IS STRESS-INDUCED Hsp EXPRESSION?

Hsp expression is up-regulated in virtually every inflammatory condition. Also in autoimmune disease this has been reported; enhanced expression of Hsp60 has been shown in synovial and mononuclear cells of juvenile idiopathic arthritis (JIA) patients (Boog et al., 1992; de Graeff-Meeder et al., 1995). In addition, increased expression of inducible Hsp70 and HSF1 has been shown in the inflamed joint of RA patients (Schett et al., 1998). This has also been seen for BiP, an ER restricted Hsp70 family member (Blass et al., 2001) and interestingly enhanced expression in RA synovium was also seen for the constitutive Hsc70 (Schick et al., 2004).

As mentioned before, stress-induced Hsp expression has been proposed to be important for induction, maintenance, and activation of Hsp-specific Treg. If indeed so, reduced expression of Hsp – like with aging, as also depicted in **Figure 2**, where a reduced HSF activity leads to a relatively poor capacity to up-regulate Hsp (Rao et al., 1999; Njemini et al., 2003) – can be expected to influence Hsp mediated immune homeostasis and therefore might contribute to development of chronic inflammatory diseases. In fact, Hsp70 polymorphisms have been associated with inflammatory or autoimmune diseases such as Crohn's disease (Debler et al., 2003), Alzheimer's disease (Clarimon et al., 2003), pancreatitis (Balog et al., 2005) and with development of graft versus host disease upon allogeneic hematopoietic stem cell transplantation (Bogunia-Kubik and Lange, 2005).

Decreased Hsp expression has been observed in several immune disorders. A low Hsp70 response has also been described in a subtype of Biobreeding (BB) rats with a high susceptibility for development of autoimmune (Bellmann et al., 1997). Similar results have been found in human PBMC from patients with newly diagnosed type-1 diabetes. In that study, stress responses were found to become re-established again in patients with longstanding diabetes, more than 8 months after disease manifestation. So, defective Hsp70 induction coincided with beta cell directed inflammatory activity, and seemed modulated by pro-inflammatory cytokines rather than metabolic factors (Burkart et al., 2008).

To amplify stress-induced Hsp70 expression, a study tested multiple food-derived compounds for their effect on Hsp70 expression (Wieten et al., 2009b). One of the compounds, carvacrol, was identified as a potent enhancer of stress-induced Hsp70 both *in vitro* and *in vivo*. Also *in vivo*, intragastric (i.g.) gavage of carvacrol enhanced Hsp70 expression in Peyer's patches (Wieten et al., 2010). Carvacrol was used to boost Hsp levels in APCs and this enhanced Hsp-specific T cell hybridoma activation. We also addressed the immunomodulatory potential of carvacrol *in vivo* and found that i.g. carvacrol treatment specifically boosted Hsp70-specific T cell responses. The finding that adoptive transfer of T cells, isolated from carvacrol treated donor mice, suppressed PGIA, were indicative of the induction of Treg.

The above mentioned findings suggested that the immune system can recognize and react on altered expression of these proteins.

PERSPECTIVES

Hsp expression or Hsp-specific T cell responses have been positively associated with a better disease prognosis in several inflammatory conditions (de Graeff-Meeder et al., 1991; de Kleer et al., 2003). In addition, the immunosuppressive action of Hsp has been demonstrated in multiple rodent disease models. So, it is attractive to speculate that simply enhancing Hsp mediated immunoregulation in either way could be used as therapy.

Apparently, this is oversimplified. Depending on multiple factors such as disease etiology and inflammatory status, patient age and genetic background, difficulties will be encountered. In general, defects in for example positive or negative selection in the thymus, IL-2 production by effector T cells or IL-10 or TGF-β production by Tregs can lead to loss of peripheral tolerance as a result of decreased T cell numbers or functioning (Brusko et al., 2008). Some of these defects might also influence Hsp-specific Treg. For example, the findings that Hsp70-induced suppression of arthritis failed in the absence of IL-10 (Wieten et al., 2009a), illustrated that defects in IL-10 production will also influence Hsp-specific Treg. Furthermore, as disease progresses, severe ongoing inflammation has been described to obstruct the effectiveness of antigen-specific Tregs (Valencia et al., 2006; Peluso et al., 2007). It is currently not known if Hsp-specific Treg can also be hampered by ongoing inflammation. Recent experiments performed by us (Lotte Wieten, Martijn J. C. van Herwijnen, Femke Broere, Ruurd van der Zee, and Willem van Eden) have indicated that this is not the case, however. Transfer of Hsp70 peptide-induced Tregs were found to suppress ongoing experimental arthritis (van Herwijnen et al., in preparation). Recently, it has been reported that iTreg but not natural Treg can convert into Th17 cells after exposure to IL-6 and TGF-β (Horwitz et al., 2008). Besides Th1 cells, Th17 cells are major pathogenic effector cells in many autoimmune diseases. Whether Hsp-specific Treg can convert into Th17 cells has not been studied, but if so, timing and route of boosting the Hsp response could be important to avoid exacerbation of disease instead of induction of regulation.

Earlier studies have emphasized the pro-inflammatory nature of stress proteins such as the Hsp70 family members. In this

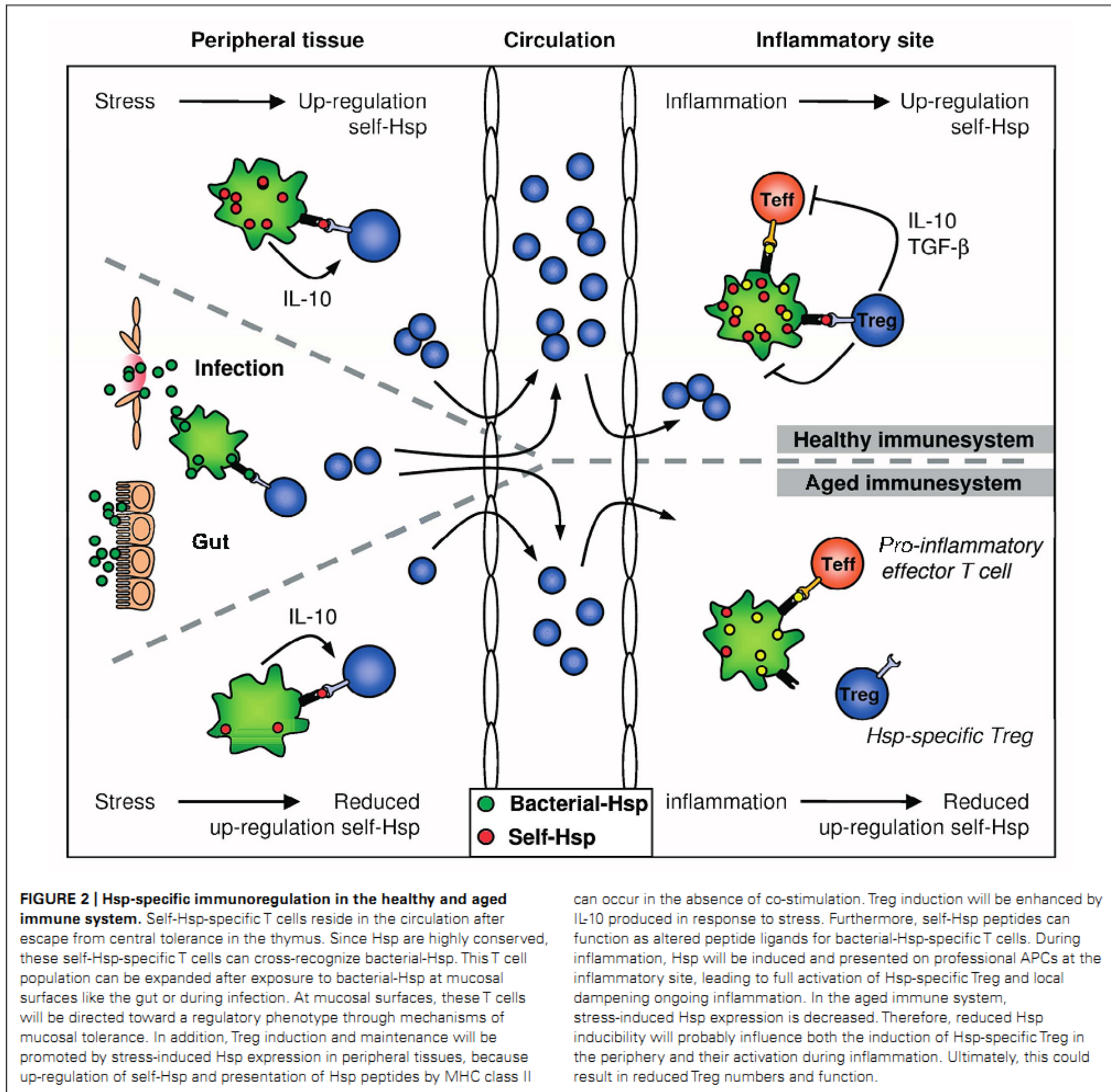

FIGURE 2 | Hsp-specific immunoregulation in the healthy and aged immune system. Self-Hsp-specific T cells reside in the circulation after escape from central tolerance in the thymus. Since Hsp are highly conserved, these self-Hsp-specific T cells can cross-recognize bacterial-Hsp. This T cell population can be expanded after exposure to bacterial-Hsp at mucosal surfaces like the gut or during infection. At mucosal surfaces, these T cells will be directed toward a regulatory phenotype through mechanisms of mucosal tolerance. In addition, Treg induction and maintenance will be promoted by stress-induced Hsp expression in peripheral tissues, because up-regulation of self-Hsp and presentation of Hsp peptides by MHC class II can occur in the absence of co-stimulation. Treg induction will be enhanced by IL-10 produced in response to stress. Furthermore, self-Hsp peptides can function as altered peptide ligands for bacterial-Hsp-specific T cells. During inflammation, Hsp will be induced and presented on professional APCs at the inflammatory site, leading to full activation of Hsp-specific Treg and local dampening ongoing inflammation. In the aged immune system, stress-induced Hsp expression is decreased. Therefore, reduced Hsp inducibility will probably influence both the induction of Hsp-specific Treg in the periphery and their activation during inflammation. Ultimately, this could result in reduced Treg numbers and function.

sense, they were often mentioned as prime examples of so-called DAMPs or damage-associated molecular patterns. It is possible that contaminating microbial components present in partially purified recombinant proteins used in the experiments have contributed to this (Bausinger et al., 2002; Gao and Tsan, 2004; Motta et al., 2007). Besides this, there are other arguments to make against a pro-inflammatory role of Hsp (Broere et al., 2011). As discussed here above, experimental evidence in favor of an immunomodulatory role for Hsp70 is accumulating and therefore Hsp70's immunosuppressive potential seems to constitute a real phenomenon. A more detailed characterization of the molecular pathways activated by Hsp70 in different cell subpopulations is needed. Such studies will allow us to understand and maximize the use of Hsp70 as an anti-inflammatory agent.

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Capítulo 3

Prolonged survival of allografts induced by Mycobacterial Hsp70 is dependent on CD4+CD25+ regulatory T cells

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Prolonged Survival of Allografts Induced by Mycobacterial Hsp70 Is Dependent on CD4+CD25+ Regulatory T Cells

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Abstract

Background: Heat shock proteins (Hsps) are stress induced proteins with immunomodulatory properties. The Hsp70 of *Mycobacterium tuberculosis* (TBHsp70) has been shown to have an anti-inflammatory role on rodent autoimmune arthritis models, and the protective effects were demonstrated to be dependent on interleukin-10 (IL-10). We have previously observed that TBHsp70 inhibited maturation of dendritic cells (DCs) and induced IL-10 production by these cells, as well as in synovial fluid cells.

Methodology/Principal Findings: We investigated if TBHsp70 could inhibit allograft rejection in two murine allograft systems, a transplanted allogeneic melanoma and a regular skin allograft. In both systems, treatment with TBHsp70 significantly inhibited rejection of the graft, and correlated with regulatory T cells (Tregs) recruitment. This effect was not tumor mediated because injection of TBHsp70 in tumor-free mice induced an increase of Tregs in the draining lymph nodes as well as inhibition of proliferation of lymph node T cells and an increase in IL-10 production. Finally, TBHsp70 inhibited skin allograft acute rejection, and depletion of Tregs using a monoclonal antibody completely abolished this effect.

Conclusions/Significance: We present the first evidence for an immunosuppressive role for this protein in a graft rejection system, using an innovative approach – immersion of the graft tissue in TBHsp70 solution instead of protein injection. Also, this is the first study that demonstrates dependence on Treg cells for the immunosuppressive role of TBHsp70. This finding is relevant for the elucidation of the immunomodulatory mechanism of TBHsp70. We propose that this protein can be used not only for chronic inflammatory diseases, but is also useful for organ transplantation management.

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Introduction

Heat shock proteins (Hsp) are highly immunogenic proteins, though conserved between mammals and microorganisms. Hsp70, originally described as a heat induced protein [1], is the most conserved of Hsps, and currently known to have immunomodulatory properties. Nevertheless, the exact mechanisms through which it exerts this effect are not completely clear [2]. *Mycobacterium tuberculosis* Hsp70 (TBHsp70) has been shown to protect from induced arthritis in rats [3,4,5]. We have demonstrated that TBHsp70 induces IL-10 production by monocytes and synovial cells of arthritis patients, leading to a reduction of TNF-α e IFN-γ levels [6]. Also, we observed that TBHsp70 can inhibit differentiation of bone marrow derived dendritic cells (BMDCs) *in vitro*, leading to the production of IL-10 by these cells [7]. It was suggested that exposure to bacterial Hsps could activate self Hsp-specific T cells that would be cross reactive with bacterial Hsps and trigger immunoregulatory pathways [8]. More recently, in a proteoglycan-induced arthritis model, TBHsp70 immunization showed a protective potential that was dependent on IL-10 [9]. In addition, the treatment with TBHsp70 upregulated IL-10 mRNA in regulatory T cells (Tregs).

Treg cells are crucial for the suppression of acute rejection in allografts [10]. These cells develop in the thymus or can be induced in peripheral sites when given appropriate signals by the antigen presenting cells. They are CD25+, and also express the transcriptional factor forkhead box 3 (FoxP3), cytotoxic T-lymphocyte antigen 4 (CTLA-4) and glucocorticoid-induced tumor necrosis factor receptor (GITR) [11]. They can suppress inflammatory responses by regulating the activity of self-reactive

conventional T cells. Tregs produce IL-10 and TGF-β and actively suppress non-Treg proliferation [12]. They have been shown to be important for inhibition of allograft rejection in several models [13,14]. In this study, we investigated whether TBHsp70 could act as an immunosuppressant in two allograft rejection systems. We also investigated if treatment with this protein would induce Treg cells. Our results suggest that TBHsp70 is capable of delaying acute allograft rejection, and this response is mediated by Tregs.

Results

TBHsp70 suppresses tumor allograft rejection

We asked if TBHsp70 could inhibit allograft rejection in two different models. We first used a transplanted allogeneic tumor model, injecting BALB/c mice (H-2^d) with B16F10 melanoma cells (H-2^b). Cells were resuspended in PBS containing TBHsp70 or no stimulus and were injected subcutaneously in mice. Tumor growth was recorded daily for 12 days. Around day 6 after injection, PBS-injected mice completely rejected the tumor cells, and the progressive elimination of the dark area occupied by melanoma cells could be observed at the injection site (Fig. 1A). However, mice that received tumor cells with TBHsp70 not only did not reject the tumor (Fig. 1A), but also allowed tumor growth through the 12-day period (Fig. 1B). These results suggested that TBHsp70 could inhibit tumor allograft rejection, supporting the immunosuppressive potential that had been previously observed in arthritis models [3,4,15,16].

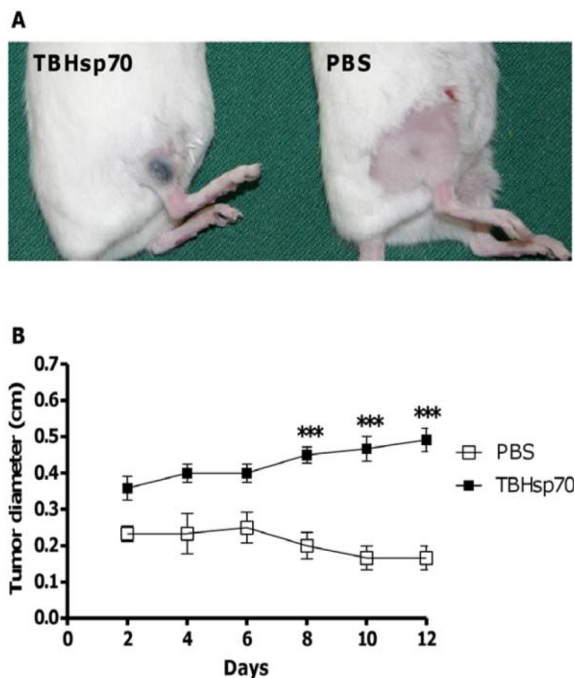

Figure 1. B16F10 tumor allograft in BALB/c mice. (A) Typical aspect of injection site in mice treated subcutaneously with tumor cells in PBS containing TBHsp70 or PBS alone on day 12 after transplant of tumor cells. (B) Sequential measurements of tumor diameter. n = 3 mice per treatment group. This experiment was performed seven times, with identical results. ***, $p<0.0001$.
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T cells with regulatory phenotype are observed at the tumor allograft site in HSP70-treated animals

We next investigated whether the inhibition of rejection of allogeneic tumor cells by TBHsp70 was associated with a local infiltration by Treg cells. We performed immunohistology on the tumor graft site, and sections were incubated with anti-CD4, anti-CD25, anti-FoxP3, and anti-GITR antibodies (Fig. 2). In mice injected with tumor cells in PBS alone, the tumor cells were not detectable by histology at the injection site, as analyzed by hematoxillin/eosin staining. Also, immunohistochemistry for CD4, CD25, FoxP3 and GITR was negative (Fig. 2A). In mice injected with tumor cells in TBHsp70 solution, staining for CD4, CD25, FoxP3 and GITR was observed in overlapping areas, mostly surrounding the tumor mass, as well as inside the tumor (Fig. 2A). This indicates that Tregs were locally infiltrating the tumor allograft site. Figure 2B shows the quantification of CD4, CD25, FoxP3 and GITR staining, captured with the Image Pro-Plus and quantified using the color range function in Adobe Photoshop tool.

Injection of TBHsp70 suppresses T cell proliferation and induces Treg cells

Tumors can develop varied immunosuppressive strategies in order to grow [17], and those include the recruitment of Tregs [18,19,20]. It was possible that the Tregs observed infiltrating the TBHsp70 treated tumors could be a result of tumor activity, rather than a direct effect of TBHsp70. To investigate this possibility, tumor-free mice were injected subcutaneously with TBHsp70 (30 μg) or PBS, and after 4 days they were sacrificed. Draining lymph nodes were excised and a single cell suspension was obtained. Cells were counted and stained for the presence of Treg cells, with anti-CD4 Cychrome, anti-Foxp3 PE and anti-CD25 FITC, and analyzed by flow citometry. The injection of TBHsp70 led to a 2.4-fold increase in the percentage of CD25+ Foxp3+ cells among CD4+ lymphocytes compared to the PBS-injected animals (Fig. 3A). Because the total number of cells was also increased two-fold in the lymph nodes of tumor-treated animals, the total increase in CD4+ CD25+ Foxp3+ was approximately four-fold, compared to the PBS-injected animals (Fig. 3A). This result supported our hypothesis that TBHsp70 could induce Treg cells in the draining lymph node.

The lymph nodes are the sites to which antigens from the periphery are drained by the lymphatic system [21], and subcutaneous injection of antigens results on the presentation of such antigens to T cells in the draining lymph nodes [22]. Cell interactions occurring in the draining lymph nodes are thus crucial to the immune responses leading to allograft rejection or acceptance [23]. To determine whether the immunosuppressive effects of TBHsp70 would affect T cell proliferation in the draining lymph node, we injected mice subcutaneously with PBS, TBHsp70 or Dexamethasone (DEXA) as a positive control for suppression of proliferation. Four days later, the draining inguinal lymph nodes were excised, a single cell suspension was obtained and the cells were cultured with PHA for another four days. Viability of proliferating cells was estimated by an MTT assay. As expected, mice injected with DEXA exhibited significant inhibition of polyclonal T cell proliferation (Fig. 3B). Surprisingly, TBHsp70 inhibition of lymph node cell proliferation was superior to the inhibition induced by DEXA (Fig. 3B). Supernatants from cell cultures were analyzed for the presence of IL-10, TNF-α, IL-4, IL-6, IFN-γ, IL-17A and TGF-β. IL-10 production was significantly upregulated by treatment with TBHsp70 as well as by the treatment with DEXA, and both treatments significantly inhibited

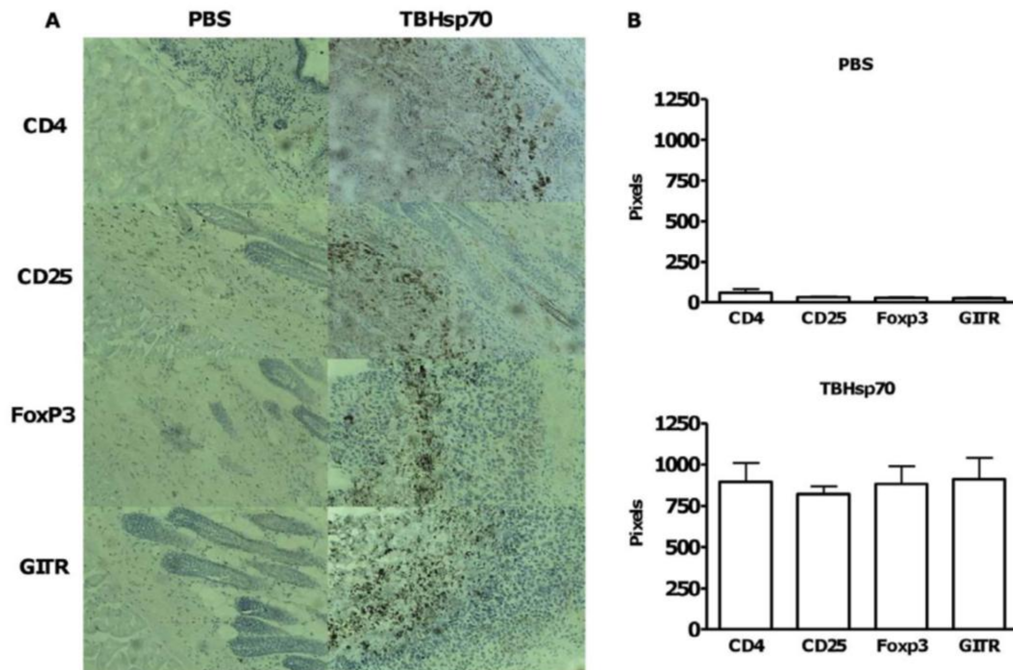

Figure 2. Treg cells are observed at the tumor allograft site. (A) Graft site (8 µm) serial sections from mice injected with tumor in PBS or TBHsp70 were stained with biotin-labeled anti-CD4, anti-CD25, anti-GITR and anti-Foxp3 antibodies, followed by streptavidin-peroxidase. Counter staining was hematoxillin. (B) Quantification of positive staining, expressed in pixels, using Image Pro Plus Software. n = 3 mice per group, experiments were performed 3 times.
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TNF-α production (Fig. 3C). No differences were observed for the other cytokines analyzed, including TGF-β. These results indicated that TBHsp70 treatment leads to suppression of lymph node T cell proliferation *in vitro*, and this is associated with the upregulation of IL-10 production and inhibition of TNF-α.

Altogether, the results suggested that TBHsp70 could induce Tregs which correlated with the induction of IL-10 in the draining lymph node, and that was independent of the presence of tumor cells.

TBHsp70 treatment delays skin allograft rejection

To further characterize the immunosuppressive effect of TBHsp70 over allograft rejection, and to eliminate the tumor cell variable, we switched to a traditional skin allograft model. We transferred a section of tail skin from C57BL/6 mice to BALB/c mice. The donor skin was immersed in a PBS solution containing TBHsp70 (30 µg in 500 µl, or 60 µg/ml) or PBS alone for 60 minutes at 4°C, and the graft site was analyzed daily. These experimental conditions were chosen because we had previously tested the injection of TBHsp70 at the graft site, either before or after performing the graft, and immersion of the donor graft in the TBHsp70 solution showed the best effect for a single TBHsp70 treatment (data not shown). We also tested immersing the graft in 20, 40 and 60 µg/ml of TBHsp70 solution, and the latter concentration presented the best results. At day 9, mice that received the skin grafts treated with PBS alone completely rejected the graft (Fig. 4A, B). On the other hand, mice receiving TBHsp70-treated skin graft presented prolonged acceptance of graft until day 17 (Fig. 4B). These results suggested that treatment with TBHsp70 significantly delays skin allograft rejection ($p = 0.0455$). To confirm the specificity of the regulatory effects observed with TBHsp70 treatment, we repeated the graft experiments using different proteins. Hsp90 was used to demonstrate that the effect is not a general property of Hsps, but rather of TBHsp70. Ovalbumin is a largely used antigen control, and was also used by us to treat the skin before grafting. Finally, we treated skin fragments with TBHsp70 boiled for 10 min at 100°C, to exclude the possibility that the effect could be due to a heat-resistant contaminant (such as LPS has been described to be [24,25]). Once again, only TBHsp70 treatment was capable to prolong the allograft acceptance (Fig. 4C, $p = 0.0246$).

Treg cells are essential for skin allograft survival induced by TBHsp70

We next asked whether TBHsp70-prolonged survival of skin allograft was mediated by Tregs. To investigate this possibility, we depleted these cells by treating recipient BALB/c mice with anti-CD25 mAb (PC61). Depletion with one injection of this antibody is known to eliminate ~70% of Tregs [26]. We performed a single mAb injection two days post-transplant (Fig. 5A). This treatment resulted in ~95% depletion of CD4+CD25+ T cells (Fig. 5B).

Depletion of CD25+ T cells in TBHsp70 treated mice completely abolished the suppressive effect (Figure 5C). Once again, TBHsp70-treated skin allografts had a significant prolonged survival compared to OVA-treated grafts ($p = 0.0295$). Interestingly, depletion of Treg cells diminished this effect. Mice that were treated with PC61 mAb and TBHsp70 showed decreased graft survival compared to the TBHsp70-treated, not depleted mice ($p = 0.0246$). Taken together, these results indicate that the

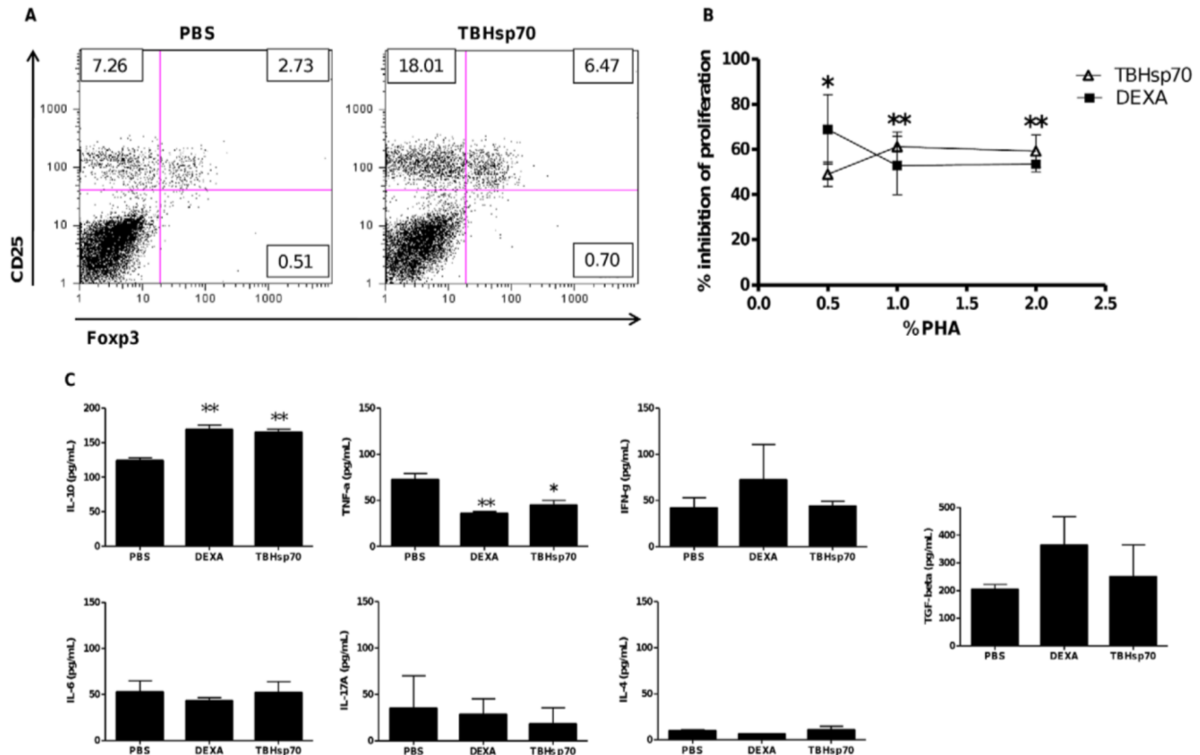

Figure 3. Local injection of TBHsp70 induces Tregs, IL-10 and leads to suppression in the draining lymph nodes. (A) Mice were injected subcutaneously in the thigh with PBS or 30 µg of TBHsp70. Draining lymph nodes were excised 4 days later, and cells were stained with fluorescent antibodies for CD4 and CD25, or CD4 and Foxp3. Plots show events gated on CD4+ cells. (B) Mice were injected subcutaneously in the thigh with TBHsp70 (1.5 mg/kg), DEXA (0.25 mg/kg) or PBS. Draining lymph nodes were excised 4 days later and single cell suspensions of lymph nodes were stimulated *in vitro* for 4 days with 0, 0.5, 1, or 2% of PHA. Viability of cells proliferating in response to PHA was estimated by an MTT assay, O.D. being read at 570 nm. Data are expressed as percentage of PHA-inhibited proliferation considering the O.D. at each PHA concentration as 100% proliferation. (C) Cytokine production of cultures supernatants was analyzed by flow cytometry, using a CBA (Mouse Th1/Th2/Th17) kit, except for TGF-β production, which was analyzed by ELISA. *, $p<0.05$; **, $p<0.01$.
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TBHsp70-induced delay of skin graft rejection is mediated by Treg cells.

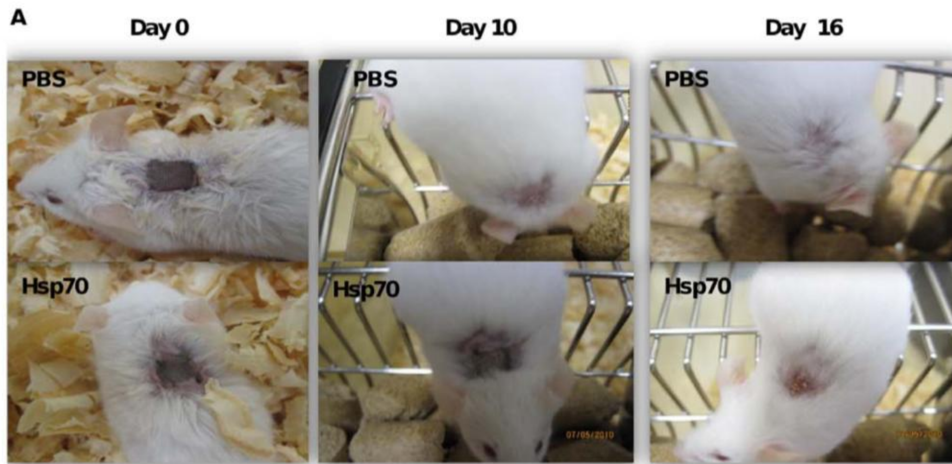
Discussion

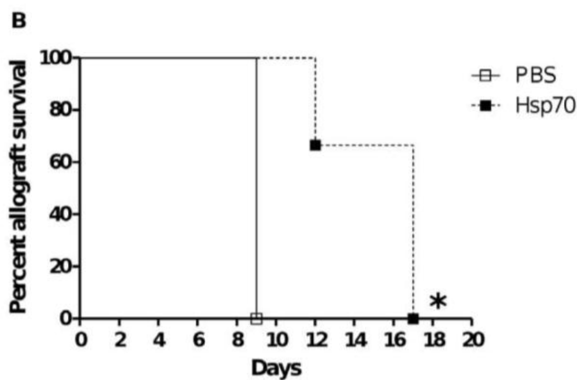
In this study, we investigated the ability of TBHsp70 to inhibit allograft rejection in two different models as well as the mechanism mediating this effect. Although TBHsp70 exerts suppressive effects in inflammatory diseases, the mechanism by which it does so has not been fully elucidated. However, the induction of IL-10 production and the suppression of inflammatory cytokines seem to be consistent findings associated with TBHsp70 immunomodulatory effects [5,9,15].

The immunoregulatory role performed by Tregs is also frequently associated with the production of IL-10 [27,28]. We demonstrated here, for the first time, that the immunosuppressive effect of TBHsp70 depends on Tregs. This was correlated with production of IL-10 in the draining lymph nodes. We have demonstrated in previous studies that TBHsp70 can induce IL-10 production by monocytes [6] and immature DCs [7]. Thus, it is possible that the IL-10 detected in our experiments is not solely produced by Tregs. This needs to be ascertained in further experiments with intracellular staining. Because Tregs can be induced locally by DCs with an immature phenotype that produce IL-10 [13,29,30], it is possible that TBHsp70 modulates the phenotype of DCs, inducing a regulatory response, and IL-10 production by DCs, leading to the recruitment and/or induction of Tregs. The importance of donor DC modulation for graft acceptance and Treg generation has been demonstrated in different systems [30,31,32].

Importantly, we verified that the induction of IL-10 in lymph nodes of TBHsp70 injected mice correlated with inhibition of TNF-α. This result corroborates an observation made by us in a previous study, in which in vitro treatment of synovial cells from arthritis patients with TBHsp70 inhibited TNF-α and induced IL-10 production [6]. This ability is likely to be determinant for the immunosuppressive effects verified for this protein both in arthritis and in the graft system. Interestingly, TGF-β, another cytokine commonly linked to Treg activity (or specific subsets of Tregs – see review in [33]) does not seem to be involved in this process, at least not in our experimental system.

We cannot conclude from the evidence presented here that the Tregs observed are specific for TBHsp70. It has, however, been hypothesized, that Hsp70 peptides constitute ligands for Treg cells [8]. This hypothesis is not completely excludent from the first one. Nevertheless, we believe that additional studies are necessary to discern from these two hypotheses, both in arthritis and transplant systems, to verify if the effect can be reproduced exclusively with





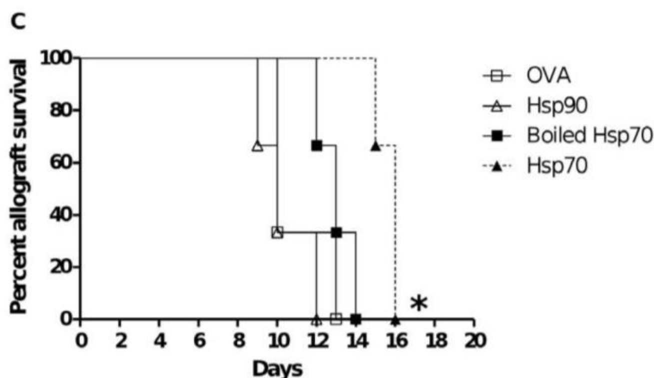


Figure 4. TBHsp70 delays skin graft rejection. (A) Skin grafts were immersed in a PBS solution (500 µl) containing TBHsp70 (30 µg) or PBS alone for 60 minutes at 4°C. After this, the skin graft was sutured to the exposed tissue of the recipient. Animals were kept in individual mini-isolators and observed daily, the state of graft acceptance being photographed and recorded. Graft rejection was confirmed by the observation of cyanosis, erythema, erosion, and loss of skin graft. (B) Survival curve of skin allograft immersed in PBS alone or TBHsp70 (30 µg). (C) Skin grafts were immersed in a PBS solution (500 µl) containing Ovalbumin (30 µg), Hsp90 (30 µg), 30 µg of TBHsp70 boiled for 10 minutes at 100°C or native TBHsp70. *, $p<0.05$. n = 3 mice per treatment group, the experiments were performed 4 times.
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TBHsp70 peptides. Because TBHsp70 immunosuppressive effects occur in rats as well as in mice, with different MHC elements, it could be predicted that multiple peptides of this protein would be candidate ligands. However, it is also possible that such peptide ligands do exist, and yet it is still necessary to initiate the response with DC modulation by TBHsp70 in its native structure. Indeed, the abrogation of suppressive effect of TBHsp70 by boiling suggests this might be a possibility. If so, the immunomodulatory

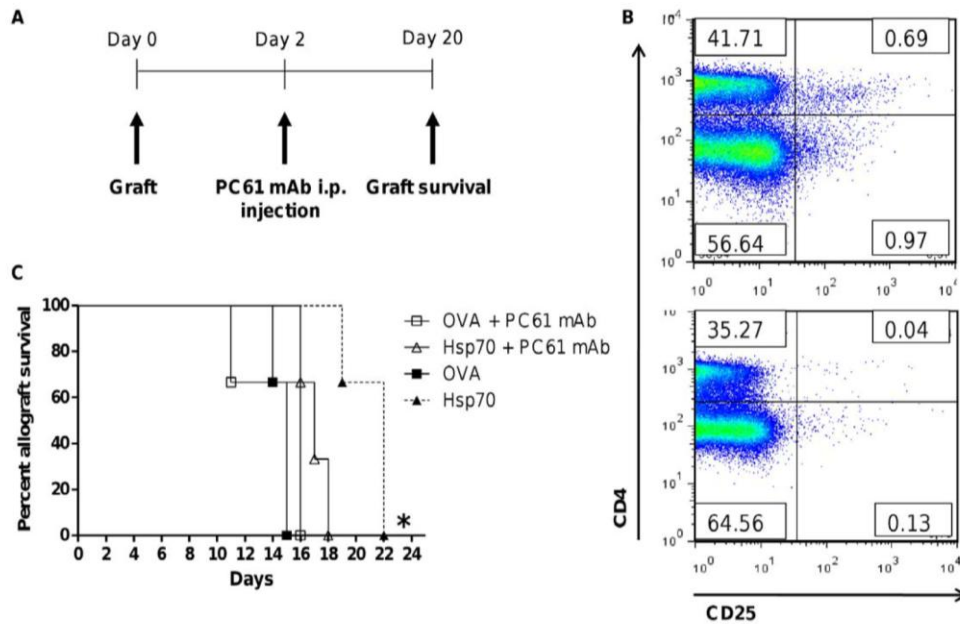

Figure 5. CD4+CD25+ regulatory T cells are crucial for prolonged survival induced by TBHsp70. Mice were injected i.p. with a single injection of 150 µg anti-CD25 mAb (PC61). (A) Schematic representation of *in vivo* Treg depletion and transplantation. (B) For depletion confirmation, lymph nodes were excised, collagenase D treated, stained with anti-CD4 and anti-CD25 and analyzed by flow cytometry. (C) Survival curve of skin allograft immersed in PBS containing 30 µg of OVA or TBHsp70. Groups were CD25+ depleted or not depleted. *, $p<0.05$. n = 3 mice per treatment group. Depletions were performed 2 times.
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effect could probably be reproduced with adoptive transfers of DCs treated with TBHsp70, and antigen processing inhibition in this system could indicate the relevance of TBHsp70 peptide presentation for the induction of Tregs. We are currently performing such experiments.

An attractive and innovative feature of TBHsp70 treatment in our system was that it was local, rather than systemic. All immunosuppressive drugs are delivered systemically, leading to undesirable side effects [34,35,36]. We verified that local treatment with TBHsp70 had a local effect, though it still remains to be determined if any systemic alterations were induced. Also, we were able to observe a significant difference with only one dose of the protein, while most immunosuppressants are used daily. Further studies need to be performed to determine how long additional doses of the protein will extend survival and acceptance of the graft.

Finally, we observed that the best effect on prolonged skin allograft survival was obtained immersing the graft in a TBHsp70 solution. The solutions used for preservation of organs before transplant are mainly buffered saline solutions that aim to protect from ischemia/reperfusion damage [37,38,39]. Although the endogenous Hsp70 protects organs against ischemia [40,41], we do not believe that this was the mechanism of action involved in the inhibition of rejection in our study, because it was abolished by depletion of Tregs. Nonetheless, our results suggest that this protein could be used in the preservation solution of solid organs, conferring an additional benefit in the use of these preparations, delaying acute rejection.

Materials and Methods

Mice

Female BALB/c and C57BL/6 mice between 6–8 weeks old were purchased from FEPPS (Rio Grande do Sul, Brazil). All animals were housed in individual and standard mini-isolators (Techniplast, Italy) in an SPF facility (Faculdade de Biociencias – PUCRS) and had free access to water and food. All procedures were previously reviewed and approved by the Ethics Committee for the Use of Animals of Pontificia Universidade Católica do Rio Grande do Sul (CEUA-PUCRS) under protocol ID CEUA 08/00048.

Protein purification and LPS extraction

Recombinant TBHsp70 was produced in XL1-blue *Escherichia coli* (*E.coli*) (a gift from Dr Douglas Young, Hammersmith Hospital, London, UK), and purified according to Mehlert [42]. Estimation of protein concentration and its purity was performed against a BSA standard curve on a 10% SDS-PAGE gel stained with Coomassie blue. To remove LPS, Triton X-114 was used according to the method described in Aida [43]. Briefly, 5 µl of Triton X-114 (Sigma) were added to 500 µl recombinant protein. After vortexing vigorously, the solution was incubated in ice for 5 min, vortexed again and incubated at 37°C for 5 min. The solution was then centrifuged for 5 minutes at 37°C and the supernatant collected and the procedure repeated 5 more times. Contaminating Triton was removed by incubating overnight with Biobeads (Bio-Rad) at 4°C with agitation. To test for remaining contaminant LPS, a bioassay was performed. BALB/c mice were injected i.v. with 100 µl volume of either PBS alone, PBS with 40 µg of LPS (Sigma) or PBS with 40 µg TBHsp70. Mice were sacrificed 6 hours after injection. The spleens were removed, collagenase D treated, and the single cell suspensions obtained were analyzed for CD11c and CD86 expression by flow cytometry, as described in Khoruts et al. [44]. Preparations were considered LPS free only when CD86 was not upregulated in splenic DCs.

Cell proliferation/viability assay

T cell proliferative responses were determined by a modified colorimetric assay [45]. To analyze inhibition of murine cell proliferation in vitro, mice were injected subcutaneously in the thigh with TBHsp70 (1.5 mg/kg), DEXA (0.25 mg/kg) or PBS. Draining lymph nodes were excised 4 days later and single cell suspensions of lymph nodes (8×10^5/ml) were cultured with 0, 0.5, 1, or 2% of PHA for 4 days.

For the MTT assay, in the last 4 h of culture, 100 µl of the supernatant was gently discarded and 30 µl of freshly prepared MTT (3-(4,5-diamethyl 2-thiazolyl) 2,5 diphenyl-2H-tetrazolium - Sigma) solution (5 mg/ml in RPMI 1640 - Sigma) was added to each well. The cell cultures were incubated for 4 h at 37°C in 5% CO_2 atmosphere. After completely removal of the supernatant, 100 µl of DMSO (Sigma) was added to each well. The optical density (OD) was determined using a Biorad ELISA plate reader at a wavelength of 570 and 620 nm. The viability of proliferating cells was expressed as the percentage of inhibited PHA-induced proliferation.

Cytokine measurement

To analyze the profile of cytokine production, mice were injected subcutaneously in the thigh with TBHsp70 (1.5 mg/kg), DEXA (0.25 mg/kg) or PBS. Draining lymph nodes were excised 4 days later and single cell suspensions of lymph nodes (8×10^5/ml) were cultured with 5 µg of conA for 4 days.

Murine cell culture supernatants were analyzed by a CBA mouse Th1/Th2/Th17 kit by flow cytometry using a FACSCalibur (Beckton Dickinson) according to manufacturer's instructions. TGF-β measurements were made using a Human/Mouse TGF-B1 ELISA Ready-Set-Go! Kit (eBioscience).

Tumor injections and measurements

B16F10 cells were cultured in complete DMEM (Sigma) with 10% fetal calf serum (FCS). BALB/c mice were anesthetized (intraperitoneally) with a 100 µl volume of PBS 34% ketamine, 10% xylazine and fur from the upper thigh was removed. Cultured tumor cells that were 80% confluent were detached from the tissue culture plate with PBS 15 mM EDTA, washed, counted and ressuspended to a density of 1.5×10^6 cells in 150 µl PBS containing either LPS-free TBHsp70 (30 µg) or PBS alone. These mixtures were injected subcutaneously in the outer region of the thigh of mice. Tumor diameter was measured with a caliper and photographed daily, for a period of two weeks. These experiments were repeated six times.

Immunohistology

Lymph nodes and thighs of the tumor-injected animals were embedded in tissue-freezing medium (Tissue-Tek - Miles Laboratories), and stored in liquid nitrogen. Serial cryostat sections were mounted on poly-L-lysine-covered glass slides and fixed for 10 min in cold acetone, washed in PBS, and incubated for 30 min in a wet chamber at room temperature with PBS and normal goat serum (Sigma-Aldrich) diluted 1/50 to reduce nonspecific binding and then incubated for 40 min with biotinylated anti-CD4, anti-CD25, anti-GITR or anti-FoxP3, according to a previous titration assay. Next, sections were incubated with avidin-biotin-peroxidase complex, the color developed with 3,3′-diaminobenzidine (Vector Laboratories). The slides were counterstained with Mayer hematoxylin, dehydrated, and mounted with Canada Balsam. The software used to capture and count the cells was the Image Pro-plus version 4.1.5 (Mediacybernetics), which uses a video camera connected to a computer card to capture the images of the selected microscopic field. Structures were selected on the computer screen with the mouse pointer and then counted manually. Histological sections were captured by ZEISS – Axioskope 40 microscope equipped with a CoolSNAP-PRO color camera. Positive cells to CD4, CD25, GITR and FoxP3 were counted at 400x magnification, and photographs had the number of pixels quantified in each section, using the Image Pro Plus Software (version 4.1.5, Media Cybernetics Inc., Bethesda) and the color range Adobe Photoshop tool. Histology was performed 3 times, in different experiments.

Skin Graft Model

For the experimental model of skin graft, the proceeding described by Billingham and Medawar was employed [46]. Briefly, C57BL/6 donor mice were sacrificed, 1 cm^2 sections of tail skin were removed and immersed in a PBS solution (500 µl) containing TBHsp70 (30 µg), or PBS alone for 60 minutes at 4°C. Control treatments were PBS solutions (500 µl) containing either 30 µg of Hsp90 (StressGen), 30 µg of Ovalbumin (Sigma) or 30 µg of TBHsp70 boiled for 10 minutes at 100°C. BALB/c recipient mice were anesthetized as described above, and fur was shaved off the dorsal trunk. At the shaved area, 1 cm^2 of skin was removed in each recipient mouse. One donor tail skin fragment was sutured to the exposed tissue of each recipient. Animals were kept in individual cages and observed daily, the state of graft acceptance being photographed and recorded. Graft rejection was confirmed by the observation of cyanosis, erythema, erosion, and loss of skin graft. Each experiment was performed four times.

In vivo Tregs depletion

Mice were injected i.p. a single injection containing 150 µg of anti-CD25 mAb, purified from PC61 hybridoma culture supernatant using a protein A column (Sigma), or with PBS. The injection was given 2 days after skin transplantation. PC61 hybridoma cells were kindly provided by Dr. Ross Kedl, National Jewish, Denver, Colorado. Efficiency of depletion was analyzed by flow cytometry of lymphoid organs, staining for CD4, CD25 and FoxP3.

Flow cytometry

Lymph nodes from mice injected with different treatments were excised after animals were sacrificed, and the organs disrupted against a nylon screen in media containing Collagenase D (Roche). Single cell suspensions were obtained, cells counted with Trypan blue and stained with antibodies against CD4-cychrome, CD25-PE and Foxp3-biotin, followed by streptavidin-FITC; or CD11c-FITC and CD86-PE (all purchased from Pharmingen). Cells were analyzed in a FACSCalibur flow cytometer (Bekton Dickinson).

Statistical analysis

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

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Author Contributions

Conceived and designed the experiments: TJB JSS CB. Performed the experiments: TJB BNP CAT MR FDM APO WRP. Analyzed the data: TJB BNP CAT APDdS FM WRP JSS CB. Contributed reagents/materials/analysis tools: JSS CB. Wrote the paper: TJB BNP CAT CB.

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Capítulo 4

Resultados Complementares

Os resultados apresentados a seguir foram obtidos nos últimos meses do mestrado e futuramente, quando complementados com dados novos, farão parte de um artigo científico que será submetido à revista *Journal of Immunology*.

Materiais e métodos

Animais

Todos os procedimentos realizados foram aprovados pelo Cômite de Ética no Uso de Animais da Pontifícia Universidade do Rio Grande do Sul (CEUA-PUCRS) sob o número CEUA 10/00220.

Camundongos C57Bl/6 e BALB/c fêmeas adquiridas da Fundação Estadual de Produção e Pesquisa em Saúde (FEPPS) com idades entre seis e oito semanas foram mantidos no vivário da Faculdade de Biociências da Pontifícia Universidade Católica do Rio Grande do Sul (FABIO-PUCRS). Água e ração autoclavadas foram fornecidas em livre demanda. Toda maravalha foi também autoclavada. A manutenção das caixas para trocas de água, ração e limpeza foram realizadas duas vezes por semana e os animais foram mantidos em ambiente com temperatura controlada de 22-23°C e ciclos de claro-escuro de 12 horas. Os tratadores sempre utilizarão luvas e máscaras cirúrgicas no manuseio dos animais. Os camundongos TLR2 KO (em *background* C57Bl/6) foram gentilmente cedidos pelo Dr. João Santana da Silva, da USP de Ribeirão Preto. No momento necessário os animais sofrerão eutanásia na câmara de sacrifício com CO_2, modelo CO_2G, Vol: 30L³, da marca Beiramar®.

Purificação da proteína e extração do LPS

A Mt Hsp70 recombinante codificada pelo plasmídeo pY3111 (gentilmente cedido pelo Dr. Douglas Young, Hammersmith Hospital, Londres), foi produzida na cepa BL21 de *Escherichia coli* e purificada de acordo com Mehlert & Young (93). Para determinar a concentração da proteína foi utilizado o teste fluorimétrico Quant-iT™ Protein Assay Kit (Invitrogen) e as amostras foram lidas no Qubit® fluorometer (Invitrogen). Para a remoção do LPS, foi utilizado um método usando Triton X-114 (Sigma) descrito em Aida e Pabst (94). O Triton contaminante foi removido através da incubação da proteína com Biobeads (Bio-Rad) à

4ºC em agitação durante 8h. Para verificar se algum LPS contaminante permaneceu na amostra um bioensaio foi realizado. Camundongos BALB/c foram anestesiados, intraperitonealmente, com 100µL de volume de uma solução em PBS com 34% de Ketamina e 10% Xilazina. Posteriormente, foram injetados i.v. com 100 µL de PBS, PBS com 30 µg de LPS (Sigma) ou PBS com 30 µg de Mt Hsp70. Após seis horas, os animais sofreram eutanásia na câmara de CO_2, modelo CO_2G, Vol: 30L³ da marca Beiramar®, e tiveram o baço removido. Foi feito um tratamento com colagenase D e, posteriormente, a suspensão celular obtida foi analisada pra a expressão de CD86 através de citometria de fluxo, como descrito em Khoruts e colaboradores (95).

Cultura de células dendríticas

As células dendríticas murinas foram geradas a partir da medula óssea, proveniente de animais selvagens ou TLR2 KO, juntamente com 40 ng/mL GM-CSF, como descrito no trabalho de Inaba e colaboradores (96). Juntamente com o GM-CSF, adicionamos 40 ng/mL de IL-4 nas culturas. As células foram usadas no dia 5 da cultura, enquando elas estão com um perfil imaturo. Elas foram incubadas com 30 µg de OVA, 30 µg de Mt Hsp70, 5 µg de DEXA ou 1 µg de PGN por 24 horas. Após esse período, o sobrenadante foi coletado e analisado para a presença de IL-10 e TNF com o kit CBA Th1/Th2/Th17 (BD), de acordo com as recomendações do fabricante.

Modelo murino de aloenxerto cutâneo

Para o modelo experimental de aloenxerto cutâneo foi utilizado o procedimento descrito por Billingham & Medawar (97). Brevemente, o camundongo doador C57BL/6 sofreu eutanásia na câmara de CO_2, modelo CO_2G, Vol: 30L³ da marca Beiramar® e, posteriormente teve a pele de sua cauda removida. O camundongo receptore foi anestesiado, intraperitonealmente, com 100 µL de volume de uma solução em PBS com 34% de Ketamina e 10% Xilazina. Após a anestesia, os pêlos do dorso foram retirados. Na área depilada do receptor, aproximadamente 1cm^2 da pele foi removida. Posteriormente, 1 cm^2 da pele da cauda do doador foi cortada. Para avaliar o efeito imunossupressor da Mt Hsp70 no prolongamento da rejeição, os aloenxertos provenientes da pele da cauda foram embebidos em uma solução contendo 60 µg/mL de Mt Hsp70 ou OVA (Sigma) durante uma hora no gelo. Após este período, o aloenxerto foi suturado no tecido exposto do

dorso do receptor. Os camundongos foram observados e fotografados diariamente. A rejeição foi confirmada pela visualização de cianoses, eritema, erosão e perda de no mínimo 80% do enxerto.

Avaliação das vias de sinalização envolvidas

As DCs selvagens e TLR2 KO foram estimuladas com 30 μg de Mt Hsp70 ou com meio por 15 minutos. Após isso, as moléculas intracelulares fosforiladas Akt (p-Akt), ERK 1/2 (p-ERK) e p38 (p-p38) foram marcadas com o auxílio do kit Phosflow (BD Biosciences), de acordo com as recomendações do fabricante. As células foram lidas no citometro de fluxo FACSCanto II (BD Biosciences) com o software FACSDiva. Todos os dados foram analisados com o programa FlowJo (versão 7.5, Tree Star Inc., Ashland, US).

Para a confirmação do papel da ERK 1/2, BMDCs WT de animais C57Bl/6 foram pré-tratadas ou não com 30 μM do inibidor seletivo PD98059 (Cayman Chemical) por 1 hora. Posteriormente, as células foram estimuladas com 30 μg de Mt Hsp70, 30 μg de OVA ou 1 μg de PGN por 24 horas. Após esse período, os sobrenadantes foram avalidos para a presença de IL-10, como descrito à cima.

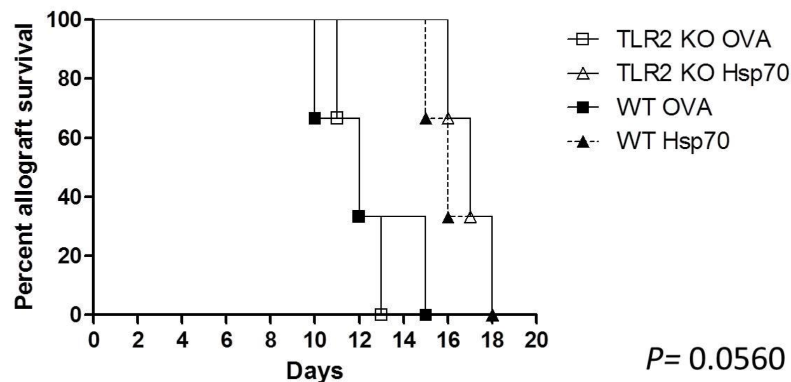
Análise estatística

As análises estatísticas foram realizadas com o auxílio do software Prism (versão 5.00, Graphpad Software Inc., San Diego, US). As comparações de parâmetros entre diferentes grupos experimentais foram realizadas por teste t de Student ou análise de variância de uma via (ANOVA), seguida de testes post-hoc adequados quando a ANOVA revelou diferenças significativas entre grupos, conforme descrito em estudos anteriores. Para a análise da sobrevida do enxerto foi utilizado o método de Kaplan-Meier. Os resultados foram expressos em média e desvio padrão da média e valores de P menores do que 0,05 indicaram diferenças significativas.

Resultados

<u>O retardo da rejeição mediado pela Mt Hsp70 é dependente de TLR2 no enxerto e não no receptor</u>

Como descrito no capítulo 3 deste trabalho, a Mt Hsp70 inibe a rejeição aguda em um modelo murino de aloenxerto cutâneo (98). Dando continuidade a esse trabalho e tentando entender o mecanismo envolvido no aumento da sobrevida do enxerto mediado pela Mt Hsp70, analisamos se o TLR2 era importante nesse processo. Primeiramente, verificamos se esse receptor era importante nos receptores dos enxertos. Todos os enxertos cutâneos eram provenientes de camundongos BALB/c selvagens e foram tratados com uma solução de PBS que continha 30 µg de OVA ou de Mt Hsp70. Esses enxertos foram transplantados em animais C57Bl/6 WT ou TLR2 KO. A evolução da rejeição foi acompanhada diariamente. Observamos que os camundongos WT e TLR2 KO que receberam enxertos tratados com Mt Hsp70 foram capazes de prolongar a aceitação do enxerto quando comparados com os animais que receberam enxertos tratados com OVA (Figura 2). Não houve diferença entre os animais WT e TLR2 KO que receberam enxertos tratados com Mt Hsp70 quando comparados entre si (*p= 0,8980*).



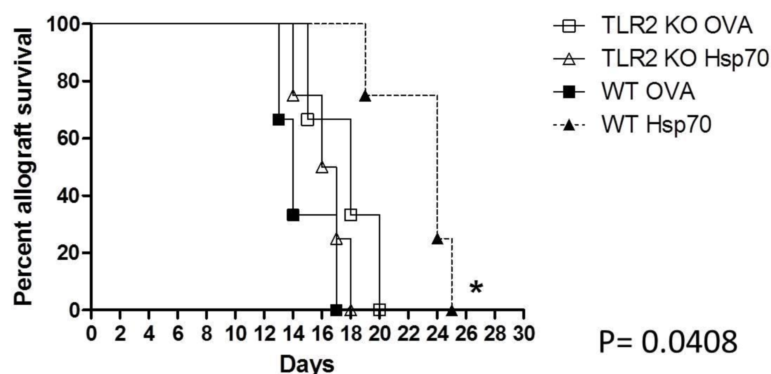
	TLR2 KO Hsp70	TLR2 KO OVA	WT OVA
WT Hsp70	P= 0.8980	P= 0.1314	P= 0.1341
TLR2 KO Hsp70	-	* P= 0.0246	* P=0.0246

Figura 1 – A inibição da rejeição aguda induzida pela Mt Hsp70 não depende da presença do TLR2 nos receptores. Enxertos cutâneos provenientes de camundongos BALB/c WT foram tratados com uma solução de PBS contendo 30 µg de Mt Hsp70 ou OVA por 1 hora à 4°C. Os enxertos foram transplantados em receptores C57Bl/6

WT ou TLR2 KO (n= 4 animais por grupo). A evolução do quadro da rejeição foi acompanhada diariamente. Dados representativos de dois experimentos independentes. * *P>0,05*.

Em seguida, verificamos se o TLR2 era importante nos enxertos, ao invés da sua presença nos receptores. Para isso, todos os receptores usados eram BALB/c WT e receberam enxertos cutâneos provenientes de camundongos C57Bl/6 WT ou TLR2 KO, tratados com uma solução de PBS que continha 30 µg de OVA ou de Mt Hsp70. Foi visto que apenas os animais que receberão enxertos WT tratados com Mt Hsp70 foram capazes de inibir a rejeição aguda (Figura 3). Nos animais que receberam os enxertos TLR2 KO não houve a inibição da rejeição quando comparadas com os WT OVA (*p= 0,3256*) e TLR2 OVA *(p= 0,2903*).

Juntos, esses dados indicam que a presença do TLR2 no aloenxerto, e não no receptor, é importante para a inibição da rejeição aguda induzida pela Mt Hsp70.



	TLR2 KO Hsp70	TLR2 KO OVA	WT OVA
WT Hsp70	** P= 0.0067	* P= 0.0476	* P= 0.0101
TL2 KO Hsp70	-	P = 0.2903	P = 0.3256

Figura 2 – A inibição da rejeição aguda induzida pela Mt Hsp70 depende da presença do TLR2 nos enxertos. Enxertos cutâneos provenientes de camundongos C57Bl/6 WT ou TLR2 KO foram tratados com uma solução de PBS contendo 30 µg de Mt Hsp70 ou OVA por 1 hora à 4°C. Os enxertos foram transplantados em receptores BALB/c WT ou TLR2 KO (n= 4 animais por grupo). A evolução do quadro da rejeição foi acompanhada diariamente. Dados representativos de dois experimentos independentes. * *P>0,05*.

Nas BMDCs, a produção de IL-10 mediada pela Mt Hsp70 é dependente de TLR2

Como mostrado pelo nosso grupo, a Mt Hsp70 é capaz de tolerizar células dendríticas *in vitro*. Isso foi caracterizado pela baixa expressão de MHC II e CD86, quando BMDCs foram estimuladas com Mt Hsp70. Também foi observada uma alta produção de IL-10 por essas células (80). Ligantes de TLR2 têm sido demonstrados como bons indutores de IL-10, em células do sistema imune inato (81, 86). Para avaliarmos o mecanismo envolvido nas DCs na produção de IL-10 induzida pela Mt Hsp70, estimulamos BMDCs provenientes de camundongos selvagens (*wild type* – WT) ou TLR2 KO com OVA, Mt Hsp70, DEXA ou PGN. Após 24 h de cultura, o sobrenadante das células foi coletado e analisado para a presença de IL-10 e TNF-α. Como podemos observar na Figura 1A, as BMDCs WT estimuladas com Mt Hsp70 produziram uma quantidade muito maior de IL-10 quando comparadas com as BMDCs TLR2 KO. Essa produção também foi maior quando comparada com as células que receberam outros estímulos. Além disso, observamos uma diminuição da produção de TNF tanto nas células WT quanto nas células TLR2 KO quando estimuladas com Mt Hsp70 quando comparadas com as células tratadas com OVA ou PGN (Figura 1B). Esse dado confirma outro achado visto pelo nosso grupo, no qual células do líquido sinovial de pacientes com artrite tiveram um decréscimo na produção de TNF-α quando tratadas com a Mt Hsp70 (82). Esses resultados nos indicam que, nas BMDCs, a produção de IL-10 induzida pela Mt Hsp70 é dependente de TLR2, enquanto que a inibição da produção do TNF não depende desse receptor.

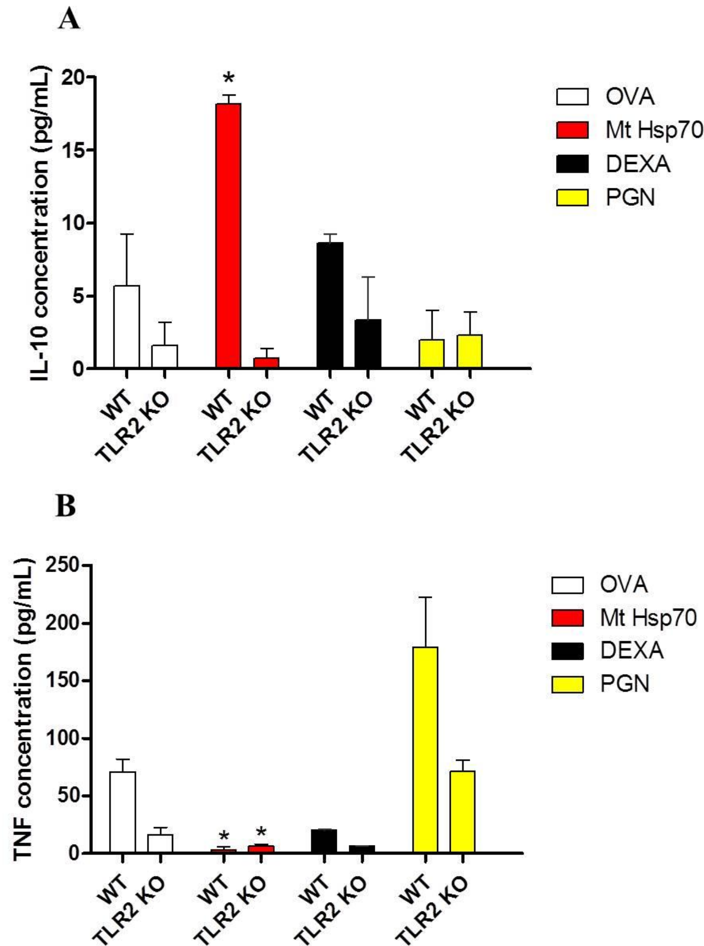


Figura 3 – A Mt Hsp70 induz a produção de IL-10 nas BMDCs em um mecanismo dependente do TLR2. BMDCs foram estimuldas no dia 5 de cultura com 30 μg de OVA, 30 μg de Mt Hsp70, 5 μg de DEXA ou 1 μg de PGN. Após 24 horas, as células foram analisadas para a presença de (A) IL-10 e (B) TNF com o kit CBA Th1/Th2/Th17. Dados representativos de dois experimentos independentes. * *P>0,05*.

<u>As MAP quinases ERK 1/2 são ativadas nas BMDCs após estímulo com a Mt Hsp70 em um mecanismo dependente do TLR2</u>

A ERK é uma MAP quinase envolvida na produção de IL-10 por diferentes células do sistema imune (85). Por isso, investigamos se essa molécula está envolvida na produção de IL-10 induzida pela Mt Hsp70 nas BMDCs. Primeiramente, BMDCs provenientes de animais C57Bl/6 WT ou TLR2 KO foram estimuladas com 30 μg de Mt Hsp70 ou não por 15 minutos. Após esse período, a ativação da ERK foi avalida através da presença de sua forma fosforilada (p-ERK) por citometria de fluxo. Observamos um aumento na expressão de p-ERK 1/2 nas BMDCs estimuladas com Mt Hsp70 quando comparadas com as células que não receberam estímulo

(Figura 4). Interessantemente, quando as células eram TLR2 KO, não houve diferença na expressão de p-ERK 1/2 nas BMDCs estimuladas com Mt Hsp70 quando comparadas com as células sem estímulo. Isso nos indica que, nas BMDCs, a ERK é ativada por um mecanismo dependente do TLR2.

Também avaliamos o envolvimento das formas ativadas da p-38 (p-p-38) e da Akt (p-Akt) nas BMDCs WT ou TLR2 KO estimuladas com Mt Hsp70, em um protocolo semelhante ao descrito a cima para a ERK. Não foi vista nenhuma diferença na expressão de p-p-38 (Figura 5) ou de p-Akt (Figura 6), tanto nas células WT quanto nas TLR2 KO, tratadas com Mt Hsp70 quando comparadas com as BMDCs que não receberam o estímulo.

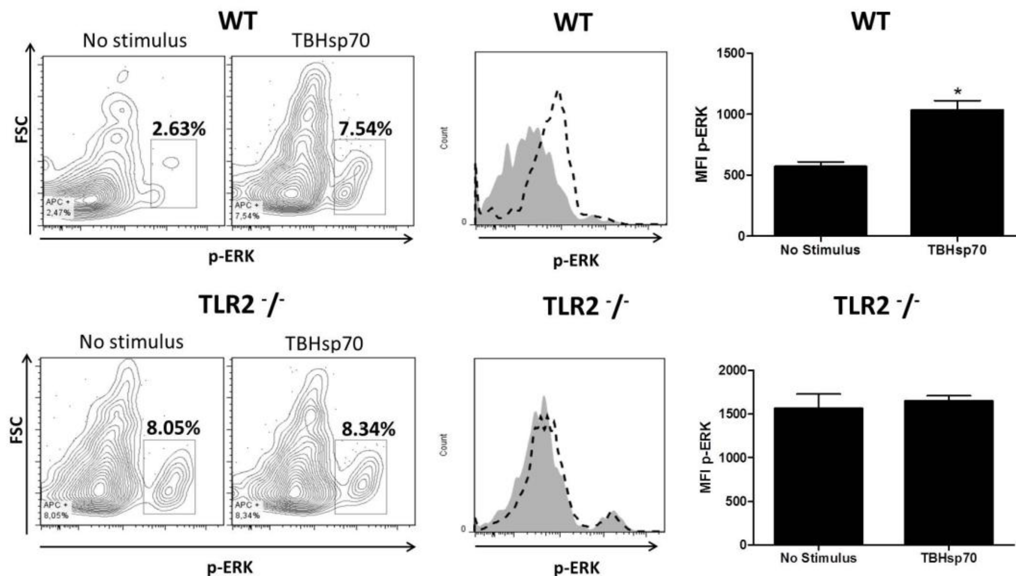


Figura 4 – A Mt Hsp70 induz a expressão de p-ERK 1/2 nas BMDCs via TLR2. BMDCs provenientes de camundongos WT ou TLR2 KO foram estimuladas por 15 min com 30 µg de Mt Hsp70 ou só meio. Posteriormente, a expressão de p-ERK 1/2 intracelular foi avaliada por citometria de fluxo com o auxílio do kit Phosflow. Dados representativos de um experimento. N = 2 animais/grupo. * *P>0,05*.

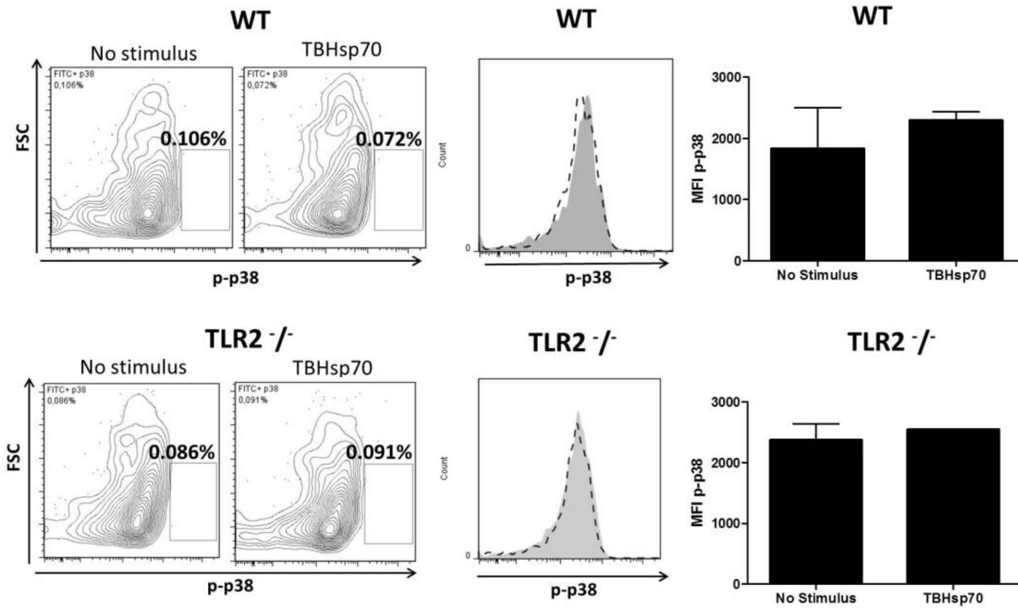


Figura 5 – A Mt Hsp70 não induz a expressão de p-p38 nas BMDCs. BMDCs provenientes de camundongos WT ou TLR2 KO foram estimuladas por 15 min com 30 µg de Mt Hsp70 ou só meio. Posteriormente, a expressão de p-p38 intracelular foi avaliada por citometria de fluxo com o auxílio do kit Phosflow. Dados representativos de um experimento. N = 2 animais/grupo.

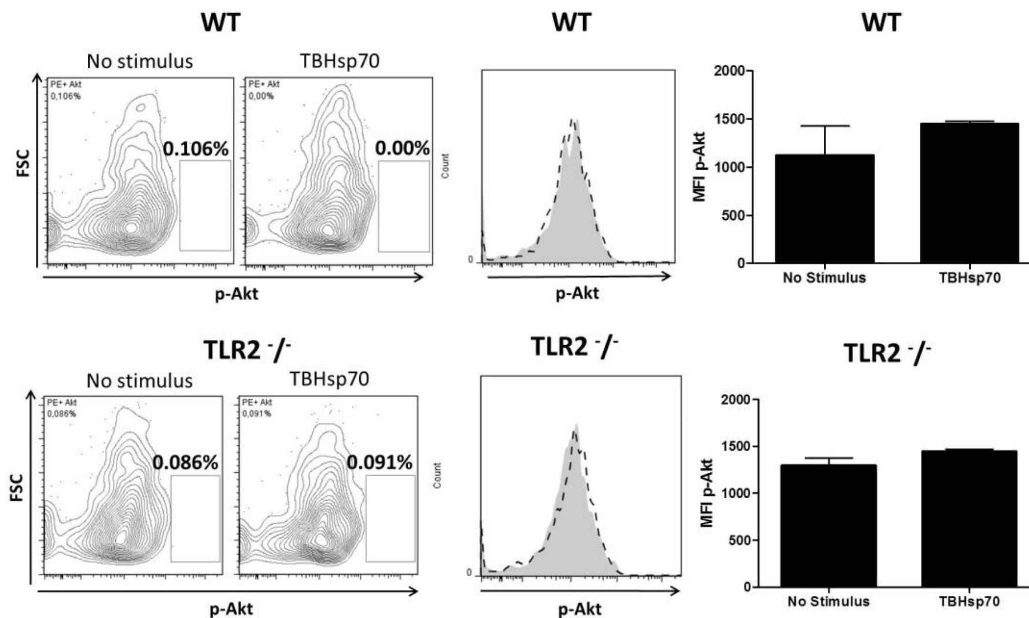


Figura 6 – A Mt Hsp70 não induz a expressão de p-Akt nas BMDCs. BMDCs provenientes de camundongos WT ou TLR2 KO foram estimuladas por 15 min com 30 µg de Mt Hsp70 ou só meio. Posteriormente, a expressão de p-p38 intracelular foi avaliada por citometria de fluxo com o auxílio do kit Phosflow. Dados representativos de um experimento. N = 2 animais/grupo.

Para confirmar o papel da ERK na indução de IL-10 pela Mt Hsp70, BMDCs WT de animais C57Bl/6 foram pré-tratadas com o PD98059, um inibidor seletivo de ERK 1/2. Após isso, as células foram estimuladas com Mt Hsp70, OVA ou PGN por 24 horas e a produção de IL-10 no sobrenandante foi avaliada por citometria de fluxo. Interessantemente, nem a OVA e nem o PGN induziu a produção de IL-10 nas BMDCs. Já a Mt Hsp70, induziu a alta produção de IL-10 nas BMDCs WT. Quando as células tiveram a ERK 1/2 inibidas essa produção foi abolida (*p<0,01*) (Figura 5). Isso nos indicas que a produção de IL-10 pelas BMDCs induzida pela Mt Hsp70 é dependente de ERK 1/2.

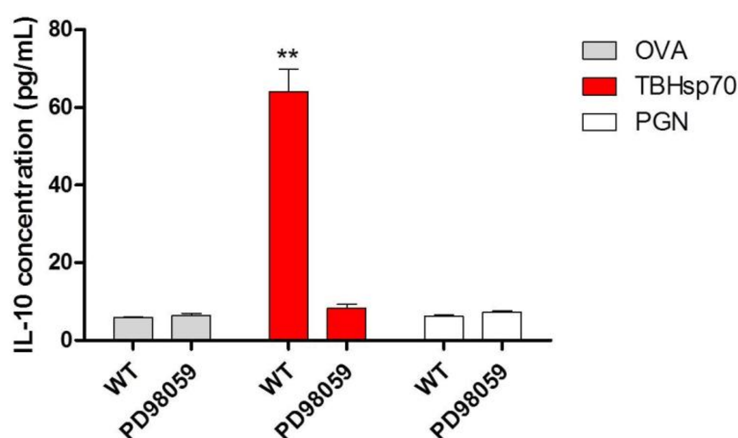


Figura 7 – Nas BMDCs, a produção de IL-10 induzida pela Mt Hsp70 é dependente de ERK. BMDCs WT de animais C57Bl/6 foram pré-incubadas com 30 µM do inibidor seletivo de ERK 1/2, o PD98059, por 1 hora à 37°C e 5% de CO_2. Após isso, foram estimuladas com 30 µg de OVA, 30 µg de Mt Hsp70 ou 1 µg de PGN por 24h. produção de IL-10 foi analisada no sobrenadante celular com o kit CBA Th1/Th2/Th17. Dados representativos de um experimento. ** *P>0,01*.

Discussão

Alguns trabalhos têm demonstrado que a Hsp70 tem um papel anti-inflamatório e protetor em modelos animais experimentais como artrite (63, 64, 90, 99), colite (66), fibrose pulmonar (67) e danos cerebrais (100). Primeiramente, confirmamos que a Mt Hsp70 livre de LPS é capaz de induzir um fenótipo tolerizado em células dendríticas. Ela aumentou produção de IL-10 em DCs diferenciadas da medula óssea de camundongos selvagens. Esse achado foi demonstrado anteriormente pelo nosso grupo e, além da produção de IL-10, a Mt Hsp70 reduziu a expressão do MHC II e da molécula co-estimuladora CD86 (80).

Também observamos que os efeitos da Mt Hsp70 sobre as BMDCs é dependente de TLR2 (Figura 1A). Corroborando com nossos achados, tem sido sugerido que ligantes do TLR2 são ótimos indutores de IL-10 em diferentes trabalhos (83-85). Por exemplo, a sinalização via TLR2 é crucial para indução de IL-10 por DCs estimuladas com *Mycobacterium tuberculosis* ou com lipoproteínas (101). O mesmo efeito foi visto em DCs estimuladas com antígenos de *Yersinia pestis* (102). Além disso, a sinalização via TLR2, em certas circunstâncias, induz o aumento da população de Tregs (86, 103, 104) e células T CD8+Foxp3+ (105). Foi sugerido que na verdade a sinalização via TLR2 não aumenta a funcionalidade das Tregs, porém, ao invés disso, aumenta sua sobrevida através de um aumento da molécula antiapopitótica Bcl-$_{XL}$ (106). Outras Hsps, como a Hsp60 humana, também podem se ligar no TLR2 das células do sistema imune inato, levando ao aumento de Tregs (107). Provavelmente, estamos vendo um fenômeno muito parecido com a Mt Hsp70.

Para excluir a hipótese de que o efeito visto pelo nosso grupo seja devido a contaminantes na Mt Hsp70, vamos também testar o papel do TLR4 em nosso sistema. O fato da diminuição do TNF produzido pelas DCs quando estimuladas com Mt Hsp70 ser independente de TLR2 será investigado melhor pelo nosso grupo. Provavelmente essa inibição da produção ocorra por um mecanismo paralelo como o bloqueio da via do NF-κB, o qual já foi reportado pela Hsp70. O NF-κB é um fator de transcrição que tem um papel chave na indução de citocinas proinflamatórias (108). Em condições fisiológicas, sem a presença de inflamação, o NF-κB é mantido inativado no citoplasma em complexo com seu inibidor IκBα (complexo NF-κB/ IκBα). Em um quadro inflamatório, o IκBα é degradado no proteassomo e o NF-κB é liberado para se translocar até o núcleo. No núcleo, ele direciona a transcrição de gene pró-inflamatórios (109). A

Hsp70 pode prevenir a produção de citocinas proinflamatórias induzida por LPS, através da interferência da transcrição dos genes dessas citocinas via NF-κB (110). Em outro estudo, a superexpressõ da Hsp70 em células mononucleares humanas, preveniu a translocação (induzida por LPS) do NF-κB para o núcleo, assim inibindo a produção das citocinas proinflamatórias (111). Foi sugerido, portanto, que a Hsp70 estabiliza o complexo NF-κB/ IκBα pela inibição da degradação do IκBα (112). Precisamos investigar em nosso modelo se algo parecido está ocorrendo, levando a inibição da produção de TNF-α.

Mostramos na Figura 4 que após da incubação com Mt Hsp70, DCs mostraram um aumento na expressão de p-ERK, dependente de TLR2, e que essa molécula é crucial para a produção de IL-10 induzida pela Mt Hsp70 (Figura 7). Em concordância com nossos achados, muitos trabalhos mostram que após o estímulo via TLRs, a expressão da ERK modula a expressão de IL-10 (113-116). Além disso, na presença de inibidores seletivos de ERK (113, 114, 116) ou em células deficientes para ERK (115), a produção de IL-10 pelas DCs ativadas via TLRs diminui. Interessantemente, a diferença na produção de IL-10 por macrófagos, DCs mielóides e pDCs está correlacionada com os diferentes níveis de ativação de ERK por essas células (116). Chalmin e colaboradores (81) demonstraram que exossomos liberados por tumores expressam Hsp72 em sua superfície interna. Essa Hsp72 se ligaria no TLR2 de MDSCs e aumentaria sua atividade imunossupressora, ajudando o tumor a bular respostas imunes do hospedeiro. Os autores também demonstraram que essas respostas ocorreram por uma via dependente de MyD88, STAT3 e ERK (81). Provavelmente essa mesma rota esteja sendo ativada nas DCs tratadas com a Mt Hsp70.

Em alguns modelos, a produção de IL-10 também pode ser compremetida pela inibição da via do p38 em macrófagos ativados com LPS ou CpG (114, 117, 118), DCs (119, 120) e monócitos humanos do sangue periférico (121). Porém nenhuma dessas células apresentava um fenótipo tolerizado. É sugerido que tanto a via da ERK, quanto a do p38 podem cooperar na produção de IL-10 induzida via TLRs (85). Em nosso modelo, vimos que as moléculas p38 (Figura 5) e Akt (Figura 6) não são fosforiladas nas DCs após o estímulo da Mt Hsp70. Provavelmente não estão envolvidas na produção de IL-10. Porém, não usamos inibidores seletivos para essas vias. Esses experimentos irão ser realizados no futuro.

Capítulo 5

Considerações Finais

Foi demonstrado nesse trabalho que a Hsp70 de *Mycobacterium tuberculosis* (TB-Hsp70) pode aumentar a sobrevida dos enxertos em dois modelos murinos de transplantes. O primeiro modelo utilizado foi um modelo tumoral onde células de melanoma B16F10 (I-A^b) foram tratadas ou não com TB-Hsp70 e, posteriormente injetadas s.c. em animais BALB/c (I-A^d). Observamos que as células tumorais tratadas com TB-Hsp70 foram capazes de se instalar e crescer nos animais BALB/c. Além disso, foi visto uma grande presença de células Tregs no tecido tumoral, demonstrando que a Hsp70 foi capaz de modular e tolerizar o microambiente tumoral. Se as Tregs migraram de outros tecidos ou foram expandidas no ambiente tumoral, como por exemplo, pela ação sobre as APCs, precisa ser verificado. Também não sabemos ainda se essas células são específicas a peptídeos presentes na sequência da Hsp70.

Para excluir a possibilidade dessa imunomodulação do microambiente tumoral ser devido a outros mecanismos apresentados pelos tumores (122), testamos o efeito imunossupressor da TB-Hsp70 em um modelo de transplante não tumoral. Foi utilizado um modelo murino bem estabelecido e estudado de aloenxerto cutâneo (97). Nesse modelo os enxertos foram tratados com TB-Hsp70 de uma maneira inovadora: foram embebidos por 1h à 4°C em uma solução que continha a proteína, mimetizando a utilização de uma solução de preservação. Esse tratamento com a TB-Hsp70 foi capaz de retardar a rejeição aguda dos aloenxertos cutâneos em um mecanismo dependente de Tregs. As soluções de preservação de órgãos são amplamente utilizadas na rotina de transplantes e permitem um maior período de armazenamento *ex vivo* (123). Estamos propondo que essa solução possua moléculas que além de manter a funcionalidade do órgão, também possa ajudar a suprimir respostas aloimunes levando a uma melhor aceitação do aloenxerto.

Por outro lado, Tesar & Golstein (124), utilizando camundongos os quais não expressavam os genes *hsp70.1* e *hsp70.3*, propuseram que a Hsp70 não possui um papel determinante na rejeição aguda de aloenxertos cutâneos. Talvez a TB-Hsp70 tem uma ação diferente da Hsp70 murina, por isso a discrepância dos dados.

Observamos que a injeção s.c. de TB-Hsp70 leva a indução de Tregs no linfonodo drenante. Esse efeito foi visto por grupos e estão em concordância com nossos achados (53, 90, 125). Porém, análises mais detalhadas sobre a origem, especificidade e fenótipo dessas células Tregs geradas com a Hsp70 precisam ser realizadas. Helios é um fator de transcrição que pertence à família Ikaros de fatores de transcrição. Esses fatores podem interagir entre si

formando homodímeros e heterodímeros (126, 127). Através dessa dimerização, Helios pode alterar a função do Ikaros (128, 129), o qual é capaz de influenciar a diferenciação de células T *helpers* (130, 131). Nas Tregs, Thornton e colaboradores demonstraram que todas células Foxp3+ geradas no timo co-expressavam o Helios, enquanto que Tregs geradas *in vitro* a partir de células T convencionais da periferia, estimuladas com anti-CD3 e anti-CD28 na presença de IL-2 e TGF-β1, não expressavam o Helios (14). Esses achados indicam que o Helios pode ser usado como um marcador molecular para distinguir as nTregs da iTregs. Precisamos analisar se essas Tregs geradas no linfonodo drenante após a administração s.c. de TB-Hsp70 são de origem tímica (que são Helios+) ou são induzidas nos tecidos periféricos (Helios-). Além disso, pretendemos colocar em cultura DCs pulsadas com a TB-Hsp70 e células T para verificarmos se o tratamento das DCs com a TB-Hsp70 induzirá Tregs, e qual subtipo dessas células irá ser gerado. Também vamos investigar outros tipos de células T reguladoras que podem estar envolvidas na resposta mediada pela TB-Hsp70, como as células T CD4+LAP+ e células T gama-delta.

Outro aspecto a ser verificado é a possibilidade da Hsp70 estar modulando células Th1 a produzirem IL-10, inibindo a produção de IFN-γ nessas células (120, 132, 133). Além a indução das Tregs, os dLNs apresentaram um aumento da produção de IL-10 e uma diminuição nos níveis de TNF-α. Esse mesmo padrão de resposta foi verificado pelo nosso grupo anteriormente (82). Nesse trabalho células sinoviais provenientes de paciente com artrie foram incubadas com TB-Hsp70 por 48h. Após esse período, essas células apresentaram uma reversão de seu perfil inflamatório, com um decréscimo da produção de TNF-α e IFN-γ e um aumeno da IL-10 (82).

Além da imunidade adaptativa, a Hsp70 parece também modular células do sistema imune inato, como mostrado na Figura 2 do capítulo 1. Nosso grupo demonstrou que essa proteína pode modular DCs (80) e monócitos (82), porém as vias de sinalização envolvida nessas células não foram elucidadas. No capítulo 4, tentamos verificar algumas moléculas que estariam envolvidas na tolerização das DCs induzida pela Hsp70. Observamos uma dependência do TLR2, *in vitro* e *in vivo*, além do envolvimento da ERK. Não foi observado um aumento na expressão de p38 e Akt. Esses achados foram melhores discutidos na seção “Discussão” desse mesmo capítulo A Figura 1B do capítulo 2 sumariza a possível rota de sinalização desencadeada pela Hsp70 em células do sistema imune inato.

Outra possibilidade é que além de alguns TLRs, a Hsp70 possa estar se ligando em receptores como os *lectin-like receptors* (LLRs), os quais são abundantes em DCs e outras células

do sistema imune inato (134). Enquanto que a ligação via TLRs resulta na ativação de DCs, contribuindo para a liberação de citocinas e quimiocinas que vão contribuir para moldar a resposta imune (135), os LRRs operam um poderoso sistema de captura e internalização de antígenos (134, 136, 137). Os receptores LOX-1, Dectin-1 e DC-SIGN são capazes de enviar sinais intracelulares, tanto sozinhos quanto em associação com TLRs, ativando as DCs e resultando em diferentes respostas de células T, inclusive Tregs (73, 83, 134, 136). Por exemplo, a ligação do DC-SIGN por diferentes patógenos pode levar a indução da diferenciação de Tregs (136, 138). Também foi visto que a ativação de DCs via Dectin-1 e TLR2 resulta em respostas mediadas por Tregs (83), e que sinias via Dectin-1 levam as DCs a produzirem IL-10 (139, 140).

Foi demonstrado que a Hsp70 humana pode se ligar no LOX-1 de células dendríticas humanas e que esse receptor está envolvido na internalização e *cross-priming* de antígenos *in vivo* (73). Em outro estudo a grp170 humana, um membro da família da Hsp70 expresso no retículo endoplasmático, conseguiu se ligar no scavenger receptor-A (SR-A) presente em DCs humanas (141). Essa ligação conferiu um sinal supressivo às células do hospedeiro. Recentemente, Li e colaboradores demonstraram um papel interessante de um LRR chmado DC-ASGPR (120). Foi visto que a entrega de antígenos, tanto próprios como estranhos, às DCs via esse receptor favoreceu a geração de células T CD4+ supressoras que eram antígeno-específicas e produziam IL-10 (120). Interessantemente, essas células T CD4+ produtoras de IL-10 eram Foxp3- e os autores sugeriram elas tinham uma origem Th1. Além disso, a geração dessas células T foi dependente da produção de IL-10 e das vias do p38 e da ERK nas DCs. Em camundongos o receptor correspondente do DC-ASGPR seria o mMGL (142). Com isso, hipotetizamos que talvez a TB-Hsp70 também possa se ligar em um LRRs, principalmente o mMGL (camundongos) e DC-ASGPR (humanos) e atuar em conjunto com o TLR2. Pretendemos realizar experimentos de imunofluorescência para verificarmos se há co-localização de algum desses receptores com o TLR2, após estímulos com a TB-Hsp70.

Outra hipótese que pretendemos investigar é que talvez a TB-Hsp70 seja internalizada pelas células do enxerto e possa exercer um papel citoprotetor e anti-apoptótico. Esse efeito citoprotetor e anti-apoptótico é bem documentado em relação a Hsp70 autóloga (56, 143) e, considerando o grau de homologia da TB-Hsp70 em relação às Hsp70s de mamífero, é plausível que isso possa ocorrer nas células do enxerto.

Esse efeito da TB-Hsp70 sobre as DCs pode servir como intervenção terapêutica em modelos de transplantes. Com as terapias administratadas atualmente, os pacientes transplantados têm apresentado uma imunossupressão sistêmica e uma série de outros diversos efeitos colaterais (33, 144-146). Por isso, tem sido sugerido que as DCs tolerizadas podem servir uma terapia alternativa e inovadora, sempre procurando uma maior sobrevida do enxerto a longo prazo e o mínimo de alterações possíveis no sistema imune do receptor (26, 32, 147). Sugerimos que possivelmente células dendríticas tratadas com a TB-Hsp70 sozinha, ou em conjunto com outros compostos, possa servir como uma terapia para a inibição da rejeição aguda em transplantes.

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