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O PAPEL DA PROTEÍNA HSPBP1 EM TUMORES E A RESPOSTA IMUNE ESPECÍFICA A ANTÍGENOS NÃO TUMORAIS

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Tese apresentada como requisito na obtenção do grau de Doutor pelo Programa de Pós-Graduação em Biologia Molecular e Celular da Faculdade de Biociências da Pontifícia Universidade Católica do Rio Grande do Sul.

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“Há duas formas para viver a vida:

Uma é acreditar que não existe milagre.

A outra é acreditar que todas as coisas são um milagre”.

Albert Einstein

RESUMO

O câncer é uma das maiores causa de morte no mundo e o desenvolvimento de novas abordagens terapêuticas faz-se necessário. No presente trabalho estudamos dois pontos importantes relacionados com a interação entre e o câncer o sistema imune que podem fornecer dados fundamentais para imunoterapia anti-tumoral. Estudamos primeiramente a proteína de choque de calor HspBP1, descrita como uma co-chaperona da Hsp70. Vimos que a expressão de HspBP1 está elevada em amostra de tumores de pacientes com câncer de mama comparando com o tecido normal adjacente. Além disso, verificamos que níveis reduzidos de HspBP1 nestes tumores está relacionado com pior prognóstico da doença. Em seguida, demonstramos que aumentando a expressão de HspBP1 em melanoma murino regredimos o crescimento tumoral *in vivo* e este mecanismo possivelmente está relacionado com Hsp70 e a resposta imune adaptativa. Concluindo, estes dados sugerem que HspBP1 pode ser um alvo promissor para uma nova terapia antitumoral. Além disso, avaliamos a imunossupressão da resposta imune pelo tumor, o que pode ser um obstáculo para a eficiência das imunoterapias e para bom manejo do paciente com câncer. Observamos que a resposta imune $CD4^+$ T específica *iv vivo* frente ao antígeno não expresso pelo tumor esta preservada no micorambiente tumoral. Avaliamos o *priming* e *recall* das células de memória e não observados diferença significativa entre os camundongos que possui tumor comparando com o controle. Acreditamos que os dados apresentados no presente trabalho fornecem informações importantes que podem contribuir para o melhor entendimento das interações entre câncer e o sistema imune.

Palavras chaves: HspBP1, Hsp70, câncer, Células T CD4.

ABSTRACT

Cancer is one of the main causes of death in worldwide and development of new strategies of therapy is urgent. In the present study we evaluated two important issues related to the interactions between tumors and immune response that can provide essential information to improve anticancer therapy. Initially, we study HspBP1, an Hsp70 co-chaperone. We found that the expression of HspBP1 is elevated in human breast cancer comparing to normal adjacent tissue. Also, we demonstrated that lower levels of HspBP1 in the tumor samples correlated with poor patient's outcome. Followingly, we found that over-expression of HspBP1 in melanoma murine can impair tumor growth *in vivo* and this function is probably associated with Hsp70 and adaptative immune responses. Collectively, these results suggested HspBP1 as a new target for tumor therapy. In addition, we evaluated if the immunosuppressive microenvironment created by the tumor presence can influence in $CD4^+$ T cells immune responses. We found that in regard to priming and memory recall, there is no alteration in tumor bearing mice comparing to control mice. We believe that the data presented in this study contribute to a better understanding of complex interactions between immune system and tumor.

Key word: HspBP1, Hsp70, Cancer, $CD4^+$ T cells.

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CAPÍTULO 1

1.1 Introdução

A despeito das múltiplas abordagens para terapia e prevenção, o câncer continua sendo uma das maiores causa de morte no mundo. O processo de formação do tumor é complexo e envolve múltiplos fatores que facilitam as mutações nas células, o que determina a expressão de oncogenes e a supressão de genes que previnem o desenvolvimento de tumor. As diferentes mutações conferem diversas vantagens seletivas para as células tumorais, permitindo seu crescimento (Hanahan e Weinberg, 2000). A maioria das terapias antitumorais não-cirúrgicas disponíveis tem como alvo as células que estão proliferando rapidamente, também afetando as células normais, resultando em efeitos colaterais que limitam o tratamento, consequentemente novas estratégias de terapia são necessárias. Atualmente está bem definido que o sistema imune pode reconhecer as células tumorais, e este fenômeno foi recentemente denominado de imunoedição (Dunn, Koebel *et al.*, 2006; Bui e Schreiber, 2007). Conseqüentemente, várias novas estratégias de terapia estão sendo estudadas objetivando uma resposta imune anti-tumoral eficiente a fim de eliminar o tumor. Diferentes tipos de vacinas vêm sendo testadas, incluindo a utilização de células tumorais inteiras (Eaton, Perry *et al.*, 2002), de antígenos tumorais na forma de peptídeos (Rosenberg, 1998), proteína ou inseridos em vetores (Rosenberg, 1999) e células dendríticas pulsadas com antígenos tumorais (Engleman, 2003). Outra estratégia de vacinação é o uso

de proteínas de choque de calor (*heat shock proteins* (Hsp)) purificadas das células tumorais dos pacientes, objetivando a apresentação de antígenos tumorais e geração de resposta imune específica, vez que foi demonstrado que estas proteínas podem carregam antígenos tumorais (Srivastava, 2005). Além disso, imunoterapias como a transferência adotiva de células T ativadas em pacientes com câncer (Dudley, Wunderlich *et al.*, 2002) e uso de anticorpos humanizados específicos contra antígenos tumorais vêm sendo utilizadas (Bargou, Leo *et al.*, 2008). Estas diferentes estratégias têm demonstrado que é possível gerar resposta imune antitumoral nos pacientes, entretanto a eficácia destas novas terapias ainda não esta atingindo os parâmetros esperados. Diversos fatores podem ser responsáveis pela baixa eficiência, mas principalmente o que ocorre é uma tolerização da resposta imune devido à presença do tumor (Rabinovich, Gabrilovich *et al.*, 2007). Estes resultados sugerem que a imunoterapia contra tumores pode ser muito promissora, entretanto um melhor entendimento de todo o processo envolvido na resposta imune tumoral, as proteínas relacionadas e a possível tolerização, é necessário a fim de aprimorar as estratégias primeiramente propostas e fornecer informações importantes para o desenvolvimento de novas terapias. Portanto, no presente trabalho estudamos dois pontos importantes que podem fornecer dados fundamentais para terapia anti-tumoral. Estudamos primeiramente a HspBP1, uma proteína de choque de calor descrita como uma co-chaperona da Hsp70, a qual pode ser um alvo promissor para uma nova terapia antitumoral e, além disso, avaliamos a imunossupressão da resposta imune pelo tumor, o que

pode ser um obstáculo para a eficiência das imunoterapias e para bom manejo do paciente com câncer.

1.1.1 As proteínas de choque térmico em tumores

As proteínas de choque térmico ou estresse (Hsps) foram primeiramente descritas em 1962 (Ritossa, 1962) e são um grupo de proteínas altamente conservadas, 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 5 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 (Jolly e Morimoto, 2000). 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 à rotas de degradação e auxiliar na dissolução de complexos protéicos (Jaattela, 1999b).

1.1.1.1 A Hsp70 e resposta imune antitumoral

A família Hsp70 é a mais conservada e a melhor estudada entre as outras famílias (Garrido, Gurbuxani *et al.*, 2001; Daugaard, Jaattela *et al.*, 2005). A expressão da Hsp70 é regulada pelo fator de transcrição HSF1 (*heat*

shock factor 1), e 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 anti-tumorais (Morimoto, 1998). A Hsp70 utiliza dois domínios funcionais para realizar sua atividade de chaperona, o domínio C-terminal de 18kDa que se liga ao substrato protéico e o domínio N-terminal de 44kDa que possui atividade ATPásica e controla a abertura e o fechamento do domínio C-terminal (Flaherty, Deluca-Flaherty *et al.*, 1990). 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 (Hartl, 1996). 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. Um exemplo de co-chaperona é Hsp40, que estimula a atividade ATPásica da Hsp70 acelerando a hidrólise do ATP, resultando no aumento da forma da ligada ao ADP em complexo estável com o substrato (Freeman, Myers *et al.*, 1995; Minami, Hohfeld *et al.*, 1996). Outras co-chaperonas, como GrpE, presente nos procarióticos e Bag-1 presente nos eucarióticos, aceleram a troca de nucleotídeo no domínio ATPásico da Hsp70, catalisando a liberação do ADP e re-ligação do ATP, com subseqüente liberação do substrato peptídico (Hohfeld e Jentsch, 1997). Outros fatores de troca de nucleotídeo de eucarióticos são Fes1p e Sls1p (Kabani, Beckerich *et al.*, 2000; Kabani, Beckerich *et al.*, 2002). A proteína HspBP1 (*Hsp70 binding protein 1*), é uma co-chaprerona, presente no citoplasma, que possui atividade de fator de troca de nucleotídeo por um mecanismo de ação diferente de Grpe ou Bag-1 (Shomura, Dragovic *et al.*,

2005). Possui 25% de identidade e 38% de similaridade com Fes1p (Kabani, Mclellan *et al.*, 2002). A HspBP1 é necessária para eficiente dobramento das proteínas no citosol, podendo inibir a Hsp70 quando presente em altas concentrações (Raynes e Guerriero, 1998).

Níveis elevados de expressão de Hsp70 têm sido amplamente descritos em câncer de mama, cólon de útero, renal, endometrial, osteosarcoma, bem como em várias leucemias (Jaattela, 1999a; Helmbrecht, Zeise *et al.*, 2000; Jolly e Morimoto, 2000; Torronteguy, Frasson *et al.*, 2006). A vantagem seletiva de sobrevivência que esta proteína fornece, contribui para o processo de formação do tumor (Calderwood, Khaleque *et al.*, 2006). A Hsp70 citoplasmática é uma proteína anti-apoptótica, atuando em diversos pontos, pode bloquear os estágios pré-mitocondriais, mitocondriais e pós-mitocondriais da cascada apoptótica (Jaattela, Wissing *et al.*, 1992; Mosser, Caron *et al.*, 1997). Em modelos murinos, altas concentrações da Hsp70 aumentam o potencial oncogênico de linhagem celulares cancerosas (Jaattela, 1995), do mesmo modo que, regulação negativa de Hsp70 diminui a oncogênese (Nylandsted, Rohde *et al.*, 2000; Gurbuxani, Bruey *et al.*, 2001). Entretanto, quando a Hsp70 encontra-se fora da célula tumor ou na sua superfície pode apresentar um efeito modulador da resposta imune antitumoral. A Hsp70 facilita a aquisição de antígenos tumorais pelas células dendríticas, facilitando a apresentação e processamento de antígenos pela via de MHCI, levando a indução de resposta de células T CD8 tumor específica (Arnold-Schild, Hanau *et al.*, 1999). Alguns prováveis receptores para complexos Hsp70 peptídeos

tumorais nas células tumorais vem sendo indicados como exemplo LOX e SR-A (Delneste, Magistrelli *et al.*, 2002). Além disso, foi demonstrado que Hsp70 quando presente na membrana das células tumorais pode ativar células NK (Gastpar, Gehrmann *et al.*, 2005). Tivemos a oportunidade de revisar o papel da Hsp70 em tumores e sua complexa interação com o sistema imune e publicar um capítulo de livro, demonstrado no Capítulo 2.

1.1.1.2 O papel da HspBP1 em tumores

A expressão da co-chaperona HspBP1 está elevada em células tumorais murinas, semelhante ao que ocorre com a Hsp70 (Raynes, Graner *et al.*, 2003). A HspBP1, ao interagir com a Hsp70 citosólica, inibe sua atividade ATPásica e de redobramento, alterando a conformação estrutural do domínio ATPásico (Raynes e Guerriero, 1998; Mclellan, Raynes *et al.*, 2003). Esta co-chaperona foi primeiramente descrita após triagem em uma biblioteca de cDNA humano, buscando proteínas que ligavam-se ao domínio ATPásico da Hsp70. A seqüência de aminoácidos derivada do cDNA hibridizado não possuía homologia com outras seqüências conhecidas (Raynes e Guerriero, 1998). A proteína recombinante produzida com uma cauda de histidinas a partir desta nova seqüência de cDNA ligou-se ao domínio ATPásico da Hsp70, sendo o complexo destas purificado por afinidade em uma matriz resinosa ativada com Ni^{2+}, evidenciando assim a interação entre a HspBP1 e a Hsp70 (Raynes e Guerriero, 1998). Entretanto estas duas proteínas aparentemente não possuem um mecanismo de regulação de expressão associado (Gottwald, Herschbach *et al.*, 2006). Foi determinado em estudos *in vitro* que para ocorrer 50% de

inibição da Hsp70 pela HspBP1 a proporção molar entre estas duas proteínas deve ser de 1 para 4, respectivamente (Raynes, Graner *et al.*, 2003).

Uma vez que a HspBP1 está elevada em tumores murinos semelhante ao que ocorre com a Hsp70, decidimos avaliar se esta proteína esta também expressa em grandes quantidades em amostras de tumores humanos. Além disso, a Hsp70 tem um papel importante na tumorigenese (Jaattela, 1995) e muitas vezes sua expressão esta associada com prognóstico dos pacientes com câncer (Ciocca, Clark *et al.*, 1993; Thanner, Sutterlin *et al.*, 2003; Torronteguy, Frasson *et al.*, 2006), deste modo avaliamos se a expressão de HspBP1 também pode estar relacionada com prognóstico dos pacientes. Nossos resultados demonstraram que níveis reduzidos de HspBP1 estão associados com pior prognóstico dos pacientes com câncer de mama (Artigo científico demonstrado no Capítulo 3). Conseqüentemente, baseando-se nestes dados levantamos a hipótese níveis elevados HspBP1 pode estar relacionados com melhor prognóstico e redução do tumor, conferindo a esta proteína uma atividade anti-tumoral. Para testar esta hipótese utilizamos um modelo de melanoma murino no qual podemos controlar a expressão de HspBP1. Vimos que o crescimento tumoral diminui quando a expressão de HspBP1 está elevada. Observamos que este fenômeno pode estar relacionado à interação com o sistema imune, uma vez que o efeito da HspBP1 ocorre apenas *in vivo*, e está diminuído em camundongos RAG -/- (Artigo científico demonstrado no capítulo 4). Entretanto, uma terapia anti-tumoral baseada na

resposta imune pode ser afetada pelas diferentes propriedades imunossupressoras do tumor reduzindo com isso sua eficiência.

1.1.2 A atividade immunossupressora dos tumores

O tumor dispõe de diversos mecanismos que podem acarretar na supressão da resposta imune anti-tumoral. O processo de tolerização no microambiente tumoral é complexo e envolve diferentes tipos celulares e fatores secretados por estas células. O tumor pode secretar proteínas com atividade imunossupressoras, tais como: VEGF (Gabrilovich, Chen *et al.*, 1996), IL-10 (Roncarolo, Levings *et al.*, 2001; Kawamura, Bahar *et al.*, 2002), TGF-β (Rodeck, Bossler *et al.*, 1994; Conrad, Ernst *et al.*, 1999), M-CSF (Menetrier-Caux, Montmain *et al.*, 1998; Duluc, Delneste *et al.*, 2007) e Gangliosideos (Mckallip, Li *et al.*, 1999; Shurin, Shurin *et al.*, 2001). A presença destas proteínas pode alterar a maturação das células dendríticas (DCs) presentes no local do tumor o que leva a diminuição da resposta anti-tumoral. Além disso, DCs com fenótipo imaturo podem recrutar células T regulatórias que tem um papel fundamental na supressão das células T efetoras e estão presentes em níveis elevados no sangue periférico de pacientes com diferentes tipos de tumores (Dhodapkar, Steinman *et al.*, 2001; Ghiringhelli, Puig *et al.*, 2005). Outras células com atividades imunossupressoras podem estar presente no ambiente tumoral, como células mielóides supressoras (MDSC) (Almand, Clark *et al.*, 2001). O tumor também é capaz de expressar moléculas que podem estar relacionada com a supressão da resposta imune, como IDO

(Brody, Costantino *et al.*, 2009), PDL-1 (Blank e Mackensen, 2007), HLAG (Tripathi e Agrawal, 2006), Galectin-1 (Liu e Rabinovich, 2005).

Através destes diferentes mecanismos está bem definido que o tumor é capaz de suprimir a resposta específica a antígenos tumorais, e isto foi demonstrado tanto para resposta células T $CD4^+$ (Farzad, Mcbride *et al.*, 1997), como para células T $CD8^+$ (Palmowski, Salio *et al.*, 2002). Entretanto, existem poucos estudos avaliando a resposta imune a antígenos não expressos pelo tumor no microambiente tumoral (Radoja, Rao *et al.*, 2000; Tassi, Gavazzi *et al.*, 2008). Tivemos a oportunidade de revisar a literatura relacionada com os diferentes mecanismos de imunossupressão utilizados pelos tumores e sua influência na resposta tumor específica e não tumor específica (Artigo científico demonstrado no Capítulo 5). Até o presente momento não existem evidências demonstrado que o tumor é capaz de suprir a resposta não relacionada com antígenos tumorais. Além disso, os poucos estudos que testaram esta hipótese utilizaram para avaliar a resposta imune re-estimulação *in vitro*. Baseando nestes fatos, resolvemos avaliar a resposta não especifica ao tumor dentro do ambiente tumoral, *in vivo*, sem utilizar nenhum tipo de re-estimulo *in vitro.* (Artigo científico demonstrado no Capítulo 6)

1.2 Objetivos

1.2.1 Objetivo geral

Avaliar as interações do sistema imune com câncer, focando principalmente no papel da HspBP1 em tumores e na imunossupressão causada pelo microambiente tumoral.

1.2.2 Objetivos específicos

1.2.2.1 Revisar a literatura relacionada com a interação da Hsp70 e câncer.

1.2.2.2 Avaliar a expressão de HspBP1 em amostras de tumores de pacientes com câncer de mama comparando com tecido normal adjacente ao tumor e associar a expressão desta proteína com prognóstico dos pacientes com um acompanhamento clinico de 7 anos,

1.2.2.3 Avaliar o potencial antitumoral da proteína HspBP1 em modelo de melanoma murino, avaliar se a superexpressão desta proteína afeta o crescimento do tumor e se esta função esta relacionada com a Hsp70 e com o sistema imune,

1.2.2.4 Revisar a literatura relacionada com os mecanismos de imunossupressão da resposta antitumoral e também a sua possível influência na resposta a antígenos não-tumorais.

1.2.2.5 Avaliar a capacidade de melanoma murino de suprimir a resposta de células T $CD4^+$ naïve e de memória específicas para um antígeno não tumoral.

CAPÍTULO 2

Capítulo de livro –

"Heat Shock proteins in cancer": Hsp70 in tumors: Friend or Foe?

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Org. Stuart Calderwood, Daniel Ciocca, Michael Sherman.

CHAPTER 10

Hsp70 IN TUMORS: FRIEND OR FOE?

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Abstract: Hsp70, the most highly conserved and abundantly induced of the stress proteins, appears to play dual and opposing roles in cancer. On the one hand, Hsp70 promotes growth and survival of tumor cells by engaging misfolded or aggregated proteins and proteins involved in cell proliferation. As such, it endows tumor cells with stress resistance. However, Hsp70 can also promote tumor immunity by stimulating innate immune mechanisms and enhancing cross-presentation of tumor antigens to lymphocytes. In this chapter, we review these opposing functions of HSP70 in the context of potential strategies for its use as a tool in cancer biology and therapy

Keywords: HSP70, tumors, immunotherapy, vaccination, apoptosis, necrosis

HHsp70 IN TUMORS70 AS TUMOR PROTECTOR: FOE?

Tumors become established when cells are able to multiply in an abnormal, uncontrolled fashion due to decisive genetic modifications. In order to grow, the resulting cell mass must successfully compete with normal surrounding tissues for nutrients and oxygen. The accumulation of genetic modifications results in the expression of damaged proteins, which aggregate and interfere with established intracellular signaling pathways, thus posing an intrinsic threat to cell survival. Moreover, damaged self-constituents are potential targets for the immune system, which can use a variety of extrinsic cytotoxic mechanisms to eliminate the tumor. By definition, tumor cells growing *in vivo* are under stress.

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Hsp70 in the Stress Response

All cells respond to stress in a much conserved fashion, inducing or sometimes enhancing the synthesis of heat shock proteins (hsps), named following the original observation that they are induced at elevated temperatures in Drosophila (Ritossa 1963). Most of these proteins have constitutively expressed counterparts that function in non-stressed cells as molecular chaperones, organizing the folding of nascent peptides and translocating molecules across organelle membranes. In response to stress, however, these proteins are expressed more abundantly, and new isoforms appear (Parsell and Lindquist, 1994). The production of inducible stress proteins is regulated by heat shock transcription factors (HSFs), particularly HSF1, which is essential for their transcription in mammalian cells. Upon heat shock, HSF1 is phosphorylated by protein kinase CK2 and translocates to the nucleus, binding to heat shock elements (HSEs), and initiating Hsp gene transcription (Soncin et al., 2003).

Following stress, hsps rescue cells from death by refolding aggregated proteins and binding the proteins mediators that relay intracellular damage signals. Later verified in cells under stresses other than heat, such as hypoxia and reperfusion or ionizing radiation, the heat shock response was renamed the stress response, and hsps are sometimes referred to as stress proteins. Stress proteins are chaperones that interact with components of the survival and apoptotic pathways, preventing the activation of both caspase-mediated and caspase-independent cell death pathways (Mosser and Morimoto, 2004).

Hsps are grouped in families according to their molecular size. Each gene family has constitutive and inducible members, the most heavily studied of which is the hsp70 family. At least eight different members of the hsp70 family have been identified that play a central role in the folding and intracellular translocation of peptides. They differ in pattern of expression and cellular localization (Table 1). This ubiquitous distribution within a given cell almost certainly endows the Hsp70 family with the ability to operate at different points in multiple apoptotic pathways (Daugaard, Jaattela and Rohde, 2005).

Table 1. The Hsp70 family (adapted from Daugaard and Jaatela, 2005)

Protein	Synonyms	Locus	Cellular localization
Hsp70	Hsp72, Hsp70i,Hsp70-1(A),Hsp70-1(B)*	HSPA1AHSPA1B	Cytosol, nucleus, membranes
Hsc70	Hsp73	HSPA8	Cytosol, nucleus, lysosomes
Bip	Grp78	HSPA5	Endoplasmic reticulum
MtHsp70	Grp75	HSPA9	Mitochondria
Hsp70-6	Hsp70B	HSPA6	Cytosol, nucleus
Hsp70t	Hsp70-Hom	HSPA1L	Cytosol
Hsp70-2	HSPA2	HSPA2	Cytosol, nucleus

*99% amino-acid identity with HSP70-1(A)

Studies on cultured cell lines provided the earliest clues that tumors might upregulate Hsp70 in response to the stress of growing *in vivo*. The heat shock response appeared to be diminished in different cultured tumor lines (Mathur, 1994). The inducibility of Hsp70 by heat or irradiation also varied among cell lines (Muramatsu 1995). Gorzowski et al. (1995) found that many cultured tumor lines failed to induce Hsp70 upon heat shock because the Hsp70 genes were silenced by methylation. CH1 lymphomas, while in culture, did not express Hsp70 upon heat shock, even though they possessed functionally intact HSF1 and hsp70 genes. However, upon transplantation, they synthesized abundant Hsp70 (Davidson, 1995). These observations suggested that while tumor cells growing *in vitro* do not necessarily require Hsp70, this stress protein can endow cells with a decisive survival advantage *in vivo*.

We have made similar observations. While studying the growth dynamics of the B16F10 melanoma, we investigated the expression of Hsp70 by B16F10 cells at different stages of *in vitro* and *in vivo* growth (Figure 1). These cells did not express detectable Hsp70 while growing *in vitro* (Figure 1A, 2). Upon heat shock,

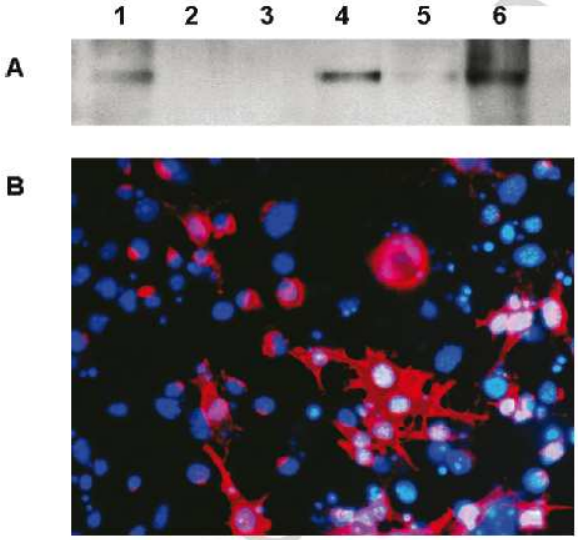

Figure 1. **In vitro and in vivo expression of Hsp70 by B16F10 cells.** A, Five micrograms of cell lysate of B16 F10 cells straight out of the culture dish, submitted for 2h to 39.9°C and 6h of recovery at 37°C. Alternatively, they were injected subcutaneously, in a C57BL/6 mouse, and after three or twenty days the mice were sacrificed and the tumor mass dissected. Samples were ran on an SDS-PAGE gel, transferred to a nitrocellulose membrane and incubated with a biotinylated anti-Hsp70 antibody, as presented: 1, B16F10 heat shocked; 2, B16F10 growing at 37°C; 3, normal skin, three days; 4, three day tumor mass; 5, normal skin, twenty days; 6, twenty day tumor mass. B, B16F10 cells growing on a coverslip placed inside the culture dish were treated as in (B), fixed in acetone, stained with Hoechst (blue) and biotin-anti-Hsp70/SA-Cy3 and visualized using a Zeiss immunofluorecence microscope (400X)

however, induction of Hsp70 was observed (Figure 1A, 1). Immunofluorescence assays showed that only a fraction of the cells in culture expressed Hsp70 in response to high temperature (Figure 1B). Within 3 days following transplantation to mice, however, production of Hsp70 was observed in the absence of any deliberate stress (Figure 1A, 3), and this persisted at least until day 20, both at the transplantation site and in lymph node metastases (Figure 1A, 6). Our observations suggest that upregulation of Hsp70 is necessary for *in vivo*, but not *in vitro*, tumor growth. Because it is likely that tumor cells in culture still produce considerable quantities of misfolded proteins, it is possible that Hsp70 is upregulated by tumor cells *in vivo* specifically to cope with major challenges associated with competition for nutrients, oxygen, and the immune system.

Protection from Apoptosis

Resistance to apoptosis is obviously a key advantage in tumorigenesis. Apoptosis can be initiated by extrinsic (receptor mediated) or intrinsic (mitochondria mediated) pathways, leading to the activation of a set of proteins known as caspases. Their activation is dependent on the oligomerization and association of these proteins in complexes, called apoptosomes. Hsps, and especially Hsp70, have been shown to bind many of the protein constituents of these complexes, and likely catalyze the conformational changes necessary for these proteins to assemble into apoptosomes (See: *Brunet et al*, Chapter 11, this volume). Different studies have shown that expression of Hsp70 by tumors is associated with protection from apoptosis by both major caspase-dependent pathways. Transfection of TNF-alpha-sensitive WEHI-S tumor cells with Hsp70 protects against apoptosis upstream of the mitochondria, after ligation of the TNF receptor (Jaattela et al., 1992). Transfection with Hsp70 also protected CCRF-CEM cells from apoptosis after Fas ligation, also acting upstream of the mitochondria, inhibiting cytochrome c release (Clemons et al., 2005). Both Saleh and collaborators (Saleh et al., 2000) and Beere and collaborators (Beere et al., 2000) showed that Hsp70 can bind to Apaf-1 and negatively regulate the recruitment of caspase 9 to the apoptosome.

Apoptosis can also occur through caspase-independent pathways. The apoptosis inducing factor (AIF) is a caspase independent death inducing factor that is released from mitochondria, like cytochrome c. In a cell-free system, AIF can bind nuclei and induce chromatin condensation and DNA loss. Hsp70 can antagonize AIF both in a cell free system, in concentrations similar to what is observed in the mitochondria intermembrane space (Ravagnan et al., 2001). Cells transiently transfected with AIF undergo apoptosis upon AIF overexpression, but this effect was inhibited by co-transfection with Hsp70, rescuing the AIF transfected cells from death. Hsp70 has also been shown to protect tumor cells from death induced from lysosomal degranulation, a caspase independent mechanism (Gyrd-Hansen, Nylandsted and Jaattela, 2004). More recently, it was shown that overexpression of Hsp70 can induce the expression of other stress proteins by enhancing HSF1 activation (Seo et al., 2006). In normal cells, excess production of hsps inhibits HSF1, in a negative feedback

mechanism. In this study, cells transfected with Hsp70 enhanced synthesis of other Hsps, in a mechanism dependent of HSF1. Hsp70 bound to MKP1and enhanced its phosphorylation, inhibiting ERK1/2 phosphorylation, which resulted in a more stable and dephosphorylated form of HSF1. That resulted in a positive feedback loop, enhancing Hsp expression. This suggests that in cancer cells that express high levels of HSF1, Hsp70 can increase resistance to apoptosis by positively regulating its own expression in cells.

If Hsp70 protects cells from apoptosis, one might expect that tumor cells will express higher quantities of inducible Hsp70 *in vivo*, relative to normal tissue. Indeed, this has been observed by numerous independent investigators (Ciocca, 1993; Protti, 1994; Lee C.S. 1994; Volm, 1995; Sugerman, 1995; (Kaur and Ralhan, 1995); (Seo et al., 1996); (Torronteguy et al., 2006). In addition, Hsp70 production was negatively correlated with apoptosis in prostate cancer cells (Jones et al., 2004). High levels of Hsp70 in tumors growing in vivo protected tumor cells from treatment with anti-cancer drugs (Ciocca, 1992; Lee, 1992), especially cisplatin (Brozovic, Simaga and Osmak, 2001). This suggests that Hsp70 may be a useful predictive marker for resistance to chemotherapy, radiotherapy and hyperthermia (Ciocca and Calderwood, 2005).

Enhancement of Tumorigenesis

There are a number of reports indicating that overexpression of Hsp70 enhances tumorigenic potential (See: *Ciocca et al*, Chapter 2). In WEHI tumor cells transfected with Hsp70, tumorigenicity was positively correlated with Hsp70 expression levels. And while transfection with Hsp70 did not enhance their ability to form colonies in agar (a property of tumor cells) it rendered WEHI cells more resistant to killing by cytotoxic T cells and macrophages in vitro (Jaattela, 1995). It also enabled Rat-1 fibroblasts to lose contact inhibition and form the characteristic foci of oncogenically transformed cells in culture (Volloch and Sherman, 1999), suggesting that Hsp70 not only protects tumor cells from death, but also enhances their proliferation. Indeed, Hsp70 expression in MCF-7 breast cancer cells enhanced cell proliferation (Barnes et al., 2001), significantly shortening the mean doubling time by approximately two fold. This observation, together with a higher frequency of cells in second and third division metaphases at 42 and 69 hours, led the authors to conclude that Hsp70 appeared to be exerting its effect on MCF-7 cells primarily by shortening of the G_0/G_1 and S phases of the cell cycle. Transgenic mice expressing Hsp70 develop malignant T cell lymphomas (Seo,et al., 1996), supporting the idea that Hsp70 confers proliferation advantages upon cells in vivo. Finally, Hsp70 can bind to proteins that are known regulators of the cell-cycle, such as p53 (Hainaut and Milner, 1992) and c-myc (Henriksson et al., 1992), so it is conceivable that Hsp70 influences cell proliferation by assisting in the proper folding of these key regulatory molecules. Given its major and ubiquitous roles in protecting tumor cells from apoptosis and enhancing proliferation, HSP70 is an attractive target for tumor therapy.

Neutralizing Hsp70 as a Therapeutic Tool for Cancer

The ability of some tumors to develop thermotolerance is attributed to Hsp70 (Subjeck, Sciandra and Johnson, 1982). Therefore, strategies to inhibit Hsp70 might enhance the efficacy of hyperthermia as a tumor therapy. Thus far, several different approaches have been applied to inhibit Hsp70, all with promising results. Nishimura et al. (Nishimura et al., 2000) found that stable transfection of a P19 carcinoma cell line with an antisense Hsp70 enhanced heat induced cell death. Inhibition of Hsp70 by antisense DNA also lead to massive death of breast cancer cultured cell lines, while it did not affect survival of non-tumorigenic breast epithelial cells or fibroblasts (Nylandsted et al., 2000). Adenovirus-encoded antisense Hsp70 erradicated human tumor xenografts in BALB/c nude mice (Nylandsted et al., 2002). Bcl-2 and Bcl-X_L, which protect tumor cells from different forms of cell death by inhibiting caspase activation, failed to rescue cells from death induced by Hsp70 depletion. However, many tumors can survive activation of caspases. Targeting the interaction between Hsp70 and the flavoprotein apoptosis inducing factor (AIF), involved in caspase-independent cell death, also sensitizes tumor cells to apoptosis induced by different stress stimuli (Schmitt et al., 2006). Consequently, a promising strategy might be to associate Hsp70 inhibition with classical anti-tumor treatments that activate caspase-dependent or –independent pathways of cell death. More recently, the inhibition of HSF-1 with RNAi (Rossi et al., 2006) lead to massive death in response to chemotherapy combined with hyperthermia. Altogether, these results indicate that Hsp70 plays a major role in tumor survival, and that neutralizing Hsp70 expression in tumors may constitute an important tool in cancer treatment.

Hsp70 AS AN IMMUNE ACTIVATOR: FRIEND?

Hsp70 is composed of at least two different structural domains: the 44 kDa N-terminal contains a nucleotide binding site and possesses ATPase activity (Chappell et al., 1987). The 30 kDa C-terminal domain can bind unfolded polypeptides (Stevens et al., 2003). It is divided into an 18 kDa N-terminal subdomain (the peptide binding site) and a 10kDa C-terminal subdomain, which constitutes a lid over the peptide binding site. The full-length structure of Hsp70 is still unknown, however different studies provided evidence that these two domains work cooperatively. While ADP binding by the N-terminal domain correlates with peptide binding by the C-terminal domain, ATP binding favors release of the peptide by the C-terminal domain (McCarty et al., 1995). Thus, in vivo, ADP/ATP concentrations are likely to regulate chaperoning function of Hsp70.

The discovery that Hsp70 extracted from tumors could elicit specific anti-tumor immune responses by chaperoning tumor peptides into antigen presentation routes inaugurated a new and unexpected role for this protein (See *Gong & Calderwood*, Chapter 18, *Tamura et al*, Chapter 19). The pioneering studies of Srivastava and co-workers demonstrated that Hsps *per se* were not immunogenic, but when

purified from tumors by ADP chromatography, consequently in a form that was still associated with tumor peptides, they could be used to immunize against the original tumor (Srivastava, 1993). These studies suggested that although Hsp70 correlated with increased tumorigenesis, enhancing tumor survival, it also correlated with tumor immunogenicity, activating anti-tumor immune responses. While the role of Hsp70 in adaptive immunity, specifically in antigen presentation, is well documented, the idea that Hsp70 can also play a role in innate immunity is disputed. Some investigators have dismissed such a role due to technical flaws in experimental procedures. However, not all studies are easily dismissed and warrant a closer look. We discuss the evidence presented by these studies below.

Hsp70 and the Adaptive Response

Antigen presentation

Menoret et al. (1995) examined the immunogenicity of different clones derived from the same parental rat colon carcinoma. Variants that expressed high levels of Hsp70, but not other proteins such as Hsc70, MHC class I or II, were more readily rejected in vivo in a manner that was dependent upon alpha-beta+ T cells. Their results suggested that production of Hsp70 by tumor cells somehow facilitated the activation of an anti-tumor immune response. Support for their findings was provided by Binder et al. (2001), who demonstrated that cytosolic Hsp70 played a role in enhancing peptide presentation by MHC I molecules. By loading cell lines with either Hsp70-peptide complexes or free peptides, using liposomes to target them directly to the cytosol, they showed that the former were significantly more likely to be presented by class I molecules. Inhibition of Hsp70 by deoxyspergualin inhibited peptide presentation, and such inhibition could be reversed by supplying additional Hsp70. This study could provide an explanation for in vivo observations: tumor cells that expressed high levels of endogenous Hsp70 would be prone to generating more MHC class I-peptide complexes on their membranes, rendering the tumor more susceptible to recognition and lysis by CD8+ T cells. It also suggested that if exogenous Hsp70 could reach the cytosol of antigen presenting cells (APC), it could enhance antigen presentation.

The clearance of tumors by the immune system is performed mainly by CD8+T cells, through the activation of cytotoxic mechanisms upon recognition of specific peptides presented by MHC class I molecules. Studies using Hsp70-peptide complexes purified from tumors (Udono and Srivastava, 1993) or reconstituted in vitro (Blachere et al., 1993) to immunize mice demonstrated it was possible to induce CD8+ T cells that specifically recognized the tumor and mediated tumor regression. Such results were somewhat surprising because exogenous antigens are generally presented by MHC class II molecules. However, they can sometimes appear in MHC class I molecules. This phenomenon was previously described and named cross-presentation by Bevan and co-workers (Bevan, 1976a, 1976b) (Bevan, 2006), while the generation of CD8+ T cell responses against antigens that reach APC via an exogenous route is referred to as cross-priming.

Additional studies provided evidence that chaperoning of peptides by Hsp70 can cross-prime T cells. Noessner and colleagues demonstrated that Hsp70 peptide complexes purified from a tyrosinase positive, but not a tyrosinase negative, human melanoma could deliver tyrosinase peptide to MHC class I molecules in dendritic cells (Noessner et al., 2002). Activation of a tyrosinase-specific CD8+ T cell clone was competitively inhibited by incubation with HSP70-peptide complexes that were purified from the tyrosinase negative melanoma. Hsp70-peptide complexes purified from tumors were shown to be internalized by bone marrow-derived DC and presented in MHC class I molecules (Ueda et al., 2004), and immunization with these complexes induced tumor specific CD8+ T cell clones. Massa and collaborators transfected tumor cell lines with secretable Hsp70, which resulted in increased tumor immunogenicity as well as generation of anti-tumor cytotoxic T cell lines (CTLs) (Massa et al., 2004). In addition to Hsp70, other hsps in tumor lysates, were necessary for cross priming against tumor antigens (Binder and Srivastava, 2005); when all hsps were depleted from a tumor lysate, cross-priming did not occur *in vivo*. Given the strong immunogenicity of Hsp-peptide complexes (very few picograms of Hsp70-peptide are sufficient to immunize mice), several groups explored the possibility that APC might possess specific receptors for stress proteins.

Receptors

While a saturable receptor-mediated mechanism seemed to apply to Hsc70 (Arnold-Schild et al., 1999) and gp96 (Wassenberg, Reed and Nicchitta, 2000), other groups found evidence for a receptor-independent pathway for Hsp70-peptide endocytosis (Fujihara and Nadler, 1999), confirming earlier observations of Hightower and Guidon (Hightower and Guidon, 1989) that Hsp70 could translocate across plasma membranes. Castellino and coworkers observed that Hsp70-peptide complexes could be transported across an endosomal membrane (Castellino et al., 2000). Their study demonstrated, however, that Hsp70-Ova complexes were endocytosed by dendritic cells in a receptor-mediated fashion, upon which it was subjected to least two distinct intracellular routes of degradation, one cytosolic and the other endocytic. The route was dependent on the sequence of the peptide, and culminated in class I presentation of the chaperoned peptide. Based on their data on saturability of Hsp70 surface binding, they predicted more that one receptor for Hsp70-peptide complexes.

Interestingly, different studies have implicated different receptors for Hsp70. The α2 macroglobulin receptor (CD91) was the first hsp receptor identified, initially for gp96 (Binder, Han and Srivastava, 2000), and later as a common receptor for Hsp70, gp96 and calreticulin in peritoneal exudate cells as well as murine bone marrow derived DC (Basu et al., 2001). In that study, following Hsp binding to CD91, Hsp-chaperoned peptides were re-presented via a proteasome-dependent mechanism, in a manner that was completely inhibited by α2 macroglobulin and anti-CD91, leading the authors to conclude that CD91 was the only receptor for these Hsps. However, CD14 (Asea et al., 2000) and TLR4 (Asea et al., 2002)

were also reported to be receptors for Hsp70. Delneste et al. observed that Hsp70 bound to the scavenger receptor LOX-1, a receptor for oxidized LDL, on human immature dendritic cells that neither expressed CD91 nor CD14 (Delneste et al., 2002), and such binding lead to internalization of Hsp70-peptide complexes and cross-presentation. Theriault and collaborators (Theriault et al., 2005) investigated binding of Hsp70 to different receptors such as CD91, TLR2 and TLR4, as well as to LOX-1. They demonstrated that only LOX-1 expression on transfected cell lines lead to significant binding of Hsp70. Surprisingly, Hsp70 was observed to bind also to non-APC cell lines, of epithelial/endothelial origin. Finally, CD40 was also reported to be an endocytic receptor for Hsp70 (Becker, Hartl and Wieland, 2002).

Other reports for multiple receptors for other mammalian hsps, as well as for their prokaryotic homologues (for a review, see (Binder, Vatner and Srivastava, 2004)) continue to puzzle investigators. The inconsistencies among the various reports on surface receptors for Hsp70 are still not completely understood. A common misconception is that, because Hsp70 is highly evolutionarily conserved, it will play the same roles in prokaryotes and eukaryotes, and consequently Hsp70 homologues should bind to the same molecules. One must consider that, if bacterial and mammalian Hsp70 present approximately 50% similarity, they also present 50% divergence. Another key issue is the uncertainty generated by the observations that one given receptor can bind different Hsps, or that different hsps can bind to the same receptor. If on the one hand these differences might be related to purification procedures (see below), on the other hand they might have structural causes. For example, if a given preparation of Hsp70 contained oligomers, instead of mainly monomers, Hsp70 could bind efficiently to low affinity receptors, due to an avidity effect. Indeed, Hsp70 has been reported to dimerize and oligomerize (Nemoto et al., 2006; Yamada et al., 2003), but it is still not clear how binding to the peptide influences dimer/oligomer formation, and which physiological conditions would influence the oligomers or monomers to be the most common extracellular forms in vivo.

In any case, the strong immunogenicity of Hsp70 suggests that this cytosolic protein can reach the extracellular milieu, and that it is advantageous for the cell (and consequently for the host) to specifically gather and internalize this protein. The ability to endocytose Hsp70 might have evolved due to issues that at least at first glance are unrelated to immunity. One example is that neurons can take up Hsp70 released by glial cells in response to stress, and that this correlates with stress protection for the neuron (Guzhova et al., 2001).

So how can we translate the results of the studies discussed above into the generation immune responses against a living tumor in vivo? Two main hypotheses guide most of the investigations on this subject: 1) that Hsp70 can somehow be released by viable tumor cells, binding to APCs; or, 2) that some tumor cells must die, releasing their intracellular contents including Hsp-peptide complexes that are taken up by APCs. There is some evidence for both scenarios. Although Hsp70 does not contain a leader sequence, a recent study shows that it can be released from viable prostate carcinoma cells through an active mechanism that involves lysosomal

translocation, as revealed by its sensitivity to lysosomal inhibitors (Mambula and Calderwood, 2006). Other studies point to a combination of Hsp70 release and cell death resulting on an important event for the generation of an anti-tumor response: the activation of innate immunity mechanisms.

Hsp70 and the Innate Response

Little is known about the generation of immune responses to tumors in vivo. Because tumor antigens are basically self proteins, central tolerance mechanisms are likely to ensure an extremely low precursor frequency of T cells able to recognize such antigens. Moreover, if tumor antigens are picked up by APC, the costimulatory signals delivered by the APC will determine the type of specific immune response generated. Antigen presentation in the presence of costimulatory molecules results in the activation of specific immune responses, while presentation in the absence of such molecules leads to tolerance (Steinman et al., 2005). Consequently, peptides chaperoned by Hsp should not be sufficient to elicit an anti-tumor immune response in vivo. On the contrary, signals that activate APCs to upregulate costimulatory molecules presumably must be provided, otherwise the presented tumor antigens will induce a state of self-tolerance in the acquired immune system and facilitate tumor growth.

Results of several studies have lead to the conclusion that Hsp70 alone could lead to upregulation of inflammatory mediators and/or costimulatory molecules after binding to receptors on APCs (Asea,et al., 2000) (Asea,et al., 2002; Basu et al., 2000) (Vabulas et al., 2002). As such, Hsp70 would could belong to a new class of natural endogenous adjuvants (Rock et al., 2005). Because many studies involving Hsps (as well as other candidate endogenous adjuvants) have demonstrated binding to TLRs 2 and 4, with activation of MyD88, the adaptor signaling molecule for several TLRs; and because most of these studies used recombinant molecules (Asea,et al., 2000) (Asea,et al., 2002) (Vabulas,et al., 2002), some critics have argued that this adjuvant effect might actually have been the result of endotoxin contamination, rather than an intrinsic biological property of Hsp70. For example, a study by Gao and Tsan (2003) demonstrated that when contaminant endotoxin was carefully removed, recombinant Hsp70 preparations lost their ability to induce TNF-alpha production by monocytes.

Consequently, conclusions from studies that used recombinant Hsp70 and verified binding to LPS receptors need to be re-evaluated. LPS is hard to remove, particularly from Hsp70, and if the preparations used were indeed contaminated by endotoxin, such studies may have actually provided some of the first evidence for a specific interaction between Hsp70 and LPS, when LPS binds its receptors. Recently, it was shown that Hsp70 can associate with LPS in membrane lipid rafts and participate in a TLR-LPS complex together with other LPS receptor molecules (Triantafilou and Triantafilou, 2003) (Triantafilou and Triantafilou, 2004). This association might have implications during the immune response to bacteria, since

Hsp70-LPS complexes appear to be targeted to the Golgi apparatus after endocytosis. However, during the generation of an anti-tumor immune response, LPS or other TLR ligands are unlikely to be present. If TLR ligation by endogenous ligands does occur, current evidence suggests it may play a role in tissue regeneration (Jiang et al., 2005) rather than CD8+ T cell priming.

So which signals activate an anti-tumor response, and how can Hsp70 participate? Because cell death is likely to be a highly frequent event in the millieu surrounding tumors growing in vivo, it has been hypothesized that the release of intracellular constituents by dead cells could provide activating signals for APC. It has been known for some time that dead cells are immunostimulatory (review in (Rock,et al., 2005)). Gallucci et al. found that a delayed type hypersensitivity reaction could be elicited upon immunization with ovalbumin mixed with killed cells (Gallucci, Lolkema and Matzinger, 1999). Shi and co-workers found that T cell responses were augmented even when dead cells were administered separately from the antigen, and that this effect was enhanced by about 10-fold when the cells were stressed or injured before dying (Shi and Rock, 2002). While Shi and Rock found uric acid to be one of the major stimulatory constituents released by dead cells (Shi, Evans and Rock, 2003), Basu et al. suggested that necrotic cell death leads to Hsp release in the supernatant (Basu, et al., 2000) and that Hsp70 purified from mouse cells induces bone marrow-derived DC maturation.

Using an engineered system that induced cell death in vivo, Vile and collaborators combined these ideas, and demonstrated that enhanced tumor immunogenicity was related to the release of Hsp70-peptide complexes from the dying tumor cells (Melcher et al., 1998). In a following study, Daniels and collaborators subcutaneously injected mice subcutaneously with a plasmid encoding Hsp70 together with DNA for the thymidine kinase of herpes simplex virus (HSVtk) (Daniels et al., 2004). Cells that express HSVtk DNA, when provided with gancyclovir (GCV) injection, transform it into a toxic form that is incorporated into the chromosomal DNA, leading to cell death. The treatment induced non-malignant melanocyte death, and induced immunity against transplanted B16 melanoma cells, while no effect was observed with plasmids encoding only HSVtk or Hsp70. Their results suggest a role for cell death signals together with Hsp70 in the activation of anti-tumor immune responses. At face value, these studies could simply indicate that cell death facilitates release of Hsp70. However, while individually proposed as endogenous adjuvants, death signals such as uric acid, DNA, nucleotides and Hsp70 could work together to generate anti-tumor responses in vivo.

Another interesting line of evidence suggests that Hsp70 can be expressed on the cell surface, serving as a target for NK cells (Botzler, Issels and Multhoff, 1996; Multhoff et al., 2001). Hsp70 has been observed on membranes of tumor cells but not normal cells (Hantschel et al., 2000). Specifically, Hsp70 on membranes of tumor cells correlated with a poor prognosis (Steiner et al., 2006), suggesting membrane expression of Hsp70 was characteristic of aggressive tumors. In principle, however, NK cells should more easily detect and lyse tumor cells with membrane Hsp70, providing signals that might activate APC. Lysis of tumor cells by NK cells

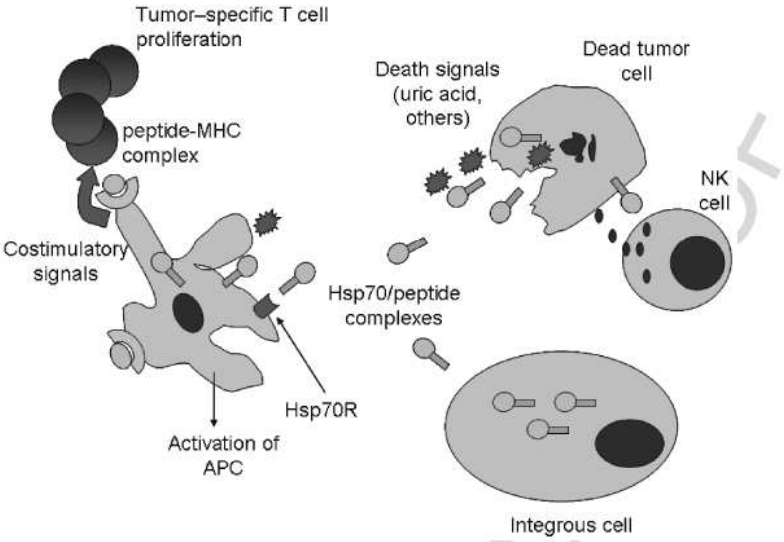

Figure 2. **Potentializing co-adjuvant effects of cell death and Hsp70.** Tumor cells that express high Hsp70 are likely to be protected against apoptosis, but cells with membrane expression of Hsp70 can be recognized and killed by NK cells. Upon tumor cell death, induced either by the intrinsic stresses of in vivo tumor growth including immune responses, Hsp70-peptide complexes are released to the extracellular medium. Also, integrous tumor cells can secrete Hsp70-peptide complexes. These complexes reach and are endocytosed by APC, through a receptor dependent or independent pathway. As a result, tumor peptides are directed to MHC molecules and presented on the cell surface. Other cell death signals like uric acid together with Hsp70 activate APC leading to upregulation of costimulatory signals, stimulating T cells in order to generate anti-tumor cell responses

might also enhance release of Hsp70-peptide complexes that could be taken up by APC, once again resulting in a favorable scenario for the generation of in vivo anti-tumor responses. A summary of the possibilities is schematically represented in Figure 2.

CONCLUSION AND PERSPECTIVES

Collectively, these observations suggest that the same Hsp70 that sometimes protects tumor growth may prove to be a useful tool for tumor elimination. Tumor cells growing in vivo face a dilemma: while the expression of high levels of Hsp70 may confer protection against apoptosis, it could also trigger tumor-specific immunity. A prediction is that primary tumors need to express sufficient Hsp70 to promote survival but low enough levels to escape immune surveillance. Advanced metastatic tumors, that successfully evade immunity, may be able to express higher Hsp70 levels, to cope with the stress associated with accumulated genetic mutations and increased division rate.

In our laboratory, we have observed that, in biopsies from breast cancer patients, expression of low levels of Hsp70 in primary tumors was significantly associated with development of metastasis, tumor relapse and/or death during a three-year follow-up (Torronteguy,et al., 2006). Hsp70 has been found to be highly expressed in poorly differentiated (more aggressive) endometrial carcinomas (Nanbu et al., 1996), in poorly differentiated chondromas (Trieb et al., 2000), as well as in more highly malignant carcinoma cells (Tang et al., 2005). However, other studies did not find correlations between Hsp70 expression and the state of differentiation of the tumor (review in (Ciocca and Calderwood, 2005), so the influence of Hsp70 on immunogenicity versus tumorigenicity of the tumor remains elusive.

Further work on the structural properties of Hsp70 is needed to clarify the interaction of extracellular Hsp70 with other cells. The interaction of Hsp70 with receptors is central for tumor therapy design. For instance, it is still not known which subpopulations of APC are critical for the generation of anti-tumor responses. If the Hsp70 receptor is not optimally expressed by these APC, Hsp70-peptide complexes will need to be targeted so that anti-tumor responses can be efficiently generated.

Ongoing clinical trials focus either on the inhibition of Hsp70 to sensitize tumor cells to apoptosis, or on the immunization of cancer patients with hsp70-peptide complexes purified from their own tumors (an autologous and costumized vaccine). It is tempting to speculate that we could combine these two approaches. Hsp70 inhibitors could induce tumor death, and those death signals combined with the clearance of dead cells by APC could lead to T cell priming against the tumor. Later, immunization with Hsp70-peptide complexes could enhance tumor antigen presentation, culminating in the expansion those T cell clones in vivo. Finally, besides designing a vaccine that will lead to the generation of an anti-tumor response, it is necessary to determine the extent of this protective effect. We must further understand the dynamics and biochemistry of those reactions in vivo, so that we can optimally harness the potential anti-cancer properties of this protein.

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CAPÍTULO 3

Artigo Científico:

HspBP1 levels are elevated in breast tumor tissue and inversely related to tumor aggressiveness

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HspBP1 levels are elevated in breast tumor tissue and inversely related to tumor aggressiveness

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ORIGINAL PAPER

HspBP1 levels are elevated in breast tumor tissue and inversely related to tumor aggressiveness

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Abstract HspBP1 is a co-chaperone that binds to and regulates the chaperone Hsp70 (Hsp70 is used to refer to HSPA1A and HSPA1B). Hsp70 is known to be elevated in breast tumor tissue, therefore the purpose of these studies was to quantify the expression of HspBP1 in primary breast tumors and in serum of these patients with a follow-up analysis after 6 to 7 years. Levels of HspBP1, Hsp70, and anti-HspBP1 antibodies in sera of breast cancer patients and healthy individuals were measured by enzyme-linked immunosorbent assay. Expression of HspBP1 was quantified from biopsies of tumor and normal breast tissue by Western blot analysis. The data obtained were analyzed for association with tumor aggressiveness markers and with patient outcome. The levels of HspBP1 and Hsp70 were significantly higher in sera of patients compared to sera of healthy individuals. HspBP1 antibodies did not differ significantly between groups. HspBP1 levels were significantly higher in tumor (14.46 ng/μg protein, $n=51$) compared to normal adjacent tissue (3.17 ng/μg protein, $n=41$, $p<0.001$). Expression of HspBP1 was significantly lower in patients with lymph node metastasis and positive for estrogen receptors. HspBP1 levels were also significantly

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The expression of HspBP1 (an Hsp70 co-chaperone) was analyzed in tumor samples and sera from breast cancer patients. HspBP1 is over expressed in these tumors and a seven year follow-up analysis found an association with a poor prognosis. Chaperones have been shown to play important roles in tumor biology and immunology; therefore, we believe the data in this study will serve as a basis for the formulation of a new hypothesis on chaperone-co-chaperone interactions and their role in tumor growth.

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lower in patients with a higher incidence of metastasis and death following a 6 to 7-year follow-up. The HspBP1/Hsp70 molar ratio was not associated with the prognostic markers analyzed. Our results indicate that low HspBP1 expression could be a candidate tumor aggressiveness marker.

Keywords Breast cancer · Co-chaperone · HspBP1 · Hsp70 · Prognostic markers

Introduction

Co-chaperones are proteins that bind and regulate the activity of chaperone proteins. The Hsp70 co-chaperone HspBP1 was first isolated from a human heart cDNA library using the yeast two-hybrid system with the ATPase domain of Hsp70 as bait (Raynes and Guerriero 1998). HspBP1 is classified as a nucleotide exchange factor and has been shown to either inhibit or stimulate Hsp70 ATPAse depending on assay conditions (Raynes and Guerriero 1998; Shomura et al. 2005). HspBP1 binding to Hsp70 results in a change in the conformation of the Hsp70 ATPase domain, and this is followed by inhibition of Hsp70-associated protein folding (McLellan et al. 2003). HspBP1 homologues can be found in other eukaryotic organisms; for example, Fes1 is the yeast cytoplasmic homologue of HspBP1 which can promote nucleotide dissociation from Ssa1p, the yeast homologue of Hsp70 (Kabani et al. 2002). Initial results have demonstrated that HspBP1 does not exhibit strict tissue specific expression; HspBP1 mRNA is expressed in all tissues examined with the highest levels in heart, brain, skeletal muscle, and pancreas (Raynes and Guerriero 1998).

Elevated levels of Hsp70 have been reported in a number of tumors including breast (Torronteguy et al. 2006), lung (Volm et al. 1995), cervical (Ralhan and Kaur 1995), prostate, and renal (Jaattela 1999; Jolly and Morimoto 2000). Hsp70 appears to play dual and opposite roles in cancer, promoting survival of tumor cells while contributing to tumor immunity. It was previously shown that HspBP1 levels were elevated in two mouse tumor models and that the molar ratio of HspBP1 to Hsp70 was within a small range in the normal and tumor tissues examined (Raynes et al. 2003). This ratio was considerably below the HspBP1 to Hsp70 ratio of 4.0 estimated to be needed for 50% inhibition of Hsp70-mediated refolding of a partially denatured protein (Raynes et al. 2003). However, it is possible that localized concentrations of HspBP1 in the cell could result in regional Hsp70 inhibition. Although both Hsp70 and HspBP1 levels increase and maintain the same molar ratio in normal tissues and tumor lines, these two proteins are not expressed in a coordinate manner (Gottwald et al. 2006).

Proteins identified in tissue and circulating blood proteins have the potential of profiling various disease states and therefore have tremendous diagnostic and treatment value. A major focus in cancer research is to identify proteomic markers for risk assessment and early detection in individuals. Nothing is known about the expression of HspBP1 in human primary tumors. In this study, the expression of HspBP1 and Hsp70 in breast cancer patient samples, both in tumor and normal adjacent tissue, was compared. In addition, the concentration of these two proteins, as well as anti-HspBP1 antibody, in the serum of patients and normal individuals was measured. The data were analyzed to determine if the expression of HspBP1, or the HspBP1/Hsp70 molar ratio, associates with aggressiveness tumor markers, as well as patient outcome. These results verified that HspBP1 is increased in human tumor tissue and found that low levels are related to a poor patient outcome.

Methods and materials

Patients

Samples of tumor and normal tissue were collected from 51 patients with primary breast cancer in 2001 and 2002. All patients were submitted to complete excision of tumor or total mastectomy. Exclusion criteria were previous radio or chemotherapy, familial cancer, hormonal therapy, and use of antidepressants or corticosteroids. Serum samples of 27 of these patients were available, and control serum samples were collected from 16 female healthy individuals. Samples were stored at −20°C. All individuals enrolled in the study signed an informed consent form, and the study protocol was previously approved by the Ethics Committee of the Sao Lucas Hospital.

ELISAS

HspBP1 enzyme-linked immunosorbent assays (ELISAs) were performed with slight modifications from what was previously described (Papp et al. 2005; Raynes et al. 2006). Briefly, for anti-HspBP1 analysis, individual wells of a 96-well microplates (Nalge Nunc, Rochester, NY, USA) were coated overnight at 4°C with 100 μL of 1 μg/mL HspBP1 (amino acids 84–359). Plates were blocked in 100 μL of blocking buffer (1% gelatin, 2% BSA, 0.01%Tween 20) for 1 h at room temperature with shaking. Serum samples were added at a 1:5 dilution in duplicates and incubated overnight at 4°C. Secondary antibody (HRP-conjugated goat anti-human IgG; Organon Teknika, West Chester, PA, USA) was used at a 1:500 dilution and incubated for 1 h at room temperature with shaking.

To measure HspBP1, plates were coated overnight at 4°C with 100 μL of 1 μg/mL sheep anti-HspBP1 antibody (Novus Biologicals, Littleton, CO, USA) in PBS. Plates were blocked with 100 μL of blocking buffer for 1 h at room temperature, and serum samples were diluted 1:5, followed by serial dilution in blocking buffer. The standard curve contained HspBP1 (amino acids 84–359) serially diluted from 2.5 to 0.15 ng in blocking solution. Aliquots, 100 μL, of the dilutions were added per well in duplicates, and the plate was incubated for 2 h at room temperature with shaking. Rabbit anti-HspBP1 (100 μL per well at 0.1 μg/mL; Delta Biolabs, Campbell, CA, USA) was added in blocking solution and incubated at room temperature for 1 h with shaking. Goat anti-rabbit IgG HRP conjugated (Zymed, San Francisco, CA, USA), 100 μL per well of 1:10.000 dilution was added, and the plate was incubated at room temperature for 1 h with shaking.

To measure Hsp70 in serum, plates were coated with anti-Hsp70 mouse (SPA-810, Assay Designs, Ann Arbor, MI, USA) at 2 μg/ml. Plates were blocked with 5% nonfat milk in PBS + 0.05% Tween for 2 h at room temperature. Sera were diluted at 1:5, and the standard curve was started at 1,000 ng/ml. Rabbit anti-Hsp70 rabbit (SPA-812, Assay Designs) was used at 1:2,000 dilution followed by anti-rabbit IgG (Zymed) at 1:5,000 dilution.

Plates were then rinsed six times and developed with 100 μL ready-to-use TMB (BioRad, Hercules, CA, USA) per well with incubation for 10–15 min at room temperature. To stop the reaction, 100 μL of 1 N HCl was added per well. Absorbance was read at 450 nm using an Anthos Zenyth 340r microplate reader. Standard curves were plotted using the GraphPad Prism software, and unknowns were determined from the standard curve. The molar ratio HspBP1/Hsp70 was calculated in the sera of breast cancer patients and healthy individuals based on a molecular weight of 70 kD for Hsp70 and 40 kD for HspBP1.

Western blots

Fresh tissue samples from the invasive margins of the tumors and adjacent normal breast tissue, as determined macroscopically, were retrieved from each patient and placed in Roswell Park Memorial Institute media for immediate processing. Total protein was extracted as previously described, and the measurement of Hsp70 in normal and tumor tissue was done in 2003 and 2004 (Torronteguy et al. 2006). The protein concentrations of the samples were estimated by Bradford assay (Dye Reagent Concentrate BIO-RAD, cat. no. 500-0006) and adjusted for loading on gels. Hsp70 blots from some patients were repeated to confirm sample integrity and when this was not observed, samples were excluded. HspBP1 expression in tumor and normal tissue was determined by Western blots. A total of 3 μg of tissue lysate of each sample were analyzed on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, purified HspBP1 (full length) was included for a standard curve using twofold serial dilutions beginning at 0.5 μg. The membrane was blocked with 5% nonfat dry milk in PBS for 30 min at room temperature and incubated with sheep anti-HspBP1 at a concentration of 1 μg/mL for 1 h. The blot was incubated with the secondary antibody (1:1,000 HRP-Rabbit anti-sheep IgG, Zymed, San Francisco) overnight at 4°C. Actin expression was used as a control for basal constitutive protein synthesis, using an anti-human actin antibody (A4700, Sigma, St. Louis, MO, USA). Samples with nondetectable actin were discarded. For detection, blots were incubated with the ECL system (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) for 5 min and exposed to X-ray film (Kodak, Rochester, NY, USA) at different times. The concentration of HspBP1 in tumor and normal tissue bands was determined according to the area of these bands in relation to a curve of areas plotted by the Imagemaster software from Pharmacia. Data was expressed as concentration of HspBP1 per microgram of total protein. Quantitations were repeated a minimum of three times. The molar ratio HspBP1/Hsp70 was calculated in tumor and normal tissues of breast cancer patients based on a molecular weight of 70 kD for Hsp70 and 40 kD for HspBP1.

Histology

Tumor samples were fixed in formaldehyde 10%, embedded in paraffin, sectioned (4 μm), and applied to histology slides. Antigen recovery was performed in Tris–ethylenediaminetetraacetic acid buffer pH 9.0 by microwave for 25 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide and 0.1% sodium azide. Additional blocking was performed with 5% nonfat dry milk. Incubation with primary antibody 1:200 (sheep anti-HspBP1 or normal sheep serum) was performed overnight at room temperature, followed by incubation with anti-sheep IgG-HRP (Zymed). Slides were developed with DAB and hydrogen peroxide. The slides were analyzed in a BX50 Olympus Microscope, and the pictures were acquired with a DXC 107A/P CCD Iris Sony camera using the Image pro-plus 4.5.1 Software (Media Cybernetics).

Statistical analysis

Statistical analysis was performed using the SPSS software version 10 (SPSS, Chicago, IL, USA) and GraphPad prism 4 (San Diego, CA, USA). The data were tested for normality of distribution using Shapiro–Wilk's W test and Kolmogorov–Smirnov. None of the data were found to be normally distributed. The Mann–Whitney U (between two

groups) and Krustal–Wallis (three or more groups) tests were used to assess differences between patients and healthy individuals as well as normal tissue and tumor breast tissue. Also, these tests were used to assess the relationship between the data with the prognostic markers and patient's outcome. All statistical tests were two sided, and $p<0.05$ was considered to be statistically significant. Correlations between the data were evaluated using Spearman rank correlation coefficients. Kaplan–Meier survival curves were calculated from the data using the percent survival of the patients with a long-rank test.

Results

Patient and tumor characteristics

The mean age of patients was 58.39±17.01 years old, and 49.3% had a menopause status. The mean age for healthy individuals was 36±11 years. The mean of tumor size was 2.63±1.5 cm. Tumor characteristics, such as histological type of the tumor, tumor size, lymph node invasion by tumor, grade, staging (TNM classification), and lymphocyte infiltrate and estrogen and progesterone receptors status are summarized in Table 1.

Table 1 Tumor characteristics

Size (mean±SD)	2.63±1.5 cm
Auxiliary lymph node status	
Node negative	48.9%
1–3	31.3%
4–10	11.1%
>10	8.9%
Staging	
I	29.5%
IIA	26.2%
IIB	19.7%
IIIA	4.9%
IIIB	8.2%
IIIC	8.2%
IV	3.3%
Tumor histological type	
Invasive ductal	70.3%
Invasive lobular	18.8%
Invasive tubule—lobular	6.3%
Intraductal	3.1%
Mucinous	1.6%
Tumor differentiation	
Grade I	10.4%
Grade II	64.6%
Grade III	25.0%
Lymphocyte infiltrate	
Absent	23.8%
Present	76.2%
Estrogen receptor	
Negative	13.3%
Positive	86.7%
Progesterone receptor	
Negative	43.3%
Positive	56.7%

HspBP1 and Hsp70 are elevated in sera of patients compared to controls

The levels of serum HspBP1 and anti-HspBP1 antibody in patients and normal individuals were measured. HspBP1 was significantly higher in sera of patients compared with sera of healthy individuals with means of 1.25 and 0.66 ng/ml, respectively (Fig. 1A). The levels of anti-HspBP1 antibodies did not differ significantly between groups (Fig. 1b). Hsp70 levels were also measured to test if the serum HspBP1/Hsp70 molar ratio differed between patients and controls. In general, the mean levels of serum Hsp70 (63.87 ng/ml) were much higher than the mean levels of serum HspBP1 (1.25 ng/ml) and the mean Hsp70 levels (63.87 ng/ml) were significantly higher in sera of patients compared to healthy individuals (13.69 ng/ml; Fig. 1C). However, the serum HspBP1/Hsp70 molar ratio was not different between patients with breast cancer tumors and healthy individuals (Fig. 1D).

HspBP1 expression by tumor and normal tissues

It has previously been determined that Hsp70 is elevated in tumor tissue of breast cancer patients (Torronteguy et al. 2006). HspBP1 and Hsp70 are both elevated in two mouse tumor cell lines (Raynes et al. 2003); however, to date, nothing is known on the expression of HspBP1 in human primary tumors. HspBP1 expression was quantified in tumor and normal tissue by western blot analysis in breast cancer patient samples. Figure 2A shows a typical result for HspBP1 expression in normal and tumor tissue of patients. A summary of the data are presented in Fig. 2C, showing that tumor tissue presented a significantly higher expression of HspBP1 (mean value of 14.46 ng/μg protein) than normal adjacent tissue (mean value of 3.17 ng/μg protein; $p<0.001$). However, the mean molar ratio (HspBP1/Hsp70) did not differ between tumor and normal tissue (Fig. 2D).

Histology

HspBP1 levels are elevated in tumor tissue; therefore, it is possible that the distribution pattern of HspBP1 differs in tumors samples from patients with high versus low HspBP1 levels. Tumor sections from these two groups of patients were sectioned and analyzed for HspBP1. Also, tumors from patients with the two highest and two lowest HspBP1/Hsp70 molar ratios were examined for HspBP1 distribu-

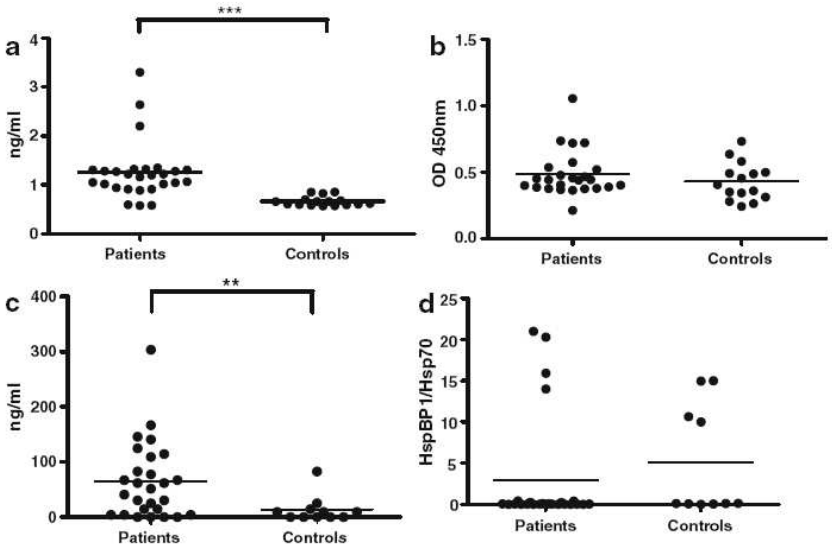

Fig. 1 Levels of serum HspBP1, Hsp70, anti-HspBP1 antibody, and serum HspBP1/Hsp70 ratios in breast cancer patients and controls. Serum samples of breast cancer patients (n=27) and healthy individuals (n=16) was used to quantify **a** Serum HspBP1 (Mann–Whitney, $p<0.001$); **b** Anti-HspBP1; **c** Hsp70 (Mann–Whitney, $p<0.001$); **d** Molar ratio of HspBP1/Hsp70. The ratio was calculated based on a molecular weight of 70 kD for Hsp70 and 40 kD for HspBP1

tion. The results, shown in Fig. 2B, confirm what was reported by Western blot—expression of HspBP1 is higher in tumor cells and low or absent in surrounding normal tissue (see Fig. 2b I and II). Tumors with high and low expression of HspBP1 (determined by Western blot) do not show differences in the distribution pattern of the protein that is localized in both the nucleus and the cytoplasm. This is similar to what has been described for Hsp70 in these patients.

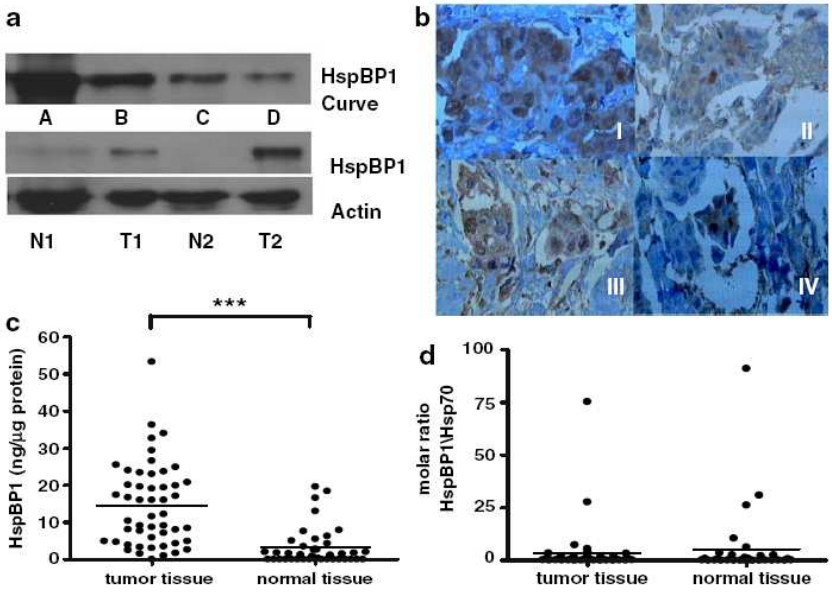

Fig. 2 HspBP1 levels in normal and tumor tissue. Tissue samples were analyzed by Western blot for HspBP1 expression and quantified using a standard curve of purified HspBP1. **a** Standard curve for HspBP1. *Lanes A–D* are serial dilutions of HspBP1 starting at 0.5 μg. *T* tumor tissue; *N*, normal tissue, probed with anti-HspBP1 or anti-actin. Numbers next to these letters denote the patient. **b** Tissue samples of breast cancer were analyzed by immunohistochemistry for HspBP1. *I* Tumor tissue with high HspBP1; *II* Tumor tissue with low HspBP1; *III* Tumor tissue with high molar ratio; *IV* Tumor with low molar ratio HspBP1/Hsp70. Magnification 400×. **c** Levels of HspBP1 in normal (n=41) and tumor tissue (n=51) of breast cancer patients quantified by Western blot analysis. **d** Data were analyzed using a t test ($p<0.001$) and the distribution is normal. The molar ratio of HspBP1/Hsp70 was calculated as described in Fig. 1

The same was observed for patients that presented a high (Fig. 2D III) versus a low (IV) HspBP1/Hsp70 molar ratio.

Associations between serum and tissue levels

The data obtained for HspBP1 and Hsp70 in the sera of patients and controls was analyzed for correlations with the data obtained in tissue samples. A Spearman correlation test was performed and the results are shown in Table 2. Serum levels of HspBP1, Hsp70 or anti-HspBP1 antibody did not significantly correlate with tissue expression of HspBP1 or Hsp70. Interestingly, we found that HspBP1 in normal tissue correlated negatively with Hsp70 in tumor tissue ($p < 0.05$). As expected, molar ratios correlated negatively with HspBP1 expression, and positively with Hsp70 expression, but only in the compartment (tissue or serum) analyzed.

Association with tumor aggressiveness markers

The serum levels of these proteins, as well as the HspBP1/Hsp70 molar ratios, were analyzed for association with tumor aggressiveness markers (progesterone and estrogen receptors, auxiliary lymph node status, tumor size, stage, histology grade, and lymphocyte infiltrate). Mann–Whitney and Kruskall–Wallis analysis of the means of tumors with different characteristics were performed. None of the tested parameters in the sera were significantly associated with tumor aggressiveness markers (data not shown).

Associations between the protein levels and tumor characteristics (progesterone and estrogen receptors, auxiliary lymph node status, tumor size, stage, histology grade, and lymphocyte infiltrate) were analyzed. Mean levels of HspBP1 in tumor samples were significantly lower in patients with tumors that were positive for estrogen receptor, at an advanced stage, or with metastatic auxiliary lymph nodes. The graphs in Fig. 3 detail the significant differences for each of these parameters. HspBP1 levels were significantly lower in tumors of patients with an increased number of compromised lymph nodes (Fig. 3b). Also, HspBP1 levels were significantly lowest in patients with tumors in an advanced stage with statistical significance when compared to intermediate stage tumors (Fig. 3C). This trend was present in early stage tumors but it was not statistically significant. The other tumor characteristics were not significantly associates with HspBP1 levels in tumor tissue (data not shown)

Follow-up

For all patients enrolled in this study 80% of patients have been under clinical follow-up for 6 to 7 years, and the other 20% do not return to clinical following in the São Lucas Hospital. All patients in the clinical follow-up received surgical treatment and tamoxifen for 5 years after the surgery. Some patients received chemotherapy and radiotherapy based on stage and treatment response. Their clinical data was obtained at the time of this analysis to determine if the levels of HspBP1 as well as the other measurements performed in this study were associated with the patients' clinical outcome. Associations were analyzed (levels of HspBP1 and Hsp70 in serum, levels of anti-HspBP1, levels of HspBP1 and HspBP1/Hsp70 in tumor and normal tissue) with the presence of distance metastasis, tumor relapse, and patient death. It was found that tumor mean HspBP1 levels were significantly lower in patients that had metastasis and died. Figure 4A and B shows graphs detailing the significant differences for each parameter. This result suggested that a low HspBP1 expression in primary tumors can be an indicator of a poor prognosis. None of the other parameters were significantly associated with the patients' clinical outcome (data not shown). Survival curves for the patients based on their tissue and serum expression of HspBP1, as well as their HspBP1/Hsp70 molar ratios were made. To categorize high and low

Table 2 Correlations between the data (Spearman correlation)

	Serum HspBP1	Serum Hsp70	Anti-HspBP1	Serum HspBP1/Hsp70	HspBP1 in normal tissue	HspBP1 in tumor tissue	HspBP1/Hsp70 molar ratio tumor tissue	HspBP1/Hsp70 molar ratio normal tissue
Serum HspBP1	1	0.353	0.053	−0.1	−0.285	−0.184	0.173	−0.277
Serum Hsp70	0.353	1	0.36	−0.941**	−0.349	0.268	0.169	0.105
Anti-HspBP1	0.053	0.36	1	−0.302	−0.503	0.088	0.02	−0.378
Serum HspBP1/Hsp70	−0.1	−0.941**	−0.302	1	0.16	−0.332	−0.091	−0.209
HspBP1 in normal tissue	−0.285	−0.349	−0.503	0.16	1	0.255	0.478**	0.666**
HspBP1 in tumor tissue	−0.184	0.268	0.088	−0.332	0.255	1	0.634**	0.212
Hsp70 in tumor tissue	−0.214	−0.058	0.26	−0.025	−0.400*	−0.005	−0.719**	−0.530**
Hsp70 in normal tissue	−0.075	0.058	0.224	−0.077	−0.137	−0.042	−0.342*	−0.796**

*$p<0.05$
**$p<0.01$

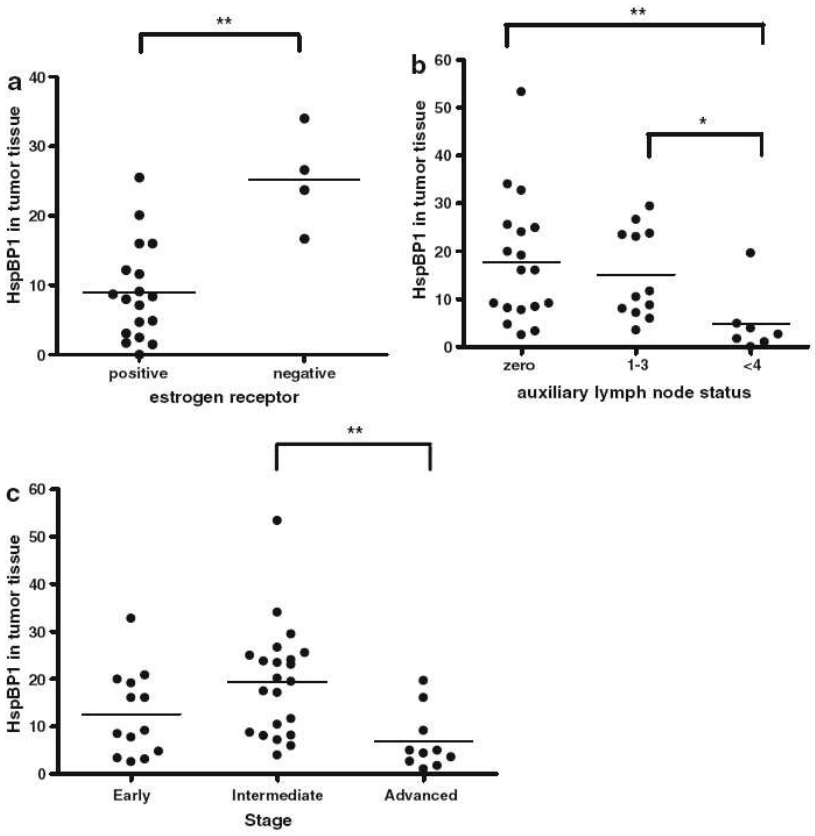

Fig. 3 Associations between HspBP1 levels in breast tumors and prognostic markers. **a** Estrogen receptor (negative and positive; Mann–Whitney, $p<0.05$). **b** Auxiliary lymph node status based on pathological classification of TNM (zero, 1–3, more than 4; Krustal–Wallis with Dunn's pos hoc test, $p<0.05$) and **c** Stage (Early=Stage I; Intermediate=Stage IIa and IIb; Advanced=Stage IIIa, IIIb, IIIc, and IV; Krustal–Wallis with Dunn's pos hoc test, $p<0.05$)

expression of HspBP1, patients were divided into two groups: below, equal, or above the mean levels. For HspBP1/Hsp70 molar ratio, patients were categorized in groups with molar ratios that were equal or above 4 (value known to result in inhibition of 50% of Hsp70 ATPase activity), versus patients with molar ratios below 4. None of the differences were statistically significant (data not shown), however, all patients with high levels of HspBP1 in tumor tissue survived, and the difference was borderline significant ($p<0.052$; Fig. 4C).

Discussion

To our knowledge, this is the first report of HspBP1 levels in human primary tumors. The results showed that HspBP1 expression was increased both in tumor tissue and sera of breast cancer patients compared to normal adjacent tissue and healthy normal individuals. These data agree with previous work on two mouse tumors models that demonstrated high expression of HspBP1 in the examined tumors (Raynes et al. 2003). Interestingly, Hsp70 is also highly expressed both in murine (Raynes et al. 2003) as well as human tumors (Kaur and Ralhan 1995; Ralhan and Kaur 1995; Torronteguy et al. 2006). This is the first study to demonstrate that serum Hsp70 levels are also increased in breast cancer patients compared to healthy individuals. A previous other study reported serum Hsp70 was elevated in lung cancer patients (Suzuki et al. 2006).

Nevertheless, no significant differences were observed when comparing the soluble HspBP1/Hsp70 molar ratio in patients and controls. Also, no differences were observed when we compared the HspBP1/Hsp70 molar ratio in primary neoplasic breast tissue and adjacent normal breast tissue. The fact that both Hsp70 and HspBP1 levels were elevated in tumor samples could at least in part explain why we did not observe any significant differences when comparing the levels of HspBP1/Hsp70 molar ratios in the groups analyzed. Overall, the HspBP1/Hps70 molar ratio in serum as well as in breast tissue was distributed within a small range; just a few samples had molar ratio greater than four (the amount previously determined to inhibit 50% of Hsp70

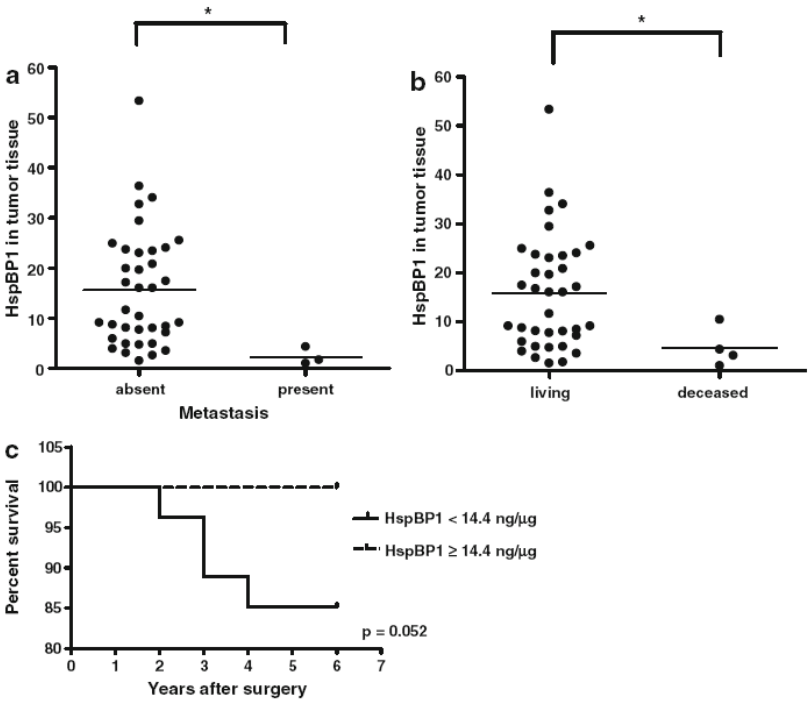

Fig. 4 Associations between HspBP1 levels in breast tumors and patient outcomes. **a** Presence or absence of metastasis (Mann–Whitney, $p<0.05$). **b** Deceased or alive (Mann–Whitney, $p<0.05$). **c** Kaplan–Meier survival curve of HspBP1 in tumor tissue (the mean was 14.4 ng/ml and groups were either below, or equal and above mean levels)

activity). This finding agrees with what was previously demonstrated by Raynes et al. (2003), who found that molar ratios in murine tumor lines also varied within a small range.

The serum levels of HspBP1, Hsp70, or anti-HspBP1 antibody did not significantly correlate with tumor or healthy breast tissue expression of HspBP1 or Hsp70. We hypothesized that the proteins found in sera could be released by other, nonanalyzed tissue, since it has previously been shown that HspBP1 mRNA is expressed in different tissues (Raynes and Guerriero 1998). Serum HspBP1 and anti-HspBP1 antibody were previously demonstrated to be present in healthy individuals (Raynes et al. 2006), HspBP1 is present in human serum at concentrations ranging between 0.74 to 3.98 ng/mL. Also, it was demonstrated that there is no significant correlation between HspBP1 and anti-HspBP1 (Raynes et al. 2006), thus agreeing with our present study.

Extracellular Hsp70 released from tumor cells undergoing necrosis, as well as circulating in the bloodstream was associated with enhancement of tumor growth (Asea et al. 2000; Calderwood et al. 2005). Also, Hsp70 expression in tumor tissue was associated with a poor prognosis in breast cancer patients (Ciocca et al. 1993; Elledge et al. 1994; Thanner et al. 2003; Torronteguy et al. 2006). However, we were not able to see any significant association between serum Hsp70 levels and prognostic markers. Tissue HspBP1 levels was associated with prognostic markers, HspBP1 levels were significantly lower in patients who had advanced stage and metastasis in auxiliary lymph nodes. Involvement of auxiliary lymph nodes is the most reliable and reproducible prognostic indicators for primary breast cancer; in general, 50% to 70% of patients with positive lymph nodes have a relapse (Fisher et al. 1983). This indicates that lower expression of HspBP1 in primary breast tissue could be related with poor patient's outcome. Mean levels of HspBP1 were also significantly lower in patients who had positive estrogen receptors (ER). The relationship with prognosis of ER expression in breast cancer is still controversial. Although ER positive tumors are more often well differentiated, associated with favorable prognostic characteristics and are predictors of a favorable response to endocrine therapy (Osborne 1998), some studies have suggested that very high levels of receptor may be associated with poor prognosis (Fisher et al. 1988; Thorpe et al. 1993; Hilsenbeck et al. 1998). Receptor data may be of greater value when combined with other prognostic factors. For this reason, it is still too early to draw conclusions from the association of HspBP1 with estrogen receptors; however, we believe that in this study, the significantly lower mean of HspBP1 in patients with positive estrogen receptor may be related to tumor aggressiveness, since

two other important tumor aggressiveness markers were correlated with HspBP1, namely, staging and lymph node status.

After a 6- to 7-year follow-up, we found that patients that had metastasis and died presented significantly lower mean levels of HspBP1 in their primary tumors. This result reinforced the hypothesis that the lower expression of HspBP1 in primary breast tissue could be related to poor patient outcome. Although we were not able to see a statistically significant difference between the groups with low or high HspBP1 using the Kaplan–Meier survival curve, a trend could be observed since the results are on the verge of significance ($p<0.051$). Studies with a higher patient number will enable us to test if HspBP1 levels in primary tumors are associated with survival. The molecular mechanisms responsible for overexpression of HspBP1 in tumor cells are unknown and may be tumor specific.

The observations that primary tumor cells have elevated levels of HspBP1 and that the levels of HspBP1 are associated with poor prognostic markers therefore raise intriguing questions regarding whether this protein confers a selective prosurvival advantage to such cells, contributing to the process of tumorigenesis. There are a number of reports indicating that Hsp70 enhances tumorigenic potential, and several candidate mechanisms have been suggested based on the molecular evidence. We demonstrated that HspBP1 was localized in cytoplasm and nucleus of breast tumor tissue cells, the same localization already described for Hsp70 expression in tumors (Vargas-Roig et al. 1998; Torronteguy et al. 2006; Ramp et al. 2007). It remains to be tested if HspBP1 can be involved in tumor survival in a manner associated with Hsp70 and if there are any differences between the activities of this protein found in the nucleus or cytoplasm.

Breast cancer is the most common malignancy for women throughout the industrialized world. In general, most individuals with cancer do not die from the tumor in the primary site, but rather from local invasion and/or distant metastasis (Woodhouse et al. 1997). Breast cancer is a heterogeneous disease, and there is a continual drive to identify markers that will aid in predicting prognosis and response to therapy. To date, relatively few markers have established prognostic power (Payne et al. 2008). This present study has shown that HspBP1 is elevated in breast cancer tumors, and lower levels are associated with poor prognosis. This inverse relationship could be a candidate for a new cancer marker.

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CAPÍTULO 4

Artigo Científico:

HspBP1 overexpression impairs tumor growth *in vivo*

Artigo Científico a ser submetido para: Molecular Cancer Research

HSPBP1 OVEREXPRESSION IMPAIRS TUMOR GROWTH *IN VIVO*

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ABSTRACT

The incidence of melanoma is increasing and new and improved therapies are urgent. HspBP1 is a co-chaperone of Hsp70 that binds to its N-terminal domain, inhibiting its ATPase activity. We have recently demonstrated that HspBP1 levels are increased in primary breast tumors compared to adjacent normal tissue. In breast cancer patients, low levels of this protein in tumor tissue were associated with poor prognosis. These results suggested that HspBP1 could have a role in the control of tumor growth. In this study, we tested this hypothesis in a murine melanoma model (B16F10 cells). Overexpression of HpsBP1 by tumor cells impaired tumor growth *in vivo*. The production of HspBP1 and Hsp70 appeared to be coordinately regulated in tumor cells - upregulation of Hsp70 expression in vivo correlated with downregulation of HspBP1. However, overexpression of HspBP1 in stressed tumor cells did not impair survival in vitro. Modulation of both HspBP1 and Hsp70 expression was observed both in co-culture with dendritic cells and TNF-α. Finally, inhibition of in vivo tumor growth by HspBP1 overexpression was significant in immunocompetent, but not in immunodeficient mice. Collectively, our results suggest that HspBP1 is a natural regulator of tumor growth, possibly through modulation of Hsp70. Nevertheless, they indicate that rather than simply modulating Hsp70 mediated mechanisms of cell survival, HspBP1 is related to anti-tumor immune responses. We propose that HspBP1 is an important tool for melanoma management, and a candidate target for melanoma therapy.

Key words: HspBP1, Hsp70, tumor, melanoma, Rag-/-.

INTRODUCTION

The worldwide incidence of cutaneous malignant melanoma, a highly aggressive skin cancer, is increasing (Parkin, Bray *et al.*, 2005; Markovic, Erickson *et al.*, 2007). About 20% of patients with primary melanomas of intermediate thickness (depth of invasion 1·0–4·0 mm) develop metastases to the regional lymph nodes, that being associated with poor prognosis (Mcmasters, Wong *et al.*, 2001; Boon, Coulie *et al.*, 2006; Amersi e Morton, 2007). The standard management for regional nodal disease combines surgery and radiotherapy, mainly in patients whose tumors have clinical and pathological features associated with high risk of regional nodal recurrence (Ballo e Ang, 2004; Berk, 2008; Mendenhall, Amdur *et al.*, 2008). New therapies are greatly required, and many current approaches focus on immunotherapy. Although melanoma is relatively immunogenic compared with other cancer types (Morton e Reiter, 1991), it may remain refractory to immunologic control (Pawelec, 2004; Boon, Coulie *et al.*, 2006). High frequencies of circulating tumor antigen–specific T cells can be successfully generated in melanoma patients through vaccination with peptide (Rosenberg, Yang *et al.*, 1998), dendritic cell (DCs) (Banchereau, Palucka *et al.*, 2001), whole tumor cells (De Gruijl, Van Den Eertwegh *et al.*, 2008), and viral vector-based vaccines (Rosenberg, Zhai *et al.*, 1998). However, these strategies alone have shown little efficacy (Fang, Lonsdorf *et al.*, 2008). Such results demonstrate that although immune responses against melanoma cells are developed, they are still not efficient, suggesting new and improved strategies are necessary.

Stress or heat shock proteins (Hsps), such as Hsp70, play an essential role as molecular chaperones by assisting the correct folding of nascent and stress-accumulated misfolded proteins. HspBP1 is an Hsp70 co-chaperone that has been shown to inhibit Hsp70 ATPAse activity (Raynes e Guerriero, 1998; Shomura, Dragovic *et al.*, 2005). HspBP1 binding to Hsp70 results in a change in the conformation of the Hsp70 ATPase domain and this is followed by inhibition of Hsp70-associated protein folding (Mclellan, Raynes *et al.*, 2003). Hsp70 is highly expressed in several different tumors (Ciocca e Calderwood, 2005; Torronteguy, Frasson *et al.*, 2006) and may have opposite roles in cancer depending on where in the cell it is expressed. When present in the cytoplasm, it can protect tumor cells from apoptosis, however when expressed in the cell surface or secreted, it can modulate the immune response (Schmitt, Gehrmann *et al.*, 2007).

HspBP1 mRNA is expressed in different tissues and cell types, with the highest levels in human heart, brain, skeletal muscle, and pancreas (Raynes e Guerriero, 1998). HspBP1 levels were shown to be elevated in two mouse tumor models, such as lung carcinoma and neuroblastoma (Raynes, Graner *et al.*, 2003). We have recently demonstrated that HspBP1 was elevated in human breast tumor tissues compared to the normal adjacent breast tissue (Souza, Albuquerque *et al.*, 2009). Another important observation was that patients who expressed less HspBP1 in primary tumors were the ones with poor outcome (Souza, Albuquerque *et al.*, 2009). These results suggested that HspBP1 could have a role in tumor control.

We investigated this hypothesis in a melanoma murine model (B16F10 cells). Overexpression of HpsBP1 impaired tumor growth *in vivo*. The production of HspBP1 and Hsp70 was coordinately regulated in tumor cells, *in vivo* and *in vitro*. However, overexpression of HspBP1 in stressed tumor cells did not impair survival after heat shock in vitro. Interestingly, modulation of both HspBP1 and Hsp70 expression was observed both in co-culture with dendritic cells and TNF-α. Finally, inhibition of in vivo tumor growth by HspBP1 overexpression was significant in immunocompetent, but not immunodeficient mice. Collectively, our results suggest that HspBP1 is a natural regulator of tumor growth, possibly through modulation of Hsp70. Also, they indicate that immune responses are involved in this process.

MATERIALS AND METHODS

Mice

C57Bl/6 (B6) mice were purchased from Fundação Estadual de Produção e Pesquisa e Saúde (FEPPS) Porto Alegre, RS, Brazil and Rag-/- mice were provided by Marc Jenkins (University of Minnesota, USA). Mice were kept under pathogen-free conditions at the PUCRS (FABIO) animal facility with *ad libitum* access to food and water. Female six–to eight week old mice were used for all experiments and these were conducted with the approval of the PUCRS ethics committee on animal research. In some experiments mice were treated with tetracycline (Sigma) in drinking water at 1mg/ml during 12 days. SCID mice were obtained from University of Arizona, USA.

Cell lines

The murine melanoma cell line B16F10 (ATTC CRL-6475) was cultured with DMEM media (Cultilab) supplement with 10% of fetal calf serum (FCS) (Cultilab), 1X essentials aas (Gibco), 1X vitamins (Gibco) and 55μM of β-mercaptoethanol at 37ºC with 5% of CO_2 atmosphere. The human fibrosarcoma cell line HT1080 (ATTC CCL-121) were culture using MEM with 10% of FCS at 37ºC with 5% of CO_2 atmosphere.

Transfections and silencing

B16F10 cells were transfected with 1μg plasmid vector pcDNA4/TO (Invitrogen) encoding either murine HspBP1 cDNA (as previously described by Raynes, 1998) or with the empty vector as a mock transfection control using the Transfast reagent (Promega). Alternatively, cells were transfected with 1μg of plasmid pcDNA6/TR from the TREX expression system (Invitrogen) and selected with 10μg/ml Blasticidin (Invitrogen). After selection these cells were also transfected with 1μg plasmid vector pcDNA4/TO (Invitrogen) encoding human HspBP1 cDNA or with the empty vector as a control, and selected with 400μg/ml Zeocin (Invitrogen). The *in vitro* expression of HspBP1 was induced adding tetracycline in the media at 1μg/ml. For silencing of Hsp70, si RNA was purchased from SantaCruz Biotech (Cat number sc 29352). Cells (5×10^5) were incubated with 1 μg Hsp70 siRNA or control siRNA for 4h, and subsequently heat shocked at 42ºC for 2h, followed by recovery at 37ºC for 4h. After that, cells were stained with Trypan blue and counted in a haemocytometer. HT1080

cells were also transfected with plasmid pcDNA6/TR pcDNA4/TO encoding human HspBP1 cDNA as described above.

Evaluation of tumor growth in vivo

Mice were implanted subcutaneously in the thigh with B16F10 or HT1080 HspBP1 transfectant cells at a concentration of $5X10^5$ or 10^5 cells, after anesthesia with 83 mg/Kg of ketamine and 17 mg/kg of xylazine. Tumor growth was evaluated using a digital caliper. Tumors were excised for analysis at different time points depend on the experiments procedure.

Western Blot

In order to analyze HspBP1 and Hsp70 expression by tumor cells *in vitro* and *in vivo,* western blots were performed. Total protein from fresh tumor tissue or cultured tumor cells was extracted with lysis buffer (10 mM Tris-HCl,pH 7.5; 1 mM MgCl2; 1 mM ethylenediamine-tetraacetic acid [EDTA]; 0.1 mM phenylmethylsulfonyl fluoride [PMSF]; 5 mM βmercaptoethanol; 0.5% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate [CHAPS]; 10% glycerol), followed by centrifugation at 13.000rpm for 1h at 4ºC. Protein concentrations of the samples were estimated by Bradford assay (Dye Reagent Concentrate BIO-RAD). A total of 8 μg of protein lysate of each sample was analyzed on 10% SDS-PAGE gels, purified HspBP1 (full length) was included for a standard curve. To measure HspBP1 expression the membrane was incubated with sheep anti-HspBP1, followed by incubation with the secondary antibody (HRP-Rabbit anti-sheep IgG, Zymed, San Francisco) and to measure

Hsp70 expression the membrane were incubated with anti-Hsp70 (Stressgen) after striping, followed by incubation with the secondary antibody (HRP-Rabbit anti-mouse IgG Zymed). Actin expression was used as a control, using an anti-actin antibody (Sigma, St. Louis, MO). For detection, blots were incubated with the ECL system (GE Healthcare Bio-Sciences Corp, Piscataway, NJ) for 5 min and exposed to x-ray film (Kodak, Rochester, NY) at different time points.

MTT assay

To assess the *in vitro* proliferation/viability of transfected tumor cells, those were plated at $4X10^4$ cells per well at 96 well plate. After 24 or 48hs, 100µl of the supernatant was discarded and it was added 40µl MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide) (Sigma) reagent solution at 5mg/ml in cell culture and incubated for 4hs. The absorbance was read at 620nm and 570nm using a microplate reader (Anthos Zenyth 340r).

Immunohistochemistry

Fresh tumor tissue was frozen emerge in isopentane using liquid nitrogen, after this it was store at -80ºC. The sections were cut in cryostat (Shandon) in 7 µm using Tissue Tek (EasyPath). The sections were thaw and fixed with acetone, following the blocking Avidin and Biotin (Vector). The sections were than stained with anti-Hsp70 biotin (StressGen) or anti-HspBP1. The stained of secondary was SA-HRP (Kit *TSA Fluorescence Systems* (PerkinElmer)) followed by CY3 (PerkinElmer) for Hsp70 and anti-sheep IgG FITC (Jackson ImmunoResearsh Laboratories) for HspBP1. Nuclei were stained using Hoesch

(Invitrogen). The sections were analyzed using a fluorescence microscopic (Olympus).

Dendritic Cell and TNF-α co-culture

To examine the effects of modulation of dendritic cells on HspBP1 expression in B16F10 cells, a co-culture of these two cell types was performed. Murine dendritic cells were grown from bone marrow tissue of C57Bl/6 mice in AIM-V media (Gibco) with 40ng of GM-CSF and IL-4 (Peprotech, Ribeirao Preto, Brazil) in 7% of CO_2 at 37ºC as previously described in (Motta, Schmitz *et al.*, 2007). The cells were cultured for 9 days with media changes every two days. B16F10 cells were plated at concentration of 10^4 cells per well and cultured overnight, and the cells were then transfected with the plasmid encoding HspBP1 or with the vector and incubated for 3h. After this the bone marrow dendritic cells (BMDCs) were added to the culture at a concentration of $3X10^5$ cells per well and this co-culture was incubated for 24hs or 48hs in 5% of CO_2 at 37ºC in DMEM media. Alternatively, B16F10 cells were plated at 10^4 cells per well and TNF-α (R&B system) was added 12h later at a concentration of 1 or 2 ηg/ml for 48hs, the cells then being collected for analysis.

RESULTS

Induction of HspBP1 dramatically impairs tumor growth in vivo

Low levels of HspBP1 were related to poor prognosis in breast cancer, suggesting that expression of this protein could be related to tumor growth. We asked if tumor progression could be affected by overexpression of HspBP1 in a melanoma model. We developed a system in which HspBP1 expression was regulated by the presence of tetracycline. B16F10 cells were stably transfected with plasmid pcDNA6/TR, following by transfection with a plasmid encoding human HspBP1, or empty vector as a control. The cell lines thus obtained were named B16huBP1 and B16ve, respectively. Increase in HspBP1 expression was verified by adding tetracycline to the culture media (Figure 1A). The transfected cells were injected subcutaneously in mice, one group of mice receiving tetracycline in the drinking water (BP1 induced) and the other not (BP1 not induced). Tumor growth was clearly impaired in the group of mice with induced overexpression of HspBP1 (Figure 1B). The mean tumor volume with HspBP1 overexpression was 2.07 mm^3 while the mean volume for mock induced tumors was 46.9 mm^3, a significant difference (1C). This result indicated that this protein could have a role in tumor progression.

Figure 1

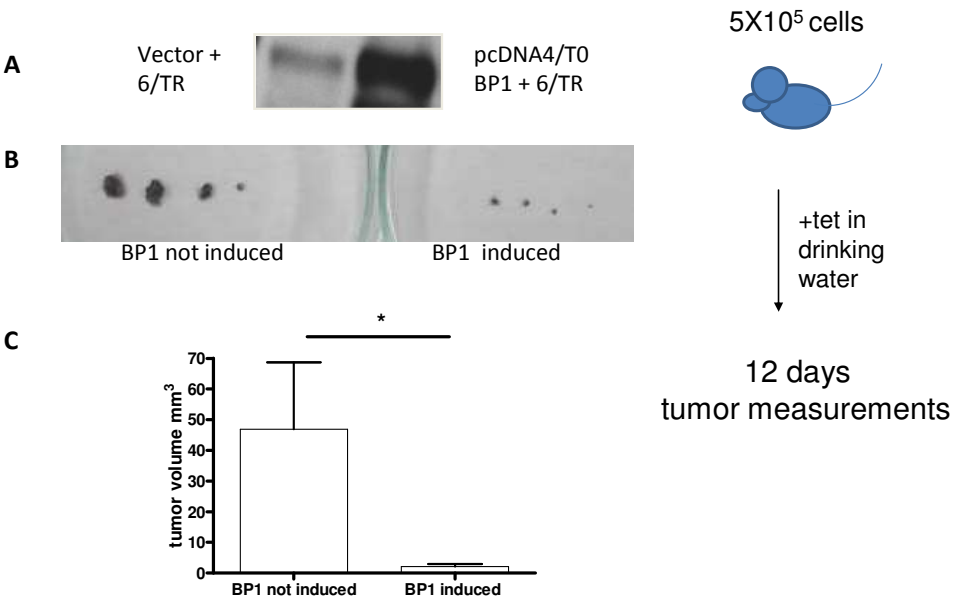

Figure 1: Human HspBP1 Overexpression Inhibits Tumor Growth /In Vivo. **A**. Western blot analysis of B16F10 cells stably transfected with plasmids pcDNA6/TR and pcDNA4/TO with or without the human HspBP1 sequence.Tetracycline (1ug/ml) was added to the media to induce HspBP1 expression for 24h. **B**. Photograph of control and over expressing HspBP1 tumors after have been removed. B16F10 cells stably transfected with pcDNA6/TR and plasmid pcDNA4/TO with human HspBP1 cDNA were induced in vivo with (BP1 induced) or without (BP1 not induced) tetracycline for 24hrs. Cells ($5X10^5$) were injected subcutaneously in mice. Mice received drinking water with (1 mg/ml) or without tetracycline for 12 days before analysis of the tumors. **C**. Quantification of tumor growth inhibition by HspBP1 (Mann-Whitney, $p < 0.05$). Tumor volume (V) was calculate using $V=d^2$ xDx0.5, where d=minor diameter and D=major diameter.

However, the dramatic impairment observed by overexpression of HspBP1 could be due to an immune response mounted by the mice against the human protein encoded by the sequence used in that experiment, since identity of human and murine HspBP1 is 87.37%. To answer that question, we cloned the murine sequence in the pcDNA4/TO expression vector, and transfected B16F10 as described earlier (Figure 2A). The transfected cells were cultured for 24 or 48hs and the proliferation/viability measured by an MTT assay. Interestingly, in vitro viability of the cells overexpressing HspBP1 did not show any impairment compared to cells which were not over-expressing this protein (Figure 2B). The same phenomenon had previously been observed for HT1080 cell and B16F10 transfected with the plasmid encoding the human HspBP1 sequence– not shown. This indicated that high levels of HspBP1 did not affect tumor growth *in vitro*, and suggested that the effect HspBP1 exerted over tumor growth was not directly related to cell proliferation.

Figure 2

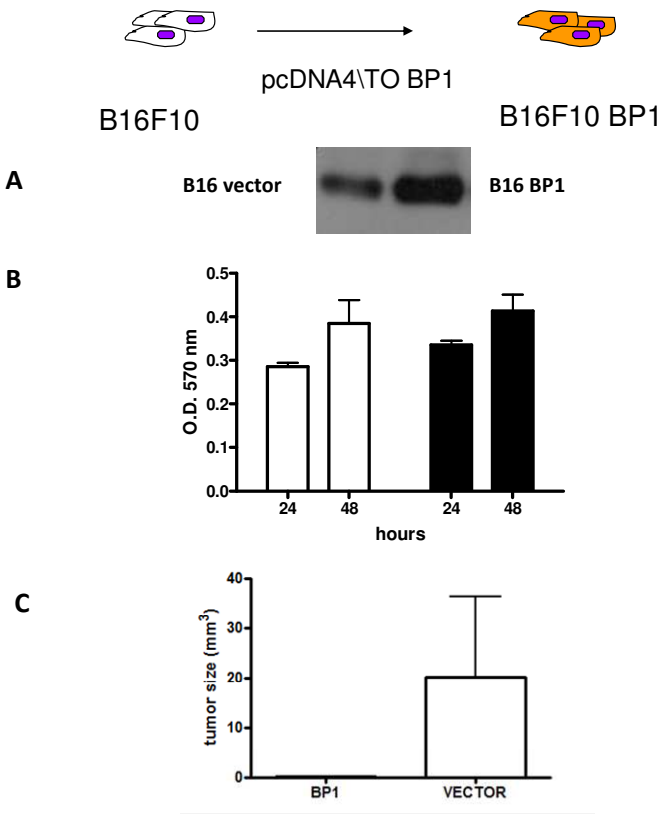

Figure 2: Tumor growth of murine HspBP1 over-expressing B16F10 cells *in vitro* and in vivo. A- Western blot analysis of expression of HspBP1 in B16F10 transiently transfected with plasmid vector encoding murine HspBP1 sequence (B16 BP1) or plasmid vector (B16 vector). **B-** MTT assay of transienly transfected cells showing the correspondent optical density (OD) for the proliferation/viability of the cells in 24hs and 48hs of culture. **C-** Tumor volume (mm^3) of B16 BP1 and B16 vector was measured after 25 days of tumor growth *in vivo* (Mann-Whitney $p < 0.05$).

Nevertheless, when the B16F10 cells transfected with the murine HspBP1 sequence (B16moBP1) were injected subcutaneously in C57Bl/6 mice, tumor growth was again greatly impaired. Growth of B16moBP1 cells (mean tumor volume 0.13 mm^3) was reduced by approximately 150-fold compared to B16ve cells (mean tumor volume 20.18 mm^3) (Figure 2C), in agreement with our previous results with B16huBP1 cells. These results suggested that

overexpression of HspBP1 can impair melanoma growth *in vivo*, and that this effect was not caused by an immune response to epitopes in the human HspBP1 or related with the transfection method, since with B16huBP1 we used an stably transfection using tetracycline to induce expression and with B16muBP1 we used an transient transfection system.

HspBP1 and Hsp70 expression is inversely regulated in murine melanoma in vitro and in vivo

HspBP1 was first described as a co-chaperone of Hsp70, inhibiting its ATPase activity, thus we hypothesized that the mechanism through which HspBP1 impaired tumor growth *in vivo* was associated with its interaction with Hsp70. To investigate this hypothesis, HspBP1 and Hsp70 expression in wild type B16F10 cells was analyzed by western blot *in vitro* as well as *in vivo* after 3, 9, 15 and 20 days of tumor growth after subcutaneous injection. Interestingly, HspBP1 expression was found to be inversely correlated with Hsp70 expression in this system. On the one hand, HspBP1 levels were high in B16F10 cells *in vitro*, however *in vivo* they were considerably decreased as early as three days after injection, as shown in Figure 3A. On the other hand, Hsp70 expression presents almost exactly opposite dynamics. In vitro, Hsp70 expression by B16F10 cells was very low, sometimes undetectable, but as soon as the cells were injected *in vivo* the levels of Hsp70 expression were greatly upregulated (Figure 3A). This suggested the existence of a coordinate expression mechanism for these two proteins.

Figure 3

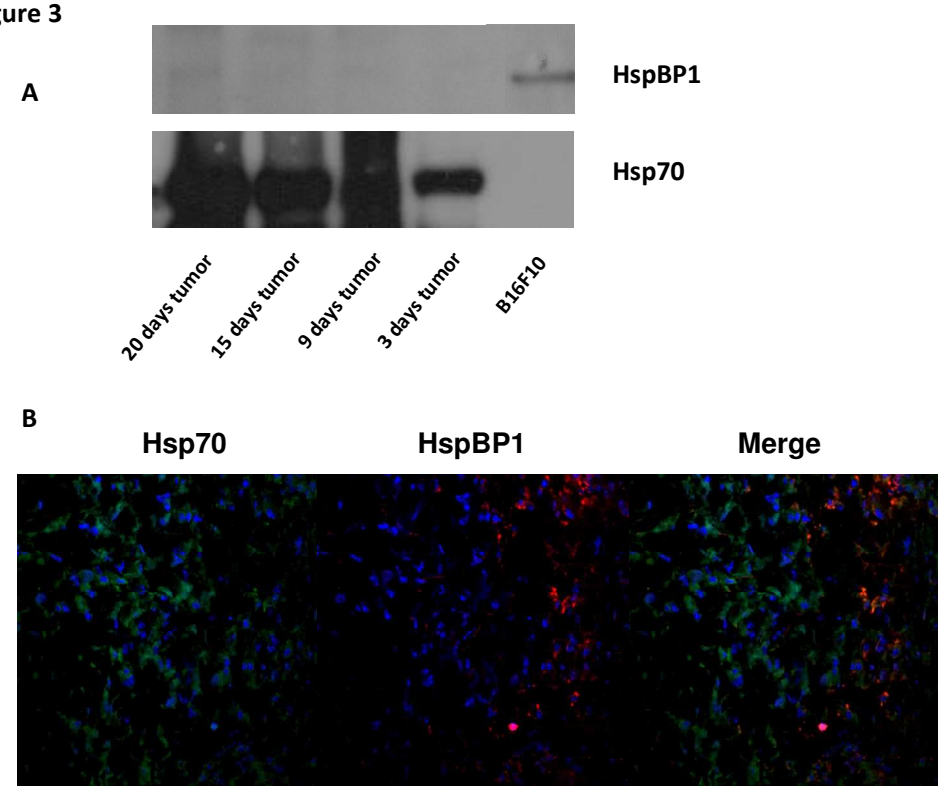

Figure 3: HspBP1 and Hsp70 expression in B16F10 melanoma *in vitro* and *in vivo*. A- Western blot analysis of expression of HspBP1 and Hsp70 in B16F10 in vitro and at different stages of tumor growth *in vivo*. B16F10 cells were injected at concentration of $5X10^5$ cells subcutaneously and at different time points (3, 9, 15 and 20 days) the mice were killed, the tumor was excised for analysis. **B**- Imunohistochemistry of 20 day- tumor sections. Staining of HspBP1 and Hsp70. Blue, Hoescht, green, Hsp70, red, HspBP1.

Because the expression of HspBP1 had a slight upregulation in 20-day tumors, we analyzed the expression of these two proteins by immunofluorescence. The results, shown in Figure 3B, indicate that while most tumor cells expressed Hsp70, only a few, possibly the expansion of a clone, were making HspBP1. Expression of HspBP1 and Hsp70 showed some co-

localization in the cells that did express HspBP1, as can be seen in Figure 3B in the merge picture.

Such results suggested that, in order for B16F10 melanoma cells to grow in vitro, HSP70 is not required. However, when these tumor cells are injected in a mouse, they have to face a series of stresses, such as shortage of nutrients and oxygen, as well as the immune responses that the mouse will develop against them. In this situation, Hsp70 is essential for tumor survival, thus expression of HspBP1 would have to be shut down. HspBP1 in vivo would probably associate with Hsp70, decreasing the survival potential of the tumor cells.

Overexpression of HspBP1 in stressed cells does not impair viability

To test this hypothesis, we analyzed the effect of overexpression of HspBP1 in stressed tumor cells. B16muBP1 cells were heat shocked for 2h at 40^{o}C, and subsequently left to recover at 37^{o}C for 6h. Some of the cells had been plated on coverslips, and immediately after recovery the coverslips were retrieved, fixed in acetone and analyzed by immunofluorescence for expression of Hsp70 and HspBP1. The rest of the treated cells remained in culture for 12h and were then assayed for survival using a MTT assay. The heat shocked B16muBP1 cells showed almost complete co-localization of Hsp70 and HspBP1 (Figure 4A). Interestingly, survival after heat shock was not affected by HspBP1 overexpression (Figure 4B). In a different assay, we heat shocked B16F10 cells that had Hsp70 expression silenced by siRNA (Figure 4C), and counted the living cells by Trypan blue exclusion in a haemocytometer. As expected, inhibition of Hsp70 production in heat shocked cells resulted in massive cell

death (Figure 4D). Taken together, these results indicated that the binding and inhibition of Hsp70 by HspBP1, and inhibiton of synthesis of Hsp70 by siRNA had different effect on cells under stress.

Figure 4

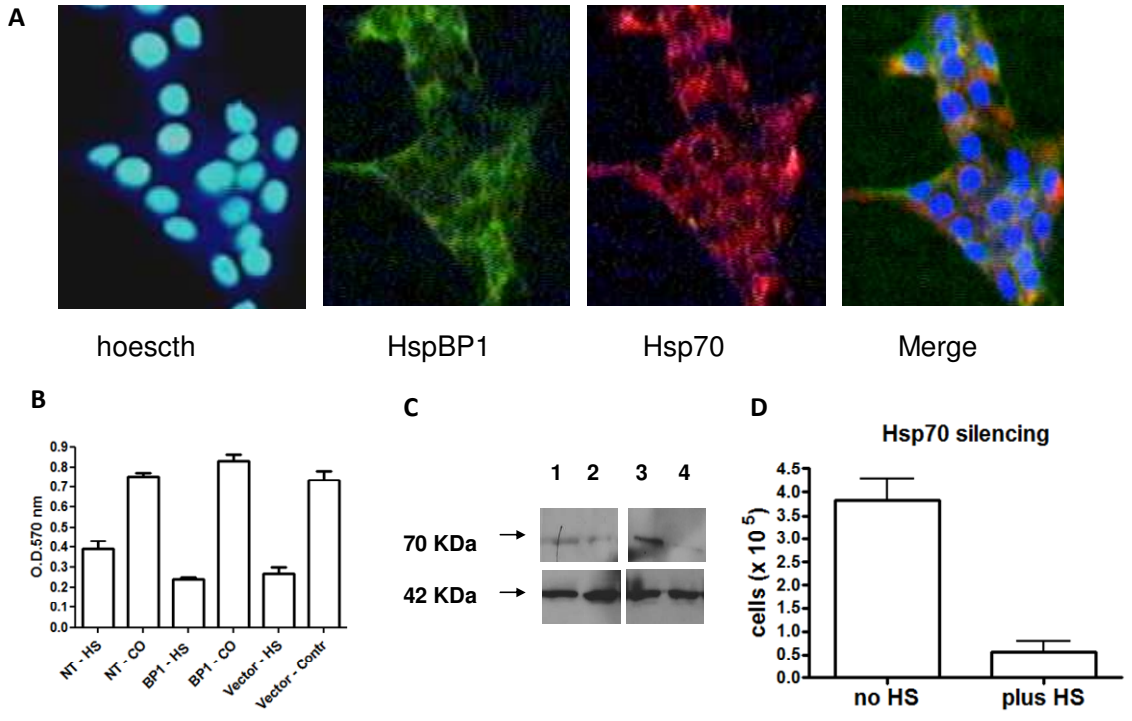

Figure 4: Inhibition of Hsp70 by HspBP1 not impairs survival of stressed cells. A- Staining for HspBP1 and Hsp70 expression in heat shocked B16F10 over-expressing murine HspBP1. Blue, Hoescht, green, HspBP1, red, Hsp70. **B.** MTT assay of B16F10 cells transiently transfected with plasmid encoding murine HspBP1 after heat shock showing the correspondent optical density (OD) for the proliferation/viability. **C**. Western blot analysis of silencing Hsp70 with siRNA in heat shocked B16F10 cells, 1 and 3, untreated cells, 2 and 4, treated cells. For 70 kDa- Hsp70 expression in the samples; 42 kDa- actin expression. **D.** Number of the viable B16F10 cells determined by trypan staining in vitro after Hsp70 silencing, with or without heat shock.

Impairment of tumor growth by HspBP1 is associated with the immune response

The expression of Hsp70 by tumors has been extensively demonstrated to confer multiple advantages for survival. Intracellular Hsp70 when can bind to different pro-apoptotic factors, inhibiting both intrinsic (Mosser, Caron *et al.*, 2000; Stankiewicz, Lachapelle *et al.*, 2005) and extrinsic (Jaattela, Wissing *et al.*, 1992; Park, Lee *et al.*, 2001; Clemons, Buzzard *et al.*, 2005; Didelot, Schmitt *et al.*, 2006) apoptotic pathways. In addition, there is evidence that Hsp70 can influence immune responses. Some studies have also indicate a stimulatory effect of Hsp70 over immune responses, Hsp70 is outside the cells or in the membrane could stimulate the immune system carrying peptides to presentation routes (Arnold-Schild, Hanau *et al.*, 1999; Binder, Vatner *et al.*, 2004) or activating NK cells (Gross, Koelch *et al.*, 2003; Gross, Schmidt-Wolf *et al.*, 2003). Conversely, others suggest that this protein might have an inhibitory effect over different immune cells (Van Roon, Van Eden *et al.*, 1997; De Kleer, Kamphuis *et al.*, 2003; Van Eden, Van Der Zee *et al.*, 2005; Wieten, Broere *et al.*, 2007).

To investigate if HspBP1 inhibition of tumor growth was related to immune modulation, we performed in vitro and in vivo experiments. We reasoned that probably one of the first immune responses faced in vivo by tumor cells was the encounter with macrophages or dendritic cells, and/or the production of TNF-α by these and other immune cells. In order to test that idea, we co-cultured B16F10 cells with bone marrow-derived DC (BMDC).

Alternatively, we incubated B16F10 cells with different concentrations of TNF-α. The results shown in Figure 5 A and B indicate that these stimuli had only a slight effect in the expression of HspBP1 (decreasing it approximately 2-fold) or Hsp70 (in which a slight upregulation was observed). That suggested that some modulation of the expression of these two proteins could be performed by these elements of the innate response.

Because we had previously observed that over-expression of HspBP1 impair tumor growth in wild type C57Bl/6 mice, we hypothesized that this could be related to *in vivo* modulation of the adaptive immune response by either HspBP1 itself or its effect over Hsp70. We tested this hypothesis in two independent experiments. First, we injected B16muBP1 cells in RAG-/- mice or control wild type mice, and followed tumor growth for 25 days. The tumors never grew in the immunocompetent mice during this time, however clearly we could observe tumor growth in the immunodeficient RAG -/- mice (Figure 5C). In the second experiment, we injected HT1080 stably transfected with the plasmid overexpressing HspBP1 under tetracycline control into SCID mice, with one group receiving the antibiotic in the water and the other not. Clearly, in the SCID mice, overexpression of HspBP1 by the tumor does not affect its growth in vivo (Figure 5D). Collectively, these results suggested that the modulation of tumor growth by overexpression of HspBP1 was connected to the presence of an intact adaptive immune

response.

Figure 5

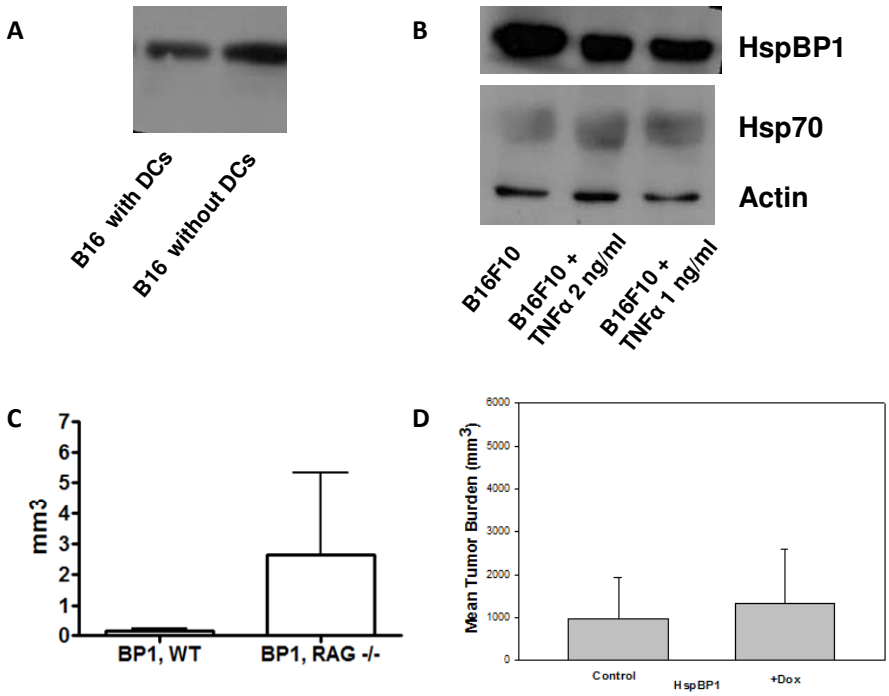

Figure 5: HspBP1 expression in B16F10 in vitro after culture with BMDCs or TNF-α and Tumor growth of HspBP1 over-expressing B16F10 cells in vivo in immunocompromise mice. A- Western blot analysis of HspBP1 expression after co-culture bone marrow dendritic cells. **B-** HspBP1 and Hsp70 expression of B16F10 after culture with TNF-α for 48hs. **C-** Tumor volume (mm^3) of B16 BP1 and B16 vector after 25 days of tumor growth in C57 and Rag¨/¨ mice (Mann-Whitney $p < 0.05$). **D.** Tumor volume (mm^3) of HspBP1 induced or not in HT1080 cells injected into SCID mice after 25 days of tumor growth.

DISCUSSION

The results of this study suggest that in order to grow *in vivo,* tumors must downregulate the expression of HspBP1. Also, they must upregulate expression of Hsp70. Finally, they indicate that this is related to modulation of the adaptive immune response. Therefore, we propose that HspBP1 could be a new therapy target in melanoma.

We suggest that the mechanism related to the role of HspBP1 in tumor growth impairment it is probably associated with its interaction with Hsp70, given that HspBP1 and Hsp70 expression are inversely regulated in this tumor model in vitro and in vivo, and that they appear to co-localize when expressed in the same cell. Tumor growth *in vivo* is certainly a complex and dynamic process, and probably the cells that express more Hsp70 and less HspBP1 will be selected for survival. These results agree with our previous observation that low levels of HspBP1 were associated to poor prognosis in breast cancer patients (Souza, Albuquerque *et al.*, 2009), confirming that HspBP1 could have a role in tumor progression. Many studies have demonstrated that Hsp70 increased the tumorigenic potential of cancer cells, due to its pleiotropic activities as a chaperone (Jaattela, 1995; Calderwood, Khaleque *et al.*, 2006) as well as its anti-apoptotic properties (Gurbuxani, Bruey *et al.*, 2001; Zhu, Xu *et al.*, 2009). Also, other studies suggested that inhibitition of Hsp70 is by siRNA in tumor cells impaired tumor growth (Xiang, Li *et al.*, 2008). In addition, inhibition of HSF1, the transcriptional factor for Hsp70, in tumor cells could be an anticancer therapy (Whitesell e Lindquist, 2009), agreeing with our observation that higher levels of Hsp70 are important for tumor growth.

Previous studies demonstrated that the expression of HspBP1 was not intrinsically linked to Hsp70 expression in cell lines (Gottwald, Herschbach *et al.*, 2006). Stimuli that usually modulated Hsp70 expression, such as heat shock and induction of apoptosis did not interfere in HspBP1 expression, and the over-expression of HspBP1 *per se* did not increase or decrease the expression of Hsp70 (Gottwald, Herschbach *et al.*, 2006). However, it was recently demonstrated that A431 human squamous carcinoma cells accumulated Hsp72 and HspBP1 in chromogranin A- positive granules following heat stress, or in the presence of an inhibitor of phospholipase C (Evdonin, Kinev *et al.*, 2009). Yet, it still remains to be evaluated what is the transcriptional factor that regulates the expression of HspBP1 and its association with Hsp70 transcription. We showed here that dendritic cells (DC) could be involved in the regulation of the expression of HspBP1 in melanoma tumor *in vivo*, since when we cultured bone marrow DC bearing a mature phenotype with melanoma cells in vitro we observed a little alteration on expression of HspBP1. DC have a pivotal role in immune responses, are the most potent antigen-presenting cells, they are distribution in peripheral tissue and upon activation they migrate into secondary lymphoid organs, where they are capable of inducing primary T-cell responses or tolerance (Banchereau, Briere *et al.*, 2000; Banchereau, Palucka *et al.*, 2001). These cells are one of the first ones to be in contact with the antigen and then produced a lot of different cytokines, including TNF-α. We also demonstrated that TNF-α could be related with this regulation of expression of HspBP1 and Hsp70, even though just a slight alteration of these protein expression occurred in B16F10 in vitro after the addition of this cytokine. TNF-α

is immune cytokine involved in primary inflammatory response (Balkwill, 2009) and is a major mediator of cancer-related inflammation (Sethi, Sung *et al.*, 2008; Balkwill, 2009); participating of the angiogenic process (Li, Vincent *et al.*, 2009) and induced directed DNA damage (Yan, Wang *et al.*, 2006). Furthermore, TNF-α is related to tumor-promoting by activation of NFK-β signaling (Greten e Karin, 2004; Lee, Kuo *et al.*, 2007). This is related to tumor aggressiveness given that HspBP1 was decreased in presence, similar to what happened *in vivo*.

It was recently observed that over expression of HspBP1, by antagonizing the pro-survival activity of Hsp70 sensitizes tumor cells to cathepsin-mediated cell death (Tanimura, Hirano *et al.*, 2007). Here we showed that over-expression of HspBP1 does not alter the survival rate of cells after heat shock (a common apoptotic stimulus) suggesting that in our model HspBP1 is not facilitating apoptosis by inhibiting Hsp70. Nevertheless, we demonstrated that the mechanism by which high levels HspBP1 impaired melanoma growth is related with the adaptative immune responses since this inhibition was not observed in immune-compromised mice or *in vitro.* We believe that the inhibition of Hsp70 by HspBP1 allows the tumor to become more immunogenic. It is well established that the adaptative immune response can play an important role in tumor control (Koebel, Vermi *et al.*, 2007). On the one hand, expression of Hsp70 in tumor cells has been proposed to enhance their immunogenicity. However, on the other hand, Hsp70 has also been demonstrated to prevent tumor cell death, a key process for the development of

tumor cell immunogenicity. Previous studies showed that when Hsp70 is silenced, tumor immunogenicity is increased: subcutaneous injection of cells with silenced Hsp70 induced tumors that rapidly regressed in syngeneic rats while they grew normally in nude mice (Gurbuxani, Bruey *et al.*, 2001). This study also demonstrated that tumor cells in which Hsp70 was silenced were more sensitive to NO-mediated, caspase-dependent, machophage cytotoxicity in vivo (Gurbuxani, Bruey *et al.*, 2001). Our results agreed with that study, since when we over-expressed HspBP1, the tumor did not grow in immunocompetent mice, but grew in immunodeficient mice.

We did not evaluate alterations in tumor specific immune responses to melanoma antigens in our tumor model when HspBP1 was over-expressed. An interesting point to consider is if HspBP1 can act as a tumor antigen. We cannot establish that an anti-HspBP1 immune response is present in this system. However, in our previously study with HspBP1 in breast cancer patients we did not found any alterations in the present of anti-HspBP1 antibodies in the serum of the patients comparing to healthy individuals (Souza, Albuquerque *et al.*, 2009). It is remains to be determined if the presence of anti-HspBP1 specific immune response is related to tumor regression. In conclusion, we believe, based on the information provided by this study, that as well as TNF-α and DC, other components of the immune system provide selective pressures over tumor growth. The decrease in HspBP1 expression, and the concomitant increase inHsp70 expression could provide an escape mechanism for the tumor cells. When we over expressing HspBP1, artificially, we altered this scenario

and consequently impaired tumor growth. This possibly occurred because Hsp70 is suppressed by HspBP1, dampening the survival advantage of Hsp70 expressing-tumor cells, allowing that adaptative immune response to act and consequently reducing tumor growth. We are currently investigating this possibility.

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CAPÍTULO 5

Artigo Científico (revisão)

Tumor immunosuppressive environment: effects on tumor –specific and non-tumor antigen immune responses

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Tumor immunosuppressive environment: effects on tumor-specific and nontumor antigen immune responses

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The interactions between cancer cells and host immune cells in tumoral microenvironments create an immunosuppressive network that promotes tumor growth, protects the tumor from immune attack and attenuates the efficacy of immunotherapeutic approaches. The development of immune tolerance becomes predominant in the immune system of patients with advanced-stage tumors. Several mechanisms have been described by which tumors can suppress the immune system, including secretion of cytokines, alterations in antigen-presenting cell subsets, costimulatory and coinhibitory molecule alterations and altered ratios of Tregs to effector T cells. It is well demonstrated that these mechanisms of immunosuppression can impair tumor specific immune responses. However, it is not well established whether this immunosuppressive environment can affect immune responses to nontumor antigens, specifically in regard to priming and the development of memory. The few existing studies indicate that responses to nontumor antigens seem unaffected, although there is still a deep lack of understanding of this phenomenon. This is an important issue regarding patient endurance and quality of life. Here, we review the existing evidence on immunosuppression promoted by tumors, with particular attention to its impact on specific immune responses. Understanding these interactions can help us subvert tumor-induced tolerance and optimize anti-tumor therapy.

KEYWORDS: immunosuppression • tumor • tumor-nonspecific immunosuppression • tumor-specific immunosuppression

Mechanisms of tumor immune suppression

Human tumorigenesis is a multistep process that, similarly to chronic infection, might occur over several years [1]. In 1957, the immunological resistance of the host against the development of cancer was postulated by Burnet and Thomas, and called tumor immunosurveillance. Almost immediately, this hypothesis was criticized owing to the lack of direct experimental evidence [2,3]. However, over time, data from several groups supporting the existence of anti-tumor immune responses, and the immunosurveillance hypothesis have led to the concept of immunoediting [4–7]. According to this idea, the process of cancer immunoediting has three phases, referred to as the three Es. The first phase, elimination, refers essentially to cancer immunosurveillance, in which cells of the innate and adaptive immune response recognize and destroy developing tumors, thus protecting the host against cancer. The second phase, equilibrium, is a protracted period in which the tumor and the immune system enter a dynamic equilibrium. In the third phase, escape, tumor variants that emerge from immune selection during the equilibrium phase develop into clinically apparent tumors that grow in immunocompetent hosts [4–7]. Interestingly, 40% of tumors derived from $Rag^{-/-}$ mice (mouse strains without T and B cells) are rejected when transplanted to wild-type recipients [8], suggesting that the adaptive immune system is a strong selective pressure driving tumor editing. Also, tumor rejection has been demonstrated in $Rag^{-/-}$ mice when tumor-specific T cells, but not irrelevant T cells, are adoptively transferred to these mice [9].

Indeed, it is well-known nowadays that the immune system naturally acquires the ability to recognize cancer cells [10,11], but it is still

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disputed if it is able to control malignant growth, prompting the question of how rare an event spontaneous tumor eradication is [12]. This paradox could be explained either by a generalized immunodeficiency associated with the tumor-bearing status, or by modulatory properties of cancer and/or bystander cells that hamper immune function within the tumor microenvironment [13,14]. It was originally thought that poor specific immunity to tumor-associated antigens was due to a passive process whereby adaptive immunity is prevented from detecting tumor-associated antigens because most of them are self-antigens; however, recent evidence suggests that what probably ensues is an active process of immune tolerance, directed by the tumor [13].

Thus, if the goal is to produce immunity in a cancer patient, either naturally or experimentally, against a tumor or nontumor antigen, the mechanisms employed by the tumor to achieve tolerance must first be determined in order to be overcome. While tumor immune resistance mechanisms may allow tumors to grow even in the presence of strong immune responses, anti-tumor therapy must have a comprehensive design, targeting the diversity of adaptations that tumors can display, owing to the impressive plasticity of cancer cells.

Soluble immunosuppressive factors secreted by tumors

The development of immune tolerance within the tumor microenvironment is a complex process and involves interactions between several different T cell types and soluble factors secreted by these cells. Tumor microenvironments express abundant cytokines and growth factors with immunosuppressive effects, such as VEGF [15], IL-10 [16,17], TGF-β [18,19], macrophage colony-stimulating factor [20,21] and gangliosides [22–25].

The cytokines produced within the tumor microenvironment block differentiation and maturation of dendritic cells (DCs) [13]. DCs are the most potent antigen-presenting cells (APCs), capable of inducing primary and boosting secondary T-cell responses, when bearing a mature phenotype [26,27]. There are two main subsets of DCs, myeloid and plasmacytoid [28]. VEGF was the first tumor-derived molecule reported to suppress DC differentiation and maturation, as described previously [15]. VEGF affected the ability of hemopoietic progenitor cells to differentiate into functional DCs during the early stages of their maturation by directly binding to APCs and inhibiting nuclear factor-κB (NF-κB) dependent activation of reporter gene transcription during the first 24 h in culture [29]. Cancer cells can be major producers of VEGF [30,31], such as ovarian cancer [31], esophageal squamous cell carcinoma [32], hepatocellular carcinoma [33], lymphoma [34] and nasopharyngeal carcinoma [35].

Another important secreted factor is tumor-derived IL-10, which can act directly on DCs and T cells, suppressing the production of TNF-α and INF-γ, yielding tolerogenic DCs that induce tumor-specific anergy [16,36]. When murine colon carcinoma (Colon 26) cells that have been retrovirally transduced with the murine *IL-10* gene (Colon 26/IL-10) were inoculated in syngeneic immunocompetent or T-cell-defective nude mice, growth of Colon 26/IL-10 tumors was augmented in immunocompetent and, to a lesser extent, in nude mice compared with that of wild-type tumors developed in the respective mice. Tumor production of IL-10 may inhibit a broad range of macrophage functions, including cytotoxicity and production of IL-12 [37]. Plasmacytoid DCs have been shown, within the tumor environment, to induce significant IL-10 production by T cells. This suppresses myeloid DC-induced specific T-cell effector function, and can lead to the differentiation of T cells with regulatory phenotype (see later) [38].

It has been shown that TGF-β is produced by many different tumors, such as melanoma [18,19], glioma [39], esophageal cancer [40], colorectal cancer [41] and breast cancer [42]. TGF-β regulates the development and function of DCs in the tumor microenvironment and it is involved with the hyporesponsiveness of $CD4^+$ (T helper) and $CD8^+$ (cytotoxic T lymphocytes) T cells, natural killer (NK) cells and B cells [43,44]. TGF-β has pleiotropic effects, influencing the expression of several genes that are important for T-cell function. It can suppress T-cell proliferation by inhibiting IL-2 production [43], as well as preventing the release of lytic granules by $CD8^+$ cytotoxic T cells [45]. Although the expression of different cyclin and kinase genes is also modulated by TGF-β in T cells, the actual mechanisms and effects of this modulation are still not completely understood. In DCs, TGF-β can downregulate the expression of MHC class II, as well as costimulatory molecules [46]. Tumors that express virus antigens, such as in cervical cancer caused by infection of human papillomavirus (HPV), can also develop immunosuppressive mechanisms to suppress tumor-specific immune responses [47]. It has been demonstrated that cervical cancer cells can secrete both IL-10 and TGF-β [48,49].

Human neuroblastoma [23], melanoma [24] and lung cancer [50] cells express high levels of gangliosides, which also suppress DC differentiation [22–25]. Gangliosides are sialic acid-containing glycosphingolipids that modulate several cellular functions, such as proliferation, cell adhesion, signal transduction and apoptosis. In addition, tumor glycoproteins, such as carcinoembryonic antigen (CEA) and mucin 1 released by breast cancer cells, interact with C-type lectins on the surface of the DCs. The glycoprotein–lectin complex is endocytosed by the DCs; however, it is mostly retained in early endosomes leading to inefficient processing and presentation of these tumor antigens to T cells [51,52], and consequently to a poor anti-tumor T-cell response.

Also, paradoxically, production of inflammatory mediators can lead to local immunosuppression by tumors, for example, COX-2 [53,54], prostaglandin $(PG)E_2$ [55–57] and IL-6 [58]. A range of human tumors express high levels of COX-2, which has been implicated in tumor progression, such as glioma [53,54], breast cancer [59], oral cancer [60], endometrial carcinoma [61] and ovarian cancer [62]. COX-2 expression has been demonstrated to be a strong predictor of poor outcome for glioma patients [53]. The strong correlation between COX-2 and angiogenesis suggests that tumor vasculature is involved in this process, and COX-2 inhibitors strongly improved the response to radiation in murine gliomas [63].

COX can promote PGE_2 production in the tumor environment. Inflammatory factors, including arachidonic acid metabolites such as PGE_2, might contribute to malignant progression through enhancing angiogenesis [64] and inhibiting apoptosis

[65], thus constituting one of the chronic inflammatory processes that are associated with cancer promoton [66]. However, it was demonstrated that PGE_2 also contributes to cancer progression by inhibiting DC differentiation and function, acting paradoxically as an immunosuppressive factor [55,56,67,68]. Immature DCs have an impaired ability to migrate to the lymph node in the presence of PGE_2 compared with DCs generated *in vitro* in the absence of PGE_2, showing a cytokine production profile and phenotypical features of tolerogenic DCs [57]. PGE_2 has been demonstrated to affect the migration potential of DCs in cervical preneoplastic lesions [57], as well as to induce a tolerogenic cytokine profile in local resident DCs.

IL-6 derived from tumor cells and macrophages from the tumor microenvironment was shown to have an inhibitory effect over DC differentiation, rather favoring differentiation of monocytes into macrophages, with high phagocytic capacity but poor APC function [20,21,69]. This effect was found to be associated with local production of macrophage colony-stimulating factor, itself a macrophage differentiation-promoting cytokine. IL-6, the major acute-phase cytokine, has also been found to suppress DC maturation *in vivo* [70]. Involvement of IL-6 in impairment of DC functions has also been shown in patients with multiple myeloma [58]. High levels of IL-6 can be found in the peripheral blood and malignant ascites of patients with ovarian cancer [71]. IL-6 is thus another example of an inflammatory factor that can present immunoinhibitory properties within tumor microenvironments. Although this cytokine can inhibit suppressive Treg induction [72] and stimulate the $CD4^+$ T cells into IL-17 producing (Th17) effector cells instead [73,74], it can also act as a DC suppressor. In a model of colitis-associated cancer, IL-6 has been demonstrated to enhance proliferation of tumor-initiating cells and protect promalignant T cells from apoptosis [75]. IL-6 can enhance tumor attachment and proliferation of tumor cells, most probably through PI3K activation mechanisms, thus interfering with the differentiation of monocytes into DCs [76,77]. IFN-γ is an effector cytokine released by tumor-specific effector T cells: however, it has been demonstrated that this immunostimulatory cytokine can also stimulate B7-H1 expression on different tumor cells *in vitro*, leading to immunosuppression [78]. In addition, monocyte-derived macrophages upregulate indoleamine 2,3-dioxygenase (IDO) upon activation with IFN-γ [79]. Finally, it was demonstrated that the presence of IFN-γ was responsible for nitric oxide (NO) production by myeloid suppressive cells, contributing to its immunosuppressive mechanisms [80–82] (see next section).

More recently, the expression of soluble CD83 by tumor cells was described to induce immunosuppression. It was demonstrated that local secretion/shedding of CD83 by melanoma cells could impair the activation of T cells [83]. CD83, a marker of DC activation, can be expressed by lung cancer cells and several human tumor cell lines [84].

These results suggest that the presence of inflammatory mediators in the tumor microenvironment does not always result in the induction of APC activation, which should result in an anti-tumor immune response. Such puzzling findings suggest that we are still far from understanding the intricacy of cytokine interactions in a tumor patient.

Recruitment of cells with immunosuppressive functions

Many tumor types have been shown to promote the expansion of myeloid-derived suppressor cells (MDSCs), such as head and neck squamous-cell carcinoma, non-small-cell lung carcinoma and metastatic adenocarcinomas of the pancreas, colon and breast [85,86]. In mice, MDSCs commonly coexpress the CD11b and Gr-1 differentiation markers [87,88], and can promote immunosuppression by the expression of VEGF, IL-10, IL-6, colony-stimulating factor 1 and PGE2 [89–92]. Different phenotypic subsets of MDSC-like cells have been identified in human cancer patients, including those that express CD11b and CD15 [91,93] or others that lack CD11b but express CD33 and CD34 [86,94]. MDSCs infiltrate tumor masses and migrate to

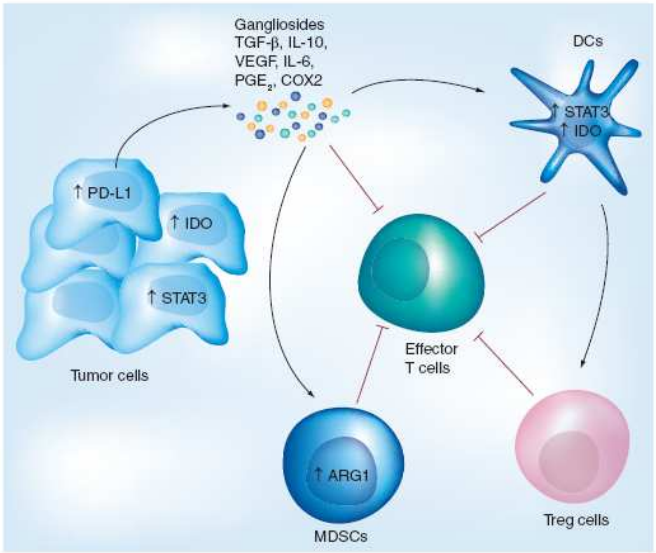

Figure 1. Summary of the immunosuppressive mechanisms used by tumors that act in concert to counteract effective immune responses.
DC: Dendritic cell; IDO: Indoleamine; MDSC: Myeloid-derived suppressor cells; PD: Programmed death; PG: Prostaglandin.

tumor-draining lymph nodes resulting in deficient anti-tumor T-cell priming [95,96]. This occurs through different mechanisms, including secretion of TGF-β [97] and production of inhibitory enzymes involved in L-arginine metabolism, such as arginase 1), which leads to arginine depletion. L-arginine is essential for T-cell function, including the optimal use of IL-2 and the development of a T-cell memory phenotype [96]. Tumor-educated regulatory DCs were recently described that express ARG-1 induced by PGE_2, suppressing T-cell response in a model of murine lung tumor [98]. Other studies verified that MSDCs may overexpress inducible NO synthase 2, the enzyme that directs NO synthesis, resulting in the generation of reactive oxygen species such as NO, which ultimately alter T-cell signaling, activation and eventually their survival [87,88,91,99].

Thus, DCs recruited into tumor microenvironments undergo changes that endow them with a regulatory phenotype, favorable to the tumor. Immature or partially differentiated DCs induce either T-cell unresponsiveness [100] or Tregs [101,102]. Also, melanoma and colon carcinoma cells convert immature myeloid DCs into TGF-β-secreting cells, and these induce Treg proliferation [103]. Tregs are distributed into two main categories [104]. The first group includes those that are continuously produced by the thymus and express the specific Treg transcription factor forkhead box 3 (Foxp3). These are mainly $CD4^+CD25^+$ Tregs, which can also express the cytotoxic T-lymphocyte antigen 4 and glucocorticoid-induced TNF receptor inhibitor markers, best defined by their ability to suppress effector T-cell function in both animal tumor models and patients bearing a variety of tumor types [105,106]. The second group includes the Tregs that arise as a result of peripheral encounters with regulatory DCs, and these Tregs are IL-10-producing, Foxp3-negative Tr1 cells [17] or TGF-β-producing Th3 cells [107]. Tregs are activated in an antigen-specific manner, but are generally believed to suppress T cells in an antigen-nonspecific manner [108]. Tumor-induced Tregs primarily reside in tumor tissues and, to a lesser extent, in local draining lymph nodes and circulating blood, and Treg production in tumor patients is found to be independent of thymic function [109]. Recently, studies demonstrated a new subset of Tregs, $CD69^+CD4^+CD25^-$ cells that increase dramatically along tumor progression and suppress T-cell proliferation through membrane-bound TGF-β1 in BALB/c mice [110]. It was demonstrated that $CD4^+$ Tregs are the major regulators of concomitant tumor immunity, a phenomenon in which a host with a progressive tumor rejects the same tumor at a remote site, described for weakly immunogenic tumors, such as murine melanoma B16F10 [111]. Depletion of $CD4^+CD25^+$ T cells *in vivo* by anti-CD25 antibody (PC61) before tumor challenge enhances natural tumor immunosurveillance and induces rejection of multiple immunogenic tumors in multiple strains of mice [112]. In summary, several mechanisms have been described for Treg-mediated inhibition of tumor immunosurveillance, as well as of vaccine-induced anti-tumor immunity in mouse models [113].

Supporting these observations in murine studies, it has been found that patients with different tumors, such as lung cancer [114], pancreas and breast cancer [115], leukemia [116] and melanoma [117], have increased numbers of both peripherally circulating and tumor-associated $CD4^+CD25^+$ Tregs compared with healthy controls [114,115,118]. $Foxp3^+$ Tregs are also recruited in cervical cancer associated with HPV infection [119]. Furthermore, in patients with gastrointestinal malignancies of different stages, the percentage of Tregs in peripheral blood, but not their absolute number, correlated inversely with disease prognosis [120]. The mechanisms that recruit Tregs to the ovarian tumor site probably include alteration in the expression of chemokines, such as CCL22 [106]. Different strategies have been used, aiming to disturb $CD4^+CD25^+$ T-cell activity, such as depletion of these cells using monoclonal anti-CD25 antibodies. However, a recent study showed that intratumoral injection of the agonist anti-OX40 monoclonal antibody OX86, but not anti-CD25 antibody, induced tumor rejection in mice, an effect that was abrogated by treatment with anti-CD8 antibody [121]. Upon anti-OX40 injection, DCs migrated to the draining lymph node resulting in anti-tumor $CD8^+$ T-cell depletion, suggesting that OX-40 ligation has multiple effects that converge to achieve tumor regression.

Finally, similarly to $CD4^+CD25^+$ Tregs, it has recently been shown that $CD8^+CD28^-$ cells also have a suppressive effect on T-cell function in animal models of autoimmunity [122,123]. Several distinct subtypes of $CD8^+$ suppressor cells have been described in these models [124]. In a xenograft model of human synovium, $CD8^+CD28^-CD56^+$ T cells effectively suppressed rheumatoid inflammation. Underlying mechanisms for the immunosuppressive action of these cells involve conditioning of APCs via secretion of TGF-β and IL-16, as well as interference with tryptophan metabolism and programmed death receptor (PD)1 expression (see next section for details). These $CD8^+$ regulatory cells were shown to present immunosuppressive properties in patients with prostate cancer, inhibiting T-cell proliferation [125] and are present in higher amounts in the peripheral blood of advanced lung cancer patients [126]. Fortunately, immunosuppression by these cells appears to be reversible through ligation of Toll-like receptors [125].

Membrane/cytosolic immunosuppressive molecules induced by tumors

Many human tumors (particularly melanoma [127], prostate [128], colon, gastric and renal [42], uterine [129] and pancreatic carcinomas [130], but also DCs [131] constitutively express IDO, a cytosolic enzyme that depletes the amino acid tryptophan, reducing access to free tryptophan, essential for T-cell proliferation, and consequently blocking cell cycle progression of T-cells. This, in turn, prevents the clonal expansion of T cells and promotes T-cell death by apoptosis, anergy or immune deviation [79,129,132]. Locally produced IDO can impair the proliferation of $CD8^+$ T cells at the tumor site [132] and promote the apoptosis of $CD4^+$ T cells, resulting in resistance to immune-mediated rejection of the tumor cells [133]. IDO^+ DCs have been found in breast tumor tissue and tumor-draining lymph nodes in patients with melanoma and patients with breast, colon, lung and pancreatic cancers [79]. An important role for IDO in the regional downregulation of immune function in early-stage melanoma is indicated by the presence of IDO-expressing DCs in tumor-draining lymph nodes

[79]. Studies *in vitro* have shown that the induction of immunosuppressive IDO$^+$ DCs can be coregulated by IFN-γ and IL-10; the stimulation of mature DCs with IFN-γ decreasing IDO expression, while IFN-γ stimulation of DCs matured in the presence of IL-10 increases IDO expression [79]. Interestingly, positive correlations between IFN-γ, IL-10 and IDO expression in the tumor-draining lymph nodes of a patient with melanoma were recently described [134].

Programmed death receptor-1 is a molecule expressed on membranes of a broad range of immune cells, including mature T and B cells, thymocytes and myeloid cells [135]. Upon interaction with either of its two ligands, PD-L1 (also known as B7-H1) or PD-L2, PD1 has been described to negatively regulate the proliferation and cytokine production of T cells [136,137]. PD-L1 is strongly expressed by a variety of tumors, including esophageal carcinoma [138], renal cancer [139], melanoma [140], hepatocellular carcinoma [141], brain tumors [142], leukemia and lymphoma [143], promoting apoptosis of CD8$^+$ T cells, and its expression inversely correlates with patient prognosis [78,144]. PD-L1 (B7-H1) is a recently described member of the B7 family that abrogates T-cell immunity and is expressed in certain cancer types, including ovarian, renal and mammary carcinomas [145–148]. In addition, B7-H3 inhibits immune responses and is expressed on several tumor cell lines [149,150]. Soluble factors, such as IL-10 within the tumor microenvironment, selectively modulate the expression of B7 family members so as to incline the balance towards immune suppression: coinhibitory molecules (B7-H1 and B7-H4) are upregulated and costimulatory molecules (B7.1 and B7.2) are downregulated [13].

Expression of HLA-G [151] and HLA-E [152] by tumor cells can also contribute to tumor immunosuppression. HLA-G belongs to the family of nonclassical MHC class I (class Ib) genes, plays a pivotal role in maternal–fetal tolerance and is characterized by a broad spectrum of immunosuppressive functions, which include either the inhibition of effector cells or prevention of immune responses through interactions with DCs and Tregs [153]. In cancer, HLA-G expression has been found in various malignancies, such as renal carcinoma [154], ovarian tumors [155], breast cancer [156] and leukemia [157].

Galectin-1, a glycan-binding protein, is present in different tumor types and cancer-associated stroma, and its expression levels correlate with the aggressiveness of these tumors and the acquisition of a metastatic phenotype [158]. Galectin-1 inhibits T-cell effector functions by inducing T-cell apoptosis [159], sensitizing T cells to FasL-induced cell death [160] and blocking proximal T-cell receptor (TCR) signaling [161].

Tumor cells of different histological types can release intact vesicular membrane organelles known as microvesicles or exosomes [162,163], characterized by a specific protein profile. Human melanoma and colorectal carcinoma cell-released microvesicles can promote the differentiation of myeloid cells with TGF-β-mediated suppressive activity on T cells [164].

In a similar manner to that discussed in the previous section for soluble cytokines, tumor molecules that are immunostimulatory can also display immunosuppressive effects. NKG2D is an activating receptor that is expressed by T cells, as well as by NK cells and macrophages. NKG2D-ligand expressing tumor cells (as well as soluble NKG2D ligands that are shed from tumor cells) can downregulate NKG2D expression by CD8$^+$ T cells and NK cells, or uncouple NKG2D signaling from intracellular mobilization of Ca^{2+} or cell-mediated cytolysis, thereby contributing to suppression of the immune response [165]. The expression of NKG2D ligands (which stimulate an immune response at the initial stages of oncogenesis in methylcholanthrene-induced fibrosarcoma [166]) seems to be immunosuppressive when it is sustained and localized in a skin cancer model induced by the chemical carcinogen dimethylbenzanthracene. [167].

Tumor-induced transcription factors with immunosuppressive effects

An additional mechanism of tumor immunosuppression is the expression of transcription factors by tumor and immune cells. Signal transducer and activator of transcription (STAT)3 is constitutively activated in diverse cancers [168], including melanomas [169], and it is also activated in DCs and macrophages [170]. STAT3 has been shown to inhibit Th1 immune responses and to favor Th17 immune responses through increased IL-23 production, a cytokine recently shown to significantly inhibit cancer immunosurveillance by CD8$^+$ T cells [171]. STAT3 may also play a role in the generation of tumor infiltrating Tregs. Indeed, mice with STAT3-deficient hematopoetic cells have significantly fewer tumor infiltrating Tregs [170]. Tumor-associated Tregs express IL-23 receptor, which activates STAT3 in this cell type, leading to upregulation of Foxp3 and the immunosuppressive cytokine IL-10 [172]. Constitutive STAT3 activity in tumor cells induces production of factors such as VEGF and IL-10 [15,173].

Recent studies have also identified the suppressor of cytokine signaling 1 as playing an important role in the regulation of APC function, as inhibition of this molecule by specific siRNA resulted in breaking of tolerance towards a tumor antigen [174]. Other biochemical signaling events, seen in a subset of advanced melanomas, include activation of Akt [175] and constitutive cleavage of Notch family members [176], which may be functionally linked [177]. It will be of interest to determine whether activation of these signaling events is related to the establishment of an immune evasive tumor microenvironment. Moreover, Snail is thought to be a major transcription factor frequently involved in various cancers. It was recently demonstrated that after Snail transduction murine and human melanoma cells induced Tregs and impaired DCs *in vitro* and *in vivo* [178].

Immunosuppression in tumor-bearing hosts

Given all the reports on immunosuppressive molecules produced by tumors reviewed above (for a summary see Figure 1), it is surprising that little evidence is available regarding an overall immunosuppressed condition for cancer patients or tumor-bearing animals. In general, tumor patients are not considered immunosuppressed individuals, with the exception of those who were already immunocompromised by other causes when cancer developed, or cancer patients that underwent chemotherapy. Only recently has the possibility that a general state of

immunosuppression was present in tumor-bearing hosts received some attention. While most of the studies on this subject focus on tumor-specific immunosuppression, practically no information is available regarding a compromised ability of tumor-bearing individuals to mount immune responses to nontumor antigens.

Anti-tumor responses in tumor-bearing hosts: evidence of immunosuppression

Different studies have indicated that T cells are, indeed, rendered tolerant to tumor antigens. Several dysfunctions of tumor-infiltrating, as well as peripheral blood, T cells and NK cells have been described in patients with cancer. These include poor proliferative responses in ovarian carcinoma [179,180] and in oral carcinoma [180], and increased propensity for spontaneous apoptosis in oral carcinoma and in squamous cell carcinoma of the head and neck [180–182]. Reduced levels of the ζ-chain, a molecule expressed by T and NK cells, resulted in failures in activation, proliferation and cytokine production of these cells [183,184]. Studies showed decreased expression of the ζ-chain in the tumor-infiltrating and peripheral T and NK cells isolated from patients with various types of solid tumors [185–190] and hematological malignancies [191,192].

It has been demonstrated that tumor growth and lethality were unchanged in mice even after adoptive transfer of large numbers of T cells specific for an MHC class I-restricted epitope of the self/tumor antigen gp100 in a melanoma model [193]. Also, in a pancreatic tumor model it was suggested that the tumor cannot provide enough inflammatory stimuli to support $CD8^+$ T-cell effector functions [194]. Experimental evidence supporting the hypothesis that antigen-specific $CD4^+$ T cells were also rendered tolerant during tumor growth *in vivo* was provided by adoptive transfer of naive transgenic $CD4^+$ T cells specific for influenza hemaglutinin (HA) into mice with established A20 B-cell lymphoma expressing HA as a model tumor antigen (A20HA). This resulted in a transient expansion of clonotype-positive T cells, as well as phenotypic changes associated with antigen recognition. However, these T cells had a diminished response to cognate peptide *in vitro* and were unable to be primed following vaccination with a potent immunogen *in vivo* [195]. These results suggested that tumor antigen recognition by antigen-specific T cells occurs *in vivo*, but anergy rather than T-cell priming is the default outcome of such an encounter in tumor-bearing hosts [195]. These observations where extended to memory tumor-specific $CD4^+$ T cells in B cell lymphoma [196] and could be verified in solid tumor models, such as renal tumor [197] and prostate tumor [198].

This phenomenon was also observed in myeloma patients whose $CD4^+$ T cells were also functionally unresponsive [199]. In melanoma patients, lymphocytes from lymph nodes at different distances from the tumor varied in their capacity to inhibit or enhance melanoma-cell growth *in vitro* [200]. Also, the HPV16-specific $CD4^+$ T-cell response in cervical cancer patients has been proposed to be either absent or severely impaired, despite a relatively good immune status of the patients, as indicated by intact responses against recall antigens [201,202]. Decreased $CD8^+$ T-cell responses against viral antigens in women with HPV-16 infection have been detected [203].

Recent studies have shown that effector $CD8^+$ T-cell responses to common melanoma epitopes are generally weak and localized in patients with advanced metastatic disease [204]. Other studies in melanoma patients have confirmed these observations, demonstrating that $CD8^+$ T cells specific for the tumor-associated antigens MART-1 or tyrosinase are tolerant, as determined by their poor cytolytic and proliferative capacities upon *in vitro* stimulation [205]. In addition, although $CD8^+$ T cells displaying an antigen-experienced phenotype were detected in tumor-draining lymph nodes or in nonlymphoid sites where the antigen was present, these $CD8^+$ T cells were typically deficient in one or more effector functions, a phenomenon termed split anergy [206]. Human tumor-infiltrating $CD8^+$ T cells have been shown to be anergic owing to non-co-localization of TCR and the CD8 molecule on the surface of the cells [207], and anergy could be reversed when the TCR and CD8 molecules were again able to co-localize.

Responses to nontumor antigens in tumor-bearing hosts: local, rather than systemic, immunosuppression

It has been demonstrated that the number of circulating total T cells, particularly $CD8^+$ T cells, is decreased in head and neck squamous cell carcinoma patients [208]. However, a recent study demonstrated that the frequency of NK cells, T cells and DCs in patients does not change significantly across stages of melanoma. Plasma concentrations of Th2 cytokines, IL-4, -5, -10 and -13, in tumor-bearing patients were significantly higher than in those with resected melanoma, indicating that patients with metastatic melanoma experience a state of Th2-mediated 'chronic inflammation', suggested to be a result of VEGF overproduction by malignant tumors [209].

The tumor-draining lymph nodes are partially immunosuppressed in larynx, breast, colon and lung carcinoma patients [210,211]. It can be observed that lymphocytes from lymph nodes nearest to the tumor are generally less reactive to mitogenic or alloantigenic stimuli than lymphocytes from more remote nodes [212]. The changes in lymphocyte subpopulations were complex: the decrease in the number of $CD4^+$ T cells was particularly marked, with a consequent change in the ratio of CD4:CD8 T cells and an increase in the number of $CD56^+$ NK cells [213,214]. Some studies have shown that the concentration of IL-10 in the lymph node microenvironment is higher in tumor-draining lymph nodes than in other, nondraining lymph nodes [134,200].

Another line of evidence of immunosuppression in cancer patients is the presence of immature DCs in the tumor tissue in more than 90% of breast cancer patients, whereas mature cells, recorded in 60% of samples, are confined to peritumoral areas [215]. Mature (functional) myeloid DCs, which induce strong Th1 responses, are rare in human ovarian tumor [15], prostate cancer [216] and renal cell carcinomas [217]. Recently, a high frequency of immature DCs and altered *in situ* production of IL-4 and TNF-α were described for patients with lung cancer [218]. A large number of plasmacytoid DCs, but not functionally mature myeloid DCs, accumulate in the ovarian carcinoma microenvironment [38]. The expression of MHC class II molecules and CD83 on mature DCs is profoundly reduced in tumor-draining lymph

nodes from patients with melanoma or breast cancer [219]. Using RT-PCR, a markedly reduced expression of the costimulatory molecules (77% reduction in CD80 and CD86 expression, 88% in CD40, 85% in cytotoxic T-lymphocyte antigen 4 and 85% in CD28 expression) was shown in tumor-draining lymph nodes compared with other lymph nodes from patients with melanoma [220]. In a murine melanoma model, it was verified that MHC class II presentation by DCs is defective in the tumor-draining lymph node, limiting the $CD4^+$ T-cell help to anti-tumor $CD8^+$ T-cell responses [221]. Even more interesting are the observations that not only do tumor-infiltrating DCs show functional deviations, but DC precursors obtained from peripheral blood in cancer patients also present defects that could contribute to the same effect [58,86].

The existence of so many tumor immunosuppressive mechanisms could lead to the prediction that immune responses to nontumor antigens would also be affected. Paradoxically, however, in a number of cases, even when APC function and/or tumor-specific lymphocyte function is compromised in tumor-bearing hosts, what has been demonstrated until now is that there is often no generalized immune deficiency, indicating that tumors can specifically dampen the induction of anti-tumor immunity. A notable exception is the case of patients of glioma and glioblastoma multiforme [222] who develop severe lymphopenia, especially in the $CD4^+$ T-cell compartment, often presenting concomitant opportunistic infections. Different hypotheses have been raised to explain this phenomenon, including altered thymic output [223], increased Treg fraction [224] and even tumor-induced immunosuppression [225]; however, the mechanism behind it remains unknown.

A recent study demonstrated that CEA antigen-specific, but not antiviral $CD4^+$ T-cell, immunity is impaired in pancreatic carcinoma patients as assessed by a recall assay [226]. In this study an *ex vivo* restimulation assay was used, in which $CD4^+$ T cells were purified from the blood of patients and healthy donors, and then cultured either with CEA peptide to test anti-tumor immunity or with Epstein–Barr nuclear antigen (EBNA)2 or influenza HA peptide to test antiviral immunity. After 13 days, culture supernatant was analyzed for cytokine production and T-cell proliferation was evaluated. Comparing the CEA-specific response between cancer patients and normal donors, they demonstrated that anti-CEA $CD4^+$ T-cell proliferation was more vigorous in normal donors than in pancreatic cancer patients. By contrast, when comparing the antiviral-specific cytokine production between these two groups, they found that pancreatic cancer patients and normal donors had a similar Th1 response for EBNA and HA; however, in pancreatic cancer patients CEA-specific $CD4^+$ T cells produced mostly IL-5, while HA- and EBNA2-specific $CD4^+$ T cells produced mostly IFN-γ and/or granulocyte–macrophage colony-stimulating factor. Consequently, quantitative and qualitative $CD4^+$ T-cell immunity against viral proteins, but not tumor antigen, was preserved in tumor patients, suggesting the existence of a local, rather than systemic, immunosuppressive/regulatory phenomenon [226].

A study by Radoja *et al.* supported these ideas, showing that mice bearing different late-stage tumors, such as adenocarcinoma, melanoma, sarcoma, thymoma, a transgenic model of spontaneous breast cancer, colon carcinoma, fibrosarcoma and lymphoma, have normal functional systemic T-cell responses *in vitro* and *in vivo* [227]. They demonstrated that the total number of splenocytes increased progressively in tumor-bearing mice and that by 2 weeks of growth, spleens had approximately threefold more cells than controls, with major accumulation of neutrophils and macrophages and a decrease of B and T cells. Splenomegaly was present in all kinds of tumors tested. They found that the proliferation of splenocytes from tumor-bearing mice was impaired *in vitro* and this deficiency increased over time with tumor growth, but when the T cells were purified by magnetic anti-CD3 immunobeads and stimulated *in vitro* with mitogen, this impairment of proliferation was overcome. Indeed, purified cells obtained from tumor-bearing mice proliferated better than control mice. This also demonstrated that mice bearing 2- or 3-week tumors, when injected with soluble keyhole limpet hemocyanin (KLH) not expressed in tumor, produced anti-KLH $CD4^+$ T cells that proliferated upon *in vitro* challenge equivalently to control mice, showing that tumor-bearing mice can prime nontumor antigen-specific $CD4^+$ T cells *in vivo*. In addition, they showed that tumor-bearing mice can develop $CD8^+$ T cells with cytotoxic activity after *in vitro* priming with a nontumor antigen.

Unfortunately, in these two studies, because T cells were not purified by negative selection, it is very possible that the anti-CD3 or anti-CD4 in the beads altered the proliferative capacity of the purified T cells. In another study, however, evaluating the $CD8^+$ T-cell response specific to viral antigens (Epstein–Barr virus) in melanoma patients, $CD8^+$ T cells from blood samples were enriched by negative selection, using a depletion antibody cocktail that prevented T-cell stimulation [205]. They demonstrated that $CD8^+$ T cells from melanoma patients were able to lyse Epstein–Barr virus-pulsed target cells after antigen stimulation *in vitro* showing specific cytolytic activity. Also, the cells of these melanoma patients showed robust allogeneic responses in a mixed lymphocyte reaction assay, as well as in proliferation assays after recall antigen stimulation [205].

In our hands, priming of adoptively transferred transgenic $CD4^+$ T cell specific for a soluble, nontumor antigen in tumor–bearing mice for 1 week was not different from priming observed in control mice. *In vivo* antigen-specific T-cell proliferation during the priming phase was similar in the two groups [Bonorino C, de Souza AP, Unpublished Data]. Our data agree with other studies described throughout this review, which show that although the tumor exerts several mechanisms of immunosuppression, responses to non-tumor antigens seem unaffected. However, there is still a deep lack of understanding of this phenomenon.

Expert commentary

The abundance of described mechanisms of tumor immunosuppression clearly shows that immune responses do exert a significant selection pressure over tumor growth. A direct conclusion appears to be that, in order to treat cancer patients, we should not ignore the contribution of the immune system. This is the opposite of what has been developed so far for anticancer therapy. The available strategies based on drugs that preclude

cell proliferation have the immune cells as their main collateral target, eradicating not only the ability to form immune responses to new tumors, but also impairing the host's ability to fight infection.

The variety of immunosuppressive strategies employed by tumors suggests that the interaction of tumor and host is a complex system that includes several variables. The main implication of this finding for anticancer therapy is that there is probably no key molecule that can work as a unique target for cancer therapy. Tumor growth in a host is a dynamic process of selection and adaptation and, if so, we can predict that tumor treatment should be designed to fit this dynamic.

The combination of chemotherapeutic agents is an approach already used in cancer therapy, because tumors can rapidly acquire resistance to treatment with a single drug. This is also true for immunotherapeutic approaches, such as monoclonal antibodies, that will invariably select clonal variants of the tumor that do not express the target molecule. The combination of therapies based on more specific drugs, that would preserve the immune system, and immunotherapy approaches could probably substitute the present models of cancer treatment, enhancing quality of life for the patient and preserving their ability to mount immune responses. In fact, the immune response is probably an important factor in the elimination of residual cancer cells after conventional chemotherapy, maintaining micrometastasis cells in check. It is probable that chemotherapy influences immune responses either by leading to the release of stress signals from the dying cancer cells or by causing lymphopenia followed by homeostatic expansion of the remaining T cells. Either way, chemotherapy can alter the relative state of escape between the tumor and the immune system, setting it back to the equilibrium or even elimination stage [228].

The varied strategies used by tumors to immunosuppress the host suggests that a strong selective pressure is put on the tumor by the immune response. A logical prediction of this hypothesis would be that the expression of immunosuppressive factors changes when the tumor is transferred from an immunocompetent host to a $Rag^{-/-}$ mouse. These experiments have not yet been performed.

Another important conclusion of all these studies is that, although tumors employ different strategies to suppress immune responses, targeting different cells of the immune system, the effects appear to be mostly focused on tumor antigen responses. Although this hypothesis still needs further evidence, the prediction would be that understanding of this mechanism might lead us to more than the identification of targets for anticancer therapy. It could help us find the ideal ways to improve graft acceptance without the burden of systemically immunosuppressing the host.

Five-year view

Clarification on the relevance of the immune response in cancer patients will be decisive in shaping new therapeutic approaches. Although over 30 years were necessary to take monoclonal therapy from the laboratory to patients, the recognition of the importance of translational research will significantly shorten this period.

Several approaches focusing on the complex tumor immunosuppressive network described above are currently being developed for clinical use. Current strategies are being investigated aiming to impair TGF-β activity using either antibodies [229]; compounds that interfere with receptor–ligand interactions, such as recombinant fusion proteins containing the ectodomains of the type II and type III (β-glycan) receptors; drugs that block intracellular signaling, such as SB-431542 and SB-505124 (GlaxoSmithKline, Australia); and finally antisense oligonucleotides, such as AP-12009 and AP-11014 (Pharma, Germany) both TGFβ-specific phosphorothioate antisense oligonucleotides [229,230]. Preclinical studies using TGF-β inhibitors have demonstrated efficacy in reducing metastasis and have shown improvements in cytotoxic drug delivery. Results of Phase I/II clinical trials of TGF-β inhibitors in patients with glioblastoma suggest improved survival rates compared with conventional chemotherapy. The predominant cellular target, whether cancer or stromal cell, immune cell or angiogenesis, may differ between tumor types. Different individuals may show variable responses to drug therapy dependent on both germline genetic variation and the somatic mutation profile of the tumor.

Furthermore, studies are underway to block IL-10 activity using an anti-IL10 receptor antibody to rescue the T-cell response [231], alone and combined with Toll-like receptor agonists. Studies on blocking VEGF with anti-VEGF antibody [232,233] are also under way. Depletion of $CD4^+CD25^+$ Tregs may also achieve good results, at least temporarily [234]. Another strategy is the use of STAT3 inhibitors to augment the expression of chemokines [235]. Blocking STAT3 signaling in tumors *in vivo* results in tumor growth inhibition that involves killing of tumor cells and infiltration of immune cells. Thus, either STAT3 activity in tumor cells affects immune cell recruitment or dying tumor cells can attract immune cells; in both cases, STAT3 inhibitors can be a good strategy to reverse tumor tolerance.

More studies on the potential of DC therapy are necessary, especially in individuals who have not undergone extensive chemotherapy. Chemotherapy leads to a dramatic drop in the levels of T lymphocytes, and the results of DC therapy might be warped since it is unclear exactly what cells are being stimulated by the adoptively transferred tumor-pulsed DC.

We believe that, in the coming years, the challenge to reach clinically relevant anticancer immune responses, overcoming the immunosuppressive network, will be met by therapeutic strategies that will be based on integration of several components, leading to minimum damage to the immune system.

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Key issues

- Tumors can produce different soluble factors that have immunosuppressive activities, blocking maturation and differentiation of DCs and recruiting Treg cells.
- Many tumor types promote the expansion of myeloid derived suppressor cells (MDSC).
- Tumors can constitutively expresses membrane-associated Indoleamine 2,3-dioxygenase, which can impair proliferation and induce apoptosis of T cells at the tumor site.
- PD-L1 is strongly expressed a variety of tumors, promoting apoptosis of T cells.
- Tumors can express transcriptional factors which can inhibit T-cell responses.
- The number of circulating tumor-specific $CD8^+$ T cells is decreased in tumor patients, and their proliferation ability is decreased.
- Tumor antigen-specific $CD4^+$ T cells are rendered tolerant during tumor growth.
- The tumor-draining lymph nodes are partially immunosuppressed.
- Dendritic cells and dendritic cell precursors from tumor patients have shown function impairment.
- In pancreatic cancer patients, the percentage of responders to a tumor antigen was lower than in normal donors, while the percentages of responders to virus antigens were similar.
- Mice bearing late-stage tumors have normal functional systemic T-cell responses *in vitro* and *in vivo* to a soluble non tumor antigen.

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CAPÍTULO 6

Artigo Científico:

Priming and differentiation of $CD4^+$ T cells against a non-tumor antigen in melanoma bearing mice

PRIMING AND DIFFERENTIATION OF CD4$^+$ T CELLS AGAINST A NON-TUMOR ANTIGEN IN MELANOMA BEARING MICE

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ABSTRACT

The tumor microenvironment is complex and creates an immunosuppressive network in order to tolerize tumor-specific immune responses, however little information is available on what happens to the response against non-tumor antigens in tumor bearing individuals. The goal of the present study was to evaluate if tumor burden could influence a $CD4^+$ T cell response against a soluble protein, not expressed by the tumor, specifically regarding priming and memory recall responses in the absence of *in vitro* stimulation. Mice bearing B16F10 melanoma tumors for 7 days received an adoptive transfer of transgenic TEa T cells and 24 hours later were injected with soluble EaRFP antigen in a site next to the tumor. Priming of the TEa cells was not affected by tumor presence based on the total number of cells recovered and proliferation assessed by CFSE dilution. To investigate if tumor burden could influence recall responses of already differentiated effector cells, we immunized mice with EaRFP antigen and a after a few days injected the tumor cells, later challenging the mice with the non-tumor antigen. We found that the number of $CD4^+CD90.1^+$ producing IFN-γ in tumor bearing mice was not different compared to tumor-free mice. No differences in antigen presentation, assessed by YAe antibody staining, were verified in the draining lymph node of these two groups. Collectively, our data indicate that tumor burden does not affect immune responses to non-tumor antigens. These results have important implications in the design of anti-cancer therapy.

Key words: $CD4^+$ T cells, tumor, antigen specific, dendritic cells, tolerance

INTRODUCTION

Many tolerization mechanisms of anti-tumor immune responses have been described for melanoma, such as the secretion of immune-suppressive factors TGF-β (Rodeck, Bossler *et al.*, 1994), IL-10 (Kawamura, Bahar *et al.*, 2002) and gangliosides (Mckallip, Li *et al.*, 1999; Kawamura, Bahar *et al.*, 2002), or the expression immune-suppressive molecules such as PD-L1 (Blank e Mackensen, 2007) and IDO (Brody, Costantino *et al.*, 2009). Furthermore, an increased frequency of suppressive regulatory T cells (Tregs) has been reported in murine melanoma (Turk, Guevara-Patino *et al.*, 2004) as well as in patients (Nicholaou, Ebert *et al.*, 2009). Recent studies have shown that effectors $CD8^+$ T cell responses to common melanoma epitopes are generally weak and localized in patients with advanced metastatic disease (Palmowski, Salio *et al.*, 2002). Other studies in melanoma patients have confirmed these observations, demonstrating that patients with metastatic melanoma experience a state of $CD4^+$ T helper type 2 Th2-mediated "chronic inflammation" suggested to be a result of VEGF overproduction by malignant tumors (Nevala, Vachon *et al.*, 2009), that modulates dendritic cell function. A markedly reduced expression of co-stimulatory molecules was demonstrated in tumor-draining lymph nodes compared to other lymph nodes from patients with melanoma (Essner e Kojima, 2001). In a murine melanoma model, it was verified that MCH

class II presentation by dendritic cells (DCs) of a non-tumor antigen injected intratumorally is defective in the tumor draining lymph node (Gerner, Casey *et al.*, 2008), and we have verified that prevention of traffic between lymph node and tumor site specifically affects anti-tumor $CD4^+$T cell differentiation (Maito, Souza et al, submitted), thus preventing the generation of $CD4^+$ T cell help for anti-tumor $CD8^+$ T cells.

The existence of so many tumor immunosuppressive mechanisms could lead to the prediction that immune responses to non-tumor antigens would also be affected in tumor bearing individuals. Nevertheless, interestingly, the few existing studies on the subject suggest that this is not the case. In general, cancer patients are not considered immunosupressed individuals, unless they were already so when they developed the tumor, or are in a chemotherapy-induced state of leucopenia (Souza and Bonorino, in press). Mice bearing late-stage different types of tumors, including melanoma, have been showed normal functional systemic T cell responses (Radoja, Rao *et al.*, 2000). A recent study demonstrated that CEA antigen specific, but not antiviral $CD4^+$ T cell immunity is impaired in pancreatic carcinoma patients (Tassi, Gavazzi *et al.*, 2008). However these two studies re-stimulated the cells *in vitro* perform such analysis. The goal of the present study is to evaluate if tumor burden can also tolerize the $CD4^+$ T cell response against a non-tumor antigen. We found that priming of adoptively transferred transgenic $CD4^+$ T cells specific for a soluble, non-tumor antigen in mice bearing one week tumors was not different from priming observed in control, tumor-free mice. We also analyzed the *in vivo*

recall proliferation of memory $CD4^+$ T cells specific to this non tumor antigen and we found no evidence of immune-suppression against this antigen on the tumor lymph node. Such results indicate that a fine regulation of immune responses is exerted by tumors, so as to not interfere with a non-tumor immune response.

MATERIALS AND METHODS

Mice

C57Bl/6 (B6) mice were purchased from Fundação Estadual de Produção e Pesquisa e Saúde (FEPPS) Porto Alegre, RS, Brazil and TEa transgenic mice backcrossed into a RAG-/- background, expressing CD90.1 were provided by Marc Jenkins (University of Minnesota, USA). Mice were housed under pathogen-free conditions at PUCRS (FABIO) animal facility with *ad libitum* access to food and water. Female six – to eight week old mice were used for all experiments and these were conducted with the approval of the PUCRS committee on animal research.

Cell lines

The murine melanoma cell line B16F10 (ATTC CRL-6475) was cultured with DMEM media (Cultilab) supplement with 10% of fetal calf serum (FCS) (Cultilab), 1X essentials aas (Gibco), 1X vitamins (Gibco) and 55μM of β-mercaptoethanol at 37ºC with 5% of CO_2 atmosphere.

EaRFP protein

This protein was produced as previously described (Itano, Mcsorley *et al*., 2003). Briefly, the plasmid vector pTcrHis2 TOPO (Invitrogen) encoding the fusion protein EaRFP was transformed into *E. coli* BL21 competent cells. These cells were growth in LB media at 37ºC with agitation of 250 rpm in presence of Ampiciline and 1 mM of IPTG (Sigma). After 24hs the cells were lyses by sonication in cell lysis buffer (20mM Tris pH8.0; 500mM NaCl; 0.01% Tween

20). The protein was purified from bacterial lysate using $Ni^{+}2$ resin His-Bind Kit (Novagen) with few modifications. The protein concentration was estimated measuring the density optic (DO) in a Spectrophotometer (Shinadzu model UV-1201) at 558 nm and using its extinction coefficient (52) and molecular weight (30kDa). The protein buffer was change to PBS using a PD-10 desalting column (GE).

Adoptive transfers

Pooled spleen cells from naive TEa transgenic donor mice were used for adoptive transfer, after red cells lysis with RBC lysis buffer. In some experiments, these cells were labeled before transfer with 5 μM of 5- and 6-carboxy-fluorescein diacetate succinimidyl ester (CFSE) (Invitrogen) for each $5X10^6$ cells The cells were transferred by intravenous injection in caudal vein into naive B6 mice a total of 10^5 splenocytes from donor mice.

Mice immunizations and tumor injections

In order to analyze priming of $CD4^+$ T cells in tumor bearing mice $5X10^5$ B16F10 cells were injected subcutaneously in mice. After 7 days of tumor growth TEa cells were adoptively transferred into tumor bearing mice and into others mice without tumor as a control and 24hs later the two groups of mice received 20μg of EaRPP subcutaneously in a site next to the tumor The responses were evaluated after 10 days of tumor growth in the draining inguinal lymph node. In order to analyze memory recall of $CD4^+$ T cells Tea cells were adoptively transferred into mice and 24hs later mice receive 50μg EaRPP

subcutaneously. After 5 days mice received subcutaneously $5X10^5$ B16F10 or PBS then 10 days later mice receive a recall with 20μg EaRFP subcutaneously and after 4 days the cells were analyzed in draining lymph node. All subcutaneously injections were done after anesthesia with 83 of mg/Kg ketamine and 17 mg/kg of xilazine.

Flow cytometry

The cells from inguinal lymph nodes were stained in order to evaluate the immune response at these sites by flow cytometry. Before staining, the viable cells were counted and the Fc receptors were blocked by incubating the cells with supernatant of 24G2 supplemented with 5% mouse serum and 10% rat serum for 15 min in ice. The cells were stained with anti-CD90.1 PerCP (BD Pharmingen), anti-CD4 PE (BD Pharmingen), anti-CD44 FITC (BD Pharmingen), anti-CD62L FITC (Macs), anti-CD86 PE (BD Pharmingen), anti-B220 Cy (BD Pharmingen), anti-YAe FITC (recognized the complex MHCII:Eapeptide (eBioscience)) and Streptoavidin FITC (BD Pharmingen). For intracellular staining anti-IFNγ FITC (BD Pharmingen) was used, and permeabilization was done with Perm2 (BD Pharmingen). The cells were acquired at a Beckton Dickinson FACSCalibur flow cytometer and the data were analyzed using the software FlowJo (Tristar).

RESULTS

Tumor burden does not affect priming of CD4$^+$ T cells to a non tumor antigen in vivo

CD4$^+$ T cells play a central role in the immune system, coordinating both adaptive and innate responses. Upon interaction with cognate antigen presented by antigen presenting cells such as DCs, CD4$^+$ T cells can differentiate into a variety of effectors subsets, including classical Th1 cells and Th2 cells, the more recently defined Th17 cells, follicular helper T (Tfh) cells and Treg cells (Zhou, Chong *et al.*, 2009). The differentiation decision is governed predominantly by the cytokines in the microenvironment and, to some extent, by the strength of the interaction of the TCR with antigen (Boyton e Altmann, 2002). Antigen specific CD4$^+$ T cells help macrophages to destroy vesicular and intracellular pathogens through cell–cell contact and release of immunomodulatory cytokines. Also, it was described that CD4$^+$ T cells can interact with DCs promote its maturation and longevity (Smith, Wilson *et al.*, 2004; Mueller, Jones *et al.*, 2006). At the same way, they provide help to B cells and CD8$^+$ T cells in their response to antigen (Wang e Livingstone, 2003). Indeed, CD4$^+$ T cells are required for the maintaince of memory CD8$^+$ T cells after acute infection (Sun, Williams *et al.*, 2004).

In the present study we asked if proliferation and differentiation of the primed TEa cells into an effectors phenotype would be affected by the presence of a tumor that not expresses the T cell specific epitope in the host. To determine if CD4$^+$ T cell priming to a non-tumor antigen was modulated *in vivo*

by the presence of a tumor, B16F10 cells were subcutaneously injected at concentration of $5X10^5$ in C57Bl/6 mice, in the upper flank of the thigh. After one week of tumor growth, when a solid tumor mass could be visualized at the site of injection, naïve TEa cells stained with CFSE were adoptively transferred to tumor bearing mice or control mice, and 24h later, 20µg of EaRFP protein was injected subcutaneously in a site adjacent to the first injection (as shown on Figure 1A). The optimal dose of this protein used to prime and recall the cells was previously described by others studies that demonstrated to be efficient to development of effector and memory Tea cells in vivo (Catron, Rusch *et al.*, 2006).

Figure 1

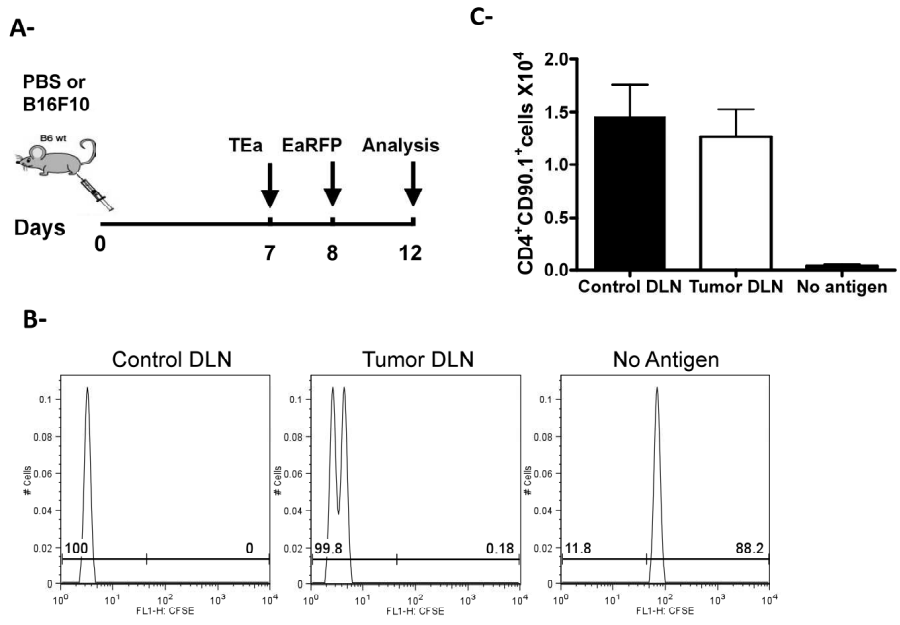


Figure 1: *In vivo* priming of $CD4^+$ T cell non tumor specific in tumor bearing mice comparing to normal mice. $5X10^5$ B16F10 cell were subcutaneous injected in the leg of a group of mice and the other one received PBS. After 7 days of tumor growth all groups received 10^5 TEa $CD4^+$ T cells stained with CFSE by IV injection and after 24hs were injected with 20ug EaRFP protein subcutaneously in a site next to the tumor. After 3 days the responses were evaluated in the inguinal draining lymph node. **A-** Experimental design. **B**- CFSE dye dilution of transferred $CD4^+CD90.1^+$ T cells**. C**- Number of $CD4^+CD90.1^+$ transgenic T cell in the tumor draining lymph node of tumor bearing mice and in control mice. DLN – Draining lymph node.

Antigen specific cell proliferation was evaluated after 3 days later (Figure 1B) by CFSE dye dilution. The number of $CD4^+CD90.1^+$ T cells recovered from the lymph nodes of mice with no tumor was not different from what was recovered in the tumor bearing mice (1C). Also, while no CFSE dilution was observed in mice that did not receive antigen (Figure 1B), the injection of EaRFP resulted in complete dilution of CFSE both in tumor bearing mice and control mice.

While naive CD4+ T cells express high levels of lymph node homing receptor molecule CD62L (MEL-14, 1-selectin) and CD127 (IL-7Rα), and low CD44 expression, effector $CD4^+$ T cells express several activation markers including CD69, IL-2 receptor-α (CD25), CD44 high, and have down-regulated the cell surface expression of IL-7Rα (CD127). We analyzed the phenotype of the primed CD4+ T cells in the draining lymph nodes of the tumor bering and the tumor-free mice. As demonstrated in Figure 2, differentiation TEa $CD4^+$ T

cells into an effector phenotype was not affected in tumor bearing mice, as assessed by expression of CD44 and CD62L.

Figure 2

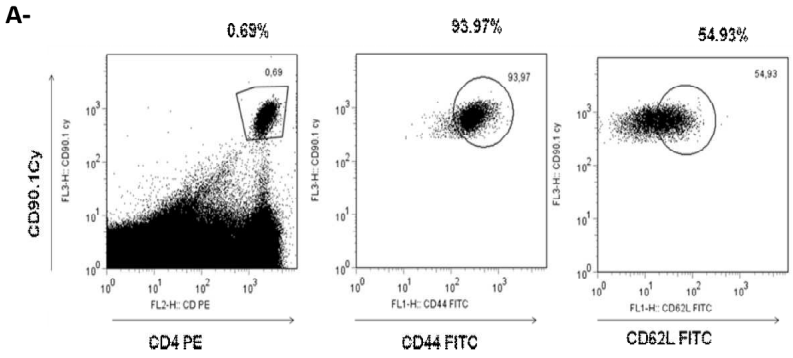


Figure 2: Effector phenotype of CD4⁺ T non tumor specific in tumor bearing mice. Dot plots showing the generation of *in vivo* TEa cells memory. Inguinal lymph nodes were analyzed after 4 days of 50ug EaRFP injection for presence of $CD90.1^{+}CD44^{high+}CD62L^{+}$ in a tumor bearing mice.

Memory recall of CD4⁺ T cells to a non tumor antigen in vivo is not impaired in tumor draining lymph nodes

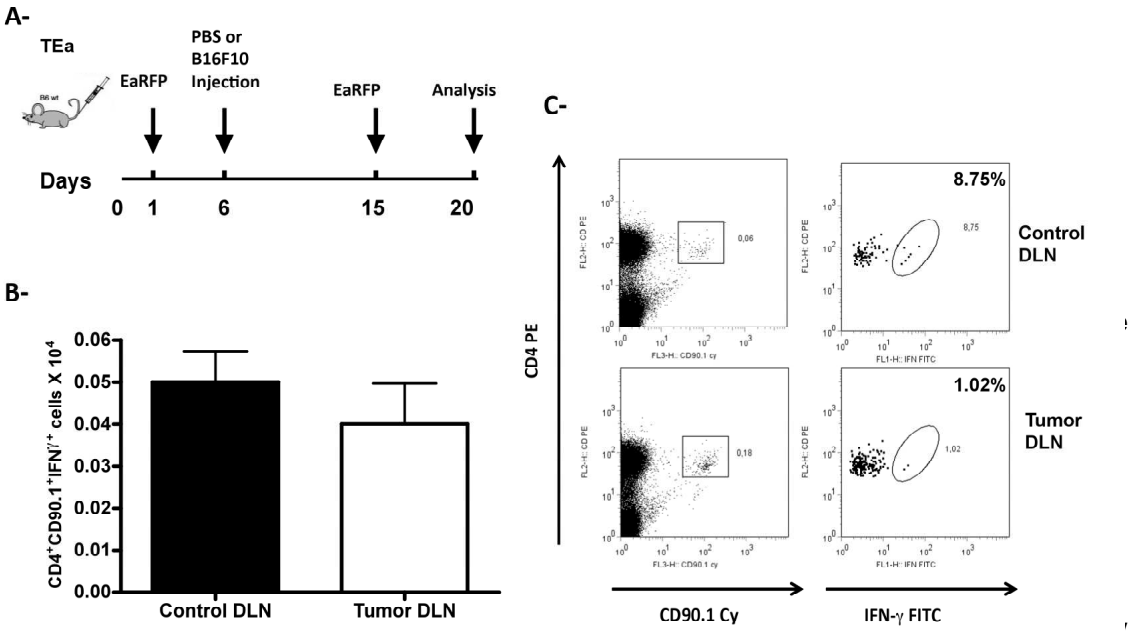
After antigen encounter, antigen-specific T cells expand as much as 50,000-fold, acquire effector function, and then 90%–95% of antigen-specific T cells die after a definite period of time, leaving behind a long-lived population of memory

T cells that provide protection (Kaech e Wherry, 2007; Williams e Bevan, 2007). Memory T cells possess several properties that are essential for their function, including higher frequencies than naive precursors, the ability to rapidly reactivate upon antigen stimulation, wide tissue distribution, and the ability to survive and self-renew for long periods in the absence of cognate antigen. Memory $CD4^+$ T cells, re-activated by antigen exposure, expand and could act to protect the host by either making early effector cytokines to direct other cells (Macleod, Mckee *et al.*, 2008). Memory cells are generally CD44-high, have regained IL-7Rα expression but have lost the expression of activation markers such as CD25 and CD69. Expression of the lymph node homing markers CCR7 and CD62L have been used to define memory T cells into two broad subsets. Central memory T cells (TCM) and effector memory T cells (TEM) (Sallusto, Geginat *et al.*, 2004; Kalia, Sarkar *et al.*, 2006). Additionally, further layers of complexity are identified within CD4 TEM and TCM subsets (Rivino, Messi *et al.*, 2004).

We asked if tumor burden could influence an already established memory immune response to a non-tumor antigen. To test that, we first differentiated TEa cells into an effectors phenotype by adoptively transferring the cells and 24h later injecting soluble EaRFP subcutaneously as described before. After 5 days, we injected $5X10^5$ B16F10 cells subcutaneously in one group of mice while the other group received PBS. After 10 days of tumor growth, the TEa memory response was recalled with 20μg of EaRFP protein and evaluated in tumor bearing mice as well as in the mice without tumor

(Figure 3A). Effector cytokine producing memory CD4 T cells have been shown to be protective in viral, bacterial and parasitic infections, therefore we assed the IFNγ production of this cells. We evaluated the number of IFNγ^{+} TEa cells in these groups and we found no difference between DLN of tumor bearing mice (mean 0.04x10^{4} cells) compared to control mice (mean 0.05x10^{4} cells) (Figure 3B and 3C). These results suggested that the tumor apparently does not influence memory recall to a non tumor antigen *in vivo*.

Figure 3



days later a group of mice received 5x10^{5} B16F10 tumor cells and other group received PBS. After 10 days the tumor group received 20µg of EaRFP in the tumor site and in a distant tumor site. The control group received 20µg of EaRFP. 4 days later the response was analyzed. **A-** Experimental design. **B-**

Number of positive IFN-γ TEa cells in a tumor mice comparing to a control mice in a secondary response in the tumor inguinal draining lymph nodes. DLN – Draining lymph node.

Tumor burden does not affect antigen presentation

DCs are the most potent antigen-presenting cells (APCs), capable of inducing primary and boosting secondary T-cell responses, when bearing a mature phenotype (Banchereau e Steinman, 1998; Banchereau, Briere *et al.*, 2000). Given that, we asked if tumor burden could influence antigen presentation of a non tumor antigen. As mentioned before, we previously demonstrated that the MHC class II antigen presentation of a tumor antigen was impaired in tumor draining lymph node when we measured the expressed Ea peptide:MHCII complex on DC using with YAe antibody staining (Maito, Souza et al., submitted). In order to evaluate antigen presentation in the tumor draining lymph node of a non tumor antigen we injected EaRFP soluble in site next to tumor and analyzed antigen presentation 4 days later. No significant difference was found between the numbers of the $CD86^{+}YAe^{+}$ in the tumor bearing group comparing to the control, tumor free group (Figure 4). These results indicated that tumor burden was not able to influence antigen presentation of a soluble antigen that it is not expressed by the tumor.

Figure 4

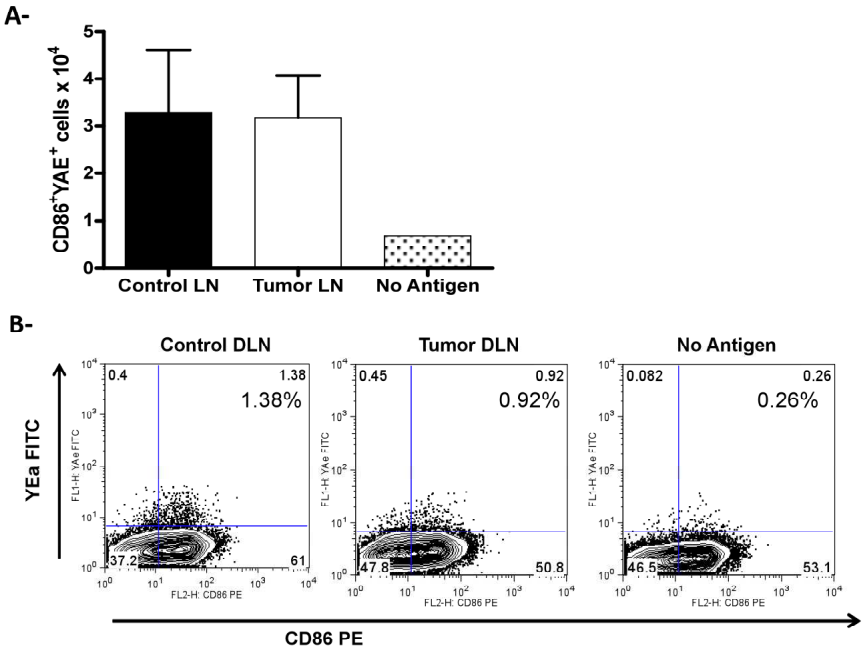


Figure 4: Antigen presentation in draining lymph node. 20µg of EaRPF was subcutaneously injected next to the tumor site in mice bearing 9 days tumor and in control mice without tumor. After 4 days the response was evaluated **A-** Number of $CD86^+YAe^+$ cells in the inguinal draining lymph node. **B-** Dot plot showing the $CD86^+YAe^+$ cells in the inguinal draining lymph node. DLN – Draining lymph node

Discussion

In the present study we established that the priming and memory $CD4^+$ T cell responses specific for a non-tumor antigen *in vivo* are not affected by tumor presence. The antigen used in this study to prime and recall memory TEa was the soluble protein EaRFP that contains the Ea peptide. This protein was not depleted of TLR ligands, in order to mimic what T cells will encounter in a real infection. Another study using soluble protein injected in the tumor site

demonstrated impairment of MHC class II presentation in the tumor draining lymph node, however they used LPS-free protein (Gerner, Casey *et al.*, 2008). We also previously demonstrated that LPS injected in the tumor site could increase the MHCII antigen presentation in the tumor draining lymph node (Maito, Souza et al., submitted) and result in effector T cell differentiation. These results support the potential of TLR ligands to reverse local suppression exerted by tumor cells.

It was recently demonstrated that persistent stimulation through the TCR and CD28 may be more important to primed $CD4^+$ T cell to ensure optimal *in vivo* cell cycle progression and differentiate into effector cells (Obst, Van Santen *et al.*, 2005; Yarke, Dalheimer *et al.*, 2008), which can in turn perform the helper functions required for the regulation of immune response. We have previously demonstrated that differentiation of anti-tumor $CD4^+$ T cells is impaired due to a gradual cessation of DC migration to the draining lymph node (DLN) and antigen presentation by these cells, preventing $CD4^+$ T cell help against the tumor and thus enhancing tumor growth. Also, that specific $CD4^+$ T cells undergo proliferate arrest, showing by stop on CFSE dilution, due tumor tolerization (Maito, Souza et al., submitted). However, these immunosuppressive mechanisms are based on tumor antigen specificity because when we used the same $CD4^+$ T cell epitope as an antigen (Ea peptide), but not expressed by the tumor, the $CD4^+$ T cells go through to a complete CFSE dilution as demonstrated in Figure 1C.

Another study using a tumor mouse model supported these ideas, showing that mice bearing late-stage different tumors, such as adenocarcinoma, melanoma, sarcoma, thymoma, a transgenic model of spontaneous breast cancer, colon carcinoma, fibrosarcoma and lymphoma, have normal functional systemic T cell responses *in vitro* and *in vivo* (Radoja, Rao *et al.*, 2000). They found that the proliferation of splenocytes from tumor-bearing mice was impaired *in vitro* and this deficiency increased over time with tumor growth. However, when the T cells were purified by magnetic anti-CD3 immunobeads and stimulated *in vitro* with mitogen, this impairment of proliferation was overcome. Indeed, purified cells obtained from tumor bearing mice proliferated better than control, tumor-free mice cells. This study also demonstrated that mice bearing 2 or 3 week tumors, when injected with soluble KLH (Keyhole Limpet Hemocyanin) not expressed in tumor, produced anti-KLH T $CD4^+$ cells that proliferated upon *in vitro* challenge equivalently to control mice, showing that tumor bearing mice can prime non-tumor antigen specific $CD4^+$ T cells *in vivo.* In addition, they showed that tumor-bearing mice can develop $CD8^+$ T cells with cytotoxic activity after *in vitro* priming with a non tumor antigen. They also demonstrated that mice bearing late stage tumors reject syngenic tumors upon challenge, as well as non-cross-reactive tumors, which are rejected by mice with functional immune systems. One of the differences between our study and the Radoja study it that they used cells after purification with positive selection, which could interfere with their results. In addition, in the Radoja study they used mice with late stage tumors and we

used mice bearing tumors at an early stage. However, our findings were similar, that tumor presence seemed not to affect the non-tumor antigen response.

Similar observations were made in tumor patients. A recent study demonstrated that antiviral $CD4^+$ T cell immunity is not impaired in pancreatic carcinoma patients (Tassi, Gavazzi *et al.*, 2008) as assessed by a recall assay. In this study they used an *ex vivo* restimulation assay, in which they purified $CD4^+$ T cells from blood of patients and healthy donors, with Epstein Barr nuclear antigen (EBNA2) or influenza hemagglutinina (HA) peptide to test antiviral immunity. When comparing the antiviral specific cytokine production, they found that pancreatic cancer patients and normal donors had a similar Th1 response for EBNA and HA. Consequently, quantitative and qualitative $CD4^+$ T cell immunity against viral proteins was preserved in tumor patients (Tassi, Gavazzi *et al.*, 2008). In another study, evaluating the $CD8^+$ T cell response specific to Epteins Barr virus (EBV) in melanoma patients, $CD8^+$ T cells from blood samples were enrichmed by negative selection, using a depletion antibody cocktail which prevent T cell stimulation (Lee, Yee *et al.*, 1999). They demonstrated that CD8+ T cells from melanoma patients were able to lyse EBV-pulsed target cells after antigen stimulation *in vitro* showing specific cytolitic activity. Also, the cells of these melanoma patients showed robust allogeneic responses in a mixed lymphocyte reaction assays as well as in proliferation assays after recall antigen stimulation (Lee, Yee *et al.*, 1999).

The present work was performed entirely *in vivo* without secondary antigen stimulation *in vitro* and without any T cell purification, differently from

the previous studies on this subject, in which all interactions between the immune cells are intact in the lymph node microenvironment. Nevertheless, our results are in agreement with theirs, in that non-tumor specific immune responses are preserved in tumor bearing mice. Also, this was the first study were the memory response *in vivo* to a non-tumor antigen was evaluated in tumor bearing mice, demonstrating that this response was not affected by the tumor immunosuppressive microenvironment. Patients with tumors are not generally immunosuppressed before chemo and radiotherapy (Souza and Bonorino, in press). The reason why this situation happens, and cancer patients do not show tolerization of immune responses to other patogens, presenting opportunistic infections, it is probably related to the fact that in a real infection there are several TLR ligands present, ant this is suffcient to reverse the tumor immunossupressive microenviroment. A notable exception is the patients with glioma and glioblastoma multiforme that have lymphopenia and can present opportunistic infections (Learn, Fecci *et al.*, 2006). Further studies are necessary to describe the exact mechanisms involved in this process that can affect also the tumor non-specific immune response.

It still remains to be determined how $CD4^+$ T cells primed or already present in a tumor bearing host will respond to a different challenge such as bacteria, virus or parasite expressing the cognate $CD4^+$ T cell antigen. Also, if different time periods of tumor growth will influence resting memory $CD4^+$ T cells behaviour, The present work suggest that priming and memory T $CD4^+$ T

cells *in vivo* are not being affected by the tumor immunosuppressive microenvironment.

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CAPÍTULO 7

7. Considerações finais e perspectivas

Demonstramos neste trabalho que níveis reduzidos de HspBP1 presentes no tumor primário de pacientes com câncer de mama está relacionado com pior prognóstico da doença. Além disso, demonstramos que níveis elevados de HspBP1 em modelo de melanoma murino pode direcionar a regressão do tumor *in vivo*, e este mecanismo provavelmente está associado com funções da HspBP1 sobre a Hsp70. Também verificamos que este fenômeno não ocorre em camundongos imunodeficientes, sugerindo que a resposta imune adaptativa tem uma importante função neste cenário. Entretanto, existe ainda um longo caminho a ser seguido a fim de elucidar todo o processo que envolve a redução do tumor direcionado pela HspBP1 e sua aplicação na terapia anti-tumoral. Um importante ponto ainda a ser avaliado que pode contribuir significantemente para ratificar a utilização desta proteína na terapia anti-tumoral é a analise da regressão tumoral após tratamento com HspBP1 em um tumor pré-estabelecido.

Em relação à interação da resposta imune na regulação da expressão da HspBP1 e Hsp70 nas células tumorails acreditamos que outros fatores, além do TNF-α e células dendríticas, podem estar envolvidos neste processo. Testamos se a presença TNF-α e células dendríticas maduras diferenciadas de medula óssea podem contribuir na regulação da expressão destas proteínas, entretanto outras citocinas poderiam estar envolvidas neste processo e também outros tipos celulares, como exemplo das células T regulatórias. Ao

mesmo tempo, ainda falta ser determinado se o tumor transplantado em camundongo imunodeficiente (Rag¨/¨) mantém o mesmo padrão de regulação da expressão de HspBP1 e Hsp70, ou seja, inversamente proporcional como encontrato em camundongos imunocompetentes.

Alguns estudos demonstraram que existe diferença na função da Hsp70 quando está expressa na membrana das células tumorais e também foi recentemente descrito que HspBP1 solúvel pode se ligar na Hsp70 expressa na superfície das celulas tumorais. Consequentemente, como perspectiva deste trabalho também sugerimos a avaliação da expressão da HspBP1 e Hsp70 na superfície das células de melanoma e das células do sistema imune a fim de verificar alguma função importante relacionada com estas proteínas sendo expressas em diferentes compartimentos celulares.

Além disso, neste estudo avaliamos a influência do ambiente imunossupressor gerado pela presença do tumor na resposta imune não tumor específica. Verificamos que o tumor, apesar de dispor de diferentes mecanismos imunossupressores, não interfere na resposta de células T $CD4^+$ especifica para um antígeno não tumoral. O real mecanismo envolvido com a imunossupressão direcionada pela presença do tumor diretamente para resposta tumor especifica permanece ainda por ser desvendado.

Concluímos com este trabalho que as interações entre o sistema imune e o tumor são complexas, entretanto quando pensarmos em tratamento eficiente para os diferentes tipos de tumores com certeza o sistema imune não deve ser negligenciado, dado a sua importância no controle do crescimento

tumoral. Além disso, concluímos que as proteínas de choque de calor, como exemplo da HspBP1 tem um grande potencial a ser investigado na terapia anti-tumoral.

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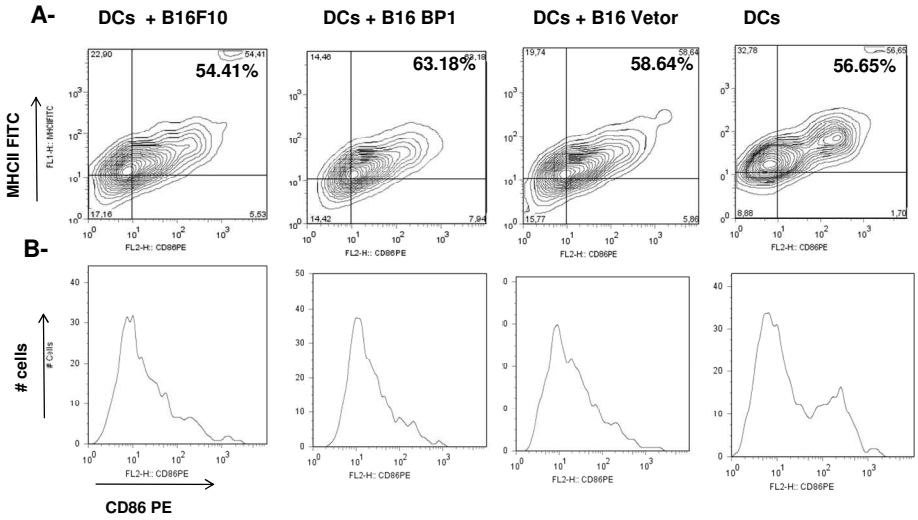
APÊNDICE I

Resultados suplementares relacionados com a super-expressão de HspBP1 em modelo de melanoma murino

Figure 1



Co-culture of bone marrow dendritic cells and B16F10. B16F10 cells were plate at concentration of 10^4 cells per well in overnight culture and the cells were transfected or not with the plasmid encoding HspBP1 and incubated for 3h. After this the bone marrow dendritic cells (BMDCs) with 9 days of differentiation with IL-4 and GM-CSF were added in the culture at concentration of $3X10^5$ cells per well and this co-culture were incubated for 24hs or 48hs in 5% of CO_2 at 37ºC in DMEM media.**C-** Dot plot of staining CD86 and MHCII in $CD11c^+$ dendritic cells. **B-** Histograms showing expression of CD86 on $CD11c^+$ cells. **D-** Dot plot of staining CD11c and HspBP1 on dendritic cells. **B**- Dot blot of staining HspBP1 on B16F10 cells **A-** Western blot analysis of expression of HspBP1 and Hsp70.

Figure 3

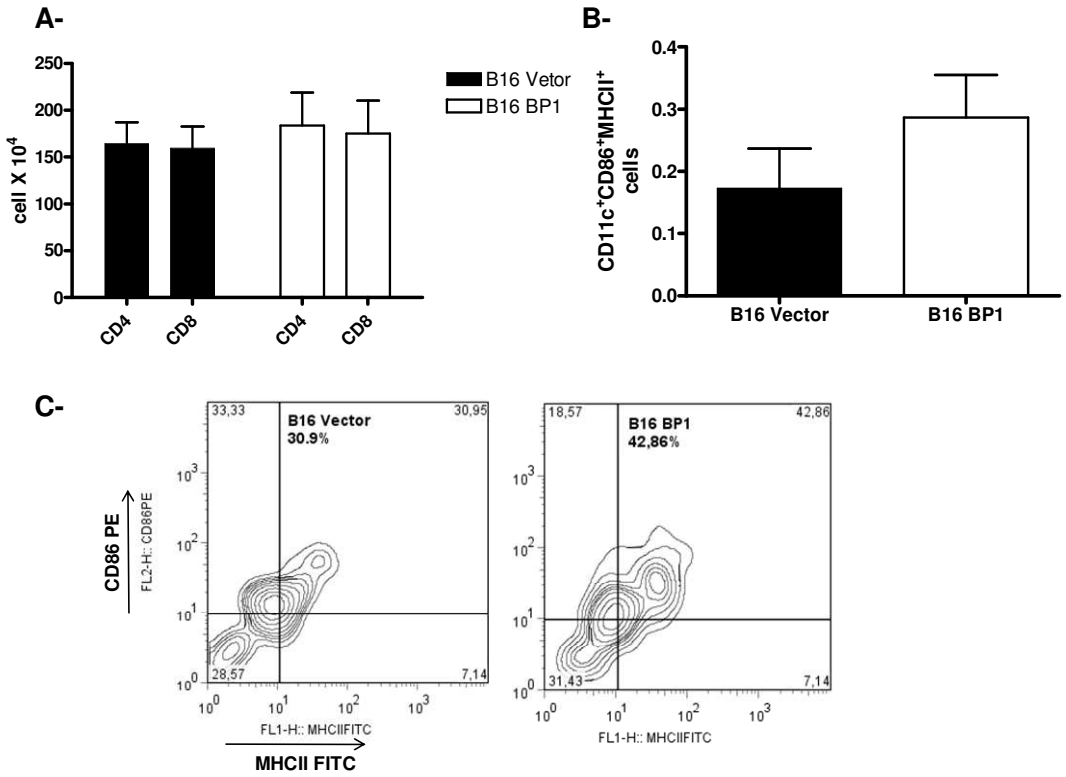


Figura 3: HspBP1 over-expressed *in vivo* and immune response. A- Number o $CD4^+$ or $CD8^+$ T cells in tumor draining lymph nodes of mice bearing tumor transfected with plasmid encoding mice HspBP1 sequence (B16 BP1) or with only plasmid (B16 vector). The transfected cells were injected subcutaneous in mice after 24hs of the transfection at concentration of 10^5 cells and the analysis was after 25 days of tumor growth, the total viable cells of lymph node were counted with trypan blue dye and stained with antibodies. **B-** Number of $CD11c^+CD86^+MHCII^+$ in tumor draining lymph nodes of B16 BP1 or B16 vector tumor bearing mice as described above. **C-** Dot plot of staining CD86 and MHC-II on $CD11c^+$ dendritic cells in the draining lymph nodes of mice bearing.