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ATIVAÇÃO DE CÉLULAS DENDRÍTICAS NA GERAÇÃO DE CÉLULAS T CD8+ E T CD4+ ANTI-TUMORAIS DE MEMÓRIA

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Orientadora: Profª.Drª. Cristina Bonorino

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Resumo

De acordo com a hipótese da vigilância imunológica, o sistema imune é relevante no controle do crescimento tumoral. Entretanto, a resposta imune não é capaz de barrar a progressão de todos os tumores. Uma possível explicação para isso é a tolerização das células imunes proporcionada pelo ambiente imunossupressor gerado por tumores, favorecendo seu desenvolvimento e diminuindo a eficácia da resposta imune contra o tumor.

O presente estudo visou analisar a formação de respostas imunes anti-tumorais *in vivo*, bem como identificar mecanismos capazes de reverter a tolerância induzida pelo tumor à resposta imune. Para realizar este trabalho foram usados três sistemas experimentais: (1) injeção subcutânea de tumor B16F10 para análise da resposta imune policlonal, (2) injeção subcutânea de B16OVA ($OVA_{257-264}$ SIINFEKL) com transferência adotiva de células OT-I para o estudo da geração de resposta de células T CD8+ e (3) construção e injeção subcutânea de uma linhagem de B16F10 expressando um antígeno restrito ao MHC classe II (B16EaRFP) com transferência adotiva de células TEa para o estudo da geração de resposta anti-tumoral de células T CD4+. Com estes modelos experimentais foi observada diminuição de células no infiltrado peritumoral e da celularidade nos linfonodos drenantes (LND) à medida que o tumor B16F10 cresce, sugerindo uma interrupção do trânsito de células imunes do sítio tumoral para o LND. A expressão de CD86 nas células dendríticas (DCs) nos linfonodos diminui com o crescimento tumoral. Com a elevação da freqüência precursora de células T CD8+ anti-tumorais no sistema B16OVA, o crescimento tumoral foi retardado mas não barrado por completo. A melhora significante da resposta contra o tumor foi obtida com a injeção intratumoral de um ligante de TLR-4, o lipopolissacarideo de E.coli (LPS), mas apenas quando foi injetado via intratumoral, após o estabelecimento do tumor *in vivo*. O sistema B16EaRFP revelou que a acessibilidade do antígeno às células dendríticas é importante na estimulação da resposta T CD4+ anti-tumoral, resultando em maior

proliferação de células TEa em resposta ao tumor lisado *versus* o tumor vivo. Quando as células tumorais foram induzidas a necrose e apoptose e injetadas subcutaneamente, houve maior porcentagem de células CD11c +YAe+ CD86+. Observou-se maior proliferação de células TEa nos linfonodos drenantes no grupo de camundongos injetados com células tumorais mortas por necrose, com um pico nove dias após a injeção tumoral, mas já apresentando contração após três dias. A divisão das células TEa também diminui gradualmente ao longo do tempo, sugerindo que nos três casos há apresentação limitada de antígeno. A diferenciação das células TEa em fenótipo de memória foi observada nos três tratamentos, com mais ênfase nos animais que receberam tumor induzido a necrose e apoptose. Contudo, as células TEa não foram capazes de fornecer ajuda para interromper o crescimento tumoral quando o B16EaRED foi injetado vivo. Em conjunto, os resultados sugerem que a diminuição da apresentação de antígeno e de co-estimulação ao longo do tempo impedem a diferenciação das células T em células de memória eficazes.

Os resultados indicam que o tumor intacto não fornece sinais suficientes para a geração de células T de memória anti-tumoral para impedir o crescimento tumoral. Ao contrário, o tumor parece gerar um ambiente que não favorece a resposta imune, impedindo o trânsito de células imunes entre o LND e o sitio tumoral, diminuindo a expressão de CD86 em DCs no LND. Ainda, sinais fornecidos por células induzidas a apoptose ou necrose aumentam a apresentação de antígenos tumorais e os níveis de co-estimulação, embora não substituam os sinais fornecidos pelo LPS, capazes de reverter o quadro imunossupressor.

Abstract

According to the immunological surveillance hypothesis, the immune system is relevant against tumor growth. However, the immune response is not completely able to block all tumors. A possible explanation is that immune cells tolerization induced by the tumor immunosuppressive environment enables tumor development and decreases immune efficiency against the tumor.

The aim of the present investigation was to better understand the *in vivo* anti-tumoral immune response as well as to identify mechanisms that play a role in modulating a reversion of tumor induced tolerance. To that end we used three experimental models: (1) Subcutaneous B16F10 tumor injection to analyze a polyclonal immune response, (2) subcutaneous B16OVA ($OVA_{257-264}$ SIINFEKL) tumor injection with OT-1 cell adoptive transfer to study a T CD8+ anti-tumoral immune response and (3) construction and subcutaneous injection of a B16F10 expressing MHC class II restricted antigen (B16F10EaRed) with TEa cells adoptive transfer to study TCD4+ anti-tumoral immune response generation. The results using these models showed a reduced number of infiltrating cells in the peritumoral area as well as a decreasing cell number in tumor draining lymph nodes as tumor grew. These findings suggested cell traffic interruption between lymph nodes and primary tumor site. Likewise, CD86 expression in dendritic cells (DCs) was decreased with tumor growth. An increased precursor frequency of anti-tumoral T CD8+ cell in the B16OVA system arrested tumor growth, but did not completely prevent it. A stronger tumor immune response was achieved with injection of intra-tumoral TLR-4 ligand, the E. coli lipopolysacharide (LPS), however only during a specific window of time after tumor injection. The B16EaRed system showed that antigen accessibility by DCs is crucial to stimulate a T CD4+ anti-tumoral immune response; as a result, there was a higher TEa cell proliferation against tumor lisates than whole tumor cells. When tumor cells were induced to

undergo necrosis or apoptosis and injected subcutaneously, the result was higher percentage of CD11c+ YAe+ CD86+ cells. There was more TEa cells proliferation in draining lymph nodes from mice injected with apoptotic or necrotic cells, showing a proliferation peak at day nine after tumor injection. However, it was observed a cell contraction three days after. TEa cell division decreased gradually throughout time, suggesting there was limited antigen cell presentation in all experimental models studied. TEa cell memory differentiation was also observed in the three treatments, with emphasis in the animals that received necrotic or apoptotic tumor cells. However, the TEa cells were not able arrest tumor growth when B16EaRED was injected live. Together, these results suggest that the decrease in antigen presentation and co-stimulation throughout time, negatively affects T cell memory differentiation.

The results point out that whole tumor does not deliver enough signals to generate anti-tumoral T cells with memory phenotype capable of blocking tumor growth. Contrarily, the tumor seems to create an environment that does not allow immune response, avoiding cell traffic between the draining lymph node (DLN) and the tumor site, decreasing CD86 expression on DCs cells in DLN. In addition, signals delivered by death cells simulating apoptosis or necrosis increased tumor antigen presentation and co-stimulation, but did not replace LPS delivered signals that were efficient to revert immunossupression.

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Capítulo 1

1.1 Introdução

Os tumores malignos são doenças que apresentam alterações no genoma resultantes de mutações acumuladas no genoma. Estas mutações são associadas ao descontrole de programas essenciais como proliferação, morte e diferenciação celular. Hanahan e Weinberg (2000) agruparam as capacidades adquiridas pelas células cancerosas em seis classes que interferem na fisiologia normal de células e tecidos: 1- auto-suficiência quanto a fatores de crescimento; 2- insensibilidade a fatores inibitórios da proliferação; 3- evasão da apoptose ou morte celular programada; 4- potencial replicativo infinito; 5- angiogênese sustentada; e 6- invasão tecidual e metástase (Hanahan e Weinberg, 2000).

Ao longo dos anos o tratamento do câncer foi baseado na combinação de cirurgia, quimioterapia e radioterapia, essas últimas com forte ação citotóxica tanto para as células malignas como para as células normais acarretando aos pacientes efeitos adversos severos. Dentro dos efeitos indesejáveis da terapia convencional do câncer está a imunossupressão, condição que deixa o paciente mais suscetível a infecções e recidivas de tumores malignos removidos anteriormente (Baxevanis, Perez *et al.*, 2008). Os tumores malignos se desenvolvem *in vivo* através de diferentes mecanismos, tendo seu crescimento relacionado com a deficiência da vigilância imunológica e com a tolerância imune que ele próprio induz (Rivoltini, Carrabba *et al.*, 2002; Mapara e Sykes, 2004). A tolerização contra o tumor é o resultado do microambiente tumoral, envolvendo células apresentadoras de antígenos (APCs), alteração nas moléculas co-estimulatórias e co-inibitórias, somando-se à razão alterada entre células T efetoras e células T regulatórias (Zou, 2005). Mecanismos locais de tolerização têm sido estudados, como a secreção de citocinas imunossupressoras IL-10 e TGF-beta que podem apresentar um efeito autócrino ou parácrino no ambiente tumoral

(Chen, Daniel *et al.*, 1994). Essas citocinas podem ser produzidas pelo tumor ou ainda por linfócitos T e células dendríticas (DCs) pertencentes ao infiltrado peritumoral, induzindo um fenótipo imunossupressivo (Polak, Borthwick *et al.*, 2007). Uma pesquisa com hepatocarcinoma mostrou que as células tumorais podem evadir a resposta imune pela diminuição da expressão do receptor de CD95, um importante mediador da citotoxidade das células T, bem como eliminar as células T CD8 através da expressão de CD95L, uma molécula pertencente à família do ligante de Fas (Strand, Hofmann *et al.*, 1996). Contudo, a expressão de Fas-L não é o único mecanismo pelo qual os tumores induzem a morte de linfócitos: foi mostrado que a não-expressão de MHC de classe I pelos tumores, além de ser uma estratégia defensiva, pode funcionar ofensivamente induzindo a apoptose de células T (Fishman, Irena *et al.*, 2001). Outro mecanismo de imunossupressão é a presença de células T reguladoras que foram descritas primeiramente por sua habilidade de suprimir doenças auto-imunes, mas em tumores, as células T CD4+CD25+ têm sido relacionadas com a diminuição da imunidade antitumoral. Níveis elevados de TGF-beta e IL-10 no sítio tumoral podem transformar células CD4+CD25- que migraram para o tumor em células T reguladoras CD4+CD25+FoxP3+, tendo papel importante na evasão tumoral (Liu, Wong *et al.*, 2007). Os tumores malignos podem também modificar as suas moléculas de superfície para se tornarem menos reconhecíveis pelas células do sistema imune e mais eficientes em induzir a morte das células do hospedeiro. As moléculas da família B7-CD28 que regulam o equilíbrio entre os sinais inibitórios e estimulatórios possuem um membro com importante papel no controle do crescimento tumoral, o receptor de morte programada (PD-1), que regula a tolerância e a imunidade provendo o segundo sinal às células T juntamente com a sinalização via receptor de células T (TCR). O PD-1 é uma molécula de superfície também expressa em linfócitos, macrófagos e células NKs que irá induzir a morte celular por apoptose quando ativada pelo seu respectivo ligante, o B7-H1, regulando negativamente a resposta imunológica. Vários

cânceres humanos e de camundongos expressam B7-H1 e isso foi relatado como mais um mecanismo que o tumor utiliza para se evadir da resposta imune e por outro lado, mostra um caminho para a imunoterapia, considerando a melhora na resposta antitumoral com o bloqueio dessa rota PD-1/B7-H1(Keir, Liang *et al.*, 2006; Azuma, Yao *et al.*, 2008).

Mesmo havendo reposta antitumoral, ela pode ser insuficiente para controlar um tumor com mutação, proliferação ou ainda expressando moléculas próprias para as quais o indivíduo pode ter sido tolerizado central ou perifericamente (Rosenberg, Sherry *et al.*, 2005). Com isso, as interações entre as células malignas e as células do hospedeiro no microambiente tumoral criam uma rede imunossupressora que promove o crescimento e protege o tumor das células de defesa prejudicado as possibilidades de tratamento (Rock, Hearn *et al.*, 2005; Zou, 2005). E ainda, o contínuo crescimento tumoral torna insuficiente o transporte de nutrientes por difusão, propiciando uma neovascularização resultante da produção de fatores angiogênicos pelo tumor, especialmente o fator de crescimento endotelial (VEGF) (Redondo, Sanchez-Carpintero *et al.*, 2003; Sharma, Sharma *et al.*, 2005).

A tolerância imunológica aos tumores malignos não acontece apenas localmente. Alguns estudos mostraram que as células T CD8+ tumor específicas recuperadas de linfonodos drenantes perderam a atividade efetora na fase tardia do crescimento tumoral. Nesses tumores avançados houve apresentação de antígeno nos linfonodos, mas a diferenciação das células T CD8+ foi incompleta, sem produção de IFN-γ (Hargadon, Brinkman *et al.*, 2006). Também foi mostrado que as mesmas células T CD8+ específicas voltaram a produzir IFN-γ quando os animais foram imunizados na forma intra-venosa com células dendríticas (DCs) incubadas com o antígeno tumoral específico, sugerindo que a incompleta diferenciação das células T CD8+ resulta provavelmente de um efeito causado pelo tumor na apresentação cruzada das APCs e não propriamente da diferenciação de células T CD8+ (Hargadon, Brinkman *et al.*, 2006). Há evidências de que os mecanismos

responsáveis pela indução de tolerância por deleção ou estabelecimento de anergia de células T CD8+ envolvem o reconhecimento de antígeno na ausência de sinais requeridos para gerar uma resposta completa. Na maioria dos casos, as células T CD8+ encontram o antígeno através da apresentação cruzada por uma subpopulação de DCs CD8+ nos linfonodos, e a resposta imune depende do estado de ativação dessas DCs (Anjuere, Martin *et al.*, 1999; Mescher, Agarwal *et al.*, 2007). Esse processo chamado de "cross-priming" foi primeiramente proposto por Bevan e colaboradores no ano de 1975 (Bevan, 1975; 1976). As DCs apresentam antígenos aos linfócitos T e por isso são capazes de induzir imunidade ao antígeno introduzido no organismo. Segundo Lin, a apresentação cruzada e′ fundamental na ativação de células T CD8+ em que as DCs apresentam uma habilidade especial de exportar o antígeno dos compartimentos endossomais ao citosol para o acesso convencional ao complexo MHC de classe I (Lin, Zhan *et al.*, 2008). As DCs residem em estágio imaturo nos tecidos periféricos onde processam os antígenos locais, tornam-se maduras e migram para os linfonodos para apresentar o antígeno às células T CD4+ e T CD8+. Possíveis defeitos em suas funções poderiam ser responsáveis em parte pela falência do hospedeiro em controlar o crescimento tumoral. Em um estudo com o tumor B16OVA, DCs purificadas da massa tumoral não induziram proliferação de células T CD8+ e T CD4+ especificas para o peptídeos $OVA_{257-264}$ e $OVA_{323-339}$ respectivamente, sugerindo que não houve adequada apresentação do antígeno tumoral. A proliferação das células T somente aconteceu quando as DCs foram incubadas com o peptídeo especifico OVA antes de serem co-cultivadas com células T CD8+ e T CD4+ (Stoitzner, Green *et al.*, 2008).

Um estudo com o tumor D459, um fibrossarcoma fracamente imunogênico, mostrou diminuição da migração de células de Langherans da pele, um subtipo de DCs, para os linfonodos indicando a diminuição da aquisição do antígeno no sitio tumoral bem como defeito no transporte e apresentação desses antígenos para as células T nos linfonodos (Ishida,

Oyama *et al.*, 1998). Analisando pacientes com melanoma, observou-se que a maioria das DCs peritumorais e intratumorais apresentou-se imatura e com baixos níveis de moléculas co-estimulatórias e a maioria das células T no tumor apresentou fenótipo virgem ou regulatório (Vermi, Bonecchi *et al.*, 2003). Outro estudo usando microscopia intravital de linfonodos observou que na presença de células T CD4+CD25+ ocorre diminuição do tempo de contato entre as DCs e as células T virgens, o que poderia prejudicar a apresentação do antígeno e ativação das células de defesa (Furumoto, Soares *et al.*, 2004; Tadokoro, Shakhar *et al.*, 2006). As DCs infiltrando o ambiente tumoral, principalmente tumores de estágio avançado, podem adquirir efeito regulatório devido ao acúmulo de moléculas supressoras como VEGF, IL-6, M-CSF, TGF-beta, IL-10, COX-2, PGE2 em contrapartida à pouca quantidade de moléculas estimuladoras GM-CSF, IL-4, IL-12 e IFN-gama. Este desequilíbrio de citocinas pode bloquear a diferenciação e maturação das DCs com conseqüente indução de células T regulatórias ou células T não responsivas (Vermi, Bonecchi *et al.*, 2003; Zou, 2005).

Entretanto, há evidências de resposta contra tumores. Foi reportado menor número de metástases e sobrevida de até cinco anos a mais em pacientes com melanoma já na fase vertical de crescimento, apresentando alta densidade de células T CD8+ infiltrando o tumor em relação àqueles apresentando menor número de células T CD8+ no tumor (Piras, Colombari *et al.*, 2005). Em pacientes com carcinoma de células escamosas grau III e IV houve menos células T CD3+ e T CD8+ do que em tumores graus I e II (Hirota, Ueta *et al.*, 1990). Como a maioria dos tumores não hematopoiéticos expressa moléculas de MHC de classe I, restringindo seu reconhecimento pelas células T CD8+ e não expressam MHC de classe II, requerido para o reconhecimento de células T CD4+, foi sugerido que o principal mecanismo antitumoral seria via linfócitos T CD8+ citotóxicos (CTL). Porém, há evidências de um papel crucial para as células T CD4+ no combate ao câncer fornecendo sinais regulatórios que ajudam na ativação de células T CD8+. Trabalhando com tumor B16F10

injetado subcutaneamente foi observado que a resposta antitumoral foi drasticamente diminuída em camungondos geneticamente modificados para não produzirem células T CD4+ (Hung, Hayashi *et al.*, 1998). Estudos em pacientes com melanoma também destacaram a importância das células T CD4+ no controle tumoral. A eficiência do tratamento quimioterápico foi maior nos casos de moderado a intenso infiltrado de células T CD4+ no sítio tumoral (Hakansson, Gustafsson *et al.*, 2001). Logo, a imunidade protetora contra tumores malignos requer células T CD8+ citotóxicas restritas ao MHC de classe I e as células T CD4+ restritas pelo MHC de classe II, resultando em uma resposta T citotóxica (CTL) específica para células tumorais associada à resposta Th1 e Th2. Nesse caso citocinas prolongam a resposta imune e recrutam mais células para o sítio tumoral (Furumoto, Soares *et al.*, 2004; Benigni, Zimmermann *et al.*, 2005).

A principal meta da imunoterapia consiste em ativar a resposta contra tumores malignos. O tema imunoterapia não é recente. Em 1890 Paul Ehrlich e William Coley propuseram imunização para doenças infecciosas crônicas e câncer. Embora os resultados de imunoterapia do câncer sejam ainda limitados, o reconhecimento dos antígenos associados ao tumor (TAA) e específicos de tumor (TSA), somados a outros perfis moleculares podem adicionar ferramentas terapêuticas contra o câncer (Rosenberg, 1999; Waldmann, 2003). Dentre as possibilidades testadas na imunoterapia do câncer, as DCs se enquadram por serem responsáveis pela apresentação de antígeno estimulando as células T CD4+ e CD8+ através das moléculas do MHC de classe II e MHC de classe I respectivamente (Banchereau e Steinman, 1998). Diversas estratégias têm sido usadas para ativar as DCs com os antígenos tumorais, incluindo peptídeo sintético derivado do antígeno do tumor em questão (Banchereau, Palucka *et al.*, 2001), RNA tumoral (Boczkowski, Nair *et al.*, 1996), lisado de tumor (Fields, Shimizu *et al.*, 1998), exosomos derivados de tumor (Wolfers, Lozier *et al.*, 2001) e células tumorais em processo de morte (Shaif-Muthana, Mcintyre *et al.*, 2000;

Goldszmid, Idoyaga *et al.*, 2003). A ativação das DCs favorece sua migração do sítio tumoral para os linfonodos onde apresentam os antígenos tumorais aos linfócitos T induzindo imunidade e não tolerância (Kaisho e Akira, 2002). Essa ativação pode ser facilitada com o contato com LPS, outras substâncias derivadas de organismos patogênicos ou citocinas inflamatórias como TNF-alpha, IL-1 beta, GM-CSF através da ligação aos receptores tipo Toll (TLRs). DCs maturadas através dos TLRs têm um aumento da expressão de moléculas MHC de classe I e II e das moléculas co-estimuladoras CD80 e CD86, por isso, apresentam papel importante no aumento expansão clonal das células T durante a apresentação de antígenos nos linfonodos (Visintin, Mazzoni *et al.*, 2001; Kaisho e Akira, 2002; Vegh e Mazumder, 2003). Os TLRs são expressos em alta densidade por macrófagos e DCs e estão envolvidos na resposta imune inata sendo importantes no reconhecimento de microorganismos invasores. O reconhecimento dos patógenos pelos TLRs induz liberação de citocinas inflamatórias aumentando a expressão de moléculas co-estimulatórias, ativando não somente a imunidade inata, mas também a imunidade adaptativa. Por isso, as substâncias estimuladoras de TLR são chamadas de adjuvantes e têm favorecido a manipulação da resposta imune no hospedeiro especialmente na imunoterapia do câncer (Kaisho e Akira, 2002). Yang et al.(1998), trabalhando com tolerância imune a tumores em camundongos C3-HA, que apresentam constitutivamente a hemaglutinina (HA) do vírus influenza como auto-antígeno, usaram uma vacina viral que foi capaz de prover não somente o sinal 1 (ligação ao TCR) mas o sinal 2 (co-estimulacão), ao fornecer sinais para a ativação de DCs através dos TLRs revertendo temporariamente a tolerância causada por células CD4+ CD25+. Nesse mesmo trabalho, as células T CD4+ e T CD8+ especificas para HA foram tolerizadas quando adotivamente transferidas para os camundongos C3-HA. Essa tolerizacão propiciou o crescimento do linfoma A20 injetado de forma intravenosa mesmo este expressando o antígeno HA. Para a reversão do quadro de tolerância imune ao linfoma A20 e para a

sustentação da secreção de citocinas inflamatórias, foi requerida a contínua estimulação das DCs através da ativação dos TLRs por LPS (Yang, Huang *et al.*, 2004). Os clássicos dois sinais necessários para a ativação de células T virgens não induziram uma forte expansão clonal, tampouco desenvolvimento de funções efetoras ou estabelecimento de uma população de células de memória contra tumores malignos. Um terceiro sinal foi necessário para haver uma resposta efetora e conseqüente transição para uma população de células de memória. Este terceiro sinal parece ser fornecido por IL-12 ou IFN-γ. Em tumores avançados a falta do terceiro sinal é mais perceptível, pois parece que no inicio do crescimento tumoral as células T podem proliferar independentemente do terceiro sinal, contudo não sustentam a produção de citocinas anti-tumorais, permitindo o avanço da doença (Mescher, Agarwal *et al.*, 2007). Alem da ativação *in situ* das DCs para combater o câncer (Furumoto, Soares *et al.*, 2004), a adequada manipulação *ex vivo* tem originado promissoras estratégias de vacina, em que vários aspectos estão sendo considerados para melhorar a imunoterapia tumoral. Estes incluem eficientes modos de maturação das DCs (Kotera, Shimizu *et al.*, 2001), apropriadas rotas de administração da vacina (Fong, Brockstedt *et al.*, 2001), modificações genéticas nas DCs que promovam melhor ativação das células T (Kirk, Hartigan-O'connor *et al.*, 2001), e associação com outras técnicas terapêuticas contra o câncer (Tong, Song *et al.*, 2001).

A geração de células T de memória contra antígenos tumorais é crucial no desenvolvimento da imunoterapia do câncer, principalmente nos tumores fracamente imunogênicos que geralmente não fornecem sinais suficientes para que o sistema imune estabeleça uma proteção a longo prazo. Para isso, há estudos que procuram estimular adequadamente as células de defesa de forma que se tornem capazes de conter possíveis recidivas e metástases. A transferência adotiva de células T tem gerado resultados experimentais positivos nos estudos de combate ao crescimento tumoral e estabelecimento de memória antitumoral (Bathe, Dalyot-Herman *et al.*, 2001). No modelo de tumor B16F10 foi

visto que a depleção de células T reguladoras durante o crescimento primário do tumor propiciou a geração de células de memória T CD8+, sugerindo uma regulação negativa da resposta antitumoral pelas células CD4+CD25+ (Zhang, Cote *et al.*, 2007). As citocinas IFN-α/β e IL-12 podem ser importantes no desenvolvimento de células T de memória porque fornecem o terceiro sinal ativando as células T CD8+ e induzindo fenótipo de memória (Mescher, Agarwal *et al.*, 2007). Células T CD8+ do baço de camundongos OT-I estimuladas com o peptídeo OVA na presença de IL-2 apresentaram fenótipo de memória setenta e cinco dias após transferência adotiva. Em conjunto esses resultados sugerem que a resposta e a memória antitumorais podem ser dependentes das citocinas usadas para gerar as células T citotóxicas (Bathe, Dalyot-Herman *et al.*, 2001; Mescher, Agarwal *et al.*, 2007).

Embora a resposta antitumoral de células T CD8+ tenha sido bastante estudada, pouco se sabe sobre a resposta de células T CD4+, principalmente no que se refere a resposta de memória. Isso surpreende dada a relevância das células T CD4+ durante a ativação, diferenciação e manutenção das células T citotóxicas durante a fase efetora da resposta antitumoral (Hung, Hayashi *et al.*, 1998). No estudo feito por Golszdmid, foi observado efetiva resposta anti-tumoral de células T CD4+ e T CD8+ em camundongos desafiados com tumor B16F10 e imunizados com DCs incubadas com células tumorais apoptóticas. Uma resposta de memória eficaz também foi observada: os animais não desenvolveram tumores quando novamente desafiados com o tumor B16F10 dez semanas depois da imunização inicial. Nesse mesmo trabalho foi relatada uma interdependência entre resposta T CD4+ e T CD8+ pois quando uma dessas populações celulares foi depletada no decorrer do desafio tumoral a reposta foi totalmente perdida mostrando a importância das células T CD4+ na ativação das células T CD8+ (Goldszmid, Idoyaga *et al.*, 2003). Em relação à importância das células T CD4+ na formação de imunidade a longo prazo, observou-se que a imunização na ausência de células T CD4+ resultou em células T CD8+ de memória que perderam a sua

funcionalidade e diminuiram em número ao longo do tempo. Com isso, parece que a interdependência das células T CD4+ e T CD8+ também é importante na formação de uma memória imunológica eficiente (Sun, Williams *et al.*, 2004).

O processamento e a apresentação do antígeno tumoral para as células T no linfonodo dependem da ativação das DCs e também de como o antígeno tumoral é fornecido a elas (Brusa, Garetto *et al.*, 2008; Melief, 2008). Com o objetivo de facilitar o acesso e fornecer de forma mais eficiente os antígenos tumorais às DCs os pesquisadores têm tentado incubações com células tumorais mortas por apoptose (Tobiasova, Pospisilova *et al.*, 2007) e necrose (Basu, Binder *et al.*, 2000; Goldszmid, Idoyaga *et al.*, 2003). Células em processo de morte por necrose e/ou apoptose liberam moléculas que podem ser interpretadas como sinais de perigo pelo sistema imune (Gallucci e Matzinger, 2001). Assim, funcionando como sinais co-estimulatórios, induzem uma adequada maturação das DCs que adquirem, processam e apresentam os antígenos `as células T nos LN (Gallucci, Lolkema *et al.*, 1999). A explicação provável para a melhora na resposta é que o sistema imune responderia melhor a substâncias que possam sugerir dano do que simplesmente a um antígeno estranho ao organismo. Matzinger et al. (2001) e (Goldman, Park *et al.*, 2005) propuseram que o sistema imunológico diferencia antígenos que representam perigo de antígenos inócuos, por isso os sinais de perigo liberados por células sob estresse, células danificadas ou em processo de morte funcionariam como adjuvantes endógenos favorecendo a adequada maturação das DCs e eficiente apresentação do antígeno às células T. Os adjuvantes endógenos CD40-L, TNF-α (Caux, Massacrier *et al.*, 1994) e IL-1β (Zepter, Haffner *et al.*, 1997) foram os primeiros as serem caracterizados e facilitam a comunicação entre as células T e DCs (Gallucci e Matzinger, 2001). A resposta imune também pode ser estimulada por adjuvantes exógenos; o mais estudado e′ o LPS que pode ativar as DCs (Murshid, Gong *et al.*, 2008) via ligação com o TLR-4 (Aderem e Ulevitch, 2000) e o TLR-2 (Darveau, Pham *et al.*, 2004), conectando a

resposta imune inata com a resposta adaptativa contra um antígeno específico. Além disso, com a morte tumoral por necrose e apoptose pode haver a liberação de maior quantidade de proteínas do choque térmico (HSPs), que formam complexos com os peptídeos tumorais facilitando sua aquisição pelas DCs e favorecendo a maturação das mesmas, através da ligação aos receptores tipo "scavengers". Esse processo poderá induzir a apresentação cruzada do antígeno tumoral (cross-priming), levando a uma melhora na resposta antitumoral (Delneste, 2004; Murshid, Gong *et al.*, 2008). Esses estudos se devem ao fato de que já se sabe que imunização somente com o antígeno integro poderá não ser suficiente para estimular o sistema imune e ainda causar tolerância (Rock, Hearn *et al.*, 2005).

1.2 Hipótese

O crescimento tumoral está relacionado a um processo de supressão da resposta imune e a imunoterapia do câncer visa a reversão desta imunossusupressão. A facilitação do acesso pelas células dendríticas aos antígenos tumorais bem como a maturação das mesmas pode tornar mais eficiente a apresentação de antígenos aos linfócitos T nos linfonodos e isso resulta em resposta T antitumoral mais eficaz.

1.3 Objetivos

1.3.1 Objetivo geral

Analisar o crescimento de um tumor maligno usando o modelo de melanoma murino B16F10, e resposta imune *in situ* e nos linfonodos drenantes, identificando mecanismos que possam ajudar no controle do crescimento tumoral e geração de resposta de células T de memória contra o tumor

1.3.2 Objetivos específicos.

1) Caracterizar o crescimento do tumor B16F10 *in vivo* associando com a análise histológica *in situ* e citometria de fluxo para marcadores de proliferação celular e de memória nas células dos linfonodos drenantes.
2) Verificar a importância dos epítopos de células T CD8+ na resposta imune ao tumor B16F10.
3) Determinar o efeito da ativação das DCs por LPS avaliando o crescimento tumoral, expressão de fenótipo de memória e de moléculas co-estimuladoras.

4) Criar um sistema experimental capaz de avaliar a resposta antitumoral de células de T CD4+ onde se possa ver ao mesmo tempo apresentação de antígeno, ativação de células dendriticas e proliferação de células T.
5) Verificar se os diferentes sinais de morte celular, necrose e apoptose, facilitam o acesso pelas células dendríticas aos antígenos tumorais.
6) Caracterizar a geração in vivo de memória T CD4+ anti-tumoral.

1.4 Aspectos Éticos

Este projeto foi aprovado pelo Comitê Científico da Faculdade de Biociências e ao Comitê de Ética em Pesquisa – processo número 04/02312

Capitulo 2

2.1 Artigo cientifico 1

ACTIVATION OF INFILTRATING DENDRITIC CELLS AFTER TUMOR ESTABLISHMENT, BUT NOT INCREASE IN T CELL PRECURSOR FREQUENCY IS CRITICAL FOR TUMOR ELIMINATION

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ACTIVATION OF INFILTRATING DENDRITIC CELLS AFTER TUMOR ESTABLISHMENT, BUT NOT INCREASE IN T CELL PRECURSOR FREQUENCY IS CRITICAL FOR TUMOR ELIMINATION

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Running title: Activation of Infiltrating DC and anti-tumor response

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Abstract

The immune system has an important role in cancer prevention, however, it is not always able to prevent malignant tumor growth, as a consequence of immune tolerization induced by tumor cells. We compared the relevance of different immune mechanisms in tumor control, using B16F10 and B16OVA melanomas, and found that tumor growth is inversely correlated with peritumoral infiltrate cell number. We also found a decreasing inflammatory cell number in draining lymph nodes (LNs), suggesting an interruption of cell traffic from the tumor site to the draining LN. Adoptive transfer of naïve CD8+ OT-I cells in C57Bl/6 mice injected with B16OVA was able to arrest tumor growth, but that was not sufficient to block it completely. Tumor growth was blocked only when dendritic cells (DCs) were activated by intra-tumoral LPS injection, reestablishing cell traffic to the LN. Pre-activation of skin dendritic cells or co-delivery of tumor cells and LPS did not have any effect on tumor progression. Our results suggest that increased precursor frequency helps tumor control; however, an efficient anti-tumor immune response can be only achieved with proper DC activation.

Introduction

The immune system has an important role in cancer prevention. This can be corroborated by the low tumor incidence in immunocompetent hosts, in which malignant cells could be eliminated before they are clinically apparent[1]. Also, tumor cells can be recognized by the immune system through overexpression of tumor associated antigens (TAA) and tumor specific antigens (TSA). Infiltration of immune effector cells occurs early in the course of tumor growth, and in some cases cell infiltration correlates with disease outcomes[2], either indicating a good[3] or poor[4] prognosis.

The process of tumor elimination includes both innate and adaptive immune responses against malignant cells. Activated NK cells play a central role in immunesurveilance against newly arising malignancy[5]. Tumor cell death by the innate immune system can release tumor antigens (TAs), which elicit adaptive immune responses. DCs phagocytose and process tumor antigen, migrating to lymphoid organs where they present tumor antigen to naive T cells[6, 7] initiating anti-tumor responses.

However, the immune system is not always able to avoid tumor growth. The probable lack of stimulatory signals provided by tumor cells, as opposed to microbial ones, does not favor DCs[8], necessary to initiate a T cell response. Consequently, it can be hypothesized that the activation of effector anti-tumor T lymphocytes is not a frequent event. When it does occur, tumors can edit their antigens, thus avoiding such established anti-tumor adaptive responses[9 10]. Finally, tumors have been shown to create an immunosuppressive environment[11] sometimes favoring the differentiation of regulatory T cells (T reg) [12], which in turn can suppress anti-tumor lymphocyte activity[2, 12, 13]

In this study we asked which immune mechanisms are more relevant to the control of tumor growth using the B16F10 melanoma. To analyze whether or not tumor antigenicity is relevant for immune control of tumor growth, we performed naive CD8+ OT-1

cell transfer in C57Bl/6 mice injected with B16FOVA tumor cells. To determine if inflammatory signals can rescue DCs activation against tumor cells to a level sufficient to result in tumor rejection, we analyzed tumor growth in an environment in which DCs were activated by LPS at different timepoints. Our results suggest that while an increased precursor frequency can help contain tumor growth, DCs activation is relevant for tumor elimination, but only in a specific window of time.

Methods

Mice and tumor cell lines

Six-to eight-week-old C57BL/6 (H-2b) mice were purchased from Fundação Estadual de Produçao e Pesquisa em Saúde (FEPPS), Porto Alegre, RS Brazil. OT-1 TCR transgenic mice encoding a TCR specific for the OVA epitope (SIINFEKL-H2K^b) were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained in specific pathogen-free conditions at 22°C under controlled light (12h light/12h darkness), and allowed free access to water and food. B16F10 melanoma cells, a kind gift from Dr. Peter Henson (National Jewish Center for Immunology, Denver, CO) were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), Gentamicin 80mg/l, (Novafarma), Fungisone, 5mg/l, (Bristol Myers Squibb), 55 μM 2-Mercaptoethanol (Sigma), 2mM L-Glutamine (Sigma) and 1X MEM amino acid solution (Gibco). The OVA – expressing melanoma cell line (B16OVA from Dr. Archie Bouwer, V.A medical center, Portalnd, OR) was maintained in DMEM plus 10% FCS and 100μg/ml G-418. The current study was approved by the Ethics Committee of the Pontifícia Universidade Católica do Rio Grande do Sul, Brazil. (PUCRS). Experimental procedures followed the recommendations for Animal Care and Use by the Ethics Committee (process number 04/02312). Some experiments were performed at PUCRS, Brazil and some at the Veteran Affairs Medical Center, Portland, OR, USA.

Tumor injections and OT-1 cell transfer

Naive C57BL/6 mice were inoculated subcutaneously with 5X10^5 B16F10 melanoma cells in the right thigh, in 100μl of PBS, and 100μl of PBS at the left thigh as a control. For all animal experiments, tumor size was measured every 3 days. Mice were killed in a CO_2 chamber at days 3, 6, 9 and 12 after tumor injection in order to harvest the draining inguinal LNs. For the

adoptive transfer experiments, $CD8^+$ OT-1 cells were obtained from spleen of OT-I mice and purified by negative selection with EasyStep Magnet Kit (StemCell Technologies, Vancouver BC Canada). Twenty-four hours before tumor injection, $2x10^4$ $CD8^+$ OT-1 cells were transferred i.v. into two mice groups of C57Bl/6 mice, and mice received either $5x10^5$ B16FOVA cells subcutaneously on both thighs, or $5x10^5$ B16F10 cells. To test the importance of LPS activation of DCs, mice did not receive any cells, but were divided in five groups: a control with PBS injection; only B16F10 cells; co-injection of B16F10 cells with 500 ng LPS; e-coli LPS (500 ng) injection at the tumor site 18 hours before tumor injection; and intra-tumoral LPS (500ng) injection 5 days after tumor injection.

Histology

Tumor samples were either snap-frozen in Tissue- Tek OCT (Sakura Finetechnical Co. Ltd., Tokyo, Japan) or fixed in formaldehyde 10% for 24 hours and embedded in paraffin wax. Paraffin embedded tissue blocks and frozen samples were cut semi-serially, 7 µm-thick slides at 200-500µm intervals. Standard procedure for haematoxylin and eosin (H/E) staining was done in paraffined blocks and immunofluorescence staining in frozen samples. For immunostaining, the commercially available signal amplification technique that is effective for amplifying the fluorescent signal (TSA, NEN Life Sciences Products Inc., Boston, MA) was used. Briefly, slides were fixed in acetone, rehydrated with PBS, and the endogenous peroxidase was blocked for 10 minutes with 3% H_2O_2 + 0.1 % azide in PBS. Fc receptors were blocked with 5% sheep serum in PBS 1X for 15 minutes. Avidin/biotin blocking was done using the respective buffers provided by TSA Kit, 10 minutes each. At this time, the sections were incubated with biotinylated anti-CD4, anti-CD8, anti-CD11c (Becton Dickinson, Hyalea FL, USA) diluted in TNB buffer provided in the TSA Kit during 30 minutes. Streptavidin, also from TSA Kit, was incubated for 30 minutes. To amplify the

signal, CY3 was added for 10 minutes. Nuclear staining was done with Hoescht 33342 (Invitrogen Corporation, Carlsbad CA, USA), for 2 minutes and the slides were mounted with Vectashield (Vector Laboratories Burlingame CA, USA). Between each step above the sections were rinsed 4 times with PBS 1X.

Quantification of tumor area and cell subsets

H/E and immunofluorescence sections were analyzed at 400 x magnification, and tumor area and peritumoral infiltrate were quantified, capturing 8 fields equidistantly surrounding the whole tumor section. H/E sections were photographed with a Sony CCD-Iris camera, connected to an Olympus BX50 microscope. Infiltrating lymphocytes were identified by morphology according to an experimented pathologist and all of them were counted in each analyzed microscopic field. The software used to capture and count the cells was the Image Pro-plus version 4.1.5 (Mediacybernetics), which uses a video camera connected to a computer card to capture the images of the selected microscopic field. Structures were selected on the computer screen with the mouse pointer and then counted manually. Immunofluorescence sections were captured by ZEISS – Axioskop 40 microscopic equipped with a CoolSNAP-PROef color camera coupled to Image Pro-Plus software. Anti-CD4, anti-CD8 and anti-CD11C (Bekton Dickinson, Hyalea FL, USA), positive cells were counted in 8 fields as described above, and photographs had the number of red pixels quantified in each section, using the color range Adobe Photoshop tool.

Lymph node analysis by Flow Cytometry

Cell suspensions were prepared from draining LN by mechanical disruption on 70μm nylon cell strainers and incubated with collagenase D (Roche, Basel-Switzerland). Fc receptors were blocked (24G2 cell supernatant supplemented with 5% mouse serum and 5% rat serum)

for 15 minutes and cells were stained for 30 minutes on ice with antibodies anti-CD4 PE, anti-CD8PacBl, anti-CD11c APCCy7, anti-CD86 FITC and anti-B220 PE-Cy5.5, in 100µl of phosphate-buffered saline (PBS) containing 1% fetal bovine serum (FBS), 0,1 % sodium azide. Alternatively, LN cells were stained with anti-CD62L FITC, anti-CD8 PE, anti-CD44 Cychrome, anti-CD11C FITC, anti-CD86PE and B220PE-Cy5.5. All the data were collected on FACScan or LSR II (BD Biosciences, San Diego, CA) and analyzed with FlowJo software (TreeStar, San Carlos, CA).

Statistical Analysis

The ANOVA test was used to compare differences between groups with Bonferroni posttest. Statistical analysis and graphs were performed using GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA). Differences with $p < 0.05$ were considered statistically significant.

Results

Tumor growth inversely correlates with infiltrate

Analysis of tumor histology showed that the subcutaneously injected B16F10 cells formed a considerable tumor mass, presenting a clear borderline between tumor and surrounding connective tissue (Figure 1 A paraffin section and 1C frozen section), which was absent in the control thigh (1B). Tumor mass started to grow faster on day 6 after injection (Figure 1H). This growth was inversely correlated with peritumoral infiltrate (1G). Analysis of cells in the peritumoral infiltrate revealed that the main infiltrating cells were DCs (1E, I), followed by CD4+ cells (Figure 1, F, I). Very few of the infiltrating cells were CD8+ (1 D, I), and most of the CD4+ and CD8+ infiltrating cells concentrated in the periphery of the tumor, while DCs were found surrounding the tumor mass, but also within it. These results suggested that the tumor was not being infiltrated by the major tumor eliminating cells, the cytotoxic CD8+ T cells. Also, the infiltrating antigen presenting cells were not in contact with the CD8+ and CD4+ T cells. Together with the continuous decrease of tumor infiltrating cells, the results pointed to an inefficient immune response at the tumor site.

Tumor growth leads to interruption of cell traffic to the lymph node and prevents activation of DC

To further investigate the apparently inefficient response at the tumor site we analyzed cell subpopulations at the tumor draining LN. The results shown in Figure 2 revealed that the frequency of all investigated cell types decreased in the lymph nodes as tumor grew (Figure 1A-E), suggesting a general cell traffic interruption between tumor site and draining LN. A continuous decrease in CD86 expression by LN DCs was observed that coincided with tumor growth (Figure 1F). This suggested not only migration of cells between tumor and LN was

being halted, but also that activation of DCs was being prevented. Such alterations were not observed in the control LN, where PBS had been injected at the corresponding site (Fig. 2, A-F). Altogether, the results indicated a specific spoliating activity of the tumor over the immune system in order to grow.

The lack of immunogenicity of B16F10 tumor is not solely due to lack of T cell epitopes.

The inefficient antitumor immune response observed has been attributed by others to the low immunogenicity of the B16F10 melanoma[14]. Indeed, due to the self nature of most tumor antigens, individuals would be expected to be centrally tolerized against them[11]. In order to test whether or not tumor elimination could be achieved by an increase in the precursor frequency of antitumor cells, we adoptively transferred $2x10^4$ OVA-specific OT-I cells into C57BL6 mice. One day later, mice were subcutaneously injected with either B16F10 cells or B16OVA cells. Tumor growth was recorded every other day, and OT-I cells analyzed for acquisition of memory markers. The results are shown in Figure 3. As expected, the B16F10 tumor grew in spite of the OT-I cells (Figure 3A). The B16OVA tumor was clearly slowed down by the naïve OT-I, however it resumed growth at the same time OT-I cells contracted in the LN – day 9 (3A, B). Analysis of memory markers in OT-I cells revealed that CD44 and CD62L were upregulated in these cells, suggesting their differentiation into a memory phenotype (Figure 3C). However, analysis of CD86 expression on CD11c cells revealed no increase throughout time, indicating that little costimulation was probably delivered to these T cells during antigen presentation. Taken together the results suggested that although increase of precursor frequency could delay tumor growth and lead to memory cell formation, these cells were not capable of stopping tumor growth, probably due to lack of costimulatory signals by the poorly activated DC.

Activation of infiltrating DC can lead to tumor rejection

Because our previous experiments indicated that tumor-specific memory cells generated in the absence of costimulation were not able to prevent tumor growth, we asked if activation of DC through a TLR could provide enough signals to reverse this scenario. Different studies have shown that TLR activation of DC is a powerful stimulus to achieve anti-tumor T cell activation[15] or reversal of anti-tumor T cell tolerance[16]. However, because we had observed that the established tumor was heavily infiltrated by CD11c+ cells, that seemed unable neither to migrate to LN nor to contact the infiltrating T cells, we targeted the DC near the tumor instead of using systemic approaches like most previous studies. We questioned if it would be more effective to activate the DC before tumor was present, at the same time tumor was injected, or after tumor was established. We thus injected B16F10 cells and provided LPS either along with the tumor (in the same injection), 18h before the tumor (which we previously determined to cause DC to be activated and migrate to LN with an upregulation of CD86 – not shown), or intratumorally, on day 5 after tumor injection and when a tumor mass was clearly established similar to what is histologically demonstrated in Figure 1. To our surprise, the only treatment that resulted in interruption of tumor growth was the intratumoral injection of LPS on day five after tumor injection (Figure 4A) – it lead to complete elimination of the tumor. Neither pre-activation of DC nor activation of DC at the time of tumor injection were sufficient to control tumor expansion – there was no difference between these two treatments and the PBS injection control. Interestingly, intratumoral LPS injection on day 5 was also the only treatment able to restore traffic of cells to the draining LN (4B). CD86 expression in CD11c was only slightly elevated in this treatment compared to the others, suggesting that is not the only explanation for the response observed. Also, CD44 was upregulated on CD8+T cells, indicating differentiation into memory cells, but that was also not different from the other treatments.

Discussion

Our data suggest that the B16F10 tumor must actively suppress the immune response in order to start growing, since its growth kinetics reproductibly correlates with the reduction of intratumoral infiltrate and cellularity of the draining LN. The initial immune response leading to infiltration is gradually suppressed locally, since infiltrating antigen presenting cells (APC) and T cells accumulate in different areas inside the tumor and in the draining LN that no longer receives cells migrating from the tumor site. These results are in agreement with different reports on immunossupression exerted by tumors on the host´s immune response[11, 17, 18]. Various mechanisms have been proposed that account for this phenomenon, such as loss of MHC class I expression by tumor cells that contributes to the ability of cancer to avoid recognition by T cell-mediated immunnity[19]. Secretion of immunossupressive cytokines can convert CD4+CD25- T cells into T(Reg) cells[12] and also may modulate immune response by inhibiting production of other cytokines involved in cell-mediated responses[17]. Expression of Fas ligand, an important mediator of T-cell cytotoxicity involved in the evasion of human carcinoma cells from immune destruction[20, 21], and B7-H1 molecules expressed by the majority of human and rodent cancer cells acting as a ligand for the receptor programmed death-1 (PD-1) to deliver an inhibitory signal to T cells, including apoptosis, anergy, exhaustion or unresponsivenes[21-23]. We found that only a small percentage of the abundant CD4+ T cells infiltrating the tumor were Foxp3+ (not shown) suggesting that the tumor employs more than one immunossupressive strategy in order to grow. Alterations in cell traffic have also been reported in different models[24], Ishida et al. (1998) observed that Langerhans cells lost the ability to migrate to the draining LN in the presence of malignant tumors[25]. Similarly, they found that DC from these draining LN downregulated the expression of CD86. Dendritic cells have been reported to be a major subpopulation infiltrating malignant tumors which sometimes present a regulatory phenotype[26] secreting

immunossupressive cytokines such as IL-10 and TGF-β[27]. Although alterations in traffic and maturation of dendritic cells appear to be crucial to prevent tumor rejection by the immune system, modifications in chemokine expression inside the tumor can prevent CD8+ T cells from efficiently infiltrating the tumor and exerting cytotoxic activity. As an example, intratumoral injection of MIP3-1alfa chemokine has been reported to return mobility to CD8+T effector cells that were then able to lyse tumor cells[28].

Increasing the precursor frequency of naïve OT-I CD8+T cells did slow down tumor growth, but it was not sufficient to completely prevent it. Although OT-I cells did differentiate into a memory phenotype in the draining LN, showing the expected expansion and contraction, tumor continued to grow, probably because no costimulatory signals were delivered by the DC in the draining LN (Figure 2, F and 3, B), rendering the anti-tumor T cells tolerant. Other studies[29, 30, 31] have not verified any impact of naïve OT-I cells transfer on B16OVA growth. That is probably related to the number of cells injected. Adoptive transfers of high numbers of CD8+ T cells leads to a poor proliferation and differentiation when compared to low transfer numbers[32]. By transferring only 2 x 10^4 OT-I cells, we might have favored the anti-tumor immune response.

Interestingly, tolerance could only be reversed by intratumoral LPS injection, activating the infiltrating cells that resumed traffic to the draining LN. Pre-activation of DC or co-immunization with tumor and LPS were not sufficient to alter tumor development, suggesting that the suppressive signals provided by the tumor can overwhelm the initial activating signals delivered through TLRs. Yang et al. (2004)[33] had previously observed that persistent TLR signals were required to reverse anti-tumor tolerance, however in that study, as well as in others[34] LPS was delivered systemically[35] or intraperitoneally [36]. In our system, one intratumoral injection, after the tumor was established, was able to result in tumor

rejection, suggesting that the local suppression on tumor infiltrating DC can be reversed, causing these cells to migrate to the DLN and efficiently stimulate anti-tumor T cells. Infiltrating DC have been reported to be able to induce anti-tumor T cell responses when stimulated *ex vivo* with the antigenic peptide[37], rapidly acquiring a mature phenotype just by manipulation. Although we did not observe any remarkable differences in CD86 expression by the DC that resumed migration to the draining LN, LPS injection is likely to have activated other costimulatory molecules[38, 39], resulting in a response able to achieve tumor elimination.

Based on the findings herein presented, we propose that an efficient anti-tumor response can be generated even prior to tumor surgery if infiltrating DC from the tumor can be activated and mobilized to migrate to the draining LN, re-initiating an anti-tumoral T cell response. Tumors that are heavily infiltrated with DC can possibly offer a good prognosis for the patients, favoring immunotherapy that would generate anti-tumor memory, aiding in the prevention of cancer recurrence.

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Figures

Figure 1

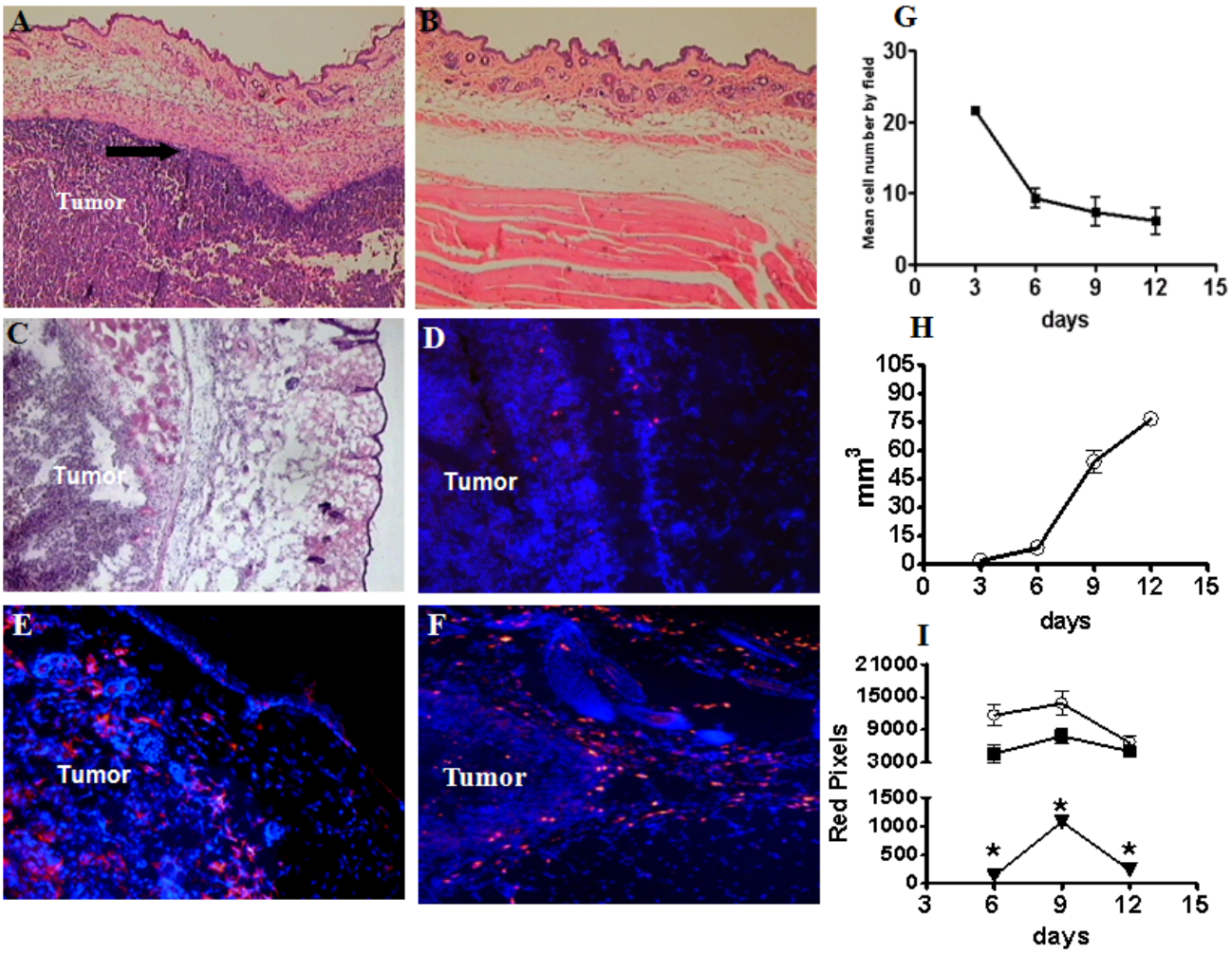
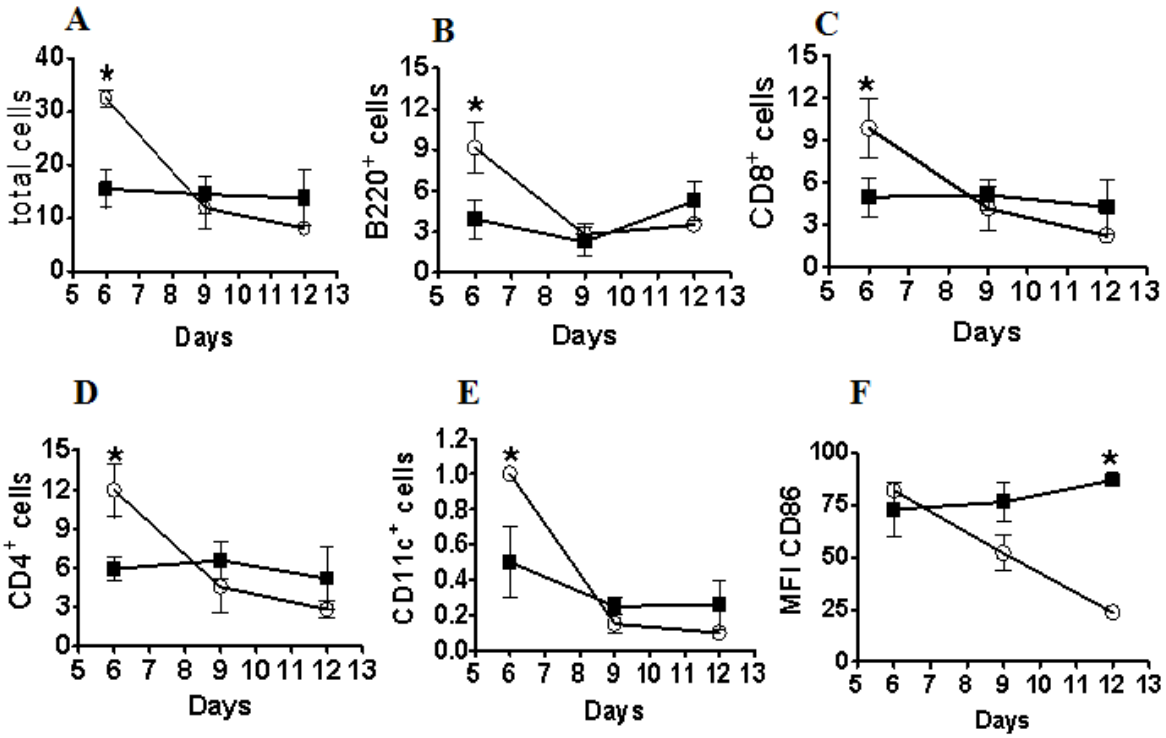


Figure 2


A
B
C
D
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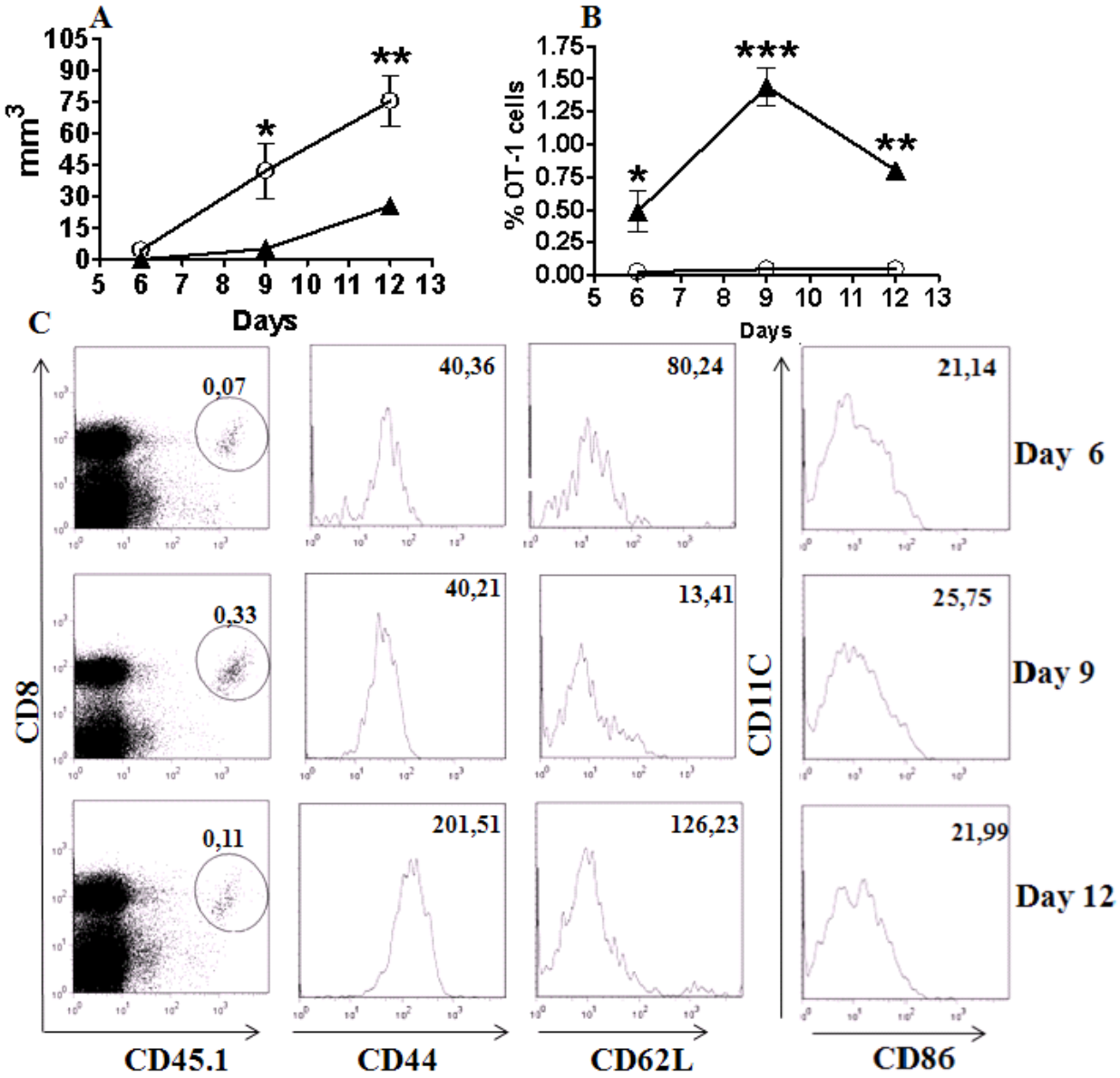
F
total cells
B220+ cells
CD8+ cells
CD4+ cells
CD11c+ cells
MFI CD86
Days
*

Figure 3


A
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Days
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C
CD8
CD45.1
CD44
CD62L
CD11C
CD86
0,07
40,36
80,24
21,14
Day 6
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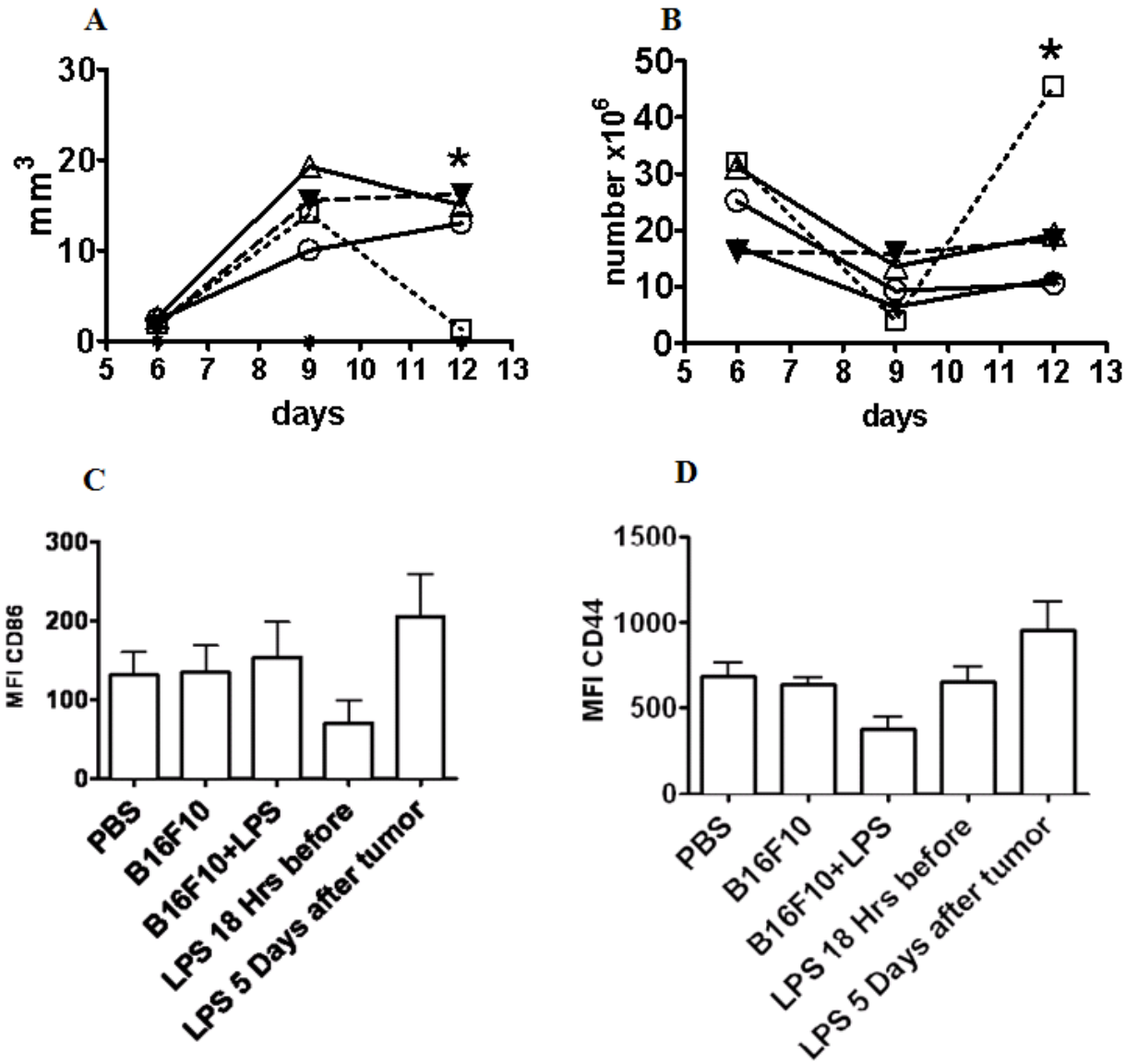
40,21
13,41
25,75
Day 9
0,11
201,51
126,23
21,99
Day 12

Figure 4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B16F10
B16F10+LPS
LPS 18 Hrs before
LPS 5 Days after tumor

Figure Legends

Figure 1. Histology and immunofluorescence analysis of peritumoral infiltrate in subcutaneously injected B16F10 tumor. **(A)** H/E paraffin skin section of B16F10, day 9 (x100). The arrow indicates the limits between tumor and peritumoral infiltrate. **(B)** H/E section of PBS injection site, day 9 (x100), evidencing normal tissue aspect without tumor. **(C)** H/E frozen section of tumor injected, base to immunofluorescence analysis. **(D, E, F)** Immunofluorescence (Red) staining of tumor frozen sections with anti-CD8 (D), anti-CD11c (E), and anti-CD4 (F), counterstained with Hoechst, blue. (G) Average number of lymphocytes per analyzed field, as described in the Methods section. (H) Tumor size (mm^3) change after injection of B16F10 cells. (I) Average numbers of Red Pixels showing CD8 ( ), CD11C ( ) and CD4 ( ) cells at the tumor site as depicted by immunofluorescence. ($*$ $P < 0,05$).

Figure 2. Flow cytometry analysis of draining lymph nodes harvested 6, 9 and 12 days after B16F10 and PBS injection. Single cell suspension of LN cells was labeled with anti-B220, anti-CD8, anti-CD4, anti-CD11c and anti-CD86. (A) Total lymph node cell numbers. (B-E) Percentages of positive cells for each marker were used to calculate absolute numbers for each subpopulation (shown as a mean x 10^5 cells). (F) Mean CD86 fluorescence in CD11c+ cells. All samples in tumor ( ) and PBS lymph node ( ). ($*$ $P < 0,05$)

Figure 3. Tumor growth and lymph node flow cytometry analysis by the time. (A) Tumor measurements in OT-1 injected mice, with ( ) B16F10 tumor and ( ) B16OVA tumor. **(B)** % cells recovered from LN of OT-1 transferred mice and injected with B16OVA tumor () and B16F10 tumor (). **(C)** Expression of CD44 and CD62L in OT-1 cells recovered from LN in different time points (first three columns) and CD86 in $CD11c^+$ cells (fourth column). Numbers on the upper right corner of histograms represent mean fluorescence (n= 3) for each marker. ($*$ $P < 0,05$)

Figure 4. Effect of intratumoral LPS injection. (A) Tumor size and (B) total LN cells in mice that received subcutaneous B16F10 injection (), LPS at the time of B16F10 injection (); Intratumoral LPS 18hours after B16F10 injection(); Intratumoral LPS five days after B16F10 injection( ) and PBS injection (); (C) CD86 MFI in $CD11c^+$ cells and (D) CD44 MFI in $CD8^+$ cell, day 6 after tumor injection. ($*$ $P < 0,05$).

Capitulo 3

3.1 Artigo cientifico 2

PROGRESSIVE DECREASE OF ANTIGEN PRESENTATION BY DENDRITIC CELLS RESULTS IN ARREST OF TUMOR SPECIFIC CD4+ T CELLS EXPANSION AND TOLERIZATION OF THE ANTI-TUMOR IMMUNE RESPONSE

SERÁ SUBMETIDO AO PERIODICO: THE JOURNAL OF IMMUNOLOGY

PROGRESSIVE DECREASE OF ANTIGEN PRESENTATION BY DENDRITIC CELLS RESULTS IN ARREST OF TUMOR SPECIFIC CD4+ T CELLS EXPANSION AND TOLERIZATION OF THE ANTI-TUMOR IMMUNE RESPONSE

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Running title: Activation of infiltrating DC and anti-tumor response

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Keywords: tumor; dendritic cells; lymph node, B16F10

Abstract

Cooperation between CD8+ and CD4+ T cells is essential in the mobilization of adaptive immune responses against tumors. Tumors do not harbor the exogenous ligands that lead to the activation of antigen presenting cells (APC). Consequently, anti-tumor CD8+ T cells probably rely on the signals provided by CD4+ T cells that can license APC to perform the effective activation necessary for cytotoxic responses. Little is known about the generation of anti-tumor CD4+ T cells *in vivo*. To study that we developed a tumor cell line that constitutively expresses the Ea peptide conjugated to a red fluorescent protein. This system allowed us to trace tumor antigen presentation using the YAe antibody that recognizes the MHC class II- peptide complex, and CD4+ T cell activation, using adoptive transfer of TEa transgenic cells. Our results show that the tumor antigen is presented in class II molecules in the draining lymph node (DLN), and T cells are activated and proliferate. However, analysis of the kinetics of presentation and proliferation suggest that after some time the antigen availability in the lymph node wanes, probably by prevention of antigen bearing dendritic cells to reach the lymph node. As a consequence, not all the tumor specific T cells proliferate, and few of them complete the development into an effector phenotype, as INF-γ producing cells, resulting in tumor growth. Taken together our results indicate that prevention of antigen presentation in the DLN is a major mechanism of tumor specific CD4+ T cell tolerization.

Introduction

Protective immunity requires both MHC class I - restrict T cytotoxic CD8+ cells and MHC class II - restrict T CD4+ cells (1). Although tumor-specific CD8+ T cell responses have been studied in animal models using the OT-I/OVA tumor system(2-5) and in patients(6, 7), limited information is available on the *in vivo* development of memory T CD4+ tumor cells(1). This could be explained by the small number of MHC class II - restricted epitopes identified and, for a long time, by the inability to trace the low number of T CD4+ cells specific for a given antigen(8), recently turned possible with the use of CD4+ T cell tetramer ligands(9).

CD4+ T cells are especially important in immune responses to tumors, due to the lack of inflammatory signals provided by tumors to dendritic cells (DC). In this case, efficient priming of CD8+T cells depends on the licensing of antigen presenting cells (APC) by CD4+ T cells(10, 11). However, tumors exert immunossupressive effects on the immune system in order to grow(12-17), and CD4+ T cell responses are probably a major target of this immunossupression. To investigate the *in vivo* generation of anti-tumor CD4+ T cell memory, as well as to identify mechanisms of tumor-CD4+ T cell interactions, we adapted the experimental system used by Itano et al. (2003) (18). We developed a B16F10 melanoma line that constitutively expresses the EaRFP fusion protein (B16EaRed), a method which allowed us to trace *in vivo* tumor antigen presentation, as well as the kinetics of tumor-specific CD4+ T cell responses without delivery of any exogenous adjuvant. Our results show that class II-restricted tumor antigens are presented in the draining lymph node (DLN), peaking 9 days after tumor injection, but that rapidly declines and is practically absent on day 15, correlating with specific CD4+ T cell expansion and differentiation into a memory phenotype (CD44+). However, these cells produced little or no INF-γ. The B16EaRed tumor grew at a similar rate to the B16Red tumor (not containing the Ea peptide), suggesting that tumor-specific CD4+ T

cells are majorly affected through inefficient antigen presentation. Delivery of apoptotic or necrotic B16EaRed cells alone allowed more efficient tumor antigen presentation by DCs at lymph nodes as well as T CD4+ expansion, however that decreased at day 15 too. CD4+ T cells presented the same phenotype as the one differentiated in the presence of the live tumor cells, suggesting that endogenous death signals are probably not able to affect that scenario.

Methods

Antigen and Tumor cell lines

EaRFP antigen is a fusion protein containing residues 52-68 of the Ea subunit of the I-E MHC molecule (18) cloned in a pDsRed1 plasmid – from Dr. Marc Jenkins (University of Minnesota, Mn). The Ea sequence was amplified with specific primers combining sites for EcoRI (GAATTC) and BamHI (GGATCC). The amplified fragment was subcloned into an eukaryotic expression vector (pDsRed monomer-C1, Clontech) under the control of a CMV promoter. This construct was later transfected with lipofectamine 2000 (Invitrogen, Carlsbad, CA.), into the B16F10 melanoma. Some tumor cells were transfected the pDsRed monomer vector, without the Ea sequence. Transfectants were selected and maintained with G418 and two stable lines were obtained, B16EaRed, expressing the fusion protein (Fig1-A, B), and B16Red, which constitutively expressed RFP as assessed by fluorescence microscopy. The peptide-MHC II complex derived from processing of the Ea antigen was visualized using the YAe antibody (19), and specific T cell proliferation was assessed by transferring transgenic TEa (20) cells to C57BL/6 host mice. All melanoma cell lines were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), Gentamicin 80mg/l, (Novafarma), Fungisone, 5mg/l, (Bristol Myers Squibb), 55 μM 2-Mercaptoethanol (Sigma), 2mM L-Glutamine (Sigma) and 1X MEM amino acid solution (Gibco). The current study was approved by the Ethics Committee of the Pontifícia Universidade Católica do Rio Grande do Sul, Brazil. (PUCRS). Experimental procedures followed the recommendations for Animal Care and Use by the Ethics Committee. Some experiments were performed at Veterans Affairs medical center, Portland OR, USA, and some at Pontificia Universidade Catolica do Rio Grande do Sul (PUC-RS), Brazil.

Mice and adoptive transfers

Six-to eight-week-old C57BL/6 (H-2^b) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or FEPPS (Fundação Estadual de Produção e Pesquisa em Saúde, Porto Alegre, RS). TEa mice expressing a transgenic specific TCR for the Ea58-62:I-A^b complex obtained from Dr. Marc Jenkins (University of Minnesota, Mn), TEa $CD4^+$ T cells express Thy1.1 (CD90.1) and Vβ6 and recognize the I-E^d α-chain peptide 52-68 $(pEa)^3$ presented by I-A^b. Mice were maintained in specific pathogen-free conditions at 22°C under controlled light (12h light/12h darkness), and allowed free access to water and food in the VA medical center animal facility or PUC-RS animal facility. Experimental procedures followed the recommendations for Animal Care and Use by the Ethics Committee (process number 04/02312).

10^5 CFSE-labeled TEa, thy1.1, $RAG^{-/-}$ cells were injected intravenously into regular C57BL/6 as adoptive transfer. After 24 hours, $5X10^5$ B16EaRED melanoma cells were subcutaneously injected in the both back legs, in 100µl of PBS. Tumor cells in suspension were injected intact (live), irradiated (100Gy) or lysed by three cycles of freezing and thawing. Control mice received either PBS or 10 µg recombinant EaRFP. Draining lymph nodes were harvested from all groups at days 6, 9 and 15 after tumor injection.

Flow Cytometry

Single cell suspensions were prepared from draining lymph nodes by mechanical disruption on 70µm nylon cell strainers and incubated with collagenase D (Roche, Basel-Switzerland). Cell were incubated with blocking buffer (24G2 cells supernatant, rat serum, mice serum and sodic azide) and divided in two groups: one stained for thirty minutes on ice with antibodies anti-CD86 FITC, anti-B220 PE-Cy5.5, anti-YAe APC, anti-CD11c Alexa fluor 750, and one with anti-CD27-PE, anti-CD90.1 PerCP, CD62L- APC and anti CD4 Pacific Blue. Data were

collected on LSR BD cytometer or FACScalibur (BD Biosciences, San Diego, CA) and analyzed with FlowJo software (TreeStar, San Carlos, CA). CFSE dilution was analyzed by gating on CD4+CD90.1+ cells.

Confocal Microscopy analysis

Lymph nodes were collected and frozen in Tissue Tek OCT (Fort Washington, PA) at -80^{o}C. Sections of seven micrometers were obtained using a cryostat (Leica) and slides were fixed in 10% acetone, air-dried and were stained with anti-B220 FITC and biotin – YAe, followed by amplification. Briefly, slides were fixed in acetone, rehydrated with PBS, and the endogenous peroxidase was blocked for 10 minutes with 3% H_2O_2 + 0.1 % azide in PBS. Fc receptors were blocked with 5% sheep serum in PBS 1X for 15 minutes. Avidin/biotin blocking was done using the respective buffers provided by TSA Kit, 10 minutes each. Slides were analyzed by Bio-Rad MRC 1000 confocal microscope with a Krypton/ argon Laser (Bio-Rad Life Sciences, Hecules, CA) and CMOS v. 7.0a (Bio-Rad Life Sciences) sotware. Separate green, red and far red (Cy5) images were collected for each section analyzed. Image processing was performed using Photoshop software (Adobe, San Jose, CA).

Statistical Analysis

The ANOVA test was used to compare differences between groups with Bonferroni posttest. Statistical analysis and graphs were performed using GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA). Values of $p< 0.05$ were considered statistically significant.

Results

Class-II peptides from tumor antigens are presented in DLNs by CD86+ DC

We obtained a B16F10 transfectant line constitutively expressing the Ea-DsRed monomer fusion protein (Fig 1, A, B), B16EaRed. When these cells are fed to bone-marrow derived dendritic cells (BMDC) grown with GM-CSF and IL-4 (21) *in vitro*, three major populations can be identified 24h later by FACS using the red channel for RFP fluorescence and green channel for the YAe-FITC antibody (1C): a red only population, that phagocytosed but not processed the antigen yet; a double positive population (phagocytosed and is presenting); and a YAe single positive population, that has processed most of the antigen. When the B16EaRed tumor is injected subcutaneously in C57Bl/6 mice, YAe+ cells can be identified by confocal microscopy 24h later, mainly in the T cell area, although a few can be spotted in the B cell follicles (1F). The negative control is presented on Figure 1D with LN section from mice that received PBS injection; as a consequence, there were not YAe+ cells. Positive control (1E) had EaRFP protein injection and positivity to YAe+ cells. Prior adoptive transfer of CFSE-labelled TEa cells enabled us to verify that these cells are being stimulated by MHC-peptide complexes in the DLN, and that division is roughly proportional to the presentation signal detected. While TEa cells divided to the point of completely diluting the dye when mice were injected with soluble EaRFP 5 days earlier (Fig 1H), an undivided population can still be detected at this point in mice that were injected with the tumor cells (Figure 1I). There was not CFSE dilution of LN TEa cells from mice that reveived PBS injection. (Figure 1G).

FACS analysis of DLN after injection of tumor cells allowed us to estimate the presentation of tumor antigen by activated (CD86+) dendritic cells. Because we had observed that TEa proliferation in mice injected with live tumor cells proliferated poorly compared to injection with the soluble antigen, we decided to investigate if injection of dead tumor cells

would result in more efficient antigen presentation. The rationale for doing this was not only that it would improve accessibility of tumor antigen to dendritic cells, but also that it could result in increased activation of DC. Several studies report that dead cells are highly immunogenic (22), and independent groups have demonstrated endogenous adjuvanticity of component released after cell death (23-25). To better understand that, we first analyzed the effect of three cycles of freezing and thawing or irradiation with 100Gy on B16F10 cells by staining with Annexin V and propidium iodide (PI). We performed this analysis with the parental tumor (non-EaRFP expressing) because the red channel had to be available for the PI stain. Staining for these markers, shown in Figure 2 (A-C), recalls what has been described by other studies (26, 27). The majority (84%) of the tumor cells that came off the culture plate did not stain for either PI or Annexin V, being viable cells (Figure 2A) (also confirmed by Trypan blue exclusion – not shown). Cells that have been freeze and thawed (a model for necrosis) are mostly PI staining (2B) – although a considerable percentage stains for Annexin only; and the majority of the irradiated cells (a model for apoptosis) were for Annexin V-stained (2C). When the same treatments were applied to the B16EaRed tumor cells, and those were subcutaneously injected in the thigh, we verified that DCs in the inguinal DLN on day 9 after injection did show an increase in tumor antigen presentation, but also CD86 expression in the groups injected with the dead cells compared to the live tumor cells (Figure 2, D-F). The highest percentage of CD11c+ CD86+YAe+ cells was consistently detected in the freeze-thaw group (2E), suggesting that the higher percentage of necrotic cells most likely provided endogenous signals capable of activating the Ea-presenting DC.

TEa T cells suffer proliferation arrest due to decrease of antigenic stimulation

Endocytosis of dead tumor cells by DC is probably a common phenomenon in vivo, and although we and others (28, 29) observed that it can lead to increased tumor antigen presentation, the impact on tumor specific CD4+ T cell activation it is still poorly understood. To investigate that, we performed an intravenously adoptive transfer of 10^5 TEa cells per mouse, and 24h later, the mice received 5 x 10^5 of live B16EaRed cells, B16Red cells (not expressing the Ea antigen, but transfected with the pDsRed monomer plasmid), B16EaRed cells treated by freeze-thaw or irradiation. Analysis of the DLN was performed on days 6, 9 and 15 after tumor injection. While only 3% or less of the DC stained for YAe in the DLN of mice that received B16Red cells (which we considered background - not shown), the other groups presented a similar kinetics for antigen presentation (Figure 3). All groups presented a peak of presentation around day 9, the percentage of CD11c+YAe+ cells rapidly decreasing to an amount sometimes lower that 10% on day 15 (Figure 3A). Similar kinetics was observed when gating was restricted to the activated (CD86+) DC that were YAe+ (3B), however, percentages of triple positive cells were generally higher in the freeze-thaw group, with a significant difference being detected on day 6. Analysis of YAe mean fluorescence on the CD11c+, CD86+ was also higher in this group, with a significant difference being observed on day 9. Noteworthy, the mean levels of CD86 in the CD11c+, YAe+, although showing a slight decrease in mean fluorescence on day 15, was not significantly altered during the whole period, and no significant differences were observed among the groups.

Analysis of CFSE dilution by TEa cells in the DLN revealed a similar pattern. All three treatments induced proliferation of TEa cells (Figure 4), which peaked for all three groups around day 9. Proliferation was more significant in the freeze-thaw group. Interestingly, all groups revealed a particular profile of CFSE dilution, with a percentage of TEa cells in all groups analyzed never showing any evidence of division. This pattern, already present on day

6 and persisting until day 15, suggested that these T cells were not receiving adequate antigen stimulation, based on observations by Obst et. al (2005) (30) on transgenic T cells, and more recently by Yarke et al. (2008) (31) for TEa cells. These two studies demonstrated that as antigen availability wanes in the DLN, CD4+T cells undergo a cessation of expansion, thus TCR signaling is required for continuous proliferation in vivo. Because the undivided population was already present on day 6, and remained so, and because presentation kinetics showed a peak on day 9, it is possible that DC bearing antigen stopped migrating to the DLN before day 6, and the peak on day 9 is the presentation peak only because it is the time necessary for the majority of the cells that migrated from the tumor site to the DLN to process tumor antigen. We have observed in a previous study (Maito et al, submitted) a gradual cessation of cell migration (not only CD11c+ cells) to the DLN occurs as the B16F10 tumor grows, and that day 6 consistently marks the point when the tumor derived from a 5 x 10^5 cells injection starts growing and becomes macroscopically detectable.

TEa cells acquire CD44 expression, but little interferon gamma production

The arrest observed in TEa proliferation could mean that these cells were not being stimulated to the point of acquiring an effector phenotype. We first investigated this hypothesis by analyzing the expression of CD44, an effector cell marker, and CD62L, a marker for central (CD62L hi) or effector (CD62L lo) memory T cells. Figure 5 shows that most of the TEa cells had already acquired CD44 expression by day 6, even the ones that had not proliferated (Figure 5A). These CD44+TEa cells also peaked at day 9, rapidly decreasing through day 15, and were more abundant in the freeze-thaw group. Similar results were obtained for CD62L expression (5B). Analysis of expansion of TEa cells showed that the overall expansion was

only significant in the freeze-thaw group, on day 9 (Figure 6). While no tumor could be detected in the animals injected with the necrotic or the apoptotic cells, the B16EaRed tumor grew in the mice injected with live cells (6B), in spite of the cells acquiring an effector phenotype. We then verified that only a small percentage of these CD90.1, CD44+ cells produced interferon-γ (6D), indicating these cells had not fully differentiated to a mature phenotype. Few of these cells were Foxp3+, suggesting also they had not differentiated toward a regulatory phenotype.

Discussion

Due to the self nature of many tumor antigens, an intense immune response is not expected. Mechanisms of central and peripheral tolerance are likely to be responsible for much of anti-tumor tolerance (32-34). However, different strategies of escape from immune responses have been described for tumors, suggesting that additional mechanisms of immune suppression are employed by these cells to expand. In our study, we asked how anti-tumor CD4+ T cell memory develops *in vivo*, and if this proccess was influenced by the growing tumor. The main mechanism we could detect that affected differentiation of TEa cells into an effector memory phenotype was the inhibition of antigen presentation in the DLN, leading to the interruption of expansion. We have previously observed that this tumor is highly infiltrated by DC and CD4+T cells, and that their migration to the LN gradually decreases as the tumor grows (Maito et al, submitted). In the latter study, tumor rejection was only observed when we injected LPS intratumorally, resuming mobilization of cells to the DLN. CD4+ T cells require continuous antigen stimulation to proliferate (30, 31), and thus inhibition of DC migration is probably a highly effective suppression mechanism used by tumors. Gerber et al. (2007) observed that injection of the Ea antigen intratumorally does not result in presentation in the DNL, indicating that DC from an established tumor no longer migrate to the DLN. This

mechanism of tolerization might be even more relevant than only preventing upregulation of CD86, because when the necrotic tumor cells were injected, a higher expression of this molecule was observed, and yet TEa cells still failed to completely dilute the CFSE. We have also observed previously that only a small percentage of the tumor infiltrating CD4+ T cells were Foxp3+. This too agrees with our present findings that the tumor is not inducing tolerance mainly by induction of regulatory T cells.

A puzzling finding was that even when tumor cells were necrotic or apoptotic, TEa cells still presented proliferation arrest. Treatment with necrotic cells has been shown by others to activate dendritic cells, and that was explained to happen via the release of proposed endogenous distress signals such as Hsps (35) or uric acid (25). We also verified a more effective antigen presentation, activation and expansion of TEa cells in the mice that received necrotic tumor cells, however they too stopped proliferating. One would expect that an active suppression mechanism that prevented DC migration would no longer be exerted by dead cells. A simple explanation for this observation is that some tumor cells are still viable at the moment of injection, as shown in Figure 2. However, tumor growth was not detected in the mice that received necrotic or apoptotic cells, suggesting that they went on to die *in vivo* after being injected. When these treatments were standardized, we performed *in vitro* controls. In cell culture, there were no survival cells treated with freeze-thaw cycles or 100 Gy of irradiation after 3 days. An intriguing possibility is that soluble factors released by tumor cells are still present even after cell death, and those influence migration of immune cells (36-38). That remains to be tested, as does the possibility that the TEa cells activated in the animals that received necrotic cells can reject the B16EaRed tumor upon challenge. This is relevant because many studies have been conducted using dying tumor cells as a source of antigens. However, none of these has showed a complete *in vivo* study about anti-tumor immunity against poorly immunogenic B16 melanoma.

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Figures

Figure 1

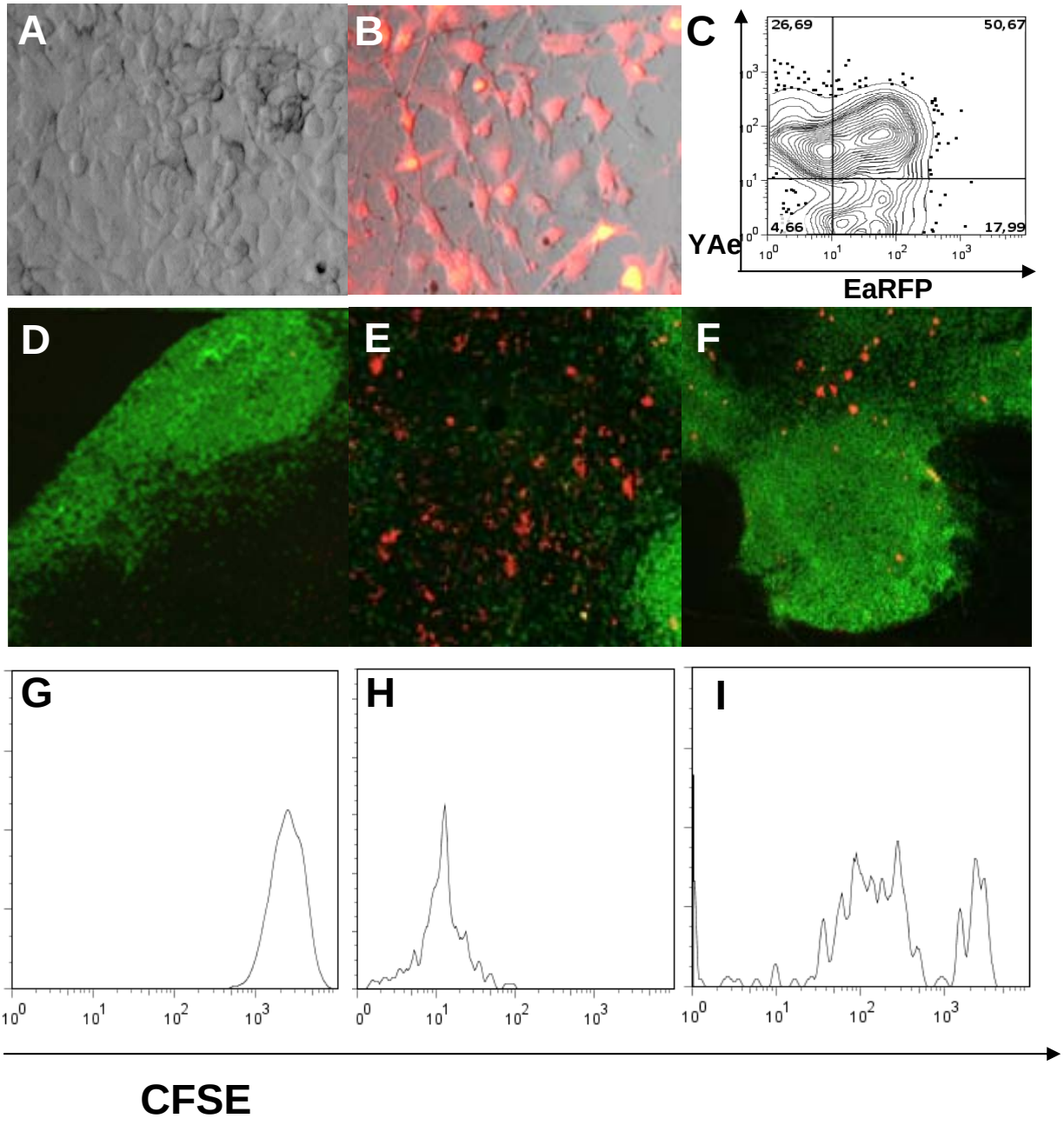
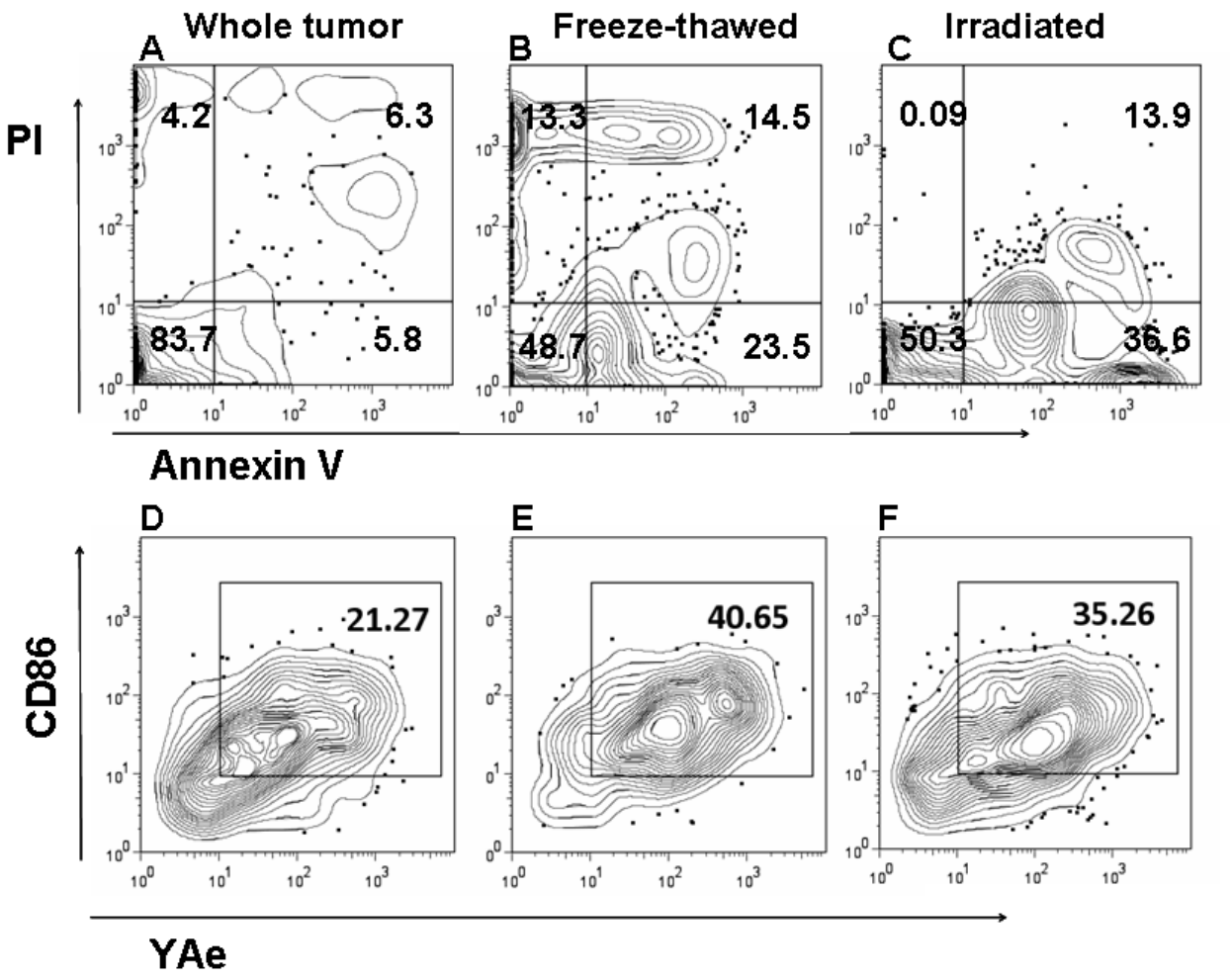


Figure 2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CD86
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YAe

Figure 3

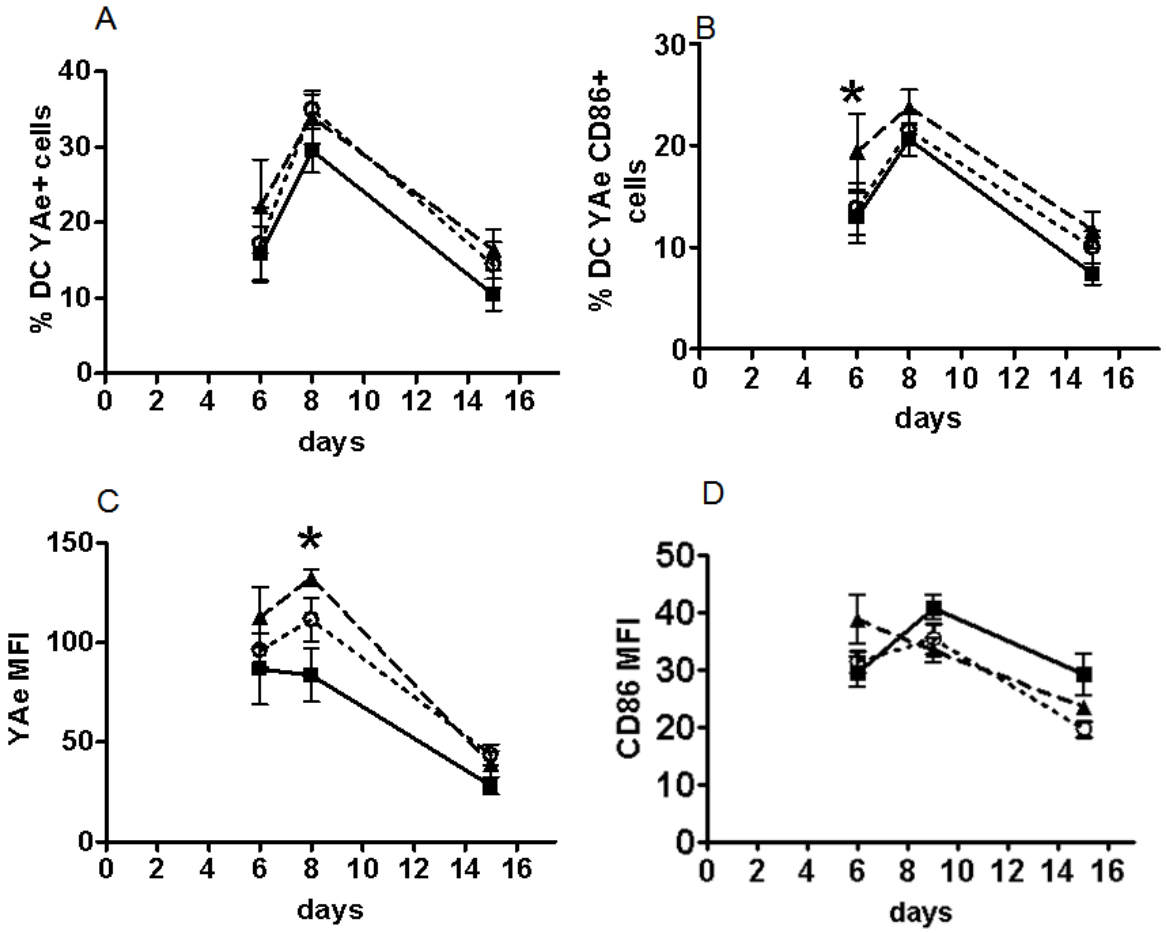
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Figure 4

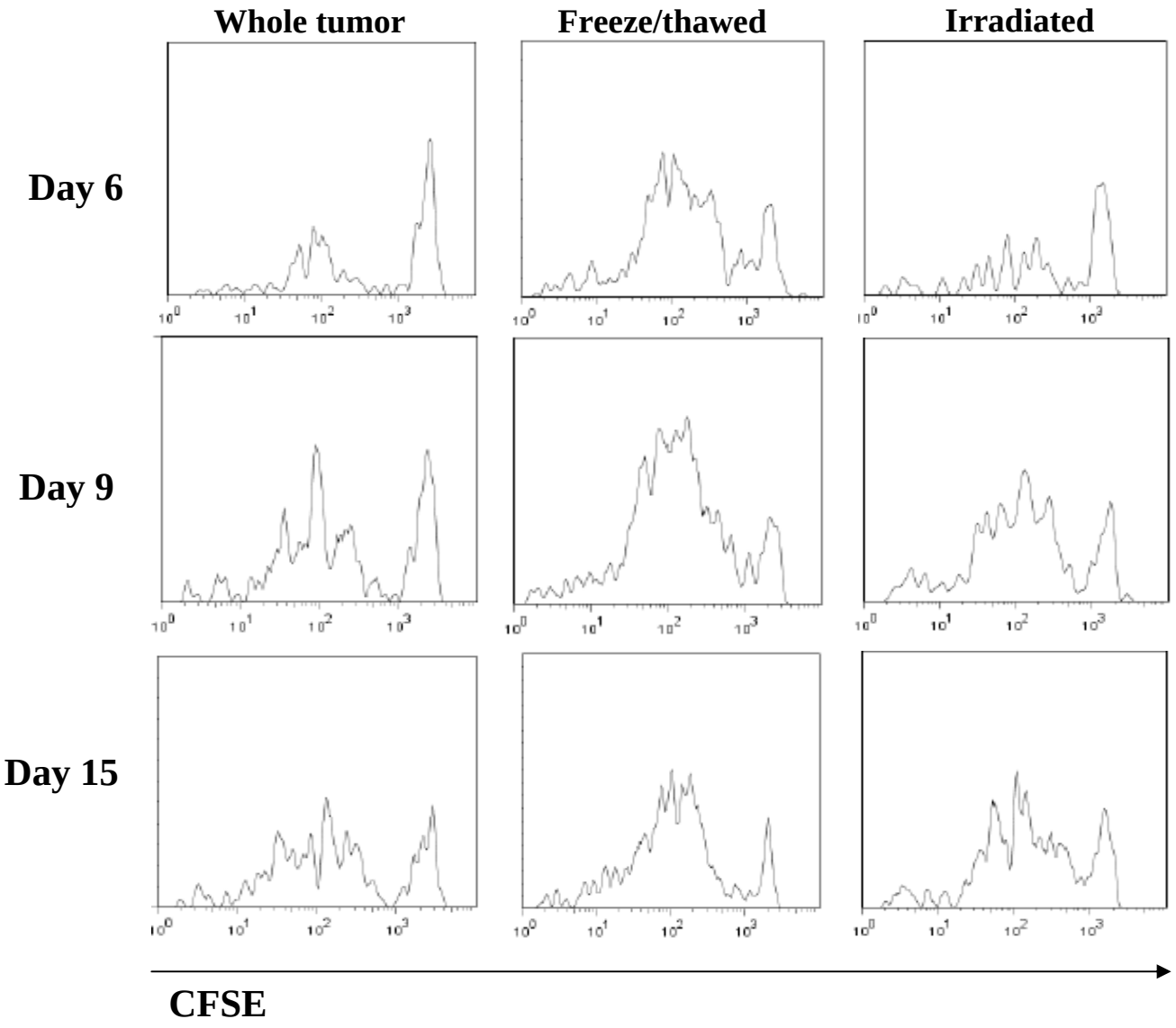
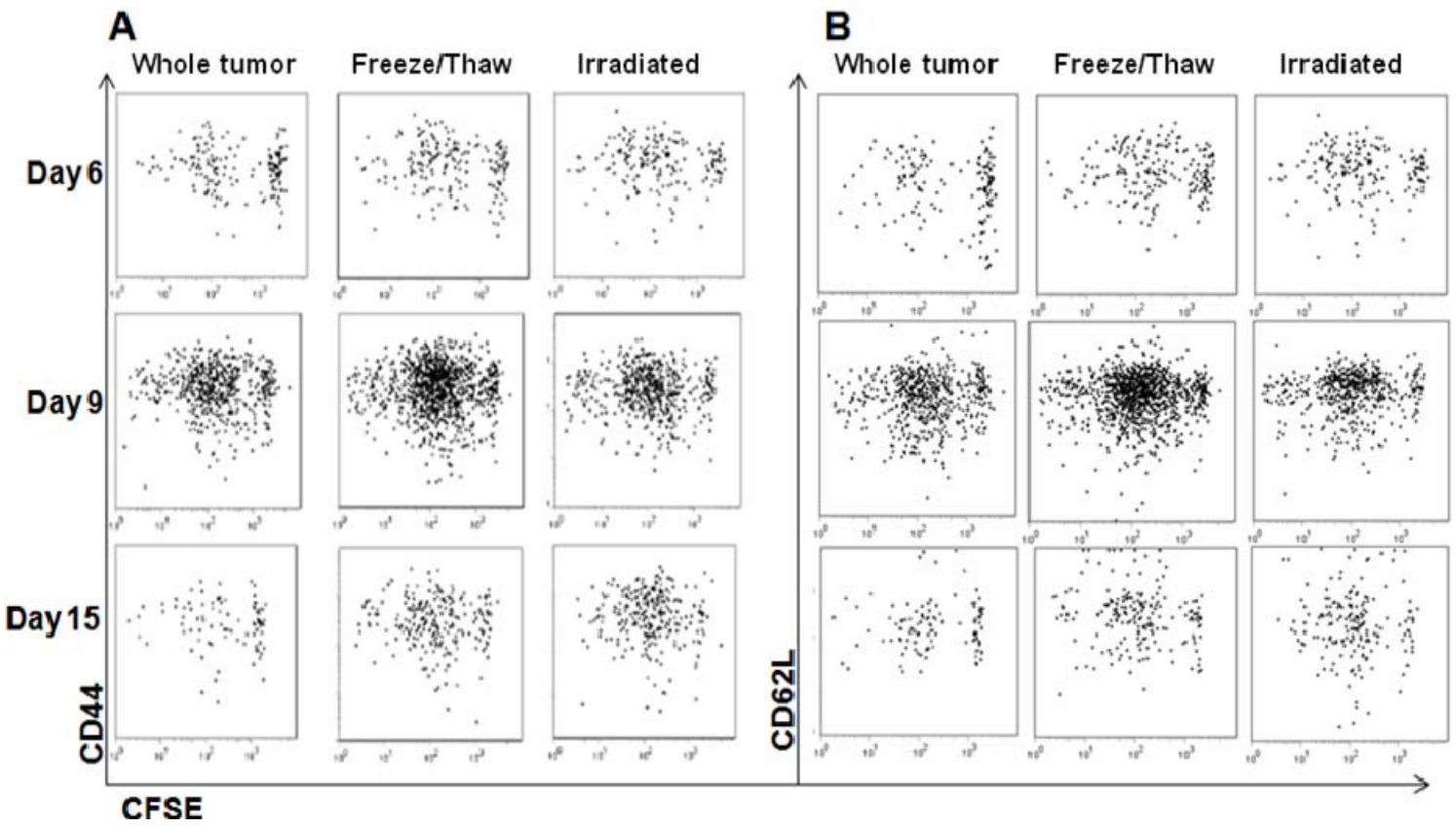


Figure 5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Whole tumor
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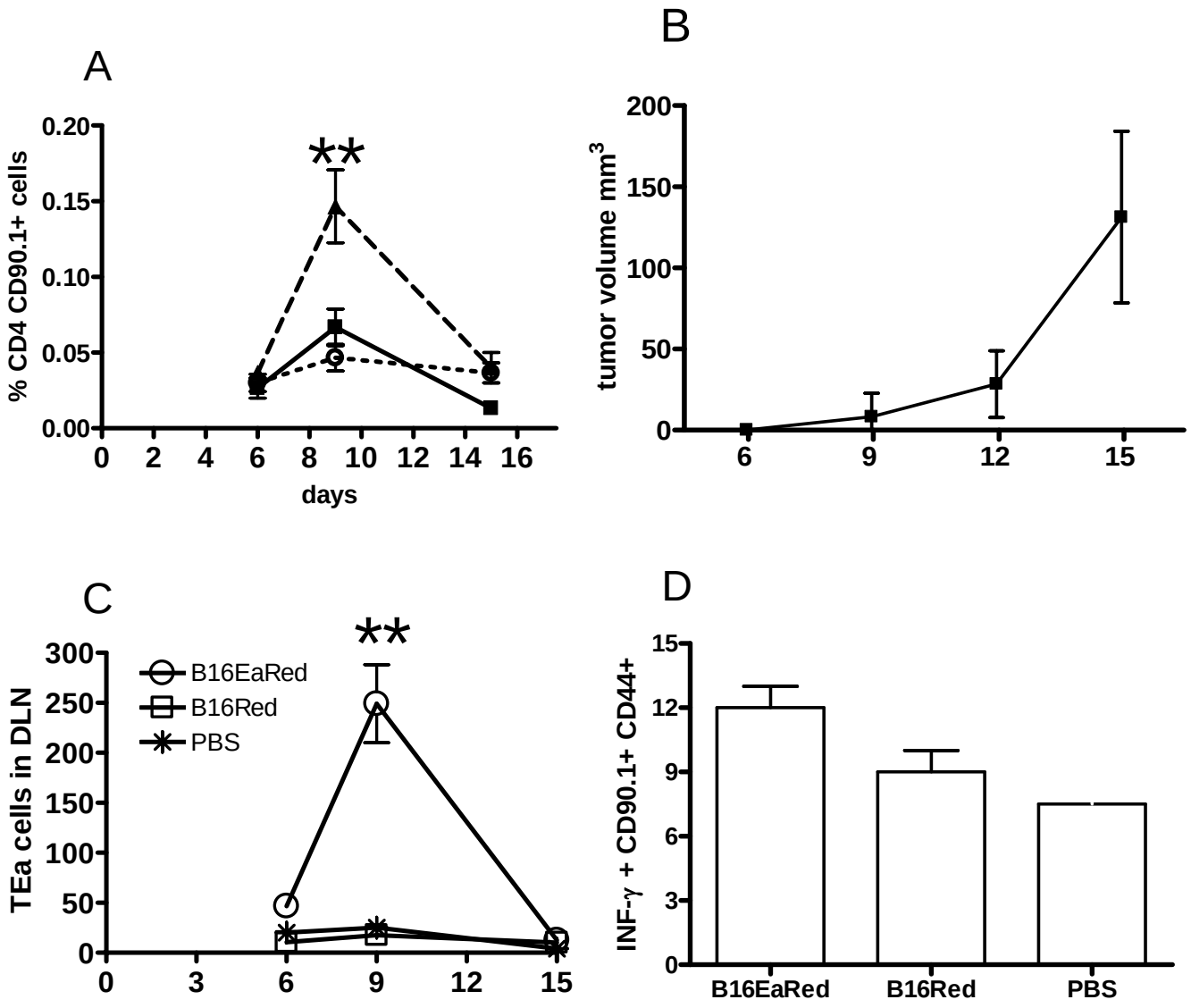
CD44
CFSE
B
Whole tumor
Freeze/Thaw
Irradiated
CD62L

Figure 6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B16EaRed
B16Red
PBS

Figure Legends

Figure 1. **Analysis of tumor antigen presentation.** (A)B16F10 cells and (B) B16EaRFP cells photographed in culture under light microscopy, 200x and merged with same field photographed under red fluorescence. (C) Representative flow cytometry analysis of BMDC after 24h incubation with B16EaRFP cells, gated on CD11c-cychrome positive cells, and stained with YAe-biotin and SA-FITC. (D,E, and F) Cervical LN sections from C57BL/6 mice 24 hours after subcutaneous PBS injection. (D) 30µg of EaRFP protein (E) or viable B16EaRFP (F) tumor cells in the ear, stained with YAe antibody and analyzed by confocal microscopy. (G, H and I) CFSE dye dilution in adoptively transferred TEa cells from cervical LN of mice that were injected 5 days earlier with PBS (G), 30µg of EaRFP protein (H) or tumor cells (I).

Figure 2. Comparison of live and dead tumor cells injection in tumor antigen presentation by DC *in vivo*. (A-C) staining with Annexin V and PI of B16F10 cells either live (A), killed by 3 cycles of freezing and thawing (B), and irradiated with 100 Gy (C). (D-F) percentage of activated (CD86+) DCs (gated on CD11c+ cells) presenting the Ea fragment detected by the YAe antibody when tumor is injected either live (D), killed by freezing and thawing (E) or irradiated (F). Results representative of 3 experiments.

Figure 3. Kinetics of tumor antigen presentation and activation of DC in the draining LN. (A) Percentage of CD11c+, presenting Ea fragment stained with YAe antibody. (B) Percentage of activated subset of DCs (CD86 hi) presenting Ea peptide. (C) YAe MFI on CD11c+, CD86+ cells. D) CD86 MFI of CD11c+, YAe+ cells. Tumor treatments are

represented by (▬) whole tumor, (▲)tumor treated with 3 cycles of freezing and thawing, and (▪⊙▪) irradiated tumor. (∗ $p < 0,05$)

Figure 4. Flow cytometry analysis of TEa cells proliferation by CFSE staining. 10^5 TEa cells were adoptively transferred to C57BL/6 mice and recovered from draining LN at days 6, 9 and 15 after 5 x10^5 B16EaRFP cells injection. Showing significant difference ($p < 0,05$) in CFSE dilution between the groups specialy at day 9 in freeze/thawed group and there are cells in all groups with no CFSE dilution.

Figure 5. Flow cytometry analysis of memory phenotype acquisition by TEa cells. 10^5 TEa cells were adoptively transferred to C57BL/6 mice and recovered from draining LN at days 6, 9 and 15 after 5 x10^5 B16EαRFP s.c. injection. Data of CFSE dilution of memory markers CD44 (panel A) and CD62L (panel B) are expressed by dot plots from different tumor treatments.

Figure 6. Kinetics of TEa differentiation and tumor growth with different tumor treatments. Mice were subcutaneously injected with 5 x10^5 B16EaRFP tumor cells. (A) Percentage of TEa cells from total LN cells recovered through time. (B) B16EaRed tumor growth. (C) Absolute number of TEa cells in the LN in mice injected with either Ea expressing tumor, Ea non-expressing tumor, or PBS. (D) Absolute number of INF-gamma producing TEa cells in the LN. Tumor treatments in **A** are represented by: (▬) Whole tumor, (▲) Freeze thaw/tumor and (▪⊙▪) Irradiated tumor. Showing that, even we had a siginificant (∗∗$p < 0,01$) recovery of TEa cell from draining LNs, when the B16EaRED tumor was injected live it grew.

Capitulo 4

4.1 Considerações finais

Para atingir os objetivos propostos neste trabalho buscou-se primeiramente entender o comportamento do tumor B16F10 enfatizando o estudo do crescimento e da resposta policlonal, porque mesmo sendo muito usado como modelo de tumor maligno não foi encontrada uma caracterização adequada in vivo. A cinética do crescimento tumoral avaliada neste primeiro experimento serviu de base para os outros estudos no que se refere a número de células injetadas, tempos experimentais e já nos deu a idéia da possível tolerização que o tumor precisa causar no sistema imune para crescer. O uso do modelo de tumor B16OVA foi importante porque com ele vimos que ao devolvermos a antigenicidade ao tumor houve uma diminuição do crescimento tumoral, mas não o bloqueio completo do seu crescimento, provavelmente porque o tumor vivo não gera sinais suficientes para que o hospedeiro monte uma resposta antitumoral eficaz.

Uma etapa relevante do trabalho foi a criação do inédito sistema B16EaRFP que estuda a resposta de células T CD4+ antitumoral, onde é possível analizar ao mesmo tempo apresentação de antígeno, maturação de DCs e proliferação de células T CD4+ *in vivo,* considerando que a maior parte dos estudos de células T CD4+ antitumorais tem sido feita com analises *ex vivo.* Mesmo analisando as respostas de células T CD8+ e T CD4+ separadamente nos modelos descritos, confirmamos a importância de ambas no controle do crescimento tumoral quando adequadamente estimuladas. Isso corrobora as pesquisas em imunologia do câncer que têm sugerido frequentemente que a cooperação entre as respostas T CD4 e T CD8 é crucial para o controle do crescimento tumoral, bem como a maturação das DCs através do adequado acesso aos antígenos tumorais. Isso pode gerar uma resposta efetora contra o câncer e também uma capacidade de responder a desafios tumorais posteriores, como por exemplo, possíveis recidivas.

Os resultados deste trabalho mostram a relevância do sistema imune no controle do crescimento tumoral e sugerem que a interrupção da migração das células imunes para os linfonodos drenantes é um mecanismo importante de supressão da resposta anti-tumoral. Quando o trafego de células entre tumor e linfonodo foi restabelecido através da injeção intratumoral de LPS, o sistema imune recebeu os sinais adequados para a melhora da resposta antitumoral, contudo, essa melhora parece não ter sido somente pela ativação das DCs. A possibilidade de outros mecanismos estarem envolvidos na redução tumoral por LPS é evidenciada por Chicoine et al. (2007) que encontraram 50% de redução do glioblastoma em camundongos "Knockout" para TLR-4 quando o LPS foi injetado intratumoralmente. Diante desses resultados, surgem novas perspectivas de estudo, como a verificação das células presentes nos linfonodos drenantes de tumor antes e depois da injeção intratumoral de LPS, bem como o perfil do infiltrado peritumoral e de citocinas, analisando marcadores de ativação celular e de memória.

Neste sistema experimental, a relevância do trânsito de células entre sitio tumoral e linfonodos drenantes se mostrou importante tanto para a resposta T CD4+ como para a indução da resposta efetora citotóxica. Segundo Obst et al.(2005) as células T CD4+ precisam de continua estimulação via TCR (receptor de célula T) para sua expansão e diferenciação em fenótipo de memória. O estudo da resposta de células T CD4+ antitumorais com o tumor B16EaRED mostrou uma expansão das células TEa nos linfonodos, bem como dos marcadores de memória, especialmente no nono dia depois da injeção tumoral e depois mostrou uma queda nesses marcadores no décimo quinto dia. Sugerindo diminuição da disponibilidade do antígeno sem a continua estimulação via TCR.

Mesmo havendo um decréscimo na proliferação das células TEa com fenótipo de memória no décimo quinto dia, as células tumorais mortas por necrose facilitaram o acesso do antígeno tumoral ás DCs. Diante disso, é necessário verificar se essa ativação das células TEa

será eficiente para rejeitar um posterior desafio com células B16EaRED em animais que receberam células tumorais necróticas previamente.

Finalizando, os estudos desenvolvidos aqui sugerem o sistema imune é importante no controle do crescimento tumoral e que a sua manipulação através da imunoterapia ou em associação com outras técnicas poderá alcançar resultados efetivos no combate ao câncer.

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