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ALTERAÇÕES EM CÉLULAS DENDRÍTICAS DERIVADAS DE MONÓCITOS E EM MONÓCITOS DE PACIENTES COM CÂNCER DE MAMA

PORTO ALEGRE

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

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"Abrir-se à *physis* é ligar-se ao problema da organização das partículas, átomos, moléculas, macromoléculas que se encontram no interior das células de cada um de nós;

"Abrir-se para a vida é abrir-se também para as nossas vidas"

Edgar Morin

RESUMO

A neoplasia mamária é uma causa importante de morbidade e mortalidade no Brasil e no mundo. Novas estratégias terapêuticas vem sendo propostas e uma delas é a utilização de terapia celular com células dendríticas (DCs), entretanto, os dados da literatura em relação ao real benefício desta abordagem apresentam grande variabilidade e inabilidade em produzir uma imunidade duradoura. No presente trabalho fizemos uma caracterização detalhada das células dendríticas derivadas de monócitos (MoDCs) das pacientes com câncer de mama e dos monócitos que as originaram. As MoDCs das pacientes são obtidas com um menor rendimento quando comparadas a controles saudáveis pareados por idade, e exibem uma diminuição nos marcadores de maturação e ativação. Além disso, as culturas de MoDCs das pacientes apresentaram altos níveis de IL-6, o que é compatível com um fenótipo pró-tumoral. Essas diferenças na produção de citocinas nos levou a postular que as vias de sinalização e/ou a expressão de *toll like receptors* (TLRs) poderiam estar alteradas. Observamos uma diminuição de TLR9 e TLR2 e um aumento na expressão de NFkBp50 nas MoDCs das pacientes, sem estímulo. Após estímulo com LPS e CPG as pacientes não aumentaram a expressão de MyD88, sugerindo uma diminuição Ada via de sinalização da resposta a esses padrões moleculares. Quando analisamos os monócitos, eles também estavam diminuídos nas pacientes, e apresentavam uma expressão de receptores para GM-CSF inferior a dos controles saudáveis. A produção de citocinas pelos monócitos das pacientes com câncer também estava alterada com uma produção aumentada de IL-6, IL-4 e IL-10. A frequência de TLR9 e TLR2 estava diminuída nos monócitos das pacientes. Esses dados conjuntamente demonstram que os monócitos já estão alterados nas pacientes com câncer, assim como as DCs diferenciadas a partir deles. O crescimento tumoral parece induzir um fenótipo de tolerância nestas células. Esse dado é de fundamental importância para o desenvolvimento de imunoterapia baseada em DCs.

Palavras chave: células dendríticas, monócitos, neoplasia mamária, câncer, IL-6.

ABSTRACT

Breast cancer is an important cause of morbidity and mortality in Brazil and worldwide. New therapeutic strategies have been proposed and one of them is the use of cell therapy with dendritic cells (DCs), however, the literature regarding the real benefit of this approach shows great variability and inability to produce a lasting immunity. In this paper we made a detailed characterization of monocyte-derived dendritic cells (MoDCs) of patients with breast cancer and monocytes that originated them. A lower yield of MoDCs was obtained from patients when compared to age matched healthy controls. Patient MoDCs exhibited decreased expressions of maturation and activation markers. Furthermore, cultures of MoDCs of patients had significantly elevated levels of spontaneous production of IL-6, which is consistent with a pro-tumor phenotype. These differences in molecule expression and cytokine production led us to postulate that the signaling pathways and / or the expression of toll like receptors (TLRs) could be altered. A decrease of TLR9 and TLR2 and an increase in the expression of NFkBp50 was found in MoDCs of patients without stimulation. After stimulation with LPS and CPG the patients did not upregulate expression of MyD88, suggesting a downregulation of the signaling pathways activated by these molecular patterns. The number of monocytes was also were decreased in patients, showing a reduced expression of GM-CSF receptors compared to monocytes of healthy controls. Cytokine production by monocytes from cancer patients was also altered, with an increased production of IL-6, IL-4 and IL-10. TLR2 and TLR9 expression was downregulated in monocytes of patients. Together these data show that monocytes are already altered in patients with cancer, and that will influence the phenotype of DCs differentiated from them. Tumor burden seems to induce a tolerogenic and pro-tumoral phenotype in patients´cells. This finding is important for the development of DC-based cancer immunotherapy.

Keywords: dendritic cells, monocytes, breast cancer, cancer, IL-6.

Índice

Alterações em monócitos e células dendríticas derivadas de monócitos de pacientes de câncer de mama

INTRODUÇÃO

Em 1909, Ehrlich postulou que o sistema imunológico seria capaz de reconhecer células tumorais como estranhas ao organismo e montar uma resposta imune antitumoral (***Ehrlich 1909)***. Sem isto, as células tumorais cresceriam infinitamente. Apenas anos mais tarde, iniciaram as evidências experimentais que confirmaram este postulado. Em 1957, Burnet demonstrou que células dependentes do timo eram capazes de gerar regressão de tumores (***Burnet 1957***). Desde então, há uma vasta literatura sobre mecanismos de resposta imune antitumoral, bem como de escape das diferentes neoplasias a essas respostas.

Faz parte do arsenal imunológico antitumoral um tipo específico de células apresentadoras de antígenos: as células dendríticas (DCs). As células dendríticas da pele humana foram descobertas em 1868 por Langherhans, e foram denominadas células de Langherhans, mas não se sabia as funções dessas células e essa dúvida permaneceu por muitos anos. As DCs de camundongos foram descritas pela primeira vez em 1973, por Ralph Steinman e Zanvil Cohn, após observarem que a população de células do baço de camundongos que aderia ao vidro ou ao plástico era muito heterogênea e que havia outro tipo celular além de "fagócitos", granulócitos e linfócitos (***Steinman and Cohn 1973***). Estas células apresentavam um formato peculiar: estrelado ou dendrítico, o que deu nome a elas. No ano seguinte, em 1974, os mesmos autores publicaram as propriedades das DCs *in vitro,* e observaram que elas aderem à placa e nela distribuem-se randomicamente. Eles observaram também que as DCs não apresentavam as propriedades funcionais dos outros tipos de células reticulares e que quando comparadas com

macrófagos, perdiam a capacidade fagocítica *in vitro* (***Steinman and Cohn 1974***). No mesmo ano foram descritas as propriedades das DCs *in vivo* e foi proposto que elas se diferenciam a partir de precursores na medula óssea e nas porções não aderentes do baço de camundongos. O mesmo trabalho demonstrou que essas DCs de órgãos linfóides podem ser destruídas por radiação ionizante e por administração de esteróides (***Steinman, Lustig et al. 1974***).

As células dendríticas do sangue periférico em humanos foram caracterizadas pela primeira vez em 1982, quando foram descritas como "as mais potentes células estimuladoras de MLR tanto alogeneico quanto singeneico" (***Van Voorhis, Hair et al. 1982***). No mesmo trabalho foi postulado que na ausência de DCs, outras populações celulares poderiam não ser estimulatórias; além disso, estabeleceram que as DCs circulantes no sangue periférico humano representam uma pequena fração (≅ 0,5%). Além da caracterização morfológica como células cujo citoplasma emite prolongamentos em todas as direções, o núcleo tem formato irregular e são ricas em mitocôndrias. Este trabalho também demonstrou que as DCs humanas expressam HLADR em sua superfície (***Van Voorhis, Hair et al. 1982***) e hoje sabemos que a alta expressão deste marcador é uma característica destas células em humanos, como bem revisado por Steinmann (***Steinman 1991***). Importante notar que essas extensões citoplasmáticas das DCs têm íntima relação com sua função, pois possibilitam o contato célula-célula; importantíssimo para a apresentação antigênica (***Kitajima, Ariizumi et al. 1996; Brocker 1997; Banchereau and Steinman 1998***).

As DCs são as principais células apresentadoras de antígenos profissionais, sendo especializadas na captura, processamento e apresentação dos antígenos para as células T (***Steinman 1991; Hart 1997; Banchereau, Briere et al. 2000; Mellman and Steinman 2001***). Elas capturam informações do mundo externo e as transferem para as células do sistema imune adaptativo podendo, a partir daí, disparar uma resposta imune efetora ou tolerante; são as DCs

que influenciam o tipo de célula T que será feito pela resposta imune (***Banchereau, Briere et al. 2000***).

As células dendríticas são uma população muito heterogênea em humanos e essa heterogeneidade se reflete em relação aos seus subtipos, sua localização anatômica, sua função e o desfecho final da resposta imune (se será efetora ou tolerogênica) (***Banchereau, Briere et al. 2000***).

Subtipos de Células Dendríticas Humanas

Os subtipos de células dendríticas, tanto em humanos quanto em camundongos, são definidos pelas proteínas expressas em sua superfície, localização anatômica e resposta funcional. Um resumo dos subtipos clássicos de DCs e sua localização pode ser visto na **Tabela 01**.

Tabela 01: Modificada de Liu e Nussenzweig 2010 (***Liu and Nussenzweig***).

Órgão	Subtipo de DC	Fenótipo	Função	Origem
Baço	DC CD8+	$CD8^+CD205^+SIRPa^-CD11b^-$	Apresentação cruzada de antígenos obtidos de células mortas e bactérias em contexto MHC I para imunidade e tolerância	Pré-DC
Linfo-nodos	DC $CD8^-$	$CD8^-CDIR2^+SIRPa^+CD11B^+$	Apresentação para MHC II	Pré-DC
	DC migratória	$CD11C^{int}CD40^{hi}MHCII^{hi}CCR7^+$	Transfere o antígeno da periferia para o linfonodo	Pré-DC
	DC residente $CD8^+$	$CD11c^{hi}MHCII^+CD8^+CD205^+$	Apresentação cruzada de auto-antígenos e antígenos virais para tolerância e resposta T citotóxica.	Pré-DC
	CD residente $CD8^-$	$CD11c^{hi}MHCII^+CD8^-CD11B^+$	Não está clara a função	Pré-DC
Pulmões, fígado, rins	DC $CD103^+$	$CD11c^{hi}MHCII^+CD103^+CD11B^-$	Apresentação cruzada para resposta antiviral	Pré-DC
	DC $CD11b^+$	$CD11C^{hi}MHCII^+CD103^-CD11B^+$	Não está clara a função	Pré-DC + monócitos?
Intesti-nos	DC $PPCD103^+$	$CD11c^{hi}MHCII^+CD103^+CD11b^-CX3CRI^-$	Não está clara a função	Pré-DC
	DC $LPCD103^+$	$CD11c^{hi}MHCII^+CD11b^+CD103^+CX3CRI^-M\text{-}CSFR^{lo}$	Não está clara a função	Pré-DC
	DC $LPCD103^-$	$CD11c^{hi}MHCII^+CD11b^+CD103^-CX3CRI^+M\text{-}CSFR^{hi}$	Captura os antígenos do lúmen intestinal e os transporta para os linfonodos mesentéricos	Pré-DC + monócitos?

As DCs humanas são subdivididas em dois grandes grupos conforme seu precursor: DCs mielóides (mDCs ou DCs convencionais) e DCs plasmocitóides (pDCs). Outra forma de defini-las seria: DCs migratórias (aquelas que residem nos tecidos periféricos não linfóides e que após capturarem o antígeno migram para os linfonodos), DCs linfóides e DCs plasmocitóides.

Em camundongos esse terceiro subtipo é bem caracterizado e denominado linfóide expressando CD8α. Existem no timo de camundongos dois subtipos de DCs linfóides: $CD11b^{low}CD8^{+}$ (em maior quantidade) e $CD11b^{high}CD8^{-}$ (em menor quantidade). As DCs linfóides dos camundongos também são chamadas de DCs residentes (aquelas que residem nos órgãos linfóides) (***Watowich and Liu***).

Mais recentemente houve avanços na resolução da controvérsia sobre a definição de DCs linfóides na espécie humana. Como revisado por Shortman, em humanos não existe o subtipo CD8+, havendo indicações de que existam DCs linfóides com outro padrão de moléculas de superfície, dadas as diferenças entre DCs de camundongos e de humanos (***Shortman and Heath***). Um fator a favor de que possa haver similaridade entre essas células em espécies diferentes vem da produção de Necl2, tanto em DCs $CD8^{+}$ de camundongos quanto nas DCs humanas localizadas na zona de células T do tecido linfóide (***Galibert, Diemer et al. 2005***). Há outra molécula que também demonstra essa correspondência: Clec9A (***Caminschi, Proietto et al. 2008; Huysamen, Willment et al. 2008; Sancho, Mourao-Sa et al. 2008***). Ela é expressa em humanos pelas DCs BDCA-3+ Necl2+Clec9A+ e acredita-se atualmente que estas DCs correspondam ao subtipo de DCs CD8+Necl2+Clec9A+ encontrado em camundongos (***Shortman and Heath***).

Vandenabeele *et al*, ao estudar o timo humano, percebeu dois subtipos de células maduras: $CD11b^{low}$ $CD33^{low}CD45RO^{low}$e $CD11b^{high}$, sendo que o primeiro apresenta marcadores de DCs maduras ($HLADR^{high}$, $CD40^{+}$, $CD86^{+}$, $CD83^{+}$) mas não apresenta marcadores da linhagem

mielóide, sendo $CD14^{-}CD64^{-}$ (***Vandenabeele, Hochrein et al. 2001***). Ambos os subtipos de DCs encontradas no timo humano possuem diferentes capacidades de produção e secreção de IL-12 (***Vandenabeele, Hochrein et al. 2001***).

As DCs plasmocitóides (pDCs) foram descritas pela primeira vez em 1958 (***Lennert and Remmele 1958***) e permaneceram muito tempo sem ter sua origem e função estabelecidas até que fossem identificadas como monócitos plasmocitóides e mais tarde como um subtipo de DCs (***Galibert, Maliszewski et al. 2001***).

As pDCs são a linha de frente da imunidade antiviral, sendo capazes de produzir rapidamente grandes quantidades de interferon tipo I em resposta a vírus via TLR7 e TLR9 (***Siegal, Kadowaki et al. 1999; Okada, Lian et al. 2003***) . As pDCs originam-se a partir de células tronco hematopoiéticas (***Manz, Traver et al. 2001***) e podem ser encontradas nos seguintes sítios: medula óssea, sangue, timo, áreas ricas em células T dos órgãos linfóides; observando que na presença de infecção ou autoimunidade, as pDCs podem estar na pele e em outros tecidos ***(Asselin-Paturel, Boonstra et al. 2001; O'Keeffe, Hochrein et al. 2002***)

Outra forma de caracterizar subpopulações de DCs seria definindo as DCs migratórias, também chamadas de DCs teciduais, encontradas em diversos órgãos, como pele, pulmões e intestinos. Este subtipo tem como principal característica funcional a captura de antígenos da periferia e transporte deles, via vasos linfáticos aferentes, para as DCs residentes nos linfonodos. Dentre elas, interessam-nos particularmente as DCs migratórias derivadas de monócitos (MoDCs) que diferem das DCs migratórias derivadas da medula óssea (BMDCs). As primeiras podem ser DC LP (lamina própria) $CD103^{-}$ e DCs inflamatórias, e as últimas podem ser: DC $CD103^{+}$, DC $CD103^{-}$ e DC LP $CD103^{+}$ (***Watowich and Liu***), **Fig 01**.

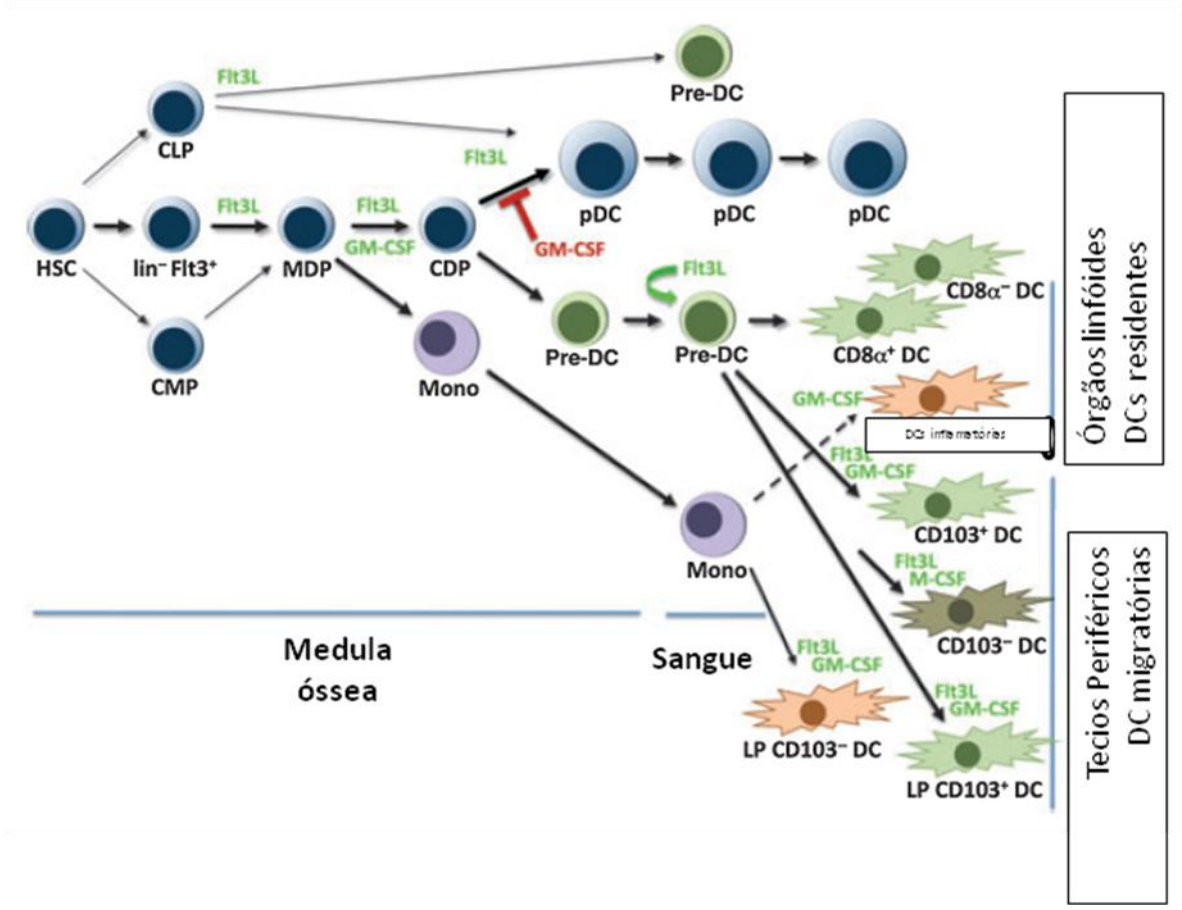

Fig 01: Modificada de Watowich (***Watowich and Liu)***.

DCs derivadas de monócitos

Ainda é controversa a questão de ocorrer *in vivo* a diferenciação de DCs a partir de monócitos. Randolph *et al* demonstraram, em um modelo de migração transendotelial *in vitro* que tenta mimetizar o movimento das DCs da periferia para o lúmen dos vasos linfáticos aferentes, a diferenciação de monócitos em MoDCs (***Randolph, Beaulieu et al. 1998***). Um ano depois, Randolph e Steinman *et al* utilizaram um modelo murino a fim de saber se essa diferenciação poderia ocorrer *in vivo*. Eles utilizaram microesferas de látex, coradas com FITC e injetadas via

subcutânea em vários lugares da pele dos camundongos a fim de gerar infiltração dos monócitos naquele sítio. Ao analisarem os cortes histológicos dos linfonodos, eles observaram que as células contendo as microesferas coradas com FITC exibiam um fenótipo bastante semelhante às DCs. Elas expressavam altíssimos níveis de moléculas MHC I e II além de CD86; uma vez que não expressavam B220, sabia-se que não seriam células B. Além disso, uma pequena fração destas células expressava DEC-205 (***Randolph, Inaba et al. 1999***). Este trabalho também teve o cuidado de verificar que as células encontradas nos linfonodos com as microesferas não eram células de Langerhans, concluindo ser possível a diferenciação in vivo de monócitos em células dendríticas.

Merad e colaboradores observaram que monócitos Gr-1^{high} infiltravam e proliferavam na pele inflamada em um modelo experimental que utilizava radiação UV (ultravioleta) como causa da inflamação. Eles observaram que no sétimo dia após a irradiação UV, os monócitos diminuíam a expressão de Gr-1 e aumentavam a expressão das moléculas MHC de classe II e logo após, deixavam de se apresentar em clusters e passavam a expressar langerina (um marcador específico das células de Langerhans). (***Ginhoux, Tacke et al. 2006***). Gr-1 é também citado como Ly6C.

Mais tarde, um estudo usando modelo murino de camundongos transgênicos CD11c-DTR tratados com toxina diftérica, nos quais foram transfectados monócitos $Ly6C^{high}$ propôs que as DCs da lâmina própria (intestinais e pulmonares) derivavam destes monócitos $Ly6C^{high}$ no *steady-state* (***Varol, Landsman et al. 2007***). Varol em outro estudo *in vivo*, em 2009, fez uma caracterização das DCs encontradas na lâmina própria do intestino de camundongos e encontrou duas subpopulações principais: DCs $CD11b^{-}CD103^{high}$ e DCs $CD11b^{+}CD103^{low}$. Neste estudo ele demonstrou que as células $CD11b^{+}$ e $CD11b^{-}$ apresentavam origens diferentes e dependiam de Fit3L e de GM-CSF respectivamente, concluindo que as primeiras originam-se a partir de pré-DCs e as últimas, de monócitos (***Varol, Vallon-Eberhard et al. 2009***). Bogunovic

também estudando a lâmina própria do intestino de camundongos, encontrou dois subtipos celulares de diferentes origens: DCs $CD103^{+}CD11b^{+}$ que derivam de pré-DCs de maneira dependente de Flt3L e DCs $CD103^{-}CD11b^{+}$ derivadas de monócitos, sob o controle do ligante de M-CSFR (***Bogunovic, Ginhoux et al. 2009***).

Mais estudos são necessários para a resolução da controvérsia sobre a diferenciação de DCs a partir de monócitos ocorrer in vivo (***Liu and Nussenzweig***), especialmente em humanos. Este trabalho considera que a diferenciação de monócitos circulantes em DCs pode ocorrer in vivo, em situações de infecção e inflamação como revisado recentemente por Ardavín (***Dominguez and Ardavin***). Considerando-se o desenvolvimento tumoral como uma condição inflamatória, é possível especular que possam existir MoDCs em pacientes com câncer. Contudo, as MoDCs são consideradas diferentes das DCs existentes *in vivo*, e entre elas, diferem quanto ao seu fenótipo de acordo com as citocinas utilizadas para sua diferenciação *in vitro* (ver item **Uso Clínico de Células Dendríticas**, mais adiante).

Funções das Células Dendríticas:

São funções das DCs: reconhecimento dos patógenos, ativação da imunidade imediata e de memória, preservação da tolerância a autoantígenos (***Watowich and Liu***).

Um fator determinante na função das células dendríticas é seu estado de maturação (também chamado de ativação) (***Palucka, Banchereau et al.***). As DCs imaturas residem nos tecidos periféricos e possuem alta capacidade endocítica/fagocítica, porém, baixa expressão de moléculas coestimulatórias como CD86, CD80, bem como baixa expressão de receptores de quimiocinas como o CCR7 (***Trombetta and Mellman 2005***). Além disso, elas "escondem" boa parte das suas moléculas HLA de classe II dentro do compartimento lisossomo/endossomo (***Trombetta and Mellman 2005***). Sendo assim, elas apresentam baixa expressão das moléculas

HLA classe II na sua superfície. Outro ponto importante é a incapacidade das DCs imaturas de liberar citocinas imunoestimulatórias para as células T (***Trombetta and Mellman 2005)***.

Desta forma, quando entram em contato com antígenos, elas os capturam. Isso pode ocorrer de várias formas: macropinocitose, endocitose mediada por receptores (como o DEC-205, o receptor de manose e receptores Fc), fagocitose de micropartículas e internalização de peptídeos carreados pelas *heat shock proteins* gp96 e HSP70 (***Banchereau, Briere et al. 2000***). Entretanto, enquanto imaturas elas são incapazes de processar esse antígeno e o apresentar para as células T. Então, as DCs imaturas, residentes nos tecidos periféricos como a derme, por exemplo, após capturarem antígenos, migram via vasos linfáticos de maneira dependente de CCR7 para os linfonodos (***Liu and Nussenzweig***). Durante essa migração, inicia a maturação das DCs, um processo no qual ocorre: perda dos receptores endocíticos e fagocíticos; *up regulation* de CD40, CD58, CD80 e CD86; desvio no compartimento lisossômico com *down regulation* de CD68 e *up regulation* de DC-LAMP; alterações no compartimento MHC de classe II e alterações morfológicas (perda de estruturas de adesão, reorganização do citoesqueleto e alta motilidade celular) (***Banchereau, Briere et al. 2000***). O que dispara essa migração periférica das DCs para os órgãos linfóides drenantes são sinais inflamatórios como LPS, TNF-α e IL-1. Os estímulos inflamatórios reduzem a captura de antígenos, estabilizam as moléculas MHCII na superfície celular e aumentam a expressão das moléculas coestimulatórias (***Banchereau and Steinman 1998***). Além disso, os estímulos inflamatórios bloqueiam a resposta das DCs ao MIP-3α (***Dieu, Vanbervliet et al. 1998***) e as DCs passam a expressar altos níveis de CCR7 ***(Yoshida, Imai et al. 1997***). Isso acarreta uma responsividade aumentada a duas importantes moléculas presentes nos órgãos linfóides secundários: MIP-3β e 6Ckine (***Banchereau, Briere et al. 2000***) Estas duas citocinas atraem tanto DCs quanto células T *näive* (***Gunn, Tangemann et al. 1998; Banchereau, Briere et al. 2000***).

Uma vez que estejam no linfonodo, as DCs em processo de maturação podem tanto encontrar as células T näive quanto as DCs residentes no linfonodo (DCs linfóides). Ao encontrarem as DCs residentes, essas DCs migratórias transferem sua carga antigênica para as DCs residentes nos linfonodos como foi demonstrado, em um modelo murino de infecção por herpes vírus, por Allan *et al* (***Allan, Waithman et al. 2006***). Esta transferência antigênica entre DCs de diferentes subtipos é crítica para a apresentação de antígenos para as células T, ou seja, para o início da resposta imune celular.

Ainda no linfonodo, as DCs migratórias, recém chegadas, encontram as células TCD4 *näive* e apresentam a elas o antígeno através da ligação do TCR (receptor de célula T) ao complexo MHC II-antígeno (***Itano, McSorley et al. 2003***). Este é o chamado primeiro sinal da ativação das células T, aquele que informa sobre o "invasor" ***(Kalinski, Hilkens et al. 1999***). Outros sinais são necessários para a ativação das células T. O segundo sinal é a ligação de moléculas coestimulatórias presentes nas DCs (CD80, CD86, CD40) aos seus respectivos ligantes nas células T (CD28 e CD40L). O segundo sinal informa sobre o potencial patogênico relacionado ao antígeno ***(Kalinski, Hilkens et al. 1999***). O terceiro sinal polariza a resposta imune e é desencadeado pelas citocinas ***(Kalinski, Hilkens et al. 1999***). Diferentes citocinas liberadas pelas DCs desencadearão diferentes respostas de diferentes subtipos de células T.

O INF-γ matura as DCs em DCs tipo 1 (alta coestimulação e alta produção de IL-12) que induzem células Th1 (***Kalinski, Hilkens et al. 1999***); ao passo que PGE2 induz as DCs a serem DCs tipo2, que na presença de alta coestimulação produzem baixas quantidades de IL-12 e induzem células Th2 (***Kalinski, Hilkens et al. 1999***).

A IL-12 é considerada o principal produto das DCs e pode direcionar tanto uma resposta Th1 quanto Th2 dependendo da quantidade de IL-12 produzida pelas DCs (***Ronchese, Hausmann et al. 1994; Hilkens, Kalinski et al. 1997; Stumbles, Thomas et al. 1998***). Quando ocorre baixa

produção de IL-12, há indução de células Th2. Quando ocorre grande produção de IL-12 pelas DCs, há indução de células Th1.

A IL-6 produzida pelas DCs pode induzir tanto células Th1 (***Yamamoto, Yoshizaki et al. 2000***) quanto Th2 (pois leva as células T*näive* a produzirem IL-4 e esta citocina polariza para Th2) (***Diehl and Rincon 2002***). Além disso, tanto a IL-6 quanto o TGF-β são cruciais para o desenvolvimento de células Th17. (***Veldhoen, Hocking et al. 2006***). O mesmo estudo mostrou que DCs oriundas de camundongos MyD88-/- não conseguiam induzir células Th17, sugerindo o envolvimento de citocinas inflamatórias nesta diferenciação (***Veldhoen, Hocking et al. 2006***).

A indução de células TCD8$^+$ pelas DCs começa pela apresentação de antígenos por moléculas MHC de classe I presentes na superfície das DCs. Esta via permite a apresentação tanto de antígenos exógenos quanto endógenos (***Banchereau, Briere et al. 2000***). Embora as DCs consigam ativar diretamente as células TCD8$^+$ (***Inaba, Young et al. 1987***), em geral é necessária a ajuda das células TCD4+ (***Banchereau, Briere et al. 2000***). Recentemente foi demonstrado em modelo murino que o acréscimo de INF-α em culturas de células T*näive* com DCs pulsadas com peptídeo em camundongos depletados para TCD4+, foi capaz de induzir resposta TCD8 independente da ajuda TCD4 ***(Ontiveros, Wilson et al.)***.

Embora tanto as DCs migratórias maduras no linfonodo quanto as DCs residentes no linfonodo e maduras tenham capacidade de apresentar antígenos para as células T, existem diferenças no tipo de apresentação entre estes dois subtipos celulares. A principal diferença é o fato de as DCs migratórias, durante o seu processo de migração/maturação, passarem a apresentar alta expressão de moléculas coestimulatórias, o que desencadeará uma resposta imune efetora.

Apesar de as DCs recém imigradas para o linfonodo serem estimulatórias, parte das DCs residentes pode ser tolerogênica (***Itano and Jenkins 2003***).

As DCs desempenham um papel muito importante na tolerância periférica. A indução de tolerância pelas DCs ocorre no *steady state* como uma consequência da apresentação antigênica na ausência de inflamação ou infecção (apenas primeiro sinal – ausência de coestimulação) (***Hawiger, Inaba et al. 2001; Bonifaz, Bonnyay et al. 2002; Liu, Iyoda et al. 2002***). A fagocitose de corpos apoptóticos produz mediadores anti-inflamatórios, como a IL-10, o que inibe a expressão de moléculas coestimulatórias e mantém a tolerância das células T, como revisado por Itano (***Itano and Jenkins 2003***).

A indução de tolerância periférica pelas DCs é muito importante na imunidade, pois existem autoantígenos que escapam à tolerância central (***Steinman, Hawiger et al. 2003***). Células dendríticas imaturas conseguem apresentar antígenos para células T desencadeando tolerância devido à ausência do segundo sinal. Isso foi demonstrado em experimentos nos quais células T alogeneicas foram colocadas em cultura com MoDCs imaturas. Como resultado disto, as células T tornaram-se refratárias a estímulos antigênicos, mesmo por DCs maduras. Estas células T tolerantes ainda podiam inibir a resposta a DCs maduras por outras células T. (***Jonuleit, Schmitt et al. 2000***). No mesmo trabalho, como um controle, foram colocadas DCs maduras em cultura com células T alogeneicas. Ao comparar o tipo de célula T induzida, o resultado do *priming* com MoDCs maduras foram células Th1 e observou-se secreção de INF-γ e IL-2 pelas DCs. Já o *priming* com MoDCs imaturas gerou células Treg, observando-se secreção de IL-10 pelas DCs (***Jonuleit, Schmitt et al. 2000***).

Essa função regulatória requer o contato célula-célula e foi demonstrado que ela pode ser bloqueada com o uso de anticorpos anti-IL10 (***Jonuleit, Schmitt et al. 2000***).

O Sistema Imune no Paciente com Câncer: Este tópico foi extensamente discutido por nós no artigo de "*The Immune System of Cancer Patients*", aceito pela revista "*Anti-inflamatory and Anti-*

Allergy Agents in Medicinal Chemistry" e o manuscrito está no ítem Manuscrito do artigo 02 desta tese, na página 59.

Células Dendríticas no Paciente com Câncer:

Em 1997, Gabrilovich *et al* descreveram diminuição da apresentação de antígenos pelas células dendríticas em pacientes com câncer de mama em um estudo no qual foram recrutadas 32 pacientes com neoplasia de mama e analisaram, inicialmente, as DCs circulantes quanto a capacidade de apresentar antígenos e estimular células TCD8 após estímulo com infuenza e toxóide tetânico. O resultado observado neste estudo foi que pacientes com câncer de mama em estágio avançado (III e IV pelo sistema TNM) não apresentavam resposta de células TCD8 ao vírus influenza nem ao toxóide tetânico (***Gabrilovich, Corak et al. 1997***). Quando eles diferenciaram DCs a partir dos monócitos das mesmas pacientes e controles, não observaram diminuição na função das DCs obtidas a partir dos monócitos das pacientes (***Gabrilovich, Corak et al. 1997***).

Gabrilovich, em 2000, num estudo que arrolou um total de 93 pacientes entre neoplasia de cabeça e pescoço, câncer de mama e carcinoma pulmonar tipo não pequenas células, observaram alterações nas DCs das pacientes. Primeiramente, a contagem de DCs obtidas diretamente do sangue periférico das pacientes era menor quando comparada com a contagem dos controles. O número de DCs das pacientes expressando CD86 também era menor que o dos controles (***Almand, Resser et al. 2000***). Outro achado importante deste estudo foi que, nos três tipos de câncer, conforme a proporção de células imaturas aumentava, a progressão da doença também aumentava. Além disso, observaram uma correlação direta entre a presença de DCs imaturas no sangue periférico e a duração do câncer de mama (***Almand, Resser et al. 2000***)

Treilleux et al observaram que carcinomas primários da mama são infiltrados frequentemente por DCs expressando marcadores mielóides e fenótipo imaturo e/ou maduro, mas raramente por pDCs; embora as pacientes que possuíam pDCs CD123+ no infiltrado peritumoral apresentassem um pior prognóstico (menor sobrevida geral e menor sobrevida livre de doença) (***Treilleux, Blay et al. 2004***).

Um estudo utilizando DCs circulantes de pacientes com câncer de mama em estágio inicial versus de controles saudáveis, demonstrou que as DCs das pacientes apresentavam níveis de apoptose espontânea significativamente maiores que as do grupo controle (***Pinzon-Charry, Maxwell et al. 2006***). O mesmo estudo, a fim de saber se isso ocorria devido a fatores liberados pelo tumor, testaram *in vitro* PBMCs, incubando-os com TDSN e mensurando apoptose das células $Lin^{-}HLADR^{+}$, observando um aumento significativo na apoptose das DCs com TDSN em relação a cultura-controle não tratada. Além disso, eles observaram que quando acrescentavam CD40L às culturas tratadas com TDSN, havia redução na apoptose. Concluíram então, que fatores liberados pelas linhagens tumorais causavam a apoptose nas células *in vitro* (***Pinzon-Charry, Maxwell et al. 2006***). O mesmo grupo, em outro estudo, também observou alterações numéricas e funcionais nas DCs circulantes de pacientes com câncer de mama tanto em estágios iniciais quanto avançados, abrangendo um total de 173 pacientes entre 40 e 83 anos de idade. Neste trabalho foi demonstrado que a contagem de DCs circulantes e de linfócitos estava reduzida nas pacientes em estágio avançado. Além disso, as DCs das pacientes em estágio avançado deste estudo apresentavam baixa expressão de CD86 e HLADR (***Pinzon-Charry, Ho et al. 2007***).

Com base nestes dados, acreditava-se então que sim, haveria uma resposta imune prejudicada nos pacientes com câncer e que não somente as células T estariam alteradas como também as DCs. O que motivava diversos grupos para tentar utilizar as vacinas de DCs autólogas como terapia para pacientes com câncer, era a ideia de que seria a presença tumoral *in vivo* a

causadora das alterações no sistema imune devido a fatores liberados pelo tumor para a corrente sanguínea. Os protocolos de vacinas autólogas de DCs as diferenciavam *in vitro* a partir dos monócitos das pacientes. Dessa forma o processo de diferenciação de monócitos em DCs seria controlado, estaria longe do microambiente tumoral e poderia recuperar a função das DCs. Entretanto, como discutido mais adiante, os resultados dos ensaios clínicos envolvendo vacinas de DCs não foram duradouros como se esperava.

Uso Clínico das Células Dendríticas:

Sallusto e Lanzavecchia foram os primeiros a diferenciar DCs a partir de monócitos obtidos no sangue periférico (***Sallusto and Lanzavecchia 1994***). Em 1995, foi publicado o primeiro ensaio clínico de vacina de DCs. Desde então, as células dendríticas têm sido objeto de estudo de muitas pesquisas com o objetivo de usá-las em vacinas terapêuticas para diversos tipos de câncer.

As principais fontes atuais de DCs para protocolos clínicos são: precursores CD34+ vindos de sangue ou medula óssea; DCs circulantes no sangue; e monócitos. As DCs derivadas de monócitos, ou MoDCs, são as mais frequentemente usadas em protocolos clínicos, sendo tanto DCs imaturas (derivadas com GM-CSF e IL-4) ou DCs maduras, derivadas de monócitos cultivados com GM-CSF e IL4 em combinação com IL-1β, IL-6, TNF-α e PGE2 (frequentemente referidas como o "padrão ouro") (***Nestle, Farkas et al. 2005***). Enquanto alguns estudos mostram que as DCs imaturas são inferiores quando comparadas às DCs maduras com respeito à sua capacidade de estimulação de células T (***Adams, O'Neill et al. 2004***), outros estudos clínicos contradizem este achado, indicando que a indução de resposta T por DCs imaturas é melhor (***Smithers, O'Connell et al. 2003***)

Alguns sugerem que as DCs "padrão ouro" maduras são piores por não produzirem IL-12 (***Kalinski, Vieira et al. 2001***) e temem que por serem maduras elas estejam exaustas e incapazes de realizar a sensibilização de células T. Assim, é necessário que outros protocolos de geração de DCs sejam investigados ***Nestle, Farkas et al. 2005***). Nosso estudo utilizou um protocolo intermediário, cultivando monócitos com GM-CSF e IL-4, fornecendo TNF-α como estímulo de maturação.

Pedersen *et al*, analisou fenotípica e funcionalmente DCs obtidas a partir de monócitos do sangue periférico de pacientes com neoplasia mamária recrutadas para um ensaio clínico em fase I/II de vacina de DCs pulsadas com peptídeo e observou que as DCs preparadas a partir das pacientes apresentavam um fenótipo de maturação intermediária (a maioria expressava CD40, CD54 e CD86) além de apresentarem uma reduzida capacidade aloestimulatória no ensaio de MLR quando comparadas com as DCs obtidas de controles saudáveis.(***Pedersen, Thorn et al. 2005***). Outro achado importante deste estudo foi o fato de eles terem testado diferentes coquetéis de citocinas para a maturação das DCs diferenciadas *in vitro* a partir de monócitos de pacientes e de controles e observaram que nenhum dos coquetéis testados foi capaz de produzir DCs maduras o suficiente para serem utilizadas como terapia.

Em um outro estudo, com ensaio clínico em fase I para mieloma múltiplo, utilizando vacina de DCs autólogas fusionadas a células derivadas do mieloma, foi demonstrado um aumento de duas vezes na porcentagem de células TCD4 e TCD8 reativas ao tumor em 11 dos 15 pacientes (***Rosenblatt, Vasir et al.***). No entanto, a maioria dos pacientes apresentou uma diminuição na resposta imune durante 6 meses após a vacinação. Onze pacientes apresentaram doença estável após a vacinação (**Rosenblatt, Vasir et al.**).

Um ensaio clínico de fase III para câncer de próstata refratário a hormônios, com células apresentadoras de antígenos autólogas (Sipuleucel-T), mostrou que não houve diferença entre a resposta clínica dos pacientes tratados com a vacina e os pacientes tratados com o placebo (***Small, Schellhammer et al. 2006***).

Alguns grupos utilizam células heterólogas para confecção das vacinas, devido a resultados encontrados de baixa estimulação de células derivadas de pacientes. Um estudo no qual incluíram pacientes com melanoma ou carcinoma de células renais em estágio avançado, com tumores volumosos e que não responderam a outros tratamentos e foram submetidos à pelo menos duas doses de vacina híbrida com DCs heterólogas e células tumorais autólogas, demonstrou que 71% dos pacientes apresentavam estabilização da doença entre 4 e mais de 19 meses. ***(Barbuto, Ensina et al. 2004***). Outro estudo, do mesmo grupo, mostrou que o uso de vacinas híbridas com DCs heterólogas de doadores saudáveis fusionadas com células tumorais autólogas induziu recuperação da função das DCs em pacientes com câncer metastático (melanoma e câncer de células renais).***(Neves, Ensina et al. 2005***). Uma metanálise recente analisou dezessete ensaios clínicos de vacinas de DCs para câncer de próstata e doze de células renais e concluiu que houve efeito estatisticamente significativo na resposta imune celular mediada por DCs e que a dose de DCs na taxa de benefício clínico pode ser demonstrada. No entanto, observaram grande heterogeneidade em relação aos seguintes parâmetros: pureza das DCs, dose da vacina, antígeno liberado na apresentação, via de vacinação e controle de qualidade. Além disso, a dose da vacina, o fenótipo maduro da DCs utilizadas e o acesso linfonodal foram fatores que afetaram o sucesso da vacinação com DCs ***(Draube, Klein-Gonzalez et al.***).

O motivo destes resultados conflitantes pode estar na interação câncer x sistema imune do paciente. Há estudos que observaram alterações nas DCs geradas a partir de monócitos de pacientes com câncer ***(Pedersen, Thorn et al. 2005; Pinzon-Charry***, ***Ho et al. 2007***); bem

como nas DCs circulantes no sangue periférico ou em linfonodos drenantes de pacientes com câncer. (***Almand, Resser et al. 2000; Pinzon-Charry, Ho et al. 2007)***. Isso pode ser explicado pelo fato de o ambiente tumoral afetar as células responsáveis pelas respostas imunes através, por exemplo, da *down regulation* de moléculas coestimulatórias, perda de expressão de MHC de classe I, indução de células T regulatórias e de células dendríticas regulatórias. (***Croci, Zacarias Fluck et al. 2007***).

Em face do acima exposto, nós hipotetizamos que o uso de células dendríticas autólogas em vacinas terapêuticas para neoplasias precise ser otimizado devido ao funcionamento prejudicado das DCs obtidas a partir de monócitos de pacientes com câncer. Contudo, os mecanismos que causadores dessas alterações das DCs geradas a partir de monócitos de pacientes ainda são pouco conhecidos.

Especula-se que a estratégia de vacinar via intradérmica é inferior do que intranodal. Muitos ensaios clínicos utilizaram DCs imaturas na confecção da vacina e sabemos que as DCs imaturas são ótimas para capturar antígenos, mas não conseguem migrar para os linfonodos nem ativar células T. Na tentativa de ultrapassar a dificuldade da migração, alguns trabalhos tentaram utilizar a via intranodal (***Figdor, de Vries et al. 2004; Nestle, Farkas et al. 2005***).

Recentemente foi publicado um ensaio clínico que utilizou da via intranodal para tratar pacientes com glioblastoma metastático com vacina de DCs autólogas associadas ao lisado tumoral. Quatro de dez pacientes não apresentaram progressão da doença e sobreviveram pelo menos dois anos (***Fadul, Fisher et al.***). É importante observar que a sobrevida esperada neste tipo de neoplasia é em média 12 meses.

Mais recentemente tem sido proposto o uso de terapias-alvo para pacientes com câncer, ao invés de vacinas de DCs. Uma molécula promissora parece ser o DEC-205 (receptor de fagocitose que media a captura do antígeno). Bonifaz *et al* demonstrou em modelo murino que o

targeting de antígenos para as DCs via DEC-205 melhorou a vacinação com células T, bem como o uso de anti-CD40 associado ao DEC-205 causou *priming* das células TCD4 e TCD8. (**Bonifaz, Bonnyay et al. 2004**).

Um trabalho recente demonstrou que células dendríticas tanto de controles saudáveis quanto de pacientes com melanoma foram eficientemente carregadas pelo *targeting* com DEC-205 (***Birkholz, Schwenkert et al.***).

Outra estratégia é a indução de imunidade antitumoral através da indução de DCs in situ, como demonstrado em modelo murino por Furumoto *et al*. Neste estudo foi utilizado um vetor retroviral para transferir o DNA do CCL20 murino para linhagens de adenocarcinoma de cólon e melanoma; os camundongos inoculados com as linhagens tumorais transfectadas com CCL20 apresentaram contagens de DCs tumorais duas ou quatro vezes maiores que os controles negativos, apesar de não terem iniciado imunidade antitumoral efetiva (***Furumoto, Soares et al. 2004***). O mesmo estudo mostrou que quando o agonista de TLR9, CpG foi acrescentado, houve indução de imunidade antitumoral, assinalando a importância de adjuvantes nas estratégias de vacinação. Além disso, a injeção intratumoral de CCL20 e CpG induziu a regressão de tumores estabelecidos em sítios distantes. (***Furumoto, Soares et al. 2004***).

Um ensaio clínico recente para melanoma metastático arrolou 16 pacientes e demonstrou melhora objetiva de 8 deles (com algumas peculiaridades em cada paciente) após vacinação com MoDCs autólogas combinadas com Tremelimumab (um anticorpo monoclonal que bloqueia o CTLA4) (***Ribas, Comin-Anduix et al. 2009***).

Os resultados dos ensaios clínicos envolvendo vacinas de DCs não foram duradouros como se esperava ou não acarretaram o nível esperado de regressões tumorais (***Figdor, de Vries et al. 2004; Nestle, Farkas et al. 2005***). O presente trabalho apresenta alguns mecanismos candidatos para explicar esses dados: os monócitos das pacientes já apresentam alterações

críticas que interferem na diferenciação deles em DCs *in vitro*, tais como baixa frequência de monócitos, baixa expressão de TLR2 e TLR9 em monócitos e MoDCs, expressão alterada de NFkBp50, além de alta produção e secreção de IL-6.

Câncer de Mama e Células Dendríticas

O câncer de mama é o segundo tipo de câncer mais frequente nas mulheres no mundo. No Brasil a taxa de incidência estimada para 2010 foi de 49 casos novos para cada 100.000 mil mulheres e no Rio Grande do Sul de 81,57 novos casos para cada 100.000 mulheres. (***INCA – Instituto Nacional do Câncer*** *http://www1.inca.gov.br/estimativa/2010/index.asp?link=conteudo_view* ***acessado em 15 de julho de 2011 8h40 min***).

A caracterização das DCs em pacientes com câncer de mama foi discutida acima, no ítem Células Dendríticas no Paciente com Câncer na página 20.

Dada a importância epidemiológica da neoplasia mamária, nós a escolhemos como objeto do nosso estudo.

Hipótese do Trabalho

As DCs derivadas de monócitos e os monócitos de pacientes de câncer de mama estão alteradas em relação a controles saudáveis e isso contribui para os resultados variáveis em vacinação baseada em DCs.

OBJETIVOS

Objetivo geral

Comparar DCs geradas a partir de monócitos, e também monócitos, de controles saudáveis, pacientes com patologias benignas da mama e pacientes com câncer de mama.

Objetivos específicos

Analisar marcadores de superfície das DCs geradas em cultura e dos monócitos, através de citometria de fluxo.

Analisar citocinas secretadas *in vitro* pelas DCs e pelos monócitos sem estímulo e após 24h de estímulos com agonistas de *toll like receptors* (PGN, LPS e CpG).

Analisar expressão de *toll like receptors* e sinalização após a interação com seus respectivos ligantes

Caracterizar alterações em monócitos usados para diferenciar as DCs

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MONOCYTE-DERIVED DENDRITIC CELLS AND MONOCYTES OF BREAST CANCER PATIENTS SPONTANEOUSLY PRODUCE IL-6 AND SHOW IMPAIRMENT IN TLR9 EXPRESSION AND FUNCTION

Short Title – Monocytes and DCs from cancer patients produce IL-6

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Abstract

Monocytes and the cells they can originate, such as macrophages and dendritic cells (DC), are often found infiltrating tumors, and have been reported to enhance malignancy. Nevertheless, the ability of monocyte-derived DCs (MoDC) to present antigen to T cells, providing signals for initiation of tumor immunity, makes them an important tool for cancer immunotherapy. Two common aspects of studies employing DC-based immunotherapy in cancer are a great variability of results and an inability to produce long-lasting immunity. In this study, we analyzed 41 breast cancer patients, 38 tumor-free controls and 22 patients with benign breast alterations, and observed a major impairment in their yield of MoDC from tumor patients. A more detailed analysis revealed that these cells show a significantly increased spontaneous production of IL-6 compared to tumor-free gender/age matched controls. Also, their expression and function of TLR 9, but not TLR-4 and 2, was significantly reduced, as evidenced by production of cytokines and MyD88 up regulation in response to TLR agonists. Importantly, we found that these alterations can already be detected in monocytes of these patients, but not in tumor-free controls or patients with benign alterations. Moreover, not only monocyte numbers are decreased in patients, but these cells also show a decreased expression of GM-CSF receptors. Together, our results indicate that tumor burden can lead to pronounced systemic alterations in monocytes and MoDCs, leading to a tolerogenic phenotype and favoring a tumor promoting environment. These findings are relevant for the design of DC-based cancer immunotherapy.

Introduction

Dendritic cells (DC) provide a crucial link between the innate and adaptive immune responses. These cells can be found both in lymphoid and non-lymphoid tissue, and express a myriad of receptors involved in surveillance and pathogen recognition, presenting antigens to T cells and conveying signals that will activate of long-term memory responses, as well as establish tolerance to self antigens (Banchereau and Steinman 1998). Such characteristics make them unique targets for all types of immune therapy, especially for cancer patients.

Different subpopulations have been characterized for DC *in vivo*, due to heterogeneity in cell surface markers, and for each subpopulation specific functions have been demonstrated (Liu and Nussenzweig; Watowich and Liu). *In vitro*, DC can be obtained from bone marrow precursors, as well as from monocytes, using in vitro differentiation protocols, in the presence of GM-CSF and other cytokines (Sallusto and Lanzavecchia 1994; Barbuto, Ensina et al. 2004; Figdor, de Vries et al. 2004; Nestle, Farkas et al. 2005); (Bernhard, Disis et al. 1995; Dauer, Obermaier et al. 2003). While the conditions for *in vivo* differentiation of DC from monocytes are still not clear (Liu and Nussenzweig; Watowich and Liu), this aspect of DC biology has been widely explored in the design of tumor immunotherapy strategies. Tumor derived antigen (MartIn-Fontecha, Sebastiani et al. 2003; Nair, McLaughlin et al. 2003) or RNA (Van Tendeloo, Ponsaerts et al. 2001; Morse, Nair et al. 2003) can be used to load monocyte-derived DC (MoDC), followed by adoptive transfer of these cells into patients, generating anti-tumor T cell responses. More recently, it seems likely that these therapies will evolve into targeting DC subpopulations in vivo, using antigenic molecules or particles conjugated to antibodies to markers such as DEC205 (Birkholz, Schwenkert et al.; Kamphorst, Guermonprez et al.) or CLEC9A (Huysamen, Willment et al. 2008)

An important issue that must be considered in tumor immunotherapy is the phenomenon of immunoediting (Schreiber, Old et al.). In order to grow, tumors employ different escape

strategies, the immune system being a target of such strategies, not only editing expression of tumor antigens, but also employing a series of immunossupressive mechanisms (Hanahan and Weinberg; de Souza and Bonorino 2009). In fact, some of the studies on DC-based therapies suggest that associating the use of adjuvants such as CpG is necessary to obtain an effective response (Zhang, Munegowda et al.; Furumoto, Soares et al. 2004). Thus, the effort to find and develop suitable adjuvants for anti-cancer therapy, many of them based on TLR agonists (Dunne, Marshall et al.; Krutzik, Tan et al. 2005; Nestle, Farkas et al. 2005), is of central importance for the optimization of DC-based protocols.

A common aspect of most of the studies with DC-based anti-tumor vaccination is that the response, although frequently significant, is not long-lasting. Although this could probably be explained by the multitude of immunossupressive strategies used by tumors, it seems probable that not only the DC vaccination effects *per se* could be targeted by the tumor, but also that the very DC used for vaccination could have previously suffered tumor-induced alterations. If so, it would be important to characterize such alterations, in order to optimize the effect obtained in any DC-based vaccine protocol. Especially, the verification of these alterations in patients´ monocytes is of interest, given the abundant monocyte/macrophage/DC infiltrate present in many tumors (Schreiber, Old et al.).

In this study, we hypothesized that tumor burden leads to alterations in monocytes and MoDC derived from patients compared to tumor-free individuals. We analyzed MoDC as well as monocytes from breast cancer patients and compared them to healthy controls. We observed that not only the generation of MoDC in patients, but also their monocytes, are impaired in comparison to controls. In addition to alterations in numbers, the cells from patients showed an altered cytokine production profile, which could be partly explained by alterations in signaling pathways and TLR expression. Importantly, MoDCs from patients show an immature phenotype and constitutively express IL-6, contributing to a tumor promoting environment. Most of the

alterations observed in MoDCs are already present in the monocytes. We discuss the implications of these findings for the design of immunotherapy for cancer patients.

Materials and Methods

Study's Design: case control study.

Patient Population: Forty one female breast cancer patients were recruited from the Mastology Outpatient Office of São Lucas Hospital in Porto Alegre. Patients were recruited at diagnosis, prior to surgery, not receiving any type of chemotherapy. At any time, any patient enrolled could exit from this study. Patients´ and Controls´ characteristics are listed in Table 01. The control population was formed by age matched healthy women from PUCRS employees. Benign Breast Pathology Controls were women with breast diseases except cancer, age matched. All subjects signed an informed consent form detailed on the protocol approved by the Ethics Committee of São Lucas Hospital, under number: 06/03143

Blood samples and cell culture: 30 ml of blood were collected from all study subjects – cancer patients, benign patients, and healthy controls. Mononuclear cells were isolated by ficoll gradient. **Dendritic Cell Culture**: mononuclear cells were cultured in AIMV medium. GM-CSF and IL-4 were added, at day 1 (1000UI/ml) and 4 (500UI/ml). TNF-α was added at day 5 for maturation of MoDCs (500UI/ml). After 7 days, cells were harvested and counted.

For analysis of NFkB and MyD88 activity, cells were plated at 1 x 10^6 cells/ml, either without stimuli, or adding LPS (lipopolysaccharide – *toll-like* receptor 4 agonist) (1mg/ml), CpG (Oligodeoxynucleotides containing unmethylated cytosineeguanosine sequence motifs – *toll-like* receptor 9 agonist) (6pg/ml), or PGN (peptidoglycan – *toll-like* receptor 2 agonist) (0.5mg/ml).

Each stimulus was given for 30 min. After that, DCs were harvested, lysed and stored at -20ºC for posterior western blot analysis.

For analysis of cytokine production, cells were plated at 1 x 10^6 cells/ml either without stimuli, LPS, CpG, or PGN, and cultured for 24 hours, when culture supernatant was harvested and stored at -20ºC for CBA analysis.

Western Blot: A standardized amount of total protein, determined using Qubit® (Invitrogen®) per sample was ran on 12% SDS-page gels, and proteins were transferred to nitrocellulose membranes using a Mini-Transblot system (Bio-Rad). Membranes were blocked with PBS 5% milk and washed ten times (shaking during 5 minutes) with PBS Tween 0.05%. After this, membranes were incubated 1h at room temperature and 30 minutes at 37ºC with primary antibodies (anti NF-kappa B and anti-MyD88; both from Santa Cruz Biotechnology®; and anti-human β-actin from Sigma®) and washed ten times (shaking during 5 minutes) with PBS Tween 0.05%. After this, membranes were incubated with secondary antibodies (Goat anti-mouse IgG, from Zymed®) for one hour at room temperature and 30 minutes at 37ºC and washed ten times (with agitation during 5 minutes each wash) with PBS Tween 0.05%. Finally, membranes were developed with Luminol (Santa Cruz Biotechnology®) and Kodak® films, and were scanned (HP Scanjet G4050®). Western blot images were analyzed by ImageQuant® TL 1D Version 7.0 software.

Flow Cytometry: DCs were harvested and stained with the following antibodies from Beckton Dinckinson® : anti HLA-DR FITC anti HLADR PE and anti HLADR Cychrome for DC compensation controls. We used anti-human CD14 FITC, anti HLADR PE and anti-human CD14 APC for monocyte compensation controls. As isotype controls, anti-human IgG PE and FITC, both from DIATEC were used. For surface markers, we stained DCs with the following antibodies from Beckton Dinckinson®,: anti-human Lin1 (CD3, 14, 16, 19, 20, 56) FITC, anti-human CD83

PE, anti-human CD40 PE ,anti-human HLADR PE and FITC, anti-human HLADR Cychrome, anti-human CD1a PE anti-human CD86 FITC, anti-human CD195 FITC (CCR5), anti-human CCR7 biotinylated. Anti-human CD11c PE was from Ebioscience®. To analyze TLRs expression, we used biotin mouse anti-human TLR4 from Beckton Dinckinson®, anti-human TLR9 FITC from Santa Cruz Biotechnologies®, anti-human CD282 (TLR2) alexa fluor 488 from Beckton Dinckinson®. In order to analyze monocytes's phenotype, we used CD14 FITC from DIATEC, CD14 APC from Beckton Dinckinson®, anti-human Lin1 (CD3, 14, 16, 19, 20, 56) FITC from Beckton Dinckinson®, anti-human HLADR PE and FITC, both from Beckton Dinckinson®, biotin rat anti-human CD124 (IL-4 receptor) from Beckton Dinckinson® and anti-human CD116 (GM-CSF receptor) from Beckton Dinckinson®. For biotin conjugates antibodies, we used Streptavidin FITC from Beckton Dinckinson®,).

FACs data were collected in a FACSCanto or FACSCalibur (Beckton Dinckinson®,) using the Cell Quest® doftware and analyzed using Flowjo® v 7.5.

CBA: Culture supernatants were analyzed for cytokines (IL-4, IL-6, IL-10 and TNF-α) according to Human CBA protocol (Beckton Dickinson, Hyalea, FL).

Statistical Analysis: Statistical analysis was made by Kolmogorov-Smirnov, Kruskal-Wallis, ANOVA. A p value less than 0.05 was considered statistically significant. Data analysis was made with GraphPad Prizm® 4.0 software.

Results

Cultures of monocytes from breast cancer patients yield fewer dendritic cells compared to healthy controls. A characterization of patients analyzed in this study and their tumors is presented in Table 1. Culture of blood monocytes (Mo) with GM-CSF, IL-4 and TNF-α can lead to their differentiation into dendritic cells (DC) (Nestle, Farkas et al. 2005), an observation that prompted a series of studies on the potential of this technology for immunotherapy (Palucka, Ueno et al.). While standardizing the cell differentiation protocol, we observed a pronounced reduction in the yield of MoDC obtained from breast cancer patients compared to controls. The difference was already apparent in FSC by SSC plots when the cultured cells were analyzed by FACS (Figure 1, A,B). Staining for HLA-DR and CD86 also evidenced this disparity (Figure 1, C,D), showing a lower percentage of HLA-$DR^{hi}CD86^{hi}$ cells in patient cultures. These monocytes derived dendritics cells presents a phenotype $HLADR^{hi}Lin^{-}$. Analysis of the mean fluorescence intensity (MFI) for different markers of differentiation and activation of DC revealed a significantly decreased expression of CD86, CD80, CD83, HLA-DR and CD40 (Figure 1, E-H). CD1a, and CCR5 and CCR7 expression did not differ between control and patient MoDC. These findings indicated an extremely impaired potential for differentiation of DC from monocytes in breast cancer patients compared to healthy controls.

Monocyte-derived DC from breast cancer patients show spontaneous increased production of IL-6. The difference in yield lead us to hypothesize that MoDC from patients would also present functional alterations. Cytokine production is an important function of DCs, especially in the modulation of T cell responses (Ueno, Schmitt et al.). To investigate if cytokine production was impaired in this population, MoDC obtained from patients and controls were counted, re-plated in equal concentrations, and incubated with different TLR agonists or no stimulus, for 24hs. Culture supernatant was then harvested and analyzed for cytokines. Remarkably, MoDC from patients showed a significantly increased production of IL-6 without any stimulation compared to

controls (Figure 2B), while controls only produced IL-6 when stimulated with LPS, CpG or PGN. In patients, such up regulation of IL-6 in response to stimuli was not observed – in fact, IL-6 production was down regulated in response to TLR agonists. No differences in production of IL-4 were evidenced, not even in response to stimulation (Figure 2A). Controls showed a significant reduction in IL-10 production in response to CpG (Figure 2C). Interestingly, only patients presented an increased production of TNF-α when stimulated with LPS, which was not observed in controls. These results pointed to a major alteration in MoDC cytokine production in breast cancer patients.

TLR expression and signaling are altered in patient MoDC. The differences observed in cytokine production by both unstimulated and stimulated patient MoDC led us to hypothesize that TLR expression or signaling could be altered in cells obtained from patients. We thus analyzed TLR 2, 4 and 9 expression by FACS. The results, shown in Figure 3, indicated a significantly decreased MFI for TLR9 in patients compared to controls (Figure 3C), as well as a decreased percentage of MoDC positive for this receptor (Figure 3F). A significant decrease was also observed in the MFI for TLR2, but not TLR4, while the percentages of MoDC expressing these two receptors did not differ between the two groups (Figure 3).

Ligation of TLRs leads to up regulation and phosphorylation of MyD88 (Gay, Gangloff et al.), as well as activation of NFkB, which can be measured by increase in the p50 subunit (Hayden and Ghosh 2004). We analyzed the up regulation of these two molecules in MoDC from patients and controls in response to TLR2, 4 and 9 agonists. The results in Figure 4 showed an increased unstimulated expression of NFkBp50 in patients compared to controls (Figure 4, A through D). Only one out of four patients up regulated p50 in response to TLR2 stimulus (Fig. 4D). Patients failed to up regulated MyD88 in response to LPS (Figure 4F), and CpG (Figure 4G). Two out of four patients up regulated MyD88 in response to PGN (Fig. 4H). Taken together, these results

suggested that tumor patients presented several alterations in the expression of TLRs as well as in the molecules involved in the response to TLR ligation.

Breast cancer patients have fewer monocytes than tumor-free controls. In vivo, DC are found in distinct subpopulations, and have different precursors, one of these being monocytes (Dominguez and Ardavin). Modifications in circulating DC from cancer patients have been reported previously (Pedersen, Thorn et al. 2005; Pinzon-Charry, Maxwell et al. 2006; Pinzon-Charry, Ho et al. 2007), and these cells are not thought of as being all derived from monocytes (Liu and Nussenzweig). However, in our system, it was possible that the low yield of MoDC could be simply due to lower numbers of monocytes in patients. To investigate that, we first analyzed the frequency of blood monocytes from patients and controls, which are found at a higher FSC and SSC than lymphocytes, and express CD14 (Figure 5A and 5B, respectively). Unexpectedly, the frequency of CD14+ cells in PBMC of patients was remarkably low (Figures 5C and 5D) compared to controls. The MFI for CD14 was not lower in monocytes from patients, indicating that this result was not due to down regulation of CD14, used to identify the monocyte population (Figure 5E,F).

An alternative, non-excluding explanation for the low MoDC yield was that the expression of receptors for GM-CSF and/or IL-4 could be down regulated in monocytes for patients. When we stained monocytes for CD14, HLA-DR, GM-CSFR and IL-4R, we verified that the percentage of cells expressing GMCSF-R, but not IL4R, was significantly decreased in patients (Figure 5 G, H). The MFI for GM-CSFR and IL-4R was not different among groups (Figure 5 K, L). Both results suggested that tumor burden had a significant impact over monocyte production and their potential for differentiation into DC.

MoDC alterations in cancer patients are already present in the monocytes used for their differentiation. The finding that monocyte frequency was altered in cancer patients prompted us

to ask if the alterations we observed in the cells derived from patients were simply a consequence of previous modifications in the monocytes that originated them. Analysis of TLR expression in PBMC of patients showed a major impairment in the frequency of TLR9+ monocytes (Figure 6F) while the MFI for this molecule was not significantly different from controls (Figure 6E). The frequency of TLR2+ monocytes was also significantly impaired (Figure 6B). When monocytes from patients were analyzed for cytokine production, an increased spontaneous production of IL-6 was again observed compared to controls as well as patients with benign alterations (Figure 7A). Unstimulated production of IL-4 and IL-10 was also higher in patient's monocytes than that observed for controls (Figure 7B and C). Together, these results showed that some of the alterations observed for patient MoDC were already present in the monocytes that originated them.

Discussion

Different studies have reported that circulating DC from cancer patients can be tolerogenic (Frick, Grunebach et al.), apoptotic (Pinzon-Charry, Maxwell et al. 2006) and dysfunctional in terms of ability to generate T cell responses (Satthaporn, Robins et al. 2004), consistently with tumor immunoediting. Generation of MoDC from cancer patients with cytokines has also demonstrated variability in their potential to induce T cell responses. Alternatively to the use of GM-CSF, IL-4 and TNF-α, other protocols including IL-1β, IL-6, PGE-2 (the "gold standard" DC used in many clinical trials – (Figdor, de Vries et al. 2004) or type I IFN Satthaporn, Aloysius et al. 2008) have reported improvements in DC function. However the origins of these alterations are still poorly understood.

In this study, we report that the alterations in cytokine production, frequency and signaling found in MoDC from breast cancer patients are already present in their monocytes. We verified that IL-6 expression is increased in monocytes and MoDC from patients, compared to controls. The addition of IL-6 to cultures of human DC skews the differentiation of monocytes from immature DC to macrophages (Chomarat, Banchereau et al. 2000). In the steady state, DC that produce IL-6, do not produce TNF-α and show low expression of TLR,CD40 and CD80, such as the ones yielded in the cultures from patients monocytes, are tolerogenic (Frick, Grunebach et al.). IL-6 plays a major role in maintaining DC immature (Park, Nakagawa et al. 2004). In IL6 -/- mice, mature dendritic cells are greatly impaired – this is a mechanism mediated through STAT3. It would therefore be predicted that in patients with IL-6 producing MoDC, immunotherapy would probably fail to yield good results.

However, the elevated expression of IL-6 in monocytes and MoDC of cancer patients is also noteworthy in light of the role described for this cytokine in tumorigenesis. An elevated expression

of IL-6 has been reported in cancer patients' sera, and linked to increased risk in development of colorectal adenomas (Kim, Keku et al. 2008). IL-6 in serum or tissue samples of cancer patients correlates with poor prognosis (Naugler and Karin 2008). Genetic ablation of IL-6 in mice reduced size and frequency of chemically induced adenomas (Grivennikov, Karin et al. 2009). It is commonly thought that source of elevated IL-6 in cancer patients is the tumor. Our results suggest that at least part of it could be produced by monocytes and monocyte-derived cells in these patients. It has been recently shown that monocytes from advanced pancreatic cancer produce elevated IL-6 and C-reactive protein (DeJong, Busquets et al. 2005), so it is possible that this is a phenomenon common to other types of cancer. The pro-tumoral effect of IL-6 is linked to its ability to activate oncogenic transcription factors such as STAT3 and NFkB (He and Karin). Consistent with this finding, we found that NFkB subunit p50 was elevated in patients MoDC. It has been reported that monocytes can be modified by tumors, for example, to express CCL2 in order to migrate to the tumor site and promote metastasis (Qian, Li et al.). Our results point to a close interaction between tumor and monocytes, which has not only local, but systemic effects, generating circulating monocytes that would potentially favor a tumor promoting environment through the production of IL-6.

We observed that not only monocyte numbers are decreased in cancer patients, but their monocytes also present a lower expression of GM-CSF receptors. These findings, which explain the lower yield in MoDC obtained from patients, are also consistent with a decreased potential to generate inflammatory immune responses. It is tempting to speculate how this would translate *in vivo*, especially because the origin of DC from monocytes is currently under debate. *In vivo*, GM-CSF has been shown by some studies to induce monocytes to differentiate into inflammatory DC (Liu and Nussenzweig), these DC being different from the DC generated in the steady state. Our observations suggest that somehow tumor presence modulates against the formation of these inflammatory DC.

Another cytokine that has been linked to promotion of DC formation in vivo is type I IFN, that leads to an increase in CD8+ DC, the major DC subpopulation that can perform cross-priming (Shortman and Heath), as well as inflammatory DC. The human counterpart for CD8+ DC is probably the BDCA3 population (Huysamen, Willment et al. 2008), and these cells do not derive from monocytes. However, our finding that monocytes and MoDC from patients expressing TLR9 are decreased compared to controls is relevant since engagement of TLR9 is an important pathway of induction of type I IFN production. This alteration in patients would also impact the generation of other DC subpopulations, affecting not only the general immune response, but their response to DC-based immunotherapy. Consistent with the low expression of TLR9, we observed an inability of MoDC from patients to up regulated MyD88 in response to CpG treatment. MoDC from patients also did not up regulated MyD88 in response to LPS. Such impairments would explain variability in responses to DC-based vaccination protocols using TLR9 or TLR4 agonists as adjuvants, respectively.

Altogether, the findings reported in this study indicate multiple alterations in monocytes of cancer patients that could contribute to a tumor promoting environment, and that these could be also present in MoDC generated *in vitro*. Thus, it could be predicted that immunotherapy approaches for tumor patients that contemplate multiple pathways of activation are more likely to succeed in generating a response than the ones that employ a single TLR agonist as adjuvant, for example. Alternatively, it could be postulated that therapy should be customized to the alterations observed in the patient. In our study, although a strong bias for production of IL-6 was observed in patients, it was not presented by all patients; TLR9 agonists should not be used if TLR9 down regulation is observed; and so forth.

Finally, most of the MoDC-based tumor vaccines have been shown to stimulate previously existing T cell responses, rather than generating new ones (Figdor, de Vries et al. 2004). This finding is expected especially if we consider that most cancer patients are older, and thus their

output of new T cells is naturally reduced (Maue, Yager et al. 2009). In order to optimize the effects of this approach, it will also be important to determine the relevance of the mechanisms described here, as well as in similar studies, in the restimulation of effector and memory cells.

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Table 01: Clinical data from patients, healthy controls and Benign Breast Disease Controls

Parameters		Patients:	Healthy Controls:	Benign Breast Disease:
Number of patients and controls		41	39	22
Histologic type	Intraductal Pappillary Carcinoma	1(2.45%)		
	Invasive Ductal Carcinoma	29(70.7%)		
	Invasive Lobular Carcinoma	5(12.2%)		
	Intraductal Carcinoma	3(7.3%)		
	Pappilary Carcionoma	1(2.45%)		
	Medullary Carcinoma	1(2.45%)		
	Tubule Lobular Carcinoma	1(2.45%)		
	Papilloma			1(4.5%)
	Fibroadenoma			8(36.5%)
	Accessory Breast			1(4.5%)
	Intraductal Ectasia			1(4.5%)
	Without Diagnosys			6(27.25%)
	Simple Breast Cysts			4(18.25%)
	Chronic Inflammation			1(4.5%)
Distant Metastasis		1(2.45%)		
Grade I		1(2.45%)		
Grade II		20(48.8%)		
Grade III		11(26.8%)		
Grade Not Applicable		4(9.75%		
Low Grade		1(2.45%)		
High Grade		4(9.75%)		
With Metastasis at Axillary Lymph Node		12(29.3%)		
Without Metastasis at Axillary Lymph Node		29(70.7%)		
Patients Who Were Tested For Hormone Receptors	Estrogen Receptor Positive	30(79%)		
	Progesterone Receptor Positive	29(76.3%)		
	p53 mutation Positive	17(51.5% From 33 Patients tested		
	Her-2 Neu Positive	7(19.5% From 36 Patients tested)		
Age (mean ± standard deviation)		56,93(±13,03)	51,58(±15,18)	49,27(±18,42)

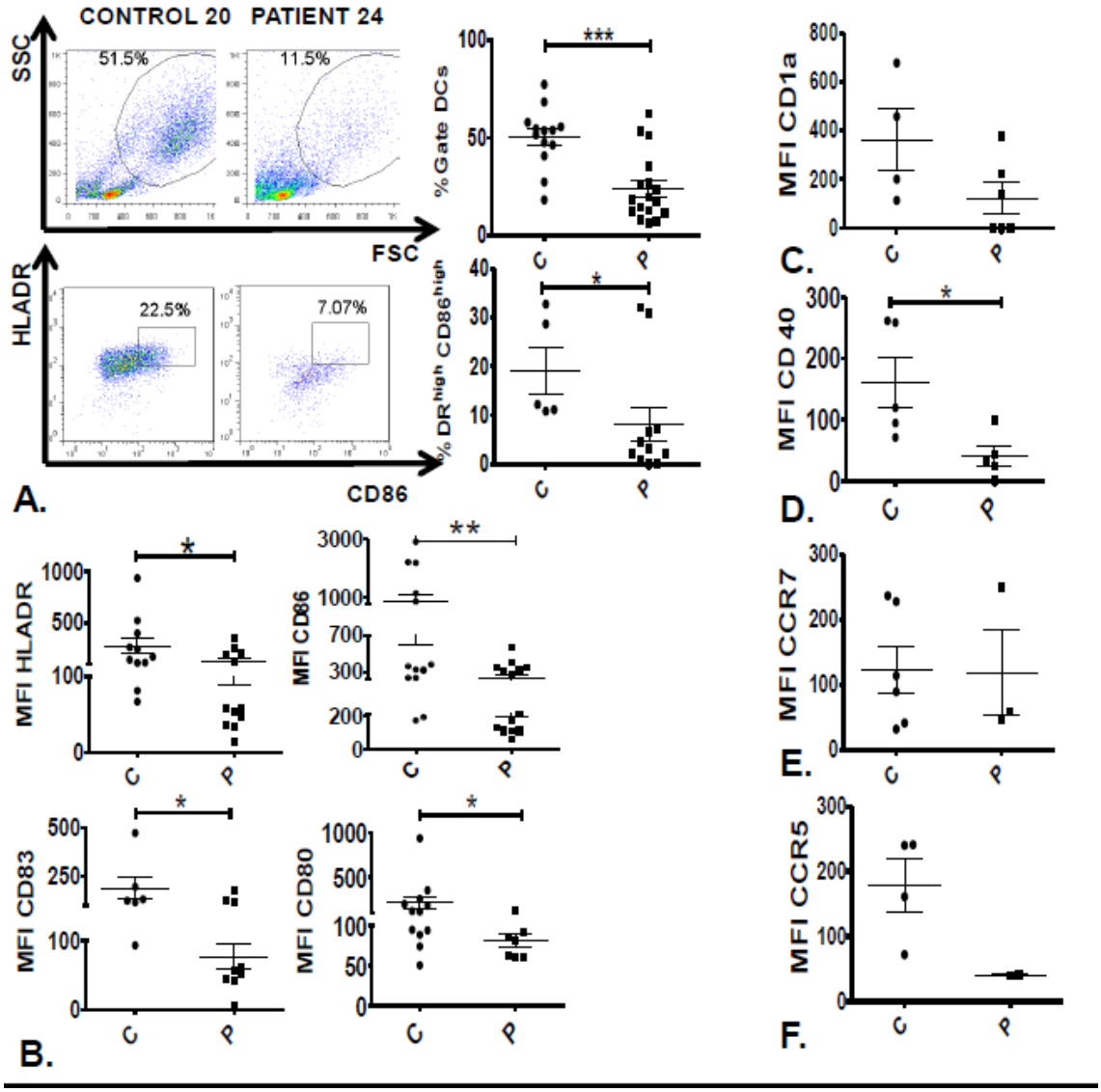

Fig. 01: **Yield and phenotype of MoDC in patients and controls.** Monocytes isolated from PBMC of patients and controls were cultivated in vitro with GM-CSF, IL-4 and TNF-α. On day 7, cells were harvested, counted, and stained for flow cytometry analysis. **A**, Representative FACs plots of MoDCs obtained from patients and controls and graphs of % cells in FSC x SSC (the gate marks region where DCs are found) and % of $HLADR^{+}CD86^{+}$ gate on MoDCs from patients and controls. **B**: Mean fluorescence intensity (MFI) for maturation surface markers HLADR (p= 0.0337 – confidence interval = 95%), CD80, CD83, CD86. **C**: MFI for CD1a. **D**. MFI for CD40 **E**. MFI for CCR7 **F**. MFI for CCR5. ***C***, controls; ***P***, patients. Data was analyzed by t-tests. * $p<0.05$; **, $p<0.01$; ***, $p<0.001$. Confidence interval = 95%

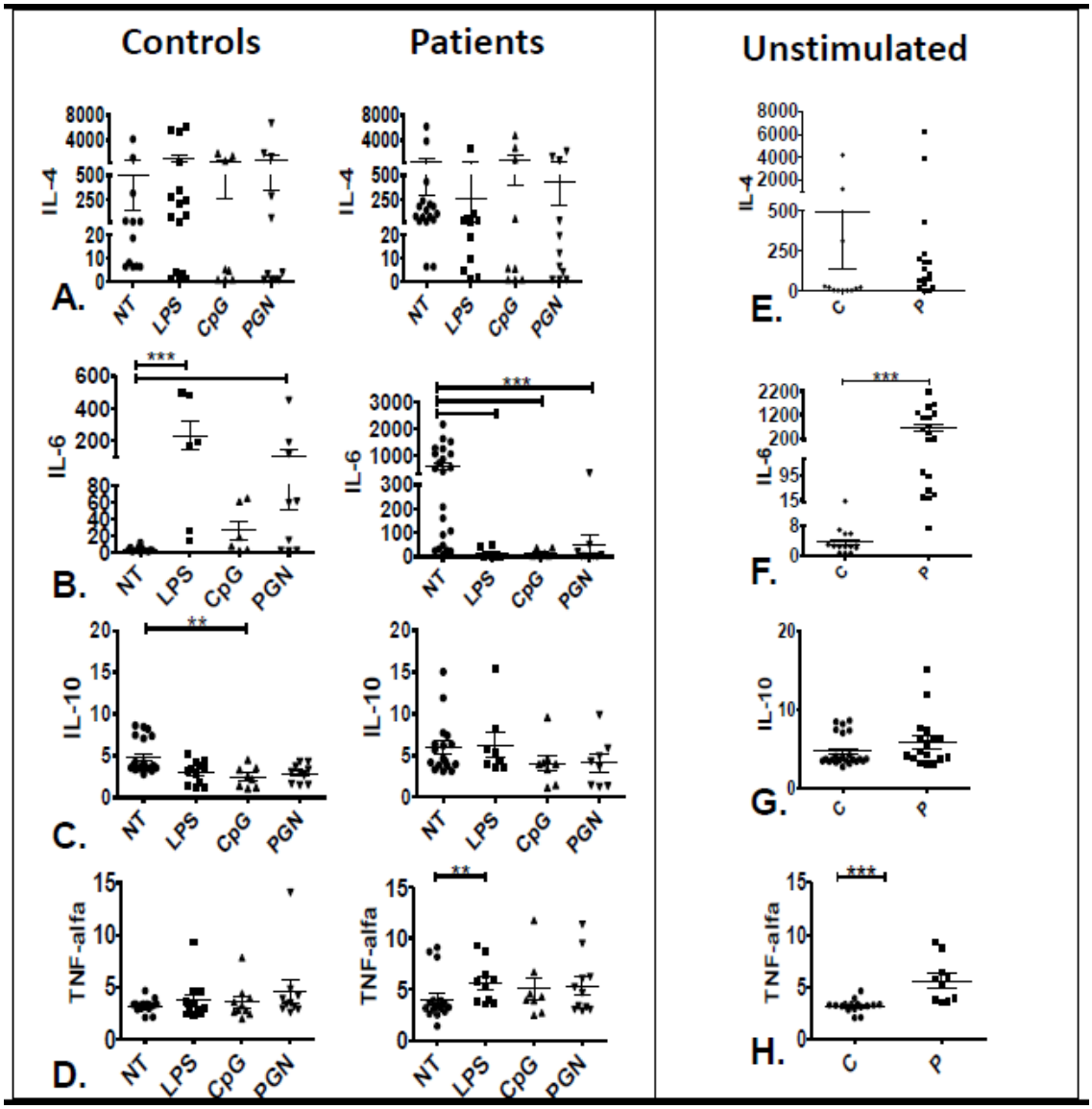

Fig 02: **Cytokine production by MoDC from patients and controls**. MoDC were harvested on day 7 of culture**,** counted and re-plated with either no stimulus, or stimulation by LPS, CpG or PGN. Supernatant was harvested 24hs later and analyzed for cytokines. **A-D**, concentration of cytokines measured in supernatant of MoDC cultures of patients or controls, in pg/ml, either unstimulated (NT – not treated) or stimulated with TLR ligands LPS, CPG or PGN. **E-H**, comparison of unstimulated cytokine production, in pg/ml, in patients (**P**) versus controls (**C**).

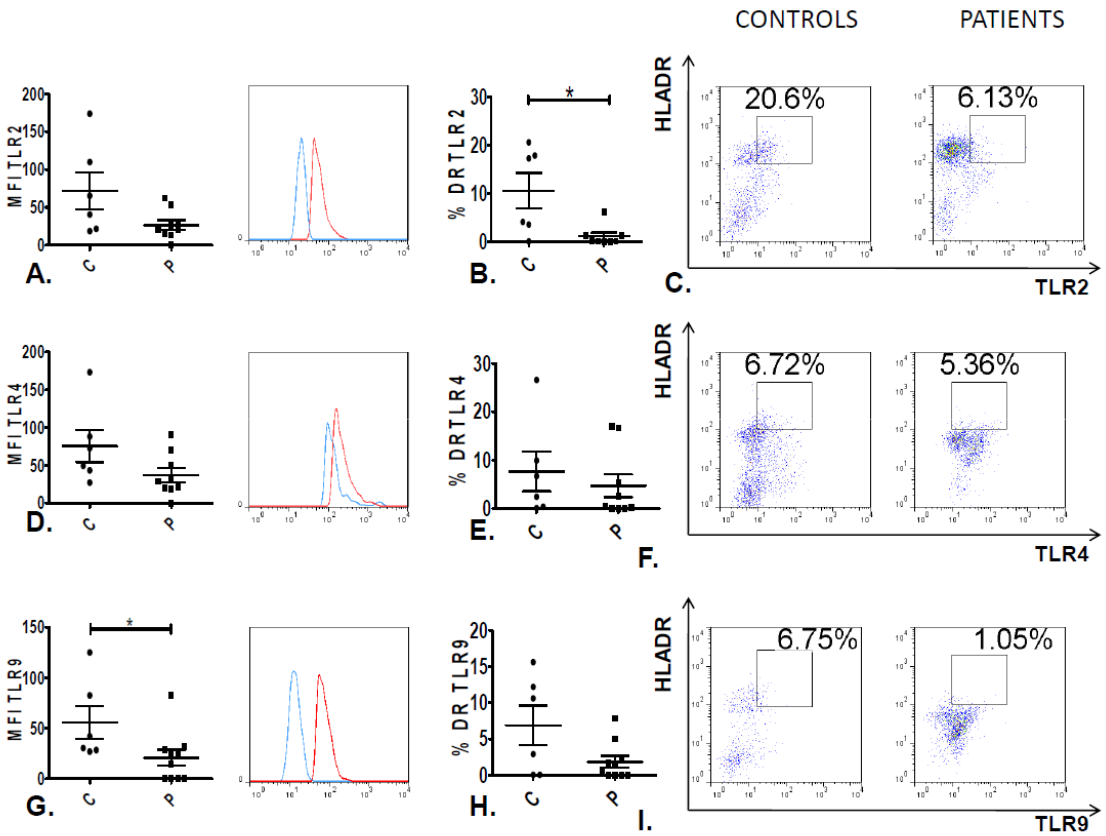

Fig. 03: **Expression of toll-like receptors (TLR) in MoDCs of patients and controls.** MoDC were harvested from culture on day 7 and stained for HLA-DR, DC86, CD1a, CD11c and TLR 2, 4 and 9. **A**, MFI for TLR2 in patients (P) and controls (C), with representative histogram overlay – blue, patients, red, controls. **B**, % of TLR2 cells in patients and controls; **C**, representative dot plots with gating on HLA- DR^{hi} TLR2+ cells. **D-E**, equivalent representation, for TLR4; **G-I**, equivalent representation, for TLR9. Data were analyzed by t-test. *, $p<0.05$.

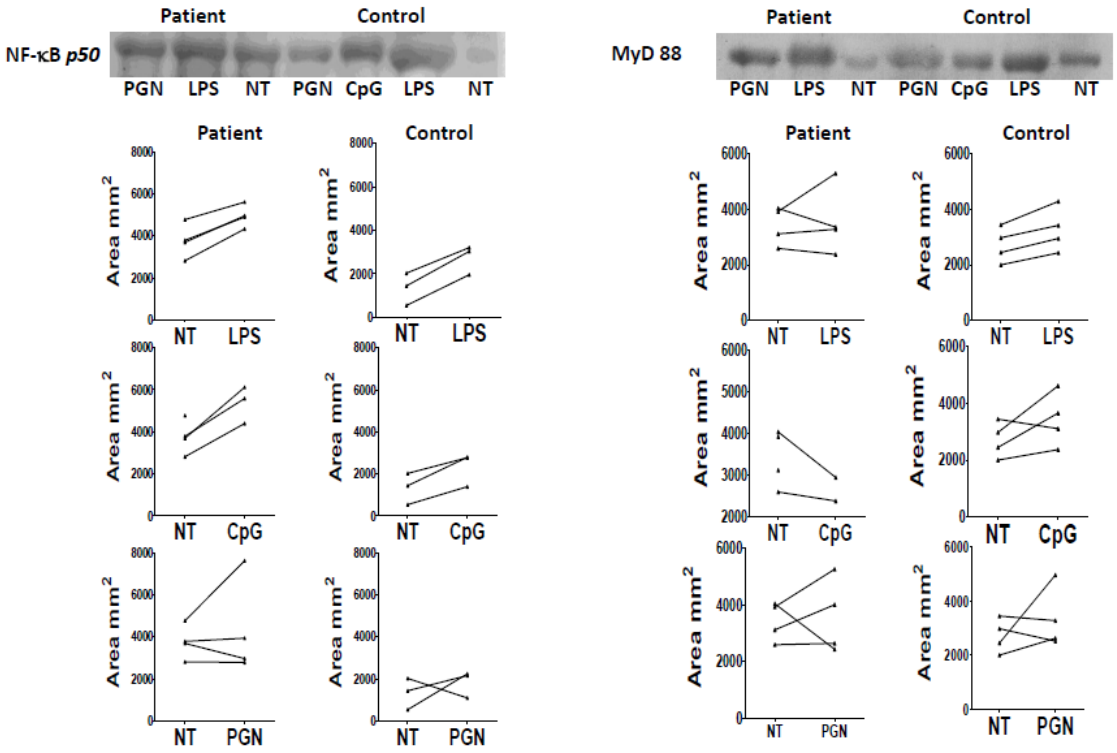

Fig. 04: **Expression of NFkBp50 and MyD88 in MoDC from patients and controls upon stimulation with TLR agonists.** MoDC were stimulated for 30 min with TLR ligands, harvested and lysed for analysis by western blot of NFkBp50 and MyD88. Upper panel, pictures show representative western blots for patients and controls. Response in up regulation of each of these mediators is represented by the area of the respective band in mm2. Each line connects the basal expression of the molecule (NT, or not treated) to the stimulated expression with the respective TLR ligand (CpG, LPS or PGN).

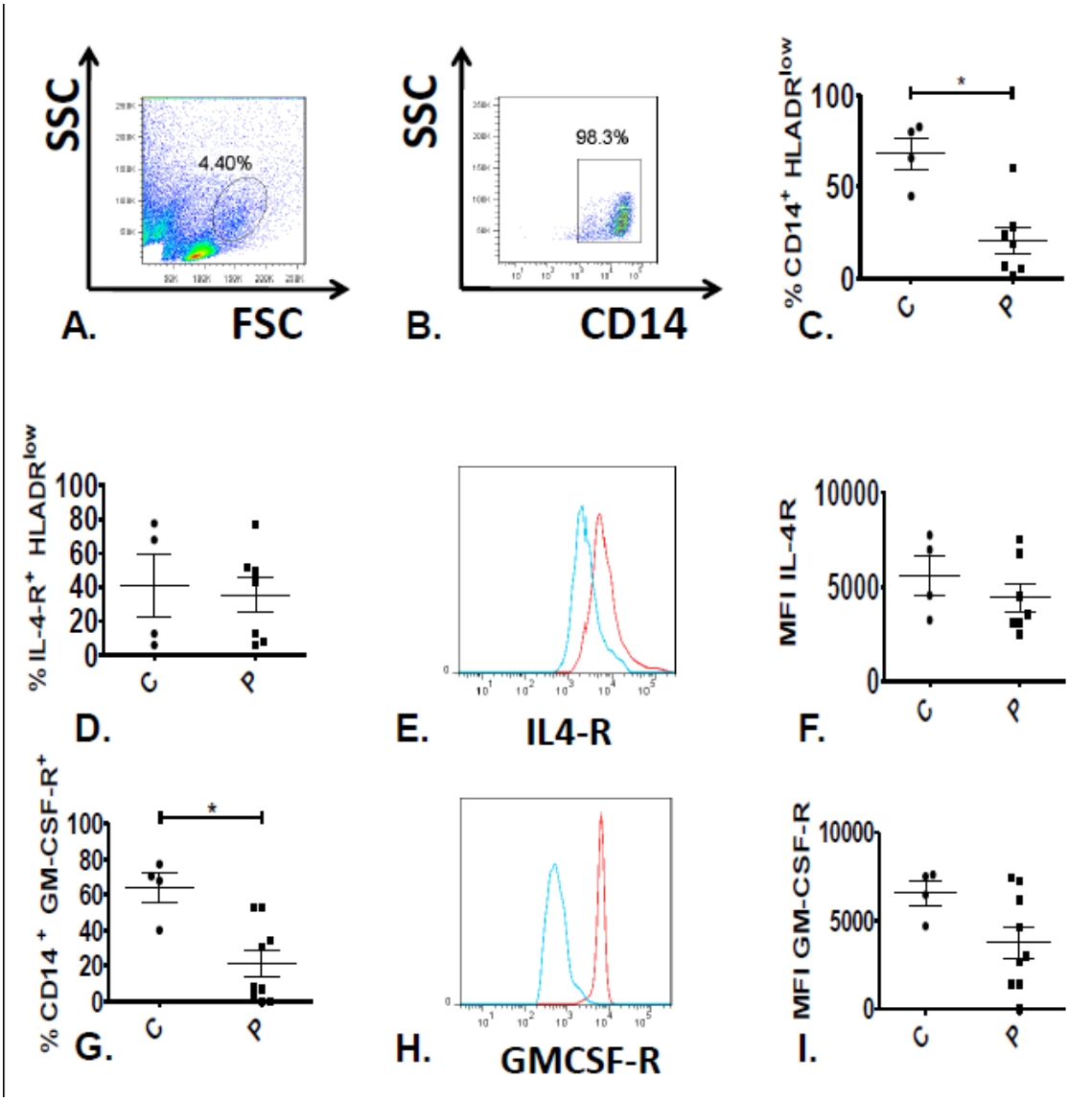

Fig. 05: **Reduction in number of CD14⁺ and GMCSF-R⁺ monocytes in patients**. PBMCs were isolated by ficoll centrifugation, and analyzed by flow cytometry after staining with specific antibodies. Monocytes distribution in FSC by SSC determined as in **A**; In **B**, Cells in that SSC region are all CD14⁺, identifying monocytes. **C**, % of CD14⁺ HLA-DR^{lo} cells (monocytes) in controls (C) and patients (P). **D**, MFI for CD14 in controls and patients. **E**, % of IL4R⁺HLADR^{lo} cells, with representative dot plots in **F. G**, MFI for IL-4 receptor. **H**, % of GM-CSFR⁺CD14⁺ cells, with representative dot plots in **I. J**, MFI for GM-CSF receptor.

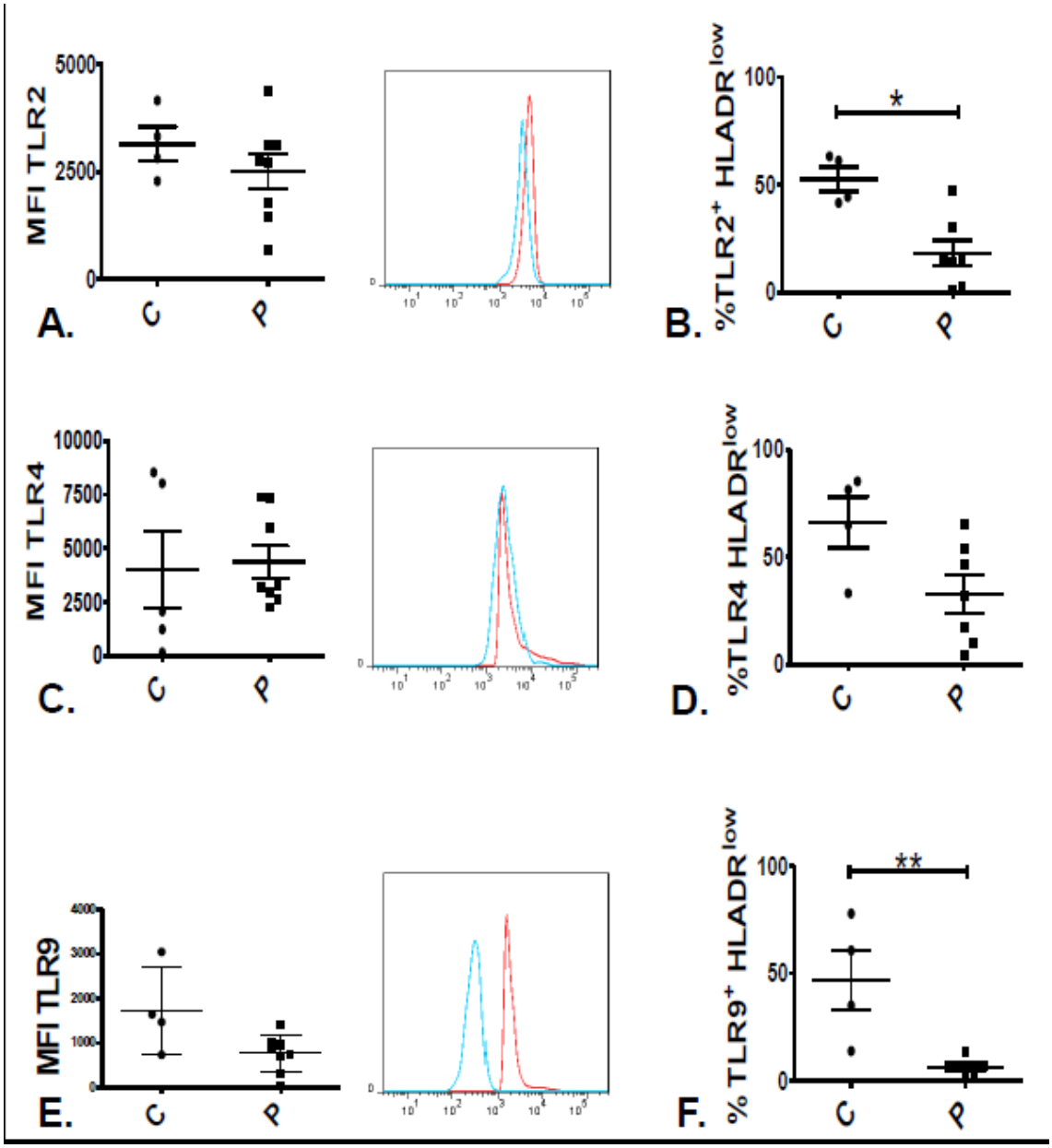

Fig. 06: **Expression of toll-like receptors (TLR) in monocytes of patients and controls.** PBMC were isolated by ficoll centrifugation and stained for CD14, HLA-DR, TLR2, TLR4 or TLR9. **A**, MFI for TLR2 in patients (P) and controls (C), with representative histogram overlay – blue, patients, red, controls. **B**, % of TLR2 cells in patients and controls; **C**, representative dot plots with gating on HLA- DR^{hi} $TLR2^{+}$ cells. **D-E**, equivalent representation, for TLR4; **G-I**, equivalent representation, for TLR9. Data were analyzed by t-test. *, $p<0.05$., **, $p<0.01$.

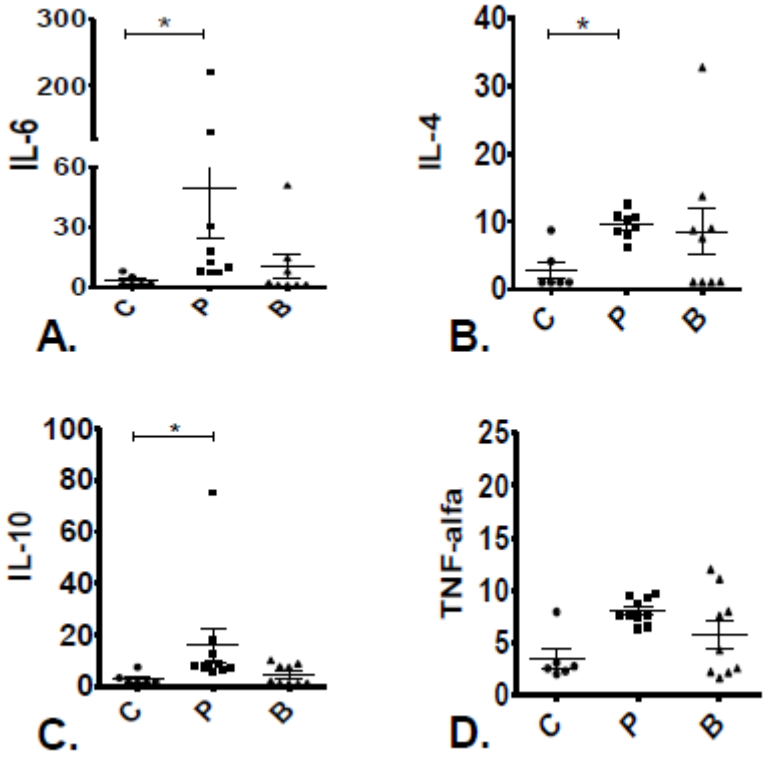

Fig. 07: **Cytokine production by monocytes from patients and controls**. Monocytes were isolated from PBMC by adherence, re-plated and cultured for 24hs in AIM-V media. **A-D**, concentration of cytokines measured in supernatant monocyte cultures of patients (P) or controls (C), or patients with benign alterations (B) in pg/ml, Data were analyzed by ANOVA (Kruskall-Wallis Test). *, $p<0.05$

From: clelia madeddu [mailto:clelia_md@yahoo.it]
Sent: Thu 1/27/2011 13:54
To: Cristina Beatriz C Bonorino
Subject: Re: RES: RES: article request

Dear Dr. Bonorino,

As Guest Editor of the Hot topic issue entitled "**Advanced neoplastic disease as immunologic, endocrine and metabolic disorder** to be published in the peer-reviewed journal "Anti-inflammatory and Anti-Allergy Agents in Medicinal Chemistry" edited by Bentham Science Publisher, I'm pleased to inform you that your revised manuscript entitled " **The immune system of cancer patients" by Carolina Torronteguy, Ana Paula Souza e Cristina Bonorino** has been accepted for publication.
According to Bentham publication plan the issue will be published in first 2011.
Your sincerely
Dr Antonio Macciò
Guest Editor

THE IMMUNE SYSTEM OF CANCER PATIENTS

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Abstract

Although a great body of evidence is available on the immunosuppressive strategies employed by tumors in order to grow, cancer patients are not considered immunosuppressed individuals. Chemotherapy used in different cancer treatments frequently leads to leucopenia and affects immune responses. Tumors of the immune system can also cause immune alterations, due to their very nature. However, in the absence of preventive routine exams, patients can bear tumors for rather long periods of time without any specific indication, not being particularly prone to contracting infectious diseases compared to cancer free individuals. In this review, we analyze the existing data on the effects of tumors on the immune system of cancer patients. An interesting pattern emerges, suggesting that immunosuppression exerted by tumors is mainly local, rather than systemic. However, some alterations in DCs of cancer patients have been recently described, indicating the interactions between tumor and immune cells may be more complex than previously imagined. This has important implications of the design of anti-tumor therapies as well as in patient quality of life.

I**mmunosurveillance – the immune responses mounted by patients against tumors and how they shape tumor development**

The idea that the immune system could recognize and attack transformed tumor cells was first envisioned by Ehrlich (Ehrlich P, 1909) when he postulated that without immune control, tumors would grow uninhibitedly However, experimental evidence for his idea was only presented more than forty years later, when Burnet (Burnet 1957) (Burnet 1964; Burnet 1967) demonstrated that thymus-dependent cells mediated tumor regression. Old and Boyse (Old and Boyse 1964) presented evidence for the existence of tumor specific antigens, and Lewis Thomas (Thomas 1982) first pointed out the high incidence of tumors in immunosuppressed transplanted patients, proposing immunosurveillance against tumors as a major role of the immune system. Since then, a myriad of studies have collected evidence of immune mechanisms of tumor control. Adaptive immunity to tumors include recognition of tumor antigens and lysis of tumor cells by CD8+T cytotoxic cells, and the engagement of CD4+T cell help has been demonstrated to be crucial in the maintenance of anti-tumor memory responses. Development of anti-tumor antibody responses led to the wide use of monoclonals for tumor therapy in recent days. Innate responses to tumors also play a major role, from the activation of NK cells to the production of TNF-α, directly killing tumor cells. The identification of DCs (DCs) and their highly specialized subpopulations, as well as regulatory T cells (Tregs) brought important insights that are being rapidly incorporated in anti-tumor immune-based therapies. More recently, recognition of endogenous ligands by pattern recognition receptors such as toll-like receptors (TLRs) have expanded our view of anti-tumor immune mechanisms. Nevertheless, the interactions between tumor and immune cells are highly dynamic, and anti-tumor responses can end up selecting tumor variants that can escape them. In fact, tumor cells can present varied immunosuppressive strategies. These interactions shape varied states of tumor development, posing an important challenge for the achievement of efficient immunotherapy to cancer, with long lasting effects. These developments led to the immunoediting hypothesis (Dunn, Bruce et al. 2002).

Immunoediting and the three Es – equilibrium, editing, evasion

Cancer immunoediting postulates that tumor development would depend on dynamic interactions between neoplastic and immune cells, occurring in three stages. The first stage would be elimination, which consists basically of the same process described in the initial theory of immunesurveillance, whereby the immune system detects and eliminates tumor cells that have developed as a result of failed intrinsic tumor suppressor mechanisms. The process of elimination includes innate and adaptive immune responses to tumour cells. For the innate immune response, several effector cells such as NK, NKT, and γδT cells are activated by the inflammatory cytokines, which are released by the growing tumour cells, macrophages and stromal cells surrounding the tumour cells (Street, Hayakawa et al. 2004). The secreted cytokines recruit more immune cells, which produce other pro-inflammatory cytokines such as IL-12 and IFN-γ (Banchereau and Steinman 1998). One mechanism by which γδT cells might regulate tumor development is through NKG2D recognition of the stress ligand retinoic acid early transcript (RAE1) (Girardi, Oppenheim et al. 2001). NK cells mediated killing of the tumor cells by perforin (Smyth, Thia et al. 2000), death-inducing molecule (FasL) (Mori, Jewett et al. 1997) and TNF-related apoptosis-inducing ligand (TRAIL) (Mori, Jewett et al. 1997) release tumour antigens (TAs) and lead to adaptive immune responses. Necrotic tumour cells are ingested by immature DCs (DCs), which will now mature upon phagocytosis and migration to tumour draining lymph nodes (TDLNs) (Colombo and Trinchieri 2002). In the TDLNs, the DCs present TAs to naive CD4+ and CD8+ TA specific T cells which will expand and differentiate into effectors and memory T cells (Dunn, Bruce et al. 2005). TA-specific CD4+ and CD8+ T cells home back to the primary tumour site, type 1 CD4+ T cells (Th1) facilitating tissue destruction and tumor rejection by providing help to cytotoxic CD8+ T cells. These will eliminate the TA-expressing tumour cells, and this process is enhanced by the secreted IFN-γ. IFN-γ exerts a limited cytotoxicity via antiproliferative (Gollob, Sciambi et al. 2005) and anti-angiogenic effects (Qin, Schwartzkopff et

al. 2003) and induces apoptosis (Wall, Burke et al. 2003). In the crosstalk between NK cells and DCs, NK cells promote the maturation of DCs resulting in the enhancement of antigen presentation to naive T cells (Zitvogel, Terme et al. 2006). The elimination phase can be complete, when all or almost all tumor cells are cleared to undetectable levels; or incomplete, when only a portion of tumor cells are eliminated.

A temporary state of equilibrium can then develop between the immune system and the tumor. During equilibrium, tumor cells either remain dormant or continue to evolve, accumulating further changes; such as DNA mutations or post-transcriptional modifications; that will modulate tumor-specific antigens and select tumour cells with reduced immunogenicity. This process may occur over a period of many years (Dunn, Bruce et al. 2002). Several studies indicated that a major selective pressure exerted by the immune system consists of lymphocytes and IFN-γ. MCA-induced sarcomas in IFN-γ-receptor-deficient mice are highly immunogenic (Shankaran, Ikeda et al. 2001). Furthermore, chemically induced sarcomas in both nude and severe combined immunodeficiency (SCID) mice were more immunogenic than similar tumors from immunocompetent mice (Svane, Engel et al. 1996), (Engel, Svane et al. 1997). Such findings suggest that the original tumor cells induced in normal mice and selected by a T-cell-mediated selection process have been adapted to grow in a host with a functional T-cell system, which has eliminated highly immunogenic tumour cells, leaving non-immunogenic tumour cells to grow. An interesting demonstration of tumor dormancy/equilibrium was documented using the BALB/c B cell leukemia/lymphoma 1 (BCL1) model (Farrar, Katz et al. 1999). In this model, mice were immunized with BCL1- derived immunoglobulin and then challenged with BCL1 cells. After 25–30 days, all control mice developed splenomegaly and were killed, and 70% of immunized mice remained protected from splenomegaly for more than 60 days (Farrar, Katz et al. 1999)). Importantly, low numbers of BCL1 cells could still be detected in these mice by flow cytometry, and mice relapsed at a steady rate over a period of more than 610 days (Vitetta, Tucker et al.

1997). In another study, mice were vaccinated with irradiated BCL1 cells and then challenged with live tumor cells. In this case, tumor cells could be detected in the spleen of 40% of the long-term survivors 250 days after tumor challenge, and throughout this period, host mice were completely asymptomatic (Vitetta, Tucker et al. 1997). Additional studies have shown that proliferating mouse lymphoma cells are kept at a low number in the bone marrow, owing to persistent antigen and memory T cells that are able to coordinate a CD4+ and CD8+ T cell-mediated response (Mahnke, Schwendemann et al. 2005), (Muller, Gounari et al. 1998). CD8+ T cells may also be the critical cell type involved in a model of surveillance of UVB-induced skin, where there is potential equilibrium between the immune system and a developing cancer (Loeser, Loser et al. 2007). Adoptive immunotherapy has also been demonstrated to protect long-term mice from prostate carcinoma in a transgenic model, and histological analysis of long-term survivors has demonstrated that tumours are not entirely eliminated but are restricted to small foci (Granziero, Krajewski et al. 1999).

The pressure exerted by the immune system during this phase would be sufficient to control tumor progression, but eventually, the process would result in the selection of tumor cell variants that would able to resist, avoid, or suppress the antitumor immune response (i.e., edited cells), leading to the escape phase. During the escape phase the immune system would no longer be able to contain tumor growth. In some cases it seems that the immune system is incapable of influencing tumor progression, but in many cases it seems that although the immune system prevents or delays tumor growth, it is eventually overwhelmed or evaded, and the tumor progresses. It is possible that escape of tumor cells from a given primary site may be due to different mechanisms. Local analysis of tumors have evidenced variants that lose MHC class I expression (Algarra, Cabrera et al. 2000) or alterations in different molecules of the class I pathway (TAP or LMP – (Seliger, Maeurer et al. 2000). One study found 4 out of 17 human adenocarcinoma cell lines to be unresponsive to IFN-γ due to varied alterations in the signalling

pathways upon engaging of the IFN-γ receptor (Kaplan, Shankaran et al. 1998). Other strategies include the tumor suppressive mechanisms, described a few sections below. Altogether, the crosstalk between tumor and immune cells sculpt tumor progression, and such interactions must be taken into account both when diagnosing and designing anti-tumor therapy. An example of the complexities of the direct crosstalk between tumor and immune cells is the tumor leukocyte infiltrate.

Tumor infiltrate and the intratumoral immune response

Infiltration of immune effectors is a well-documented observation in most if not all cancers. Emerging studies now reveal that infiltration occurs very early in the course of disease. An association between favourable patient prognosis and tumor-infiltrating lymphocytes (TILs) was first observed in patients with melanoma (Clark, Elder et al. 1989), (Clemente, Mihm et al. 1996), where it was shown that patients with high levels of CD8+ T cell infiltration survived longer than those whose tumors contained low numbers of lymphocytes. Since then, various melanoma-specific antigens have been identified, and the presence of melanoma-specific T cells recognizing a range of different antigens has been confirmed in various studies (Scanlan, Simpson et al. 2004). Taken together, these studies indicate that melanoma TILs are often tumour specific but respond to a wide range of different antigens, and the presence of tumor-specific CD8+ cells among TILs can be an indicator of enhanced survival in patients undergoing immunotherapy (Clark, Elder et al. 1989). Several other studies have shown that the high-grade density of CD8+ T cells in cancer cell nests was correlated with prognosis and the presence of TILs was able to predict survival as an independent prognostic factor in various types of cancers including colon cancer (Naito, Saito et al. 1998), (Strater, Hinz et al. 2005), oesophageal cancer (Yasunaga, Tabira et al. 2000), oral squamous cell carcinoma (Reichert, Day et al. 1998), breast cancer (Yoshimoto, Sakamoto et al. 1993) and ovarian cancer (Sato, Olson et al. 2005). Other studies

have shown a similar positive correlation between NK, or NKT cell infiltration and the survival for gastric cancer (Ishigami, Natsugoe et al. 2000), colorectal cancer (Kondo, Koda et al. 2003), squamous cell lung cancer (Villegas, Coca et al. 2002) and melanoma (Clark, Elder et al. 1989).

However, tumour infiltration by some cells from immune system, such as regulatory T cells (Tregs), can actually support tumor progression (Martin, Ladoire et al.). Tregs are significantly increased in patients with epithelial malignancies (twice the number of Tregs relative to healthy volunteers) (Buell, Gross et al. 2005). Depletion of Tregs in experimental models leads to anti-tumor immunity (Rentzsch, Kayser et al. 2003) (Disis, Knutson et al. 2000). Treg TILs have also been shown to be more represented in advanced human melanoma lesions, with more Treg TILs in metastatic lesions (Yasunaga, Tabira et al. 2000). It is possible that the increased frequency of Tregs in advanced malignant lesions (Yasunaga, Tabira et al. 2000) (Scanlan, Simpson et al. 2004) (Clark, Elder et al. 1989) may, in part, explain the anergy of tumor-specific CD8+ T cells observed in such lesions (Mantovani, Sozzani et al. 2002) (Naito, Saito et al. 1998). In a recent study (Salama, Phillips et al. 2009) FOXP3+ regulatory cells infiltrate was suggested as a prognostic marker for colorectal cancer patients. They analyzed 967 colorectal cancer patients in a follow up from 53.4 months to 69.7 months. The density of TCD8+ and TCD45RO+ cells was greater in normal tissue than in tumor infiltrate ($P<0.001$) and correlated with good prognosis. In addition, FOXP3+ density in tumor infiltrate was greater than in normal tissue and correlated with impairment in survival ($P<0.001$).

A more recent paper report that T cells infiltrated in tumor-associated lymph nodes predict survival in rectal cancer (McMullen, Lai et al.). Forty rectal cancer patients were divided by tumor stage and the percentage of T cells (CD3 and CD25), mature DCs (CD83) and immature DCs (CD1a) was estimated in the tumor associated lymph node, lymph nodes associated with normal rectal mucosa and at tumor margins. In lymph nodes associated with rectal tumor tissue, there was significant decrease of both mature DCs and TCD3+ cells. In addition, they observed

that CD3+ T cells were elevated in lymph nodes associated to tumor, predicting survival independently of tumor stage (P=0.05). Finally, immature DCs and CD25+ T cells did not seem to be either concentrated or depleted in lymphoid aggregates associated with rectal tumor areas. Consequently, TIL phenotyping, together with analysis of the lymph node infiltrate, might actually constitute important tool for diagnosis and prediction of survival of cancer patients.

Evidence of protective immunosurveillance in cancer patients

Apart from local infiltrating immune responses to tumors, systemic anti-tumor responses can be generated by patients, and such responses may correlate with disease outcome. For example, one study of ovarian cancer showed that patients (Stages III-IV) with p53 specific antibodies had a median survival of 51 months compared to 24 months for those without detectable antibody levels (Goodell, Salazar et al. 2006). Elevated T cell immunity to several other tumor antigens, such as HER-2/neu, CEA, and NY-ESO-1, has been reported (Rentzsch, Kayser et al. 2003), (Disis, Knutson et al. 2000), (Beckhove, Feuerer et al. 2004). Some tumor types exhibit a particular type of genetic instability referred to as microsatellite instability (MSI), where defects in DNA mismatch repair mechanisms lead to the duplication or deletion of short repeated sequences of DNA known as microsatellites. The high rate of mutation in MSI-H tumors has been shown to result in the generation of a number of novel tumor antigens that can be recognized by B cells (Ishikawa, Fujita et al. 2003), CD4+ T cells (Saeterdal, Bjorheim et al. 2001), and CD8+ T cells (Saeterdal, Bjorheim et al. 2001) and this is associated with a favorable prognosis. Another important finding was that patients of breast, lung and head and neck cancers (Yu, Zhan et al. 2002) recognize Cyclin B1, an aberrantly expressed antigen in these tumors, with antibodies and T cells. In the following study, this response was demonstrated to exist in disease-free individuals, and to be protective against tumors in an experimental model (Vella, Yu et al. 2009). Thus, systemic immune responses generated by cancer patients, and even by healthy

individuals, can actively protect against tumor development. And because immune responses are elicited in the draining lymph nodes, a major complication of neoplasic disease is the arrival of metastatic cancer cells to these organs.

The tumor draining lymph node – the strongest predictor of recurrence and survival

It is known that if a tumor cell can home to a lymph node, it can arrive at any other organ through blood stream or lymph flow. Consequently, lymph node status is an important prognostic factor for cancer patients, being associated with tumor size and distant metastasis evaluation composing the TNM system (Denoix 1946)**,** nowadays maintained and actualized by International Union Against Cancer (IUAC). This allows us to classify tumors into stages, tailoring the treatment to be more effective and less invasive for the patient. Lymph node dissection during tumor surgical removal has been used for over more than one hundred years (Bekker and Meijer 2008). The first draining lymph node is named the sentinel lymph node. Until recently, surgical excision of breast or melanoma tumors was accompanied by total removal of lymph nodes adjacent to the tumor site. This approach is associated with high morbidity and decreased quality of life in cancer patients. Morton *et al* revolutionized surgical cancer treatment with intraoperative lymphatic mapping for clinical stage I melanoma (Morton, Wen et al. 1992), injecting a vital dye in the tumor mass which revealed the first direct draining lymph node, subsequently removing it. This work showed that metastasis were present in 47 (18%) of 259 sentinel nodes; nonsentinel nodes were the sole site of metastasis in only 2 of 3079, showing a false negative rate lower than 1%. This technique was successfully expanded to breast (Giuliano, Dale et al. 1995) (Giuliano, Kirgan et al. 1994), colon (Saha, Wiese et al. 2000), thyroid (Kelemen, Van Herle et al. 1998), and gastrointestinal cancer (Kitagawa, Fujii et al. 2000) surgeries.

In a recent study, a follow up of 72.5 months with 790 breast cancer patients, it was observed that the size of metastasis in sentinel lymph nodes was a significant predictor of 8-year disease free survival (P<0.0001) and 8 year of overall survival (P<0.001) (Hansen, Grube et al. 2009). Studying sentinel lymph nodes and non-sentinel lymph nodes from lung patients biopsy, Manabu Ito et al, described decreased CD4+T cells numbers and increased production of TGF- β in sentinel lymph nodes when compared to non-sentinel lymph nodes. They confirmed this with semi quantitative RT-PCR showing a TGF-β1 amount 30 times higher in primary tumor than in sentinel or non sentinel lymph nodes. Following, they showed an increase in apoptotic DCs number after stimulus with TGF-β1 *in vitro*. Importantly, they used only metastasis-free lymph nodes. Consequently, they proposed that TGF-β1 can create a favorable microenvironment in lymph node for metastasis development (Ito, Minamiya et al. 2006). In a more recent study analyzing the expression of heat shock protein 70 (Hsp70) in primary breast tumors, we verified that metastasis in the sentinel lymph node was the major predictor of recurrence and death (Torronteguy, Frasson et al. 2006). Finally, different studies reported that lymph nodes close to the tumor are immune suppressed (Tsakraklides, Tsakraklides et al. 1975), (Reiss, Volenec et al. 1983), particularly when metastatic cells can be detected at the site. All these studies highlight the lymph node as a crucial site for the immune mediated control of tumor growth, raising the issue of whether or not tumor growth leads to a compromised immune system in the patient.

Paraneoplastic symptoms do not generally include susceptibility to infection

In some cases, patients with tumors experience disease symptoms that are caused by the presence of a tumor but are not the result of local tumor growth. Such symptoms are referred to as paraneoplastic syndromes; they may be caused by cross-reactivity between the anti-tumor immune responses and neurologic antigens, and the onset of neurologic symptoms often precedes diagnosis of a previously undetected tumor. Interestingly, certain paraneoplastic

syndromes are associated with particular tumor types and particular autoantibodies (Gultekin, Rosenfeld et al. 2000) and the fine specificity of the antineuronal antibodies found in patients is often related to the type of tumor present (Graus, Dalmou et al. 1997) (Darnell and DeAngelis 1993). For example, paraneoplastic cerebellar degeneration (PCD) is a neurologic syndrome that arises in some patients with gynecologic malignancies and Hodgkin disease; these patients typically exhibit high titers of Yo-specific autoantibodies (in the case of gynecologic tumors) or thioredoxin reductase 1–specific autoantibodies (in the case of Hodgkin disease) (Tanaka, Tanaka et al. 1999). Not only autoantibodies, but CTLs specific for antigens shared by tumors and Purkinje cells have been detected in the blood of PCD patients (Albert, Darnell et al. 1998), and tumors from patients with PCD often show prominent infiltration with lymphocytes and plasma cells, which is indicative of a local immune response at the tumor site. In most cases, PCD is terminal, and the only patients that generally survive this condition are those that achieve complete tumor remission in response to therapy, once again demonstrating that the tumor is the probable driver of the immune response that is both self and tumor reactive. Altogether, paraneoplastic syndromes show a crosstalk between tumors and the immune system; however, they do not include immunossupression or increased susceptibility to infection. How, then, does the tumor suppress tumor specific, but not all, immune responses?

Immunossupressive strategies employed by tumors - local or systemic?

We have recently reviewed the many strategies employed by tumors to promote immunosuppression (de Souza and Bonorino 2009). Tumor cells can express molecules that dampen immune activation, including T cell–inhibitory molecules such as B7-H1 (Dong, Strome et al. 2002), HLA-G (Tripathi and Agrawal 2006), and HLA-E (Derre, Corvaisier et al. 2006) and indoleamine 2,3-dioxygenase (IDO) (Munn and Mellor 2007). Tumor cells can resist lysis by CD8+ T cells and NK cells through mutations in the FAS encoding gene (Takahashi, Feuerhake

et al. 2006), (Wohlfart, Sebinger et al. 2004), mutations in the gene encoding the TRAIL receptor death receptor 5 (DR5) (Shin, Kim et al. 2001), and overexpression of the antiapoptotic molecules FLIP and BCL-XL (Kataoka, Schroter et al. 1998), (Hinz, Trauzold et al. 2000). Furthermore, tumors secrete soluble factors with immunossupressive properties, such as TGF-β (Zhang, Yang et al. 2005), (Chen, Pittet et al. 2005), (Gorelik and Flavell 2001), VEGF (Gabrilovich, Ishida et al. 1999), IL-10 (Kawamura, Bahar et al. 2002), and gangliosides (McKallip, Li et al. 1999), recruiting to the tumor microenvironment other cells with immunossupressive activities. The recruitment of both CD4+CD25+ Tregs and CD1d-restricted T cells suppresses antitumor immunity (Terabe and Berzofsky 2004). Tumours can also secrete prostaglandin E2 (PGE2), enhancing Treg activity (Baratelli, Lin et al. 2005).

Tumors can also lead to the expansion of immunosuppressive myeloid cell populations (Serafini, Borrello et al. 2006), (Kusmartsev and Gabrilovich 2006). Myeloid derived suppressor cells (MDSCs) are an immature myeloid population characterized by expression of CD11b, CD33, CD34; variable CD15 expression and no expression of CD 14 and HLADR ($CD14^-$ and $HLADR^-$). These cells suppress many immune effectors as DCs, macrophages, CD8+ T and CD4+T effector cells through the production of IL-10 and induction of T regulatory cells (Hanson, Clements et al. 2009). MDSC have been observed in a number of studies as being associated with profound suppression of T cell responses in both mice and humans (Kusmartsev and Gabrilovich 2006), (Mirza, Fishman et al. 2006), also being associated with tumor progression and metastasis. MDSCs reductors (gemcitabne, retinoic acid, reduced tumor bulk) enhanced immunotherapy efficacy, T cell and NK cell activation and restoration of immunesurveillance. (Kao, Ko et al.)

The mechanism of MDSC suppression is complex and seems to involve contributions from either iNOS (also known as NOS2) or arginase 1 (ARG1) (Mazzoni, Bronte et al. 2002), (Bronte, Serafini et al. 2003), (Kusmartsev, Nefedova et al. 2004). The signaling pathways

activated by these molecules enable MDSCs to inhibit T cell responses in various ways, including induction of apoptosis, inhibition of proliferation, or induction of a regulatory phenotype. Type 2 macrophages found at tumor sites have also been implicated in suppression of tumor immunity (Mantovani, Sozzani et al. 2002). From these examples it can clearly be seen that by generating the appropriate environment, tumors can skew the immune response in order to escape.

Another strategy used by tumors to escape is through alterations in dendritic cell (DCs) population. These professional antigen-presenting cells have the capacity either to trigger specific T cell responses against antigens presented by them, or to induce T regs. If a mature DC presents antigen to a T cell, an immune response will be activated; but, if an immature DC presents antigen to a T cell, tolerance ensues. Therefore, an increase in immature DC populations could constitute an immune escape mechanism. A study comparing affected tumor lung and lung not affected of the same patient demonstrated differences in the pattern of distribution of macrophages and DC between these two tissues (Baleeiro, Anselmo et al. 2008). Analysis of the number of immature DC per field (100x) showed an immature DCs ($CD1a^+$ cells) mean number range between 0 to 151 with a mean of 39.3 in affected lung; while in non-affected lungs, the frequency of these cells ranged from 0 to 52.6 cells with a mean of 6.2 (P= 0.0371). They did not find significant statistical correlation between densities of immature DCs at tumor microenvironment and tumor stage or type of tumor.

Another study showed that immature DCs (iDCs) mediate immunosuppression by recruiting TGF-β/IL-10 double positive CD4+ regulatory T cells (Cools, Van Tendeloo et al. 2008). Comparing co-culture of autologous lymphocytes with monocyte-derived immature or mature DCs, they observed increased numbers of TGF-β/IL-10 double positive CD4+ regulatory T cells in both groups. However, supernatant samples from immature DC/T cells co-cultures stimulated with cytomegalovirus (CMV) peptide pp65 presented significantly higher amount of TGF- β, when compared with T cell cultures without iDCs stimulated with same pp65 CMV peptide. In addition,

there is evidence that immature DCs play a crucial role in tumor growth and angiogenesis (Fainaru, Almog et al.), and that a semi-mature DCs state can be implicated in IL-10 production by T reg cells and T reg or T anergy induction (Frick, Grunebach et al.). These findings support the idea that DCs maturation is essential for a proper and efficient antitumor response.

Curiously, although tumor cells exert all these active processes of immunosuppression directed to tumor antigens (de Souza and Bonorino 2009), it is not generalized to other antigens (Radoja, Rao et al. 2000). We have verified, using a transgenic experimental model, that responses to a non-tumor antigen in the tumor draining lymph node are not affected significantly (Souza et al, submitted). There are, however, studies that report alterations in phenotype and function of peripheral DCs in cancer patients. Recent work demonstrates that peripheral blood DCs from advanced breast cancer patients are reduced in number when compared with healthy age matched control (Della Bella, Gennaro et al. 2003) (Pinzon-Charry, Ho et al. 2007). Moreover, blood circulating DCs from advanced breast cancer patients show decreased expression of CD86 and HLADR, thus presenting an immature profile (Pinzon-Charry, Ho et al. 2007). In this same study, DCs from controls and advanced breast cancer patients were challenged with soluble or particulate antigens conjugated with FITC and FACs analysis demonstrated that DCs from advanced breast cancer patients have impaired antigen presentation when compared with controls.

Consequently, tumors can present a myriad of immunosuppressive effects in order to grow, and the use of proper adjuvants as well as the neutralization of immunosuppressant through activation signals is probably an important element for the reversal of this immunocompromised state in the cancer patient. However, such enhancing signals need to be properly identified, since there is a great body of evidence linking inflammation to cancer, suggesting that not all immune activation may be positive for the patient.

Inflammation promoting cancer – immune response or tumor- induced phenomenon?

The hypothesis that links inflammation and cancer is old, dating from 1863, when Rudolf Virchow's reported leucocytes in neoplastic tissue (Virchow, R. 1858). It is known that inflammation may contribute to cancer progression by dependent or independent immune pathways (Ostrand-Rosenberg and Sinha 2009). Reactive species of oxygen cause mutations in the DNA and contribute to malignant cell proliferation (Federico, Morgillo et al. 2007) (Hsie, Recio et al. 1986). VEGF and other proangiogenic factors promote tumor neovascularization (Ellis and Hicklin 2008) and matrix metalloproteases facilitate invasion and metastasis (Yang, Huang et al. 2008); are examples of independent immune pathways.

Inflammation-induced tumor promotion is now a rapidly growing area of study. Many environmental causes of cancer and risk factors are associated with some form of chronic inflammation. Up to 20% of cancers are linked to chronic infections, 30% can be attributed to tobacco smoking and inhaled pollutants (such as silica and asbestos), and 35% can be attributed to dietary factors (20% of cancer burden is linked to obesity) (Nickoloff, Ben-Neriah et al. 2005).

Individuals with ulcerative colitis have a 10-fold greater risk of developing colorectal cancer compared to the risk defined for the general population (Clevers 2004), (Itzkowitz and Yio 2004), (Seril, Liao et al. 2003). Chronic exposure to irritants that cause inflammation in the bronchial track such as cigarette smoke (Takahashi, Ogata et al.), asbestos, and silica are associated with an elevated risk of lung cancers (Ballaz and Mulshine 2003), (Shacter and Weitzman 2002). Such particles can trigger inflammation through effects on prointerleukin-1b (IL-1b) processing by the inflammasome (Dostert, Petrilli et al. 2008) and this may mediate their tumorigenic activity. Many cancers are linked with chronic pathogen exposure such as gastric cancers and *Helicobacter pylori* (Prinz, Schwendy et al. 2006). The inflammatory response triggered by infection precedes tumour development and is a part of normal host defence, whose goal is pathogen elimination. However, tumorigenic pathogens subvert host immunity and

establish persistent infections associated with low-grade but chronic inflammation. Even obesity, which increases cancer risk by 1.6-fold (Calle 2007), can lead to chronic inflammation (Tuncman, Hirosumi et al. 2006) that promotes development of hepatocellular carcinoma (Park, Lee et al.). Accumulation of damaged DNA and cell senescence can also give rise to tumour-promoting chronic inflammation (Rodier, Coppe et al. 2009) (Zheng, Dai et al. 2007). Molecular, cellular, and organism studies in recent years emphasize a broad role of NF-κB pathways in mediating development and progression of cancer (Karin 2006), (Karin and Greten 2005), (Karin 2006). Several types of inflammation—differing by cause, mechanism, outcome, and intensity—can promote cancer development and progression. On the other hand, not all chronic inflammatory diseases increase cancer risk, and some of them, such as psoriasis, may even reduce it (Nickoloff, Ben-Neriah et al. 2005).

Consequently, the relationship between inflammation and cancer is probably more complex than initially thought. Further studies are necessary to distinguish the inflammation mechanisms that can mostly favor tumors from the ones which will activate immune responses and in turn support the activation of anti-tumor immunity, optimizing the design of anti-tumor therapy.

The impact of chemotherapy on the immune system of the cancer patient – it takes years

Because cancer is a complex disease and can affect different tissues, different treatment protocols are employed. It is difficult to generalize a standard chemotherapy regimen for all cancers and analyze how it would impact the immune system. However, most common chemotherapeutic agents have a direct effect on the immune system (Brunton, LL et al.2006):

- Cyclophosphamide is used for lymphocytic leukemias, Hodgking disease, non-Hodgking lymphoma, multiple myeloma, neuroblastoma, breast cancer, Wilm's tumor and others. It can cause leucopenia, thrombocytopenia and myelosuppression.
- Gemcitabine is a pyrimidine analogue used for lung, ovarian and pancreas cancers. It can cause myelosuppresion.
- Vimblastine is a vinca alkaloid used for Hodgking Disease; testicle, breast and lung cancers. It causes leucopenia and thrombocytopenia.
- Doxorubicin is used for soft tissue sarcomas, childhood sarcomas, breast cancer, and others. It causes myelossupression.

In any case, often the chemotherapy regimens using high doses of cytotoxic drugs will result in a steep decrease in leucocyte numbers, and it may be one or two years before patients recover normal cell counts (Kang, Weaver et al. 2009). This recovering period is usually a time of many infections for cancer patients, and depending on how compromised is their immune system, some of these infections are fatal.

However, it is possible to use cytotoxic drugs in a lower dose and it seems that the death of cancer cells can act as a stimulus for an immune response against the tumor. For example, when DCs are exposed to calreticulin during tumor cell apoptosis and through calreticulin binding with calreticulin receptor (present on DCs surface) DCs are activated. After calreticulin release by tumor apoptotic cell, HMGB1 is released as a late event. This HMGB1 from tumor apoptotic cells binds to TLR4 present on DCs and promotes DCs maturation, leading to tumor antigen presentation to TCD4+ and TCD8+ cells. (Kepp, Tesniere et al. 2009). On such note, Berd and Mastrangelo showed in 1987 that low dose cyclophosphamide can reduce T-suppressor function without depletion of TCD8+ cells subset (Berd and Mastrangelo 1987). More recently, new studies emerged showing that low doses of certain agents can stimulate certain pathways of the immune response.

One study evaluated immunomodulatory effects of Paclitaxel applied to ex vivo generated DCs (John, Ismail et al.). When DCs were continuously exposed to Paclitaxel for 24 and 48h at concentrations below 100uM, they showed at least 80% of viability. However, when DCs were exposed to Paclitaxel for 48h at a concentration of 100uM, the viability decreased to 60%. They observed a paclitaxel dose-dependent increase in HLA class II molecules in DCs, but no effect in CD40, CD83 or CD86 expression by these cells. An MLR assay showed that T cell proliferation was greater with low dose paclitaxel. Zhong et al (Zhong, Han et al. 2007), analyzed apoptosis in Lewis Lung carcinoma cell line 3LL after 24h or 48h co culture with three chemotherapic agents: paclitaxel, taxotere or cisplatine. Only low doses (50nmol/L) of paclitaxel induced the same apoptotic levels as negative controls, not being toxic for murine bone marrow cells. They also showed that 3LL cells inhibited murine dendritic cell maturation *in vitro*, but if the DCs were pre-treated with the low paclitaxel dose, this inhibition was reversed ($p<0.05$). In addition, chemotactic potential and motility of murine DCs was inhibited *in vitro* by 3LL cells and reversed after pre treatment with paclitaxel ($p<0.05$). This study demonstrated that injection of paclitaxel followed by intratumoral DCs injection was better than each treatment alone for inhibition of cancer growth. In addition, this approach enhanced CD4+T and CD8+T cell infiltration of the tumor. In a murine model (in this same study), tumor-draining lymph nodes of animals treated with paclitaxel were dissected and supernatants from cultures of these cells showed an increased tumor-specific IFN-γ production, correlating with significant inhibition of tumor growth (John, Ismail et al.).

Thus, it is possible to envisage a scenario in which chemotherapy could be used in low doses, activating tumor specific immune responses in patients, probably in combination with immunotherapy, creating a less toxic, but still anti-tumor environment.

Immunotherapy – it works from the outside too

Given the devastating effect that many chemotherapeutic drugs can have on the immune system of cancer patients, immunotherapy presents an attractive alternative, due to its characteristics – low toxicity, high specificity, and potential to generate a memory response. Immunotherapy can be passive, i.e., transfer of antibodies to patients, as well as active, aiming to generate in vivo an anti-tumor response. All the evidence regarding tumor immunosuppressive strategies directly targeting DCs highlight the relevance of this cell population in the generation of tumor-specific responses. Therefore it is not surprising that treatments based on DCs, together with monoclonal antibodies, constitute the most prolific areas or anti-cancer immunotherapy.

Monoclonal antibodies - In 1997 the first monoclonal antibody for cancer treatment was developed: rituximab, which reacts with CD20, present on B cells, for chronic lymphocytic leukemia. Since then, many other monoclonal antibodies have become available for clinical use in oncologic patients, namely trastuzumab (1998), gemtuzumab ozogamicn (2000), alemtuzumab (2001), Ibritumomab tiuxetan (2002), ^{131}I-tositumomab (2003), bevacizumab 2004), cetuximab (2005), panitumumab (2007), ofatumumab (2009). These antibodies are human antibodies: ^{131}I-tositumomab and Ibritumomab. Trastuzumab, alemtuzumab, gemtuzumab ozogamicin and bevacizumab are humanized monoclonal antibodies (Weiner, Surana et al.).

Cetuximab, panitumumab, necitumumab, and zymed target EGFR (epidermal growth factor receptor) (Weiner, Surana et al.). Cetuximab is used in combination with other chemotherapeutics agents. The first two are used as second and third treatment line for metastatic colorectal cancer. There are. Zymed recognizes and acts against truncate forms of EGFR: EGFRvIII, which are in-frame deletion of exons II-IV of the EGFR molecule encountered in head and neck cancer, non small lung cells cancer an glyobastoma (Li, Ji et al. 2007) (Gan, Lappas et al. 2009).

Trastuzumab (also called Herceptin) is a humanized IgG1 antibody used for treatment of invasive breast cancer, with cells expressing the marker HER2-neu. The mechanisms of action of trastuzumab have not yet been fully elucidated. A recent review examined possible mechanisms, which include activation of antibody-dependent cellular cytotoxicity, abrogation of intracellular signaling, decreased DNA repair, inhibition of extracellular domain cleavage, and reduction of angiogenesis (Spector and Blackwell 2009). An improvement in prognosis was reported for women with HER2-positive breast cancer who received trastuzumab (Ferretti, Fabi et al.). A recent report suggests the use of trastuzumab as a neoadjuvant for women with HER2-positive locally advanced or inflammatory breast cancer based on improvement in survival (free-event survival, survival and clinical and pathological responses) (Gianni, Eiermann et al.).

Another possible therapeutic target is cytotoxic T lymphocyte antigen-4 (CTLA-4). This antigen is a member of an important immunoglobulin regulatory molecule superfamily: CD28-B7 (Greenwald, Freeman et al. 2005). It binds to CD80 (B7.1) and CD86 (B7.2) with higher affinity than CD28. Binding of CTLA-4 on CD80 or CD 86 promotes T cell anergy, inhibition of IL-2 production by Tcells and reduced T cell proliferation. When CTLA-4 is blocked, its receptors (CD80 and CD86) will be free to CD28 binding and to trigger T cell activation (Weber J et al. 2006).

There is evidence that CTLA-4 blockage causes rejection of CD86 negative tumors and protection if occurs a second tumor challenge (Leach, Krummel et al. 1996), (Peggs, Quezada et al. 2006). Two monoclonal antibodies for CTLA-4 blockage are being studied: tremelimumab (CP-675, 206; Pfizer) (IgG2 isotype) and ipilimumab (MDX-010; Bristol-Meyers Suqibb/Medarex) (IgG1 isotype). Tremelimumab is being investigated for use in melanoma (Ribas 2008), (Kirkwood, Lorigan et al.), advanced breast cancer (in which demonstrated to be tolerable for patients and promoted immune activation) (Vonderheide, Lorusso et al.) and advanced gastric and esophageal adenocarcinoma (with a poor response rate) (Ralph, Elkord et al.). Ipilimumab

was tested for metastatic carcinoma. One study demonstrated that the objective response of ipilimumab was around 15%, however, both objective response and stable disease tend to be long lasting. This monoclonal antibody causes autoimmune and dose related side effects classified as immune-related adverse events (IRAEs): enterocolitis, dermatitis, diarrhea, hypophysitis, hepatitis, nephritis with azotemia and vitiligo (Peggs, Quezada et al. 2006) (Weber J 2006). These symptoms need clinical management with corticosteroids and even infliximab (a TNF-alfa inhibitor) if patients do not respond to corticosteroids (Weber J 2006). It was interesting to observe that there is evidence of a relation between immune-related adverse events and anti-tumor responses (Maker, Attia et al. 2005), (Blansfield, Beck et al. 2005). However, these data confront another study in which this relation was not clear (Downey, Klapper et al. 2007) and some patients without autoimmunity effects presented clinical responses or long term freedom from progression (Sanderson, Scotland et al. 2005). So, ipilimumab use is still controversial. Ipilimumab is being also investigated as a treatment option for men with hormone refractory prostate cancer (Small, Tchekmedyian et al. 2007).

Dendritic Cells - were first described by Ralph Steinman and Zanvil Cohn at 1973 from culture of spleen cells of mice as a "new stelate glass adherent cell type" (Steinman and Cohn 2007). Nowadays DCs are recognized as the most important antigen presenting cells because they are able to induce primary and memory immune responses and they decide between active immune response and immune tolerance both in mice and humans.

The most common approach in DCs based vaccination is in vitro to differentiate DCs from the patient's blood monocytes using GM-CSF and IL-4 (Mayordomo, Zorina et al. 1995) (Romani, Gruner et al. 1994), and later loading these differentiating DCs with tumor antigens. Finally, the loaded DCs are re-implanted in the patient, where the DCs will seek and activate tumor specific T cells *in vivo* (Barbuto, Ensina et al. 2004).

The variations on this approach include the use of peptides to load DCs, use of whole protein antigens, use of killed tumor cells that the DCs will phagocytose and process, and more recently, transfecting DCs with RNA from tumor cells or RNA encoding tumor antigens. All these strategies have advantages and caveats. The use of peptides is limited to HLA subtype binding, the use of whole antigens leaves the possibility of eliciting responses to non-conserved as well as to conserved, non-mutated epitopes that could induce autoimmune responses. Killed tumor cells as a source of antigen always leave the undesired possibility of re-injecting the patient with at least one leftover live tumor cell in the midst of the loaded DCs.

Furthermore, there is the issue of ensuing that the injected DCs will induce an effective response. Studies reveal that less than 1% of the DCs used for injection actually survives and gets to the lymphoid organs (de Vries, Lesterhuis et al. 2005), stimulating some groups to perform intranodal injections. Also, there is the activation issue. Although tumors do possess endogenous ligands to TLRs, such as HMGB1 (Lotze and Tracey 2005) and Hsps (Srivastava and Maki 1991) these do not appear to activate DCs with the same magnitude that exogenous ligands such as LPS or CpG do. To circumvent that, some DC-based vaccines include one step of in vitro activation of the cells before injections, commonly using TNF-alfa, or yet IL-15 (Mohamadzadeh, Berard et al. 2001), or even transfecting the injected DCs with plasmids encoding inflammatory cytokines.

Palucka et al vaccined twenty stage IV melanoma patients with autologous monocyte-derived DCs loaded with killed allogeneic Colo829 melanoma cells (8 vaccines were administered at monthly intervals) obtaining an estimated median survival of 22.5 months. Vaccines were safe and tolerable. Two patients who were resistant to previous therapy presented durable objective clinical responses. In addition, three patients showed T-cell immunity against melanoma antigens and, in one patient, vaccination triggered CD8+ T cell immunity specific to a novel peptide-derived from MART-1 antigen suggesting that cross-priming and cross-presentation of melanoma

antigens by DC vaccine had occurred (Palucka, Ueno et al. 2006). An alternative approach is to generate allogeneic DC-autologous tumor cell hybrid vaccines. Neves et al. compared DCs generated from cancer patients with DCs generated from healthy donors, the latter showing higher CD86 expression ($P<0.05$). When CD86 levels were analyzed before and after allogeneic DC-autologous tumor hybrid vaccination, and new DCs were generated from patients, their DCs showed recovery of CD86 expression ($P=0.03$). In MLRs (mixed lymphocyte reactions) with patient DCs obtained before and after vaccination, INF-γ levels was significantly higher after vaccination ($P<0.05$). (Neves, Ensina et al. 2005). In a recent clinical trial (Phase Ib), Redman BG et al treated twenty-four patients with autologous tumor lysate-pulsed DC vaccine with or without IL-2. Autologous DCs were obtained from patients' mononuclear cells. Vaccines were well tolerated with no subjects reporting greater than grade I toxicity at the local site. The IL-2 administration did not increase local toxicity. PMBCs from patients after vaccination presented enhanced IFN-γ response ($P<0.0011$) when compared with PBMCs before treatment. Unfortunately, there was no tumor response as defined by RECIST criteria. However, two patients that received high IL-2 dosage, presented a minor response (reduction in size of their metastatic lesions, but not enough to meet those criteria) (Redman, Chang et al. 2008). However, in a study for indolent non-Hodgking lymphoma (NHL), Di Nicola et al applied DC vaccines for eighteen NHL patients and obtained clinical responses in six (33.3%) patients in an average follow up of 50.5 months. Eight patients maintained stable disease while four had progressive disease. It is interesting to note that, only in responder patients, clinical responses were significantly associated with a reduction in regulatory T cells ($P=0.008$) and with an increase in natural killer cells ($P=0.009$). The vaccine was safe, no significant hepatic, renal, pulmonary, cardiac, hematologic, gastrointestinal or neurologic toxicity was observed (Di Nicola, Zappasodi et al. 2009).

In a phase II trial with DC vaccine for treatment of glioblastoma multiforme, 34 patients received autologous tumor lysate-pulsed DCs, and IFN-γ production was assessed before and after vaccines, showing an antigen-specific IFN-γ response with significant increase after three vaccinations ($P<0.000001$). Time of tumor survival in vaccine responders was 642+/-61 days (significantly longer than in non responders: 430+/-50 days) representing an increase of about 7 months of survival. Time of tumor progression was 308+/-55 days in vaccine responders, also longer than 167+/-22days in nonresponders representing an increase about 4.5 months. The relevance of this response is significant, because survival expected for glioblastoma multiforme patients is from 12 to 18 months. In this same trial, 19 patients received chemotherapy after DC vaccine presenting an increase of survival to tumor when compared with patients that received DCs ($P=0.072$) vaccine alone (Wheeler, Black et al. 2008).

In another study, Bonehill et al pulsed DCs with Melan-A derived peptide and transfected them with CD40 ligand, CD70 and constitutively active TLR4 mRNA (named TriMix) showing that this method is more effective than conventional approaches (Bonehill, Van Nuffel et al. 2009). Using their method in a preclinical trial, the same group demonstrated that after electroporation with TriMix mRNA, immature DCs acquired a mature phenotype (showed by up-regulation of costimulatory markers as CD40, CD80, CD83 and CD86) and enhanced cytokine secretion (IL-12p70). They showed that TriMix DCs co-electroporated with whole tumor-antigen mRNA could stimulate antigen-specific T cells against Melan-A antigen and against other TAAs (Bonehill, Van Nuffel et al. 2009).

Altogether, these results from most of these studies suggest an important, however not long-lasting effect of tumor vaccination with tumor-loaded DCs. This could be in part caused by the immune system of the patient that receives the vaccine. Many times, patients in these studies have undergone chemotherapy and their T cells are heavily depleted, thus leaving the matter of which T cells the injected DCs will stimulate. Also, the tumor induced suppressor cells (as

described previously in this article) can still be present and dampen the function of the newly activated responses.

More recently, DCs-based vaccination has considered including a combination of the antigen presenting cells with elimination of regulatory or suppressor cells, such as T-regs or MDSCs (Morse, Hobeika et al. 2008). Sunitinib is a receptor tyrosine kinase inhibitor, available in oral medication presentation that is used with significant clinical effect in metastatic renal cancer (Motzer and Bukowski 2006). In a recent study with renal carcinoma, Ko JS et al demonstrated that sunitinib can significantly reverse MSDC accumulation. In addition to the decrease in MDSCs production by bone marrow, the percentage of lymphocytes was significantly increased ($P<0.001$). These data suggest that sunitinib may have a myelospecific effect on bone marrow function, that favors anti-tumor responses. Another interesting observation from this study was that T cells produced more IFN-γ after sunitinib treatment. They observed a positive correlation between the numbers of T regs and MDSCs remaining after two cycles of therapy ($P=0.008$) (Ko, Zea et al. 2009).

Morse MA et al, in a preclinical study demonstrated enhanced T cell proliferation in vitro after Treg depletion by dinileukin diffitox. They performed a clinical study with 15 patients with CEA (carcinoembryonic antigen)-expressing malignancies in the same study, using dinileukin diffitox (an $CD25^{high}$ immunotoxin) to deplete FoxP3+ T regs before application of DCs modified with the vector rF-CEA(6D)-TRICOM vaccine. They analysed CEA-specific T-cell responses by intracellular cytokine staining and found that 14 out of 14 patients had a CD4+ and CD8+ CEA-specific T-cell response. In addition, patients' T reg levels were reduced after dinileukin diffitox while CEA-specific T cells levels were enhanced (Morse, Hobeika et al. 2008). Therefore, monoclonal antibodies may work alone or together with DCs in immunotherapy against cancer.

Conclusions – lessons to be learned

A great amount of evidence indicates that an intense crosstalk takes place between tumor and immune cells, not only locally, but also with possible systemic effects. The immune system of the cancer patient constitutes an important mechanism of control of tumor growth. In turn, it is profoundly influenced by the many molecular strategies employed by the tumor to deflect its anti-tumor action. That has to be considered when designing anti-tumor therapies. High dose chemotherapy, while toxic for tumor cells, greatly depletes the immune system, robbing the patient of an important ally in the eradication of cancer cells. Recent results obtained with immunotherapy, both passive and active, underline the importance of such effector mechanisms in tumor control, and support a view in which the immune system of the cancer patient needs to be spared and stimulated, rather than eliminated. Immunotherapy presents the important property of specificity, directing therapy against targets present exclusively or more abundantly in tumors, or yet targeting immunosuppressive mechanisms. Consequently, we propose a scenario in which combinations of low dose chemotherapy with effective immunotherapy can result in tumor control and enhanced quality of life for the patient.

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Considerações finais

Neste trabalho, nós demonstramos que monócitos e células dendríticas derivadas de monócitos de pacientes com câncer de mama apresentam múltiplas alterações consistentes com uma diminuição da resposta antitumoral e a promoção de um ambiente pró-tumoral.

A busca de novas terapias para câncer que gerem uma alta imunogenicidade e apresentem baixa toxicidade é fundamental para a qualidade de vida dos pacientes com a doença. Para avançar nesse caminho, são necessários estudos detalhados sobre a interação das células tumorais e as células dos demais sistemas, principalmente, as células do sistema imune.

A terapia baseada em células dendríticas é sem dúvida extremamente promissora, especialmente, todos os estudos indicam, quando combinada com o uso de adjuvantes e a eliminação de células T regulatórias.

Sejam quais forem os rumos dessa terapia, se permanecerem os protocolos de vacinação com célula, ou se estas evoluírem para terapias direcionadas para diferentes subpopulações de células dendríticas, é uma proposta deste estudo que o fenótipo e a funcionalidade dessas células em pacientes seja caracterizado com mais detalhe, visando maximizar os efeitos que podem ser obtidos com essa abordagem.