UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
FACULDADE DE MEDICINA
CAMPUS DE BOTUCATU

Marcela Rodrigues de Camargo

Efeito protetor *in vivo* de vacinas de células dendríticas sensibilizadas com RNA de células MC-38 pré-tratadas com agentes antineoplásicos em concentrações efetivas minímas

Botucatu – SP
2014

UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
FACULDADE DE MEDICINA
CAMPUS DE BOTUCATU

Marcela Rodrigues de Camargo

Efeito protetor *in vivo* de vacinas de células dendríticas sensibilizadas com RNA de células MC-38 pré-tratadas com agentes antineoplásicos em concentrações efetivas minímas

Tese apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Julio de Mesquita Filho", Campus de Botucatu, para obtenção do título de Doutor em Patologia.

Botucatu – SP
2014

FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO TÉC. AQUIS. TRATAMENTO DA INFORM.
DIVISÃO DE BIBLIOTECA E DOCUMENTAÇÃO - CAMPUS DE BOTUCATU - UNESP
BIBLIOTECÁRIA RESPONSÁVEL: ROSEMEIRE APARECIDA VICENTE - CRB 8/5651

Camargo, Marcela Rodrigues de.
Efeito protetor in vivo de vacinas de células dendríticas sensibilizadas com RNA de células MC-38 pré-tratadas com agentes antineoplásicos em doses efetivas mínimas / Marcela Rodrigues de Camargo. - Botucatu, 2014

Tese (doutorado) - Universidade Estadual Paulista, Faculdade de Medicina de Botucatu
Orientador: Ramon Kaneno
Capes: 21102007

1. Cólon - Câncer. 2. Reto - Cãncer. 3. Agentes antineoplásicos. 4. Celulas dendriticas. 5. Quimioterapia. 6. Vacinas.

Palavras-chave: Cancer colorretal; Celulas dendriticas; Vacina.

Dedicatória

Dedico este trabalho

Ao meu marido Pedro
por compartilhar ideais, ser meu parceiro de luta, de caminhada, de vida!

*Aos meus pais **José Luiz e Vanderli***
por serem sempre meu porto seguro, minha fonte de inspiração e exemplo!

*Dedico aos meus **amigos**, por estarem sempre ao meu lado, iluminado e guiando meu caminho!*

Agradecimentos

Agradeço meu orientador Dr. Ramon
Kaneno, por me permitir errar, me ajudar
acertar e me dar suporte para caminhar!

Agradeço também a Carolina Gorgulho, Cecília Pessoa, Juliana Frederico, Pedro Vannini, Fabiana Zambuzi, Vitoria Galvão, Carolina Almeida e Graziela Romagnolli, pela dedicação e amor ao trabalho realizado, pela amizade, paciência, carinho, confiança, alegrias e tristezas compartilhadas!

Obrigada família, por acreditar em mim até mesmo quando eu já não acreditava mais!

Agradeço as companheiras de estrada, Ana Paula, Tatiana, Bruna, Priscila, Miriane e Helga pelas risadas, desabafos e palavras de força nos momentos difíceis;

Aos meus amigos de intercâmbio, por me acolherem, me apoiarem e mais do que tudo, estarem comigo em uma das experiências mais diferentes da minha vida!

À Dra Suzanne Ostrand-Rosenberg e amigos do laboratório da UMBC, por permitirem o desenvolvimento de parte dessa tese e por essa experiência fantástica;

Agradeço os funcionários e amigos dos Departamentos de Patologia, de Microbiologia e Imunologia e também aos parceiros do Hospital Amaral Carvalho, sem vocês nada seria possível!

À ***Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP)****, pelo suporte financeiro que permitiu a realização deste trabalho. (processos nº 2009/18331-8; 2010/06013-9; 2012/17824-3);*

Ao ***Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq)*** *pela bolsa pela bolsa em parte deste trabalho. (processo nº555857/2010-07);*

À ***Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes)*** *pela bolsa em parte deste trabalho.*

Agradeço enfim todos aqueles que de uma forma ou de outra participaram dessa etapa da minha vida, me ajudando, confortando, orientando, desafiando... Por me fazer sorrir, por me fazer chorar, gritar, emudecer e até mesmo me oferecendo um cantinho pra dormir ou comer!!!

OBRIGADA A TODOS!!!

O caminho pode ser longo e difícil, mas tudo fica melhor quando não se está sozinho!

Sumário

Capítulo 1

Introduction: Immunological aspects of colorectal cancer

Introduction: Immunological aspects of colorectal cancer

Cancer represents one of the major causes of mortality worldwide [1] and the was estimated that this disease accounted for a total of 1,660,290 new cancer cases and 580,350 cancer deaths in the United States in 2013 [2]. Lung, breast, prostate, colon-recto and stomach cancers are the main causes of cancer related deaths [3]. Colorectal cancer (CRC) ranks the third main type of cancer worldwide [1, 4] with 40,340 new cases expected in 2013 [5] and growing incidences in developing countries. In Brazil it was estimated to reach 30,000 cases in 2012 [6].

CRC is classified as sporadic or familial disease, the former covering 75-85% of patients. Therefore, besides the inheritance of genetic predisposing features, prevalent in familial form, this disease is strongly influenced by environmental factors or life style, such as a diet based on high red meat and low vegetable and fruits intake, low physical activity, high body mass index[7], smoking and high alcohol intake [8]. There is also a strong association between bowel inflammatory conditions and the development of colon carcinogenesis. In this aspect, the procarcinogenic role of inflammation in CRC includes its strong association with Crohn's disease and ulcerative colitis [9-12]

The current therapy for this disease is based on the surgery (parcial or total colectomy) usually associated with chemotherapy with fluoropyrimidine, oxaliplatin, or irinotecan.[13]. Traditionally chemotherapy is based on the administration of maximum tolerated dose (MTD) regimen, as neoadjuvant or adjuvant therapies for pre-or postoperative treatments, respectively [14]. This schedule frequently promote deep myelosuppression followed by depressive effect on the functions of the immune system,

impairing both the innate and adaptive immunity [15]. This suppressive effect can facilitate the escape of drug-resistant tumor cells, increasing the possibility of relapsing disease.

An alternative schedule to avoid severe side effects - the metronomic chemotherapy - is based on the more frequent administration of lower doses of conventional drugs. This approach aims to keep effective serum levels of such drugs, preventing the development of drug resistance. Several currently used chemotherapeutic agents applied in low concentrations may also display direct stimulating effect on immune cells and fewer collateral effects [16] increasing tumor immunogenicity and/or activating the immunocompetent cells (chemoimmunomodulation) [15, 17, 18].

Paclitaxel (PAC) is one of the drugs tested for this purpose which low and ultra low doses are able to alter the tumor microenvironment and increase the antitumor efficacy of intralesionally inoculated DC vaccines by increasing $CD4^+$ and $CD8^+$ T cells and IFN-gamma production at the tumor site [19]. This drug can change the immunogenicity of human colon cancer cell lines by modulating the antigen processing machinery proteins in tumor cells [17] and stimulate the diferentiation of myeloid-derived suppressor cells (MDSC) towards dendritic cells [20]. Sevko et al. [21] showed that paclitaxel applied in ultra-low dose on C57BL/6 mice immunized with tyrosinase related protein (TRP)-2 - a model of melanoma antigen- in combination with the peptide vaccination, strongly increased the frequencies of TRP-2 specific T-cells in the spleen. The treatment also decreases the levels of regulatory T-cells and immature myeloid cells, and strongly increases the amount of effector $CD8^+$, $CD4^+$ and NK T-cells, suggesting that this agent in ultra-low, non-cytotoxic doses may potentially enhance the efficacy of anti-tumor vaccinations by neutralizing immunosuppressive T(reg) and MDSC populations in tumor-bearing hosts.

MDSC are the precursors of macrophages, granulocytes and dendritic cells in the bone marrow. However, they have also been associated with decreased antitumor response in different models, since they are able to strongly suppress $CD4^+$ and $CD8^+$ T cells by producing inhibitor cytokines IL-10 and TGF-β [22-24]. These cytokines both directly prevent T cell activation [25], and promote the development of Tregs, as well as polarize immunity towards a tumor-promoting Th2 phenotype [26, 27]. In addition, MDSC can interfere on the expression of L-selectins, preventing the homing of T cell to lymph nodes, where they would become activated [28], while their rise at the tumor microenvironment can facilitate their escape, since they affect the function of effective CTLs and NK cells [29, 30].

Low dose of cyclophosphamide was also studied in this context and it was observed that it restores T and NK cell function in patients at end-stage cancer [30], and stimulates the generation of TH1 type cytokines while suppress the production of supressive ones [31, 32]. Low dose of cyclophosphamide in combination with IL-12 therapy, selectively suppresses inhibitory cell subsets of MDSCs and Treg cells in MC-38 tumor-bearing mice through IFN- production [33].

Another chemotherapeutic and anti-mitotic drug docetaxel, is able to target MDSCs with preferential apoptosis of M2 cells and polarization of surviving cells towards M1 profile that restores T cell function and decreases tumor growth *in vivo*. Spleen $CD4^+$ and $CD8^+$ T cells isolated from docetaxel-treated mice spontaneously produced more IFNγ and displayed tumor-specific cytotoxicity than T cells from untreated tumor-bearing mice [34].

5-fluorouracil (5-FU) a chemotherapeutic agent that target the thymidylate syntase, an essential precursor for DNA biosynthesis, is the main drug used to control colon cancer, however, there are only few studies on it chemoimmunomodulatory activity when

administrated in low dose to animals or patients, or even it *in vitro* modulatory effect on dendritic cells.

Due to the high incidence of metastasis and relapsing disease, immunotherapy - including passive antibody therapy and active therapeutic vaccination - should be considered for many patients [35-37]. There are several monoclonal antibodies approved by the Food and Drug Admnistration (FDA) for passive immunotherapy, such as the anti-angiogenic antibody bevacizumab, and the anti-epidermal growth factor agents – cetuximab and panitumumab) that improve the benefits of conventional chemotherapy.

Bevacizumab or Avastin® is an inhibitor of vascular endothelial growth factor receptor-2 (VEGFR-2) developed for the treatment of primary and metastatic colorectal, lung and breast cancer [38]. In 2004 the FDA approved it use in a combination with 5-fluorouracil (5-FU)-based chemotherapy as a first-line treatment of metastatic colorectal cancer [39]. This antibody has been used for treating colorectal cancer in combination with cytotoxic agents in several other schedules, such as irinotecan/5-fluorouracil/leucovorin (FOLFIRI) that resulted in increased IL-2 and IFN-gamma production [40], and the overall survival of the patients [41, 42]. Bevacizumab was also combined with fluoropyrimidine and oxaliplatin (FOLFOX), improving in about 5 months the survival of CCR patients [43, 44]. However, some phase III trials showed that FOLFOX chemotherapy schedule alone is more effective than it combination with Bevacizumab [45]. In another approach, combining capecitabine and irinotecan (XELIRI) were combined with this antibody it was observed a 11.5-month survival and a favorably decrease of neutropenia and diarrhea events [46, 47].

Despite the successful treatment of some patient, combination of this antibody is not free of risks. In fact, VEGF inhibition due to a long term use of bevacizumab highly

increases the incidence of hypertension [48]. Patients with stage IV colorectal cancer treated with FOLFIRI and Bevacizumab showed increased risk of stroke, although they did not manifest cardiac events [49].

The anti-EGFR (Epidermal Growth Factor) Cetuximab or Erbitux® is also approved for clinical application. It is a humanized antibody that includes a human IgG1□ and murine Fv that targets EGFR and disrupts cell-signaling pathways, altering the ratio of Bax to Bcl-2 expression. It causes an increased expression of caspases and decreased tumor cells proliferation due to apoptosis [50, 51]. Nevertheless, Cetuximab therapy is very questionable, since some authors observed that metastatic colorectal cancer patients with KRAS mutations (that can affect 35-45% of patients) are resistant to the treatment with this antibody [52, 53].

EGFR can be targeted with another monoclonal antibody called Panitumumab, a fully human $IgG2_k$ anti EGFR approved for monotherapy as a third-line treatment of progressing colorectal cancer (resistant to previous chemotherapeutic schedules) [54]. Preclinical studies showed that Panitumumab binds to the EGF receptors reducing their signaling and inducing apoptosis. This antibody also binds to VEGF (vascular endothelial growth factor) and is able to decrease angiogenesis [55, 56]. In a phase III trial study, metastatic colon cancer patients with wild type K-RAS gene (WT-KRAS) treated with this antibody showed longer overall survival and increased progression free survival, supporting it efficacy [57, 58]. Similar results were observed in a phase II trials study in which such a monotherapy reached an overall survival of 13.5 months, while patients with mutanted K-RAS (MT-KRAS) reached only 7,25 months. Combination of this antibody with FOLFIRI chemotherapy increased the progression-free survival from 3.9 (FOLFIRI control) to 5.9 months [59]. Better results were obtained by Mitchell, 2010 [60] who

reported that treatment of WT and MT-KRAS patients with Panitumumab plus FOLFIRI improved the median PFS from 5.5 to 26, and from 3.3 to 19 months, respectively. However, other authors did not achieved similarly promising results with it combination with FOLFOX chemotherapeutic schedule [61].

Administration of monoclonal antibodies is frequently followed by side events such as erythema, acneiform dermatitis, pruritus, hypomagnesemia, skin exfoliation, fatigue, paronychia, abdominal pain, anorexia, nausea, diarrhea, skin rash and fissures [56, 62]. Limitations of passive immunotherapy require the continuous search for the knowledge on tumor:immune system relationship for the development of active immunotherapeutic interventions. In this aspect, one of the most prominent proposals is the development of dendritic cell-based therapeutic vaccines (DC-Vax) which main objective is to *in vivo* stimulate the adaptive immune system to achieve tumor regression [63-65].

DCs are the main professional antigen presenting cells [66],[67] and are found in most tissues [68]. Immature DCs are efficiently driven by chemotactic signals to lymphoid organs where they mature and improve their ability to present antigens for naïve T cells, and trigger an efficient immune response [68, 69]. DCs constitutively show class I and class II MHC molecules, and regularly process tumor antigens by the endocytic route for presenting peptides in association with MHC II molecules. Some of these peptides can also be associated with MHC I molecules through cross priming or cross-presentation phenomenon [65, 70, 71]. DC maturation is featured by a reduced antigen processing capacity, increased expression of MHC and costimulatory and adhesion molecules, increased expression of cytokine receptors, and reorganization of the cytoskeleton [72].

The central role of DCs on antigen presentation and induction of antitumor immunity supported the proposals of DCs-based therapeutic antitumor vaccines [73-75].

DC-vax show good results against cancer and can be prepared by different protocols that range from simple preparation of tumor cell lysates by rapid freezing and thawing [76], to the generation of hybrid cells by fusing DCs to tumor cells [77], and DC transfection with tumor RNA [78].

Results of studies on DC loaded with tumor cells lysates are controversial, since some studies have shown that this approach promotes a poor protective role, whereas other authors have been successful with their DC preparation. He *et al.* [79] observed that lysate-pulsed DC produced a poor protective effect against the development of CT26 cells, despite their ability to stimulate CTL activity, as well as INF-γ production during a CTL assay. Rather, these DC are likely to skew CD4 responses away from an optimal DC maturation and TH1 responsiveness with limited upregulation of CD80 and CD86 even after stimulation with LPS [80]. Actually, lysate-pulsed DC showed a more immature phenotype than unpulsed controls, and dramatically reduced the production of IL-12 p70 and TNF-□□while increasing IL-10. In addition, they observed that whole cell lysate inhibits the TLR-induced activation and IFN-γ production following co-culturing with T lymphocytes. Lysate was also unable to induce stimulation of specific CD8 cells against B16.OVA target cells [79]**.**

Moreover, in previous results reported by our group, we demonstrated that human DC loaded with colon cancer cell lysates efficiently stimulated allogeneic responsiveness (MLR) and induced CTL *in vitro* [81, 82]. Our results are in agreement with Larmonier *et al.* 2006, who successfully induced DC maturation and activation by pulsing them with cell lysate. Aiming to compare three different methods for preparation of tumor lysate, tumor cells were induced to necrosis by the quick freeze-thaw process, induction of syncytia by transfection with fusogenic viral vector, or treatment of HSV-transfected cells with

ganciclovir [83]. Results have shown that the manner by which tumor cells were killed did not affect the expression of CD11c, CD80 and CD86 by pulsed DC, since all methods were able to enhance the expression of these markers, as well as the expression of NF-kB and STAT1 and the production of IL-12. All the methods for antigen preparations were equally efficient at inducing T cell proliferation. Lysate-pulsed DC are even able to inhibit the growth of liver metastasis of colon cancer cells in the murine model of MC-106 cells [84].

In fact, some details can make the difference in the effectiveness of lysate-pulsed DC vaccines. For instance, the inhibitory effect of lysate on DC maturation can be reduced when tumor cells are stressed by heating at 42ºC for 25 min prior to the cell lysate preparation [85]. It is hypothesized that the expression of heat shock proteins by tumor cells can prevent the suppressive effect of cell lysate by increasing DC maturation as also evidenced by others [86-88].

Sonication of freeze/thawing lysates is another proposed method for preparing tumor antigens. Rossowska *et al.* [89] observed that DC loaded with this preparation already migrate to tumor-draining lymph nodes at the first day after injection, and the number of DC increased gradually at the tumor site until the fifth day. Most of the injected DC survived until the seventh day at the injection site whereas only Ag-loaded DC induced apoptosis and necrosis of tumor tissue.

Aiming to compare different methods for loading DC with tumor antigens, it was observed that lysate obtained from solid tumor cells homogenate poorly effects the ability of DC to stimulate antitumor activity [90]. Stressed tumor cells were obtained by freeze/thaw cycles or by irradiation at 30Gy, with the irradiation being more useful than the freeze-and-thaw process. However, for these authors, the best method for loading DC was their fusion with live tumor cells. They observed that irradiation of tumor cells with

30Gy was effective at stopping their proliferative ability and did not affect their usefulness for preparing tumor-DC hybrids. Results have shown that such hybrids produced 100% efficiency at protecting mice from tumor development.

Fusion technology was also used by Kao *et al.* 2006 [91], who prepared a vaccine by fusing DC with CCCT 26 cells. Although this vaccine was able to prevent growth of a lethal inoculum of tumor cells and induced a strong *in vitro* CTL response, it did not present a therapeutic role, failing to inhibit the growth of preexisting tumor, even after LPS-induced maturation. A TH-2–dominant response was observed in vaccinated animals, which showed a prevalence of IL-5-producing cells over IFN-γ-producing ones and increased IL-4- and IL-10-producing cells. Since the authors did not observe numerical differences in either TGF-β-producing cells or in $CD4^+/CD25^+$ cells, they concluded that their vaccine induces an aberrant TH2 response rather than increasing Treg cells. Although human DC fused with SW480 tumor cells have induced the development of specific $CD8^+$ T cell response and secretion of IFN-γ, the authors did not test them in a clinical *in vivo* trial [92].

Transfection of DC with RNA of known tumor antigens is another strategy for DC loading, with the carcinoembrionic antigen (CEA) being one of the preferred targets for this purpose in colon cancer [96-100]**.** Ojima *et al.* [101] worked with murine MC-38 tumor cells transfected with human CEA by an adenoviral vector, whereas DC were transfected to express GM-CSF, IL-12 or CEA. *In vivo* experiments showed that the antitumor response induced by transfected DC is dependent on $CD4^+$ cells rather than $CD8^+$ or NK cells. However, when both $CD8^+$ and NK cells were depleted, host resistance was completely abrogated. The role of these effector cells was confirmed by immunofluorescence microscopy, showing heavy tumor infiltration by both NK and $CD8^+$,

which surround CEA^+ target cells. This infiltration is dependent on IL-12 and GM-CSF since DC transfected with CEA alone showed only discrete infiltration by $CD8^+$ cells.

Anti-idiotype antibodies can mimic CEA for DC loading to induce specific anti-CEA cytotoxic T lymphocytes and reject CEA-transfected MC-38 cells [102]. This CTL response mediated class I-restricted lysis, IFN-γ and TNF-α production, and FAS-L expression, as well as TNF-related apoptosis-inducing ligand (TRAIL) response. *In vivo*, antitumor activity was dependent on $CD4^+$ and $CD8^+$ cells, while the expression of memory phenotype ($Ly\text{-}6C^+$ and $CD44^+$) was increased.

Rains et al (2001) [103] pulsed DC with RNA from autologous colorectal cancer cell to generate a vaccine and three months later 7 of 13 patients had reduced serum levels of carcinoembryonic antigen (CEA). It was observed that DCs derived from peripheral blood mononuclear cells (PBMC) transfected with RNA of colorectal cancer, induce antitumor response by cytolytic T cells through antigen presentation via MHC class – I [78], demonstrating the importance of the participation of CTL in antitumor response [104]. As expected, expression of class I MHC molecules highly correlates with the overall tumor immunogenicity that is a singular marker to predict the quality of response of patients to this modality of immunotherapy

Immunization of patients with DC vaccine in a phase I/II clinical trial showed that the vaccine was effective for 16.7% of patients in the phase I study and for 23% of them in phase II [93]. Messenger RNA from both the TAT protein transduction domain and calreticulin increase the immunogenicity of CEA and the effectiveness of mRNA-pulsed human DC. It is interesting that DC transfection with calreticulin mRNA seems to be associated with activation of $CD4^+$ T cells whereas TAT protein mRNA shows preferential stimulation of $CD8^+$ cells [105]. Since mRNA represents only 5% of total cell RNA, *in*

vitro amplification of mRNA was shown to be feasible for producing immunogenically active CEA-encoding mRNA [94].

Instead of using mRNA for known specific antigens such as CEA and Her2/neu, Nencione A *et al.* 2003 [78] transfected DC with total tumor RNA using an EGFP-encoding vector. They observed that DC transfected with RNA from colon cancer cells were able to induce a CTL response and that effector cells were able to recognize both the original tumor cell line used for RNA preparation (SW480) and two other cell lines, HCT-116 (colon cancer) and A498 (renal cancer). Supporting this strategy, a clinical trial using total RNA extracted from metastatic tumor cells for pulsing autologous DC (4 injections, every 4 weeks) was shown to be feasible for inducing a specific T response against CEA [106].

Systematic association of chemotherapy and immunotherapy strategies is a relatively new subject, though intuitive, especially concerning the use of low and ultra/low chemotherapy schedules. The current challenge is to find how different drugs interact with different immunotherapeutic approaches and the ideal concentrations of low/ultra low dosage, in order to combine them with immunotherapy to reduce side effects and improve the antitumor status

References

[1] Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011 Mar-Apr;61(2):69-90.

[2] Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. CA Cancer J Clin. 2012 Jan-Feb;62(1):10-29.

[3] World Health Organization. 2013 [cited 2013; Available from: http://www.who.int/gho/ncd/mortality_morbidity/cancer/en/

[4] Cunningham D, Atkin W, Lenz HJ, Lynch HT, Minsky B, Nordlinger B, et al. Colorectal cancer. Lancet. 2010 Mar 20;375(9719):1030-47.

[5] Labianca R, Beretta GD, Kildani B, Milesi L, Merlin F, Mosconi S, et al. Colon cancer. Critical reviews in oncology/hematology. 2010 May;74(2):106-33.

[6] Instituto Nacional de Câncer José Alencar Gomes da Silva MdS. Estimativa 2012, Incidência de câncer no Brasil. 2012 [cited; Available from: http://www.inca.gov.br/estimativa/2012/estimativa20122111.pdf

[7] Johnson CM, Wei C, Ensor JE, Smolenski DJ, Amos CI, Levin B, et al. Meta-analyses of colorectal cancer risk factors. Cancer Causes Control. 2013 Apr 6.

[8] Jafri SH, Mills G. Lifestyle modification in colorectal cancer patients: an integrative oncology approach. Future Oncol. 2013 Feb;9(2):207-18.

[9] Winther KV, Jess T, Langholz E, Munkholm P, Binder V. Long-term risk of cancer in ulcerative colitis: a population-based cohort study from Copenhagen County. Clin Gastroenterol Hepatol. 2004 Dec;2(12):1088-95.

[10] Farraye FA, Odze RD, Eaden J, Itzkowitz SH. AGA technical review on the diagnosis and management of colorectal neoplasia in inflammatory bowel disease. Gastroenterology. 2010 Feb;138(2):746-74, 74 e1-4; quiz e12-3.

[11] Ullman TA, Itzkowitz SH. Intestinal inflammation and cancer. Gastroenterology. 2011 May;140(6):1807-16.

[12] Lutgens MW, van Oijen MG, van der Heijden GJ, Vleggaar FP, Siersema PD, Oldenburg B. Declining risk of colorectal cancer in inflammatory bowel disease: an updated meta-analysis of population-based cohort studies. Inflamm Bowel Dis. 2013 Mar-Apr;19(4):789-99.

[13] Jonker DJ, O'Callaghan CJ, Karapetis CS, Zalcberg JR, Tu D, Au HJ, et al. Cetuximab for the treatment of colorectal cancer. The New England journal of medicine. 2007 Nov 15;357(20):2040-8.

[14] Andre T, de Gramont A, Study Group of Clinical Research in Radiotherapies Oncology OMRG. An overview of adjuvant systemic chemotherapy for colon cancer. Clinical colorectal cancer. 2004 Jun;4 Suppl 1:S22-8.

[15] Shurin MR NH, Gutkin DW, Umansky V, Shurin GV. ChemoImmunoModulation: immune regulation by the antineoplastic chemotherapeutic agents. Current medicinal chemistry. 2012;19(12):117-21.

[16] Joshua P. Landreneau MRS, Marianna V. Agassandian, Anton A. Keskinov, Yang Ma, Galina Shurin. Immunological Mechanisms of low and ultra-low dose cancer chemotherapy. Cancer Microenvironment. 2013.

[17] Kaneno R, Shurin GV, Kaneno FM, Naiditch H, Luo J, Shurin MR. Chemotherapeutic agents in low noncytotoxic concentrations increase immunogenicity of human colon cancer cells. Cell Oncol (Dordr). 2011

Apr;34(2):97-106.

[18] CL M. T-cell immunodeficiency following cytotoxic antineoplastic therapy: a review. Stem Cells. 2000;18:10-8.

[19] Zhong H, Han B, Tourkova IL, Lokshin A, Rosenbloom A, Shurin MR, et al. Low-dose paclitaxel prior to intratumoral dendritic cell vaccine modulates intratumoral cytokine network and lung cancer growth. Clin Cancer Res. 2007 Sep 15;13(18 Pt 1):5455-62.

[20] Michels T, Shurin GV, Naiditch H, Sevko A, Umansky V, Shurin MR. Paclitaxel promotes differentiation of myeloid-derived suppressor cells into dendritic cells in vitro in a TLR4-independent manner. Journal of immunotoxicology. 2012 Jul-Sep;9(3):292-300.

[21] Sevko A, Michels T, Vrohlings M, Umansky L, Beckhove P, Kato M, et al. Antitumor effect of Paclitaxel is mediated by inhibition of myeloid-derived suppressor cells and chronic inflammation in the spontaneous melanoma model. J Immunol. 2013 Mar 1;190(5):2464-71.

[22] Maloy KJ, Powrie F. Regulatory T cells in the control of immune pathology. Nature immunology. 2001 Sep;2(9):816-22.

[23] Ostrand-Rosenberg S, Sinha P. Myeloid-derived suppressor cells: linking inflammation and cancer. J Immunol. 2009 Apr 15;182(8):4499-506.

[24] Talmadge JE. Pathways mediating the expansion and immunosuppressive activity of myeloid-derived suppressor cells and their relevance to cancer therapy. Clin Cancer Res. 2007 Sep 15;13(18 Pt 1):5243-8.

[25] Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. Nat Rev Immunol. 2012 Apr;12(4):253-68.

[26] Sinha P, Clements VK, Ostrand-Rosenberg S. Interleukin-13-regulated M2 macrophages in combination with myeloid suppressor cells block immune surveillance against metastasis. Cancer Res. 2005 Dec 15;65(24):11743-51.

[27] Sinha P, Clements VK, Fulton AM, Ostrand-Rosenberg S. Prostaglandin E2 promotes tumor progression by inducing myeloid-derived suppressor cells. Cancer Res. 2007 May 1;67(9):4507-13.

[28] Hanson EM, Clements VK, Sinha P, Ilkovitch D, Ostrand-Rosenberg S. Myeloid-derived suppressor cells down-regulate L-selectin expression on CD4+ and CD8+ T cells. J Immunol. 2009 Jul 15;183(2):937-44.

[29] Ostrand-Rosenberg S. Myeloid-derived suppressor cells: more mechanisms for inhibiting antitumor immunity. Cancer Immunol Immunother. 2010 Oct;59(10):1593-600.

[30] Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, et al. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. Nat Med. 2007 Sep;13(9):1050-9.

[31] Schiavoni G, Sistigu A, Valentini M, Mattei F, Sestili P, Spadaro F, et al. Cyclophosphamide synergizes with type I interferons through systemic dendritic cell reactivation and induction of immunogenic tumor apoptosis. Cancer Res. 2011 Feb 1;71(3):768-78.

[32] Guerriero JL, Ditsworth D, Catanzaro JM, Sabino G, Furie MB, Kew RR, et al. DNA alkylating therapy induces tumor regression through an HMGB1-mediated activation of innate immunity. J Immunol. 2011 Mar 15;186(6):3517-26.

[33] Medina-Echeverz J FJ, Zabala M, Ardaiz N, Prieto J, Berraondo P. Successful colon cancer eradication after chemoimmunotherapy is associated with profound phenotypic change of intratumoral myeloid cells. J Immunol. 2011;2:807-15.

[34] Djeu J WS. Chemoimmunomodulation of MDSCs as a novel strategy for cancer therapy. Oncoimmunology. 2012;1:121-2.

[35] Riechman L CM, Waldmann H, Winter G. Reshaping human antibodies for therapy. Nature. 1988;332:323-7.

[36] Gura T. Therapeutic antibodies: magic bullets hit the target. Nature. 2002 Jun 6;417(6889):584-6.

[37] Steinman RM, Dhodapkar M. Active immunization against cancer with dendritic cells: the near future. International journal of cancer. 2001 Nov;94(4):459-73.

[38] Adjei AA MS, Dy GK, Molina JR, GandaraDR, Allen Ziegler KL et al. Phase II trial of pemetrexed plus bevacizumab for second line therapy of patients with advanced non-small-cell lung cancer: NCCTG and SWOG study. J clin oncol. 2008.

[39] Welch S, Spithoff K, Rumble RB, Maroun J. Bevacizumab combined with chemotherapy for patients with advanced colorectal cancer: a systematic review. Ann Oncol. 2010 Jun;21(6):1152-62.

[40] Tsavaris N, Voutsas IF, Kosmas C, Gritzapis AD, Baxevanis CN. Combined treatment with bevacizumab and standard chemotherapy restores abnormal immune parameters in advanced colorectal cancer patients. Investigational new drugs. 2010 Feb;30(1):395-402.

[41] Fuchs CS, Marshall J, Mitchell E, Wierzbicki R, Ganju V, Jeffery M, et al. Randomized, controlled trial of irinotecan plus infusional, bolus, or oral fluoropyrimidines in first-line treatment of metastatic colorectal cancer: results from the BICC-C Study. J Clin Oncol. 2007 Oct 20;25(30):4779-86.

[42] Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. The New England journal of medicine. 2004 Jun 3;350(23):2335-42.

[43] Saltz LB, Clarke S, Diaz-Rubio E, Scheithauer W, Figer A, Wong R, et al. Bevacizumab in combination with oxaliplatin-based chemotherapy as first-line therapy in metastatic colorectal cancer: a randomized phase III study. J Clin Oncol. 2008 Apr 20;26(12):2013-9.

[44] Giantonio BJ, Catalano PJ, Meropol NJ, O'Dwyer PJ, Mitchell EP, Alberts SR, et al. Bevacizumab in combination with oxaliplatin, fluorouracil, and leucovorin (FOLFOX4) for previously treated metastatic colorectal cancer: results from the Eastern Cooperative Oncology Group Study E3200. J Clin Oncol. 2007 Apr 20;25(12):1539-44.

[45] Allegra CJ, Yothers G, O'Connell MJ, Sharif S, Colangelo LH, Lopa SH, et al. Initial safety report of NSABP C-08: A randomized phase III study of modified FOLFOX6 with or without bevacizumab for the adjuvant treatment of patients with stage II or III colon cancer. J Clin Oncol. 2009 Jul 10;27(20):3385-90.

[46] Renouf DJ, Welch S, Moore MJ, Krzyzanowska MK, Knox J, Feld R, et al. A phase II study of capecitabine, irinotecan, and bevacizumab in patients with previously untreated metastatic colorectal cancer. Cancer chemotherapy and pharmacology. 2012 May;69(5):1339-44.

[47] Kocakova I, Kocak I, Svoboda M, Nemecek R, Rehak Z, Standara M. [Bevacizumab in combination with capecitabine and irinotecan (XELIRI) in treatment of metastatic colorectal cancer]. Klin Onkol. 2009;22(2):73-6.

[48] De Stefano A, Carlomagno C, Pepe S, Bianco R, De Placido S. Bevacizumab-related arterial hypertension as a predictive marker in metastatic colorectal cancer patients. Cancer chemotherapy and pharmacology. Nov;68(5):1207-13.

[49] Meyerhardt JA, Li L, Sanoff HK, Carpenter Wt, Schrag D. Effectiveness of bevacizumab with first-line combination chemotherapy for Medicare patients with stage IV colorectal cancer. J Clin Oncol. Feb 20;30(6):608-15.

[50] Mendelsohn J. Blockade of receptors for growth factors: an anticancer therapy--the fourth annual Joseph H Burchenal American Association of Cancer Research Clinical Research Award Lecture. Clin Cancer Res. 2000 Mar;6(3):747-53.

[51] Vincenzi B, Zoccoli A, Pantano F, Venditti O, Galluzzo S. Cetuximab: from bench to bedside. Current cancer drug targets. 2010 Feb;10(1):80-95.

[52] Papa A, Rossi L, Lo Russo G, Giordani E, Spinelli GP, Zullo A, et al. Emerging role of cetuximab in the treatment of colorectal cancer. Recent patents on anti-cancer drug discovery. May 1;7(2):233-47.

[53] Karapetis CS, Khambata-Ford S, Jonker DJ, O'Callaghan CJ, Tu D, Tebbutt NC, et al. K-ras mutations and benefit from cetuximab in advanced colorectal cancer. The New England journal of medicine. 2008 Oct 23;359(17):1757-65.

[54] Ciardiello F, Tortora G. EGFR antagonists in cancer treatment. The New England journal of medicine. 2008 Mar 13;358(11):1160-74.

[55] Hoy SM, Wagstaff AJ. Panitumumab: in the treatment of metastatic colorectal cancer. Drugs. 2006;66(15):2005-14; discussion 15-6.

[56] Messersmith WA, Hidalgo M. Panitumumab, a monoclonal anti epidermal growth factor receptor antibody in colorectal cancer: another one or the one? Clin Cancer Res. 2007 Aug 15;13(16):4664-6.

[57] Amado RG, Wolf M, Peeters M, Van Cutsem E, Siena S, Freeman DJ, et al. Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. J Clin Oncol. 2008 Apr 1;26(10):1626-34.

[58] Van Cutsem E, Lang I, Folprecht G. Cetuximab plus FOLFIRI in the treatment of metastatic coloretal cancer (mCRC): the influence of KRAS and BRAF biomarkers on outcome. gastrointestinal cancers symposium. 2010.

[59] Peeters M, Price TJ, Cervantes A, Sobrero AF, Ducreux M, Hotko Y, et al. Randomized phase III study of panitumumab with fluorouracil, leucovorin, and irinotecan (FOLFIRI) compared with FOLFIRI alone as second-line treatment in patients with metastatic colorectal cancer. J Clin Oncol. Nov 1;28(31):4706-13.

[60] Mitchell EP, Piperdi B, Lacouture ME, Shearer H, Iannotti N, Pillai MV, et al. The efficacy and safety of panitumumab administered concomitantly with FOLFIRI or Irinotecan in second-line therapy for metastatic colorectal cancer: the secondary analysis from STEPP (Skin Toxicity Evaluation Protocol With Panitumumab) by KRAS status. Clinical colorectal cancer. Dec;10(4):333-9.

[61] Douillard JY, Siena S, Cassidy J, Tabernero J, Burkes R, Barugel M, et al. Randomized, phase III trial of panitumumab with infusional fluorouracil, leucovorin, and oxaliplatin (FOLFOX4) versus FOLFOX4 alone as first-line treatment in patients with previously untreated metastatic colorectal cancer: the PRIME study. J Clin Oncol. 2010 Nov 1;28(31):4697-705.

[62] Lopez-Gomez M, Merino M, Casado E. Long-term treatment of metastatic colorectal cancer with panitumumab. Clinical Medicine Insights. 2012;6:125-35.

[63] Kalinski P, Okada H. Polarized dendritic cells as cancer vaccines: directing effector-type T cells to tumors. Seminars in immunology. 2010 Jun;22(3):173-82.

[64] Lee HJ, Hong CY, Kim MH, Lee YK, Nguyen-Pham TN, Park BC, et al. In vitro induction of anterior gradient-2-specific cytotoxic T lymphocytes by dendritic cells transduced with recombinant adenoviruses as a potential therapy for colorectal cancer. Experimental & molecular medicine. 2012 Jan 31;44(1):60-7.

[65] Steinman RM. Decisions about dendritic cells: past, present, and future. Annu Rev Immunol. 2012;30:1-22.

[66] Shurin MR. Dendritic cells presenting tumor antigen. Cancer Immunol Immunother. 1996 Nov;43(3):158-64.

[67] Steinman RM. The dendritic cell system and its role in immunogenicity. Annu Rev Immunol. 1991;9:271-96.

[68] Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature. 1998 Mar 19;392(6673):245-52.

[69] Lipscomb MF, Masten BJ. Dendritic cells: immune regulators in health and disease. Physiol Rev. 2002 Jan;82(1):97-130.

[70] Tittarelli A, Gonzalez FE, Pereda C, Mora G, Munoz L, Saffie C, et al. Toll-like receptor 4 gene polymorphism influences dendritic cell in vitro function and clinical outcomes in vaccinated melanoma patients. Cancer Immunol Immunother. 2012 Nov;61(11):2067-77.

[71] Arina A, Tirapu I, Alfaro C, Rodriguez-Calvillo M, Mazzolini G, Inoges S, et al. Clinical implications of antigen transfer mechanisms from malignant to dendritic cells. exploiting cross-priming. Exp Hematol. 2002 Dec;30(12):1355-64.

[72] Trombetta ES, Mellman I. Cell biology of antigen processing in vitro and in vivo. Annu Rev Immunol. 2005;23:975-1028.

[73] Palucka AK, Ueno H, Fay J, Banchereau J. Dendritic cells: a critical player in cancer therapy? J Immunother. 2008 Nov-Dec;31(9):793-805.

[74] Grabbe S, Beissert S, Schwarz T, Granstein RD. Dendritic cells as initiators of tumor immune responses: a possible strategy for tumor immunotherapy? Immunol Today. 1995 Mar;16(3):117-21.

[75] Gilboa E. DC-based cancer vaccines. J Clin Invest. 2007 May;117(5):1195-203.

[76] Reyes D, Salazar L, Espinoza E, Pereda C, Castellon E, Valdevenito R, et al. Tumour cell lysate-loaded dendritic cell vaccine induces biochemical and memory immune response in castration-resistant prostate cancer patients. British journal of cancer. 2013 Sep 17;109(6):1488-97.

[77] Koido S, Homma S, Okamoto M, Namiki Y, Takakura K, Uchiyama K, et al. Strategies to improve the immunogenicity of anticancer vaccines based on dendritic cell/malignant cell fusions. Oncoimmunology. 2013 Sep 1;2(9):e25994.

[78] Nencioni A, Muller MR, Grunebach F, Garuti A, Mingari MC, Patrone F, et al. Dendritic cells transfected with tumor RNA for the induction of antitumor CTL in colorectal cancer. Cancer Gene Ther. 2003 Mar;10(3):209-14.

[79] He XZ, Wang L, Zhang YY. An effective vaccine against colon cancer in mice: use of recombinant adenovirus interleukin-12 transduced dendritic cells. World J Gastroenterol. 2008 Jan 28;14(4):532-40.

[80] Hatfield P, Merrick AE, West E, O'Donnell D, Selby P, Vile R, et al. Optimization of dendritic cell loading with tumor cell lysates for cancer immunotherapy. J Immunother. 2008 Sep;31(7):620-32.

[81] Kaneno R, Shurin GV, Tourkova IL, Shurin MR. Chemomodulation of human dendritic cell function by antineoplastic agents in low noncytotoxic concentrations. J Transl Med. 2009;7:58.

[82] Roux S, Apetoh L, Chalmin F, Ladoire S, Mignot G, Puig PE, et al. CD4+CD25+ Tregs control the TRAIL-dependent cytotoxicity of tumor-infiltrating DCs in rodent models of colon cancer. J Clin Invest. 2008 Nov;118(11):3751-61.

[83] Larmonier N, Merino D, Nicolas A, Cathelin D, Besson A, Bateman A, et al. Apoptotic, necrotic, or fused tumor cells: an equivalent source of antigen for dendritic cell loading. Apoptosis. 2006 Sep;11(9):1513-24.

[84] DeMatos P, Abdel-Wahab Z, Vervaert C, Seigler HF. Vaccination with dendritic cells inhibits the growth of hepatic metastases in B6 mice. Cell Immunol. 1998 Apr 10;185(1):65-74.

[85] Jack AM, Aydin N, Montenegro G, Alam K, Wallack M. A novel dendritic cell-based cancer vaccine produces promising results in a syngenic CC-36 murine colon adenocarcinoma model. J Surg Res. 2007 May 15;139(2):164-9.

[86] Dai S, Wan T, Wang B, Zhou X, Xiu F, Chen T, et al. More efficient induction of HLA-A*0201-restricted and carcinoembryonic antigen (CEA)-specific CTL response by immunization with exosomes prepared from heat-stressed CEA-positive tumor cells. Clin Cancer Res. 2005 Oct 15;11(20):7554-63.

[87] Qiu J, Li GW, Sui YF, Song HP, Si SY, Ge W. Heat-shocked tumor cell lysate-pulsed dendritic cells induce effective anti-tumor immune response in vivo. World J Gastroenterol. 2006 Jan 21;12(3):473-8.

[88] Matera L, Forno S, Galetto A, Moro F, Garetto S, Mussa A. Increased expression of HSP70 by colon cancer cells is not always associated with access to the dendritic cell cross-presentation pathway. Cell Mol Biol Lett. 2007;12(2):268-79.

[89] Rossowska J, Pajtasz-Piasecka E, Szyda A, Zietara N, Dus D. Tissue localization of tumor antigen-loaded mouse dendritic cells applied as an anti-tumor vaccine and their influence on immune response. Folia Histochem Cytobiol. 2007;45(4):349-55.

[90] Yasuda T, Kamigaki T, Nakamura T, Imanishi T, Hayashi S, Kawasaki K, et al. Dendritic cell-tumor cell hybrids enhance the induction of cytotoxic T lymphocytes against murine colon cancer: a comparative analysis of antigen loading methods for the vaccination of immunotherapeutic dendritic cells. Oncol Rep. 2006 Dec;16(6):1317-24.

[91] Kao JY, Zhang M, Chen CM, Pierzchala A, Chen JJ. Aberrant T helper cell response in tumor-bearing mice limits the efficacy of dendritic cell vaccine. Immunol Lett. 2006 May 15;105(1):16-25.

[92] Xu F, Ye YJ, Cui ZR, Wang S. Allogeneic dendritomas induce anti-tumour immunity against metastatic colon cancer. Scand J Immunol. 2005 Apr;61(4):364-9.

[93] Yasuda T, Kamigaki T, Kawasaki K, Nakamura T, Yamamoto M, Kanemitsu K, et al. Superior anti-tumor protection and therapeutic efficacy of vaccination with allogeneic and semiallogeneic dendritic cell/tumor cell fusion hybrids for murine colon adenocarcinoma. Cancer Immunol Immunother. 2007 Jul;56(7):1025-36.

[94] Pajtasz-Piasecka E, Rossowska J, Dus D, Weber-Dabrowska B, Zablocka A, Gorski A. Bacteriophages support anti-tumor response initiated by DC-based vaccine against murine transplantable colon carcinoma. Immunol Lett. 2008 Feb 15;116(1):24-32.

[95] Kikuchi T, Moore MA, Crystal RG. Dendritic cells modified to express CD40 ligand elicit therapeutic immunity against preexisting murine tumors. Blood. 2000 Jul 1;96(1):91-9.

[96] Eppler E, Horig H, Kaufman HL, Groscurth P, Filgueira L. Carcinoembryonic antigen (CEA) presentation and specific T cell-priming by human dendritic cells transfected with CEA-mRNA. Eur J Cancer. 2002 Jan;38(1):184-93.

[97] Ojima E, Inoue Y, Watanabe H, Hiro J, Toiyama Y, Miki C, et al. The optimal schedule for 5-fluorouracil radiosensitization in colon cancer cell lines. Oncol Rep. 2006 Nov;16(5):1085-91.

[98] Nair SK, Boczkowski D, Morse M, Cumming RI, Lyerly HK, Gilboa E. Induction of primary carcinoembryonic antigen (CEA)-specific cytotoxic T lymphocytes in vitro using human dendritic cells transfected with RNA. Nat Biotechnol. 1998 Apr;16(4):364-9.

[99] Morse MA, Nair SK, Mosca PJ, Hobeika AC, Clay TM, Deng Y, et al. Immunotherapy with autologous, human dendritic cells transfected with carcinoembryonic antigen mRNA. Cancer Invest. 2003 Jun;21(3):341-9.

[100] Bergant M, Meden L, Repnik U, Sojar V, Stanisavljevic D, Jeras M. Preparation of native and amplified tumour RNA for dendritic cell transfection and generation of in vitro anti-tumour CTL responses. Immunobiology. 2006;211(3):179-89.

[101] Ojima T, Iwahashi M, Nakamura M, Matsuda K, Naka T, Nakamori M, et al. The boosting effect of co-transduction with cytokine genes on cancer vaccine therapy using genetically modified dendritic cells expressing tumor-associated antigen. Int J Oncol. 2006 Apr;28(4):947-53.

[102] Saha A, Chatterjee SK, Foon KA, Bhattacharya-Chatterjee M. Anti-idiotype antibody induced cellular immunity in mice transgenic for human carcinoembryonic antigen. Immunology. 2006 Aug;118(4):483-96.

[103] Rains N, Cannan RJ, Chen W, Stubbs RS. Development of a dendritic cell (DC)-based vaccine for patients with advanced colorectal cancer. Hepato-gastroenterology. 2001 Mar-Apr;48(38):347-51.

[104] Boczkowski D, Nair SK, Nam JH, Lyerly HK, Gilboa E. Induction of tumor immunity and cytotoxic T lymphocyte responses using dendritic cells transfected with messenger RNA amplified from tumor cells. Cancer Res. 2000 Feb 15;60(4):1028-34.

[105] Kim SG, Park MY, Kim CH, Sohn HJ, Kim HS, Park JS, et al. Modification of CEA with both CRT and TAT PTD induces potent anti-tumor immune responses in RNA-pulsed DC vaccination. Vaccine. 2008 Nov 25;26(50):6433-40.

[106] Nair SK, Morse M, Boczkowski D, Cumming RI, Vasovic L, Gilboa E, et al. Induction of tumor-specific cytotoxic T lymphocytes in cancer patients by autologous tumor RNA-transfected dendritic cells. Ann Surg. 2002 Apr;235(4):540-9.

[107] Lechner MG, Karimi SS, Barry-Holson K, Angell TE, Murphy KA, Church CH, et al. Immunogenicity of murine solid tumor models as a defining feature of in vivo behavior and response to immunotherapy. J Immunother. Nov-Dec;36(9):477-89.

Capítulo 2

Artigo - Transfection of murine dendritic cells with RNA from colon cancer cells pretreated with 5-fluorouracil activate DC and increases IFN-g production

Transfection of murine dendritic cells with RNA from colon cancer cells pretreated with 5-fluorouracil activate DC and increases INF-g production

Marcela R de Camargo[1,2], Carolina M Gorgulho[1,2], Cecília P Rodrigues[2], Marcimara Penitenti, Juliana C F Longo[1,2], Fabiana A Zambuzi[2], Victória E Galvão[2], Ramon Kaneno[1,2*].

[1]Department of Pathology, School of Medicine of Botucatu, UNESP – Univ Estadual Paulista; [2]Department of Microbiology and Imunology, Institute of Biosciences of Botucatu, UNESP – Univ Estadual Paulista, Botucatu, SP, Brasil

*Correspondence author: Departamento de Microbiologia e Imunologia, Instituto de Biociências - UNESP, Cx. Postal 510, Distr. Rubião Júnior s/n, 18618-970 - Botucatu, São Paulo, Brazil, Fax: +55-1438153744; phone: +55-1438800432 e-mail: rskaneno@yahoo.com.br

Research area: Tumor Immunology

ABSTRACT

We have recently observed that treatment of colon tumor cells with low concentration of paclitaxel increased the expression of several genes associated with antigen-presenting machinery. Since 5-fluoruracil (5-FU) is the main antineoplastic agent for colon cancer, in this study we aimed to evaluate immunomodulatory properties analyzing: a) if the DC transfection with drug-treated tumor cells RNA, enhances the effectiveness of DC-based vaccine; b) if the modulatory effects of vaccine can be observed *in vivo*, and c) if the combination of DC with low dose chemotherapy schedule improves the antitumor responsiveness. To achieve these goals s.c. MC-38 bearing C57/Bl-6 mice were treated with DC sensitized with RNA from tumor cells pre-treated with the minimum effective concentration (MEC) of 5-fluorouracil. Our results of studies show that vaccination with tumor RNA-transfected DC delays the tumor growth, increases the percentage of $CD86^+$ (35%) $CD40^+$ (63%) and MHC class II^+ (47%) DC and significantly increases the *in vitro* production of IFN-ɣ. These results suggest that treatment of tumor cells with 5-FU induces transcriptional changes that can be transfered to DC by RNA transfection, enhancing their ability to stimulate the antitumor response

Keywords: chemotherapy; colorrectal cancer; dendritic cell; immunomodulation; vaccine.

1. INTRODUCTION

Colorectal cancer (CRC) ranks the third place in cancer frequency in the world, and is the second cause of mortality among cancer patients in USA (1, 2). Malignant transformation of intestinal epithelial cells is has different causes, including genetic factors, exposition to food and environmental compounds and previous inflammatory diseases such as Crohn´s disease and colites (3-5). The incidence of CRC in Crohn's disease patients, is about 20 times higher than in subjects free of this condition and, it develops on average 20 years earlier in those patients than in the general population (5, 6). CRC leads to the development of both local and systemic immune response that may delay tumor progression (7, 8). Special attention is paid to cytotoxic T cells that specifically recognize and kill tumor cells in a MHC-restricted fashion (7, 9). Dendritic cells (DCs) are essential for the establishment of this specific antitumor immune response, but in most CRC patients, these cells are suppressed or remain poorly differentiated in the tumor site, whose density is three times lower than in normal colonic mucosa. Expressions of DCs activation markers or co-stimulatory structures are also in lower frequency, especially in metastatic tumors (10).

Examination of the relationship between the density of DCs and intratumoral cytokine expression, shows that tumors with high proportion of infiltrating T cells expressing TNF, have a higher number of mature DCs (10), indicating the role of this cytokine in the maturation and antigen presentation function. The DC:CD4 and DC:CD8 interactions in the tumor border, is related with a better prognosis and reduced metastasis in CRC patients, reinforcing the view of the central role of DCs in control of antitumor immunity (11, 12). Thus, treatments for patients with CRC should be conducted to restore immune system ability of recognition and specific reaction against tumor antigens. In this scenario, DCs

are described as an important tool for immunotherapy against cancer (Hafid, 2010), especially through therapeutic vaccines development (DC-vax), prepared with DC by different protocols.

One of the approaches for its generation is transfection of tumor nucleic acids into DC, as previously reported by Nencioni *et al* (2003) (9) and Boczkowski *et al*. (2000) (13) who achieved a protective immunity against CRC in tumor-bearing mice, using tumor RNA. We have previously observed that DCs can be modulated *in vitro* by exposure to ultralow concentrations of antineoplastic agents (14). Thus, although drugs in such concentrations are not able to induce apoptosis or necrosis of tumor cells, they can modulate the production of cytokines at the tumor microenvironment, and stimulate the *in vitro* maturation and function of DC (15, 16). Recently, we have observed that sensitization of human DCs with tumor lysate is enhanced by previous incubation with low concentration of Paclitaxel (PAC) (14). In addition, the pre-treatment of tumor cells with low concentrations of PAC, increases their immunogenicity, facilitating the generation of specific cytotoxic T lymphocytes (CTL) (17). In the clinical field some centers propose the metronomic chemotherapy - based on the more frequent administration of moderate doses of chemotherapeutic agents - that contrasts with the maximal tolerable dose treatment, which are usually followed by severe side effects (18).

Aiming to continue this line of investigation, we consider that it is necessary to *in vivo* evaluated the feasibility of chemoimmnomodulation. Since 5-fluoruracil (5-FU) is the main antineoplastic agent for colon cancer, this project was designed to evaluate the imunomodulatory role of DC transfected with RNA from tumor cells, pre-treated with the minimum effective dose of 5-fluorouracil. Our results showed the transfection increases the CD40, CD86 and MHCII expression on DC, and that animals treated with vaccine *in vitro* produced higher levels of IFN-γ.

2. MATERIAL AND METHODS

2.1 Animals

C57Bl/6 mice from the animal Facility at University of São Paulo, Ribeirão Preto, were separated into four groups of four animals and maintained at the Department of Pathology, Faculty of Medicine of Botucatu - UNESP, under controlled temperature and humidity, water and food supply *ad libitum* and 12/12 h light/dark periods. All the proceedings followed the rules of Brazilian College of Animal Experimentation and were approved by the local Ethics Committee.

2.2 MC-38 cells maintenance and treatment with 5-fluorouracil

Murine colorrectal tumor MC-38 cells were maintained in complete culture medium (10% fetal bovine serum, 2 mM L-glutamine, 1% nonessential amino acids, 25 mM HEPES, sodium pyruvate and 40mg/l gentamycin) at 37 ° C, under 5% CO^2 for 24-72 h. Cells were detached by treatment with trypsin for 3 min and washed with complete medium or PBS (when used to obtain RNA).

MC -38 cells at $2x10^4$cells/ml were cultured in a 96 flat bottoned well culture plate with different concentrations of 5-fluorouracil and 0.5 mM corresponded to a citostatic concentration, called by us as minimum effective concentration (MEC).

2.3Tumor RNA extraction

Forty million cells were cultured for 72h with the minimum effective concentration of 5-FU. Cells were detached with trypsin, washed with PBS and the pellet stored at -80^0C. Total RNA was isolated from tumor cell culture after TRIZOL extraction using RNeasy

TM Maxi anion-exchange spin columns (Qiagen) according to the protocol provided by the manufacturer and quantified by spectrophotometry at NanoVue (GE Healthcare).

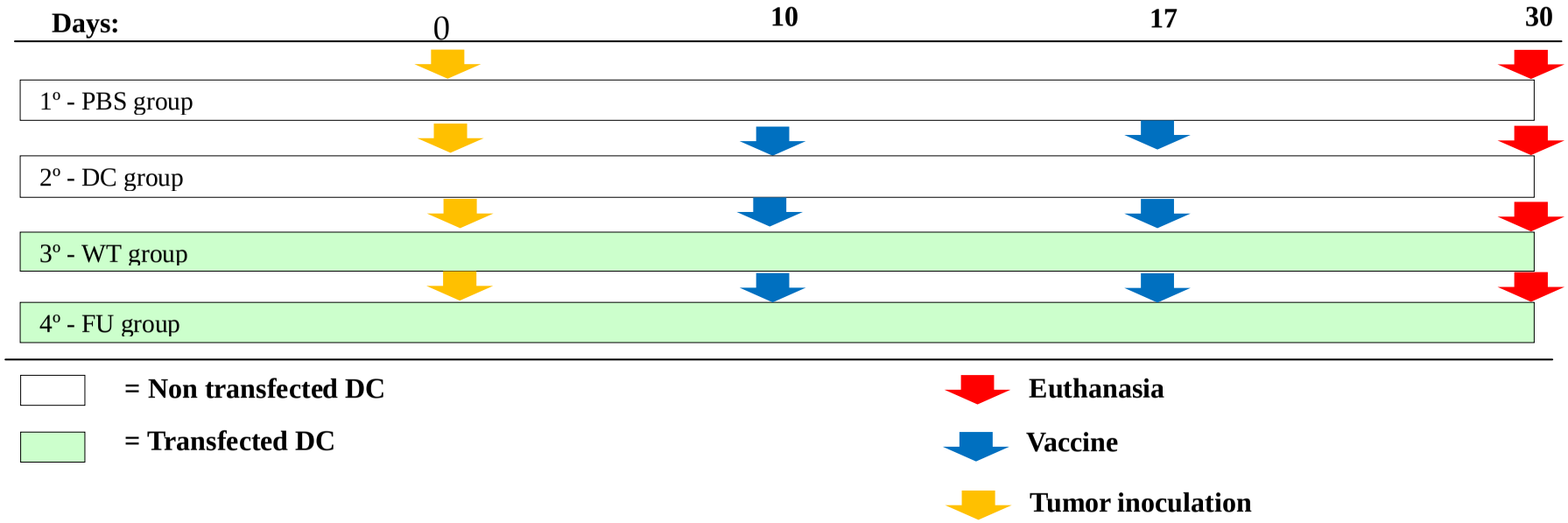
2.4. Preparation of DCs suspension

Bone marrow was collected from the tibiae and femurs of healthy mice for the generation of myeloid DC. Erythrocytes were lysed with hemolytic solution and T and B lymphocytes were depleted with anti-CD4, CD8, and B220 antibodies plus complement. Cells were cultured in 6-well culture plates with 80 ng/ml of GM-CSF and IL-4 at 37ºC in a 5% CO_2 chamber. After 6 days, immature DCs were collected, washed with fresh RPMI, adjusted to $2.4x10^6$/ml and resuspended in AIM-V medium. DCs were transfected (lipotransfection) with tumor total RNA (5µg/10^6cells) using TransMessenger Kit (Qiagen) adapted for murine dendritic cells. After 24 h, DCs preparations were stained and analyzed by flow citometry (FACS Calibur) for surface expression of MHC class II molecules, co-stimulatory (CD80), and maturation marker (CD40) (Biologend antibodies) . As control, we used non transfected dendritic cells (DC) and DCs transfected with RNA from wild type MC-38 cells (WT) or just PBS.

2.5 Animal treatment

Transfected DCs were harvested, washed with PBS and adjusted to 10^7 cells/ml for s.c. inoculation in tumor site. Animals were treated with about 10^6DC/100µl administered by the 10^{th} and 17^{th} days after tumor implantation. Freshly prepared DC were inoculated at the first application and frozen cells were inoculated at the second.

Experimental Design



- **PBS:** untreated tumor-bearing mice
- **DC:** non transfected dendritic cells
- **WT:** dendritic cells transfected with RNA from wild type tumor cells
- **FU:** dendritic cells transfected with RNA from tumor cells pre-treated with 5-fluorouracil.

2.6 Euthanasia and material collection

Animals were euthanized in a CO_2 chamber thirty days after tumor inoculation for spleen and tumor mass excision. A small tumor fragment of each animal was fixed in 10% formalin for 48 h, washed in running water and kept in 70% ethanol for subsequent histological processing.

2.7 Mixed lymphocyte reaction (MLR)

For mixed lymphocyte reaction, allogeneic T lymphocytes were obtained from BALB/C mice spleens. Spleen cells were ressuspended in complete medium and incubated in a nylon wool chamber for 30 minutes. The column was then washed with 15ml of complete medium to remove non-adherent lymphocytes. T lymphocyte enriched suspensions was set to 10^6 cells/ml and cryopreserved.

In order to verify the ability of DCs to promote proliferation of allogeneic T lymphocyte, co-cultures were prepared with the following DCs:

- FU: DCs transfected with RNA from MC-38 treated with minimal effective concentration of 5-fluorouracil.
- WT: DCs transfected with RNA from wild type MC-38 cells.
- DC: non-transfected DCs were used as controls.

DCs were adjusted to 10^6cells/ml and distributed into 96 flat-bottomed well plates and co-cultured with allogeneic T lymphocytes at 1:1, 1:3, 1:10, 1: 30, 1:100 and 1:300 DC:T ratio. The culture was incubated for 4 days under an atmosphere of 5% CO^2 at 37° C for further analysis by MTT assay.

2.8 Cytokine determination

Development of specific antitumor T lymphocytes by tumor-bearing mice was measured by the ability of spleen cells to produce IFN- ɣ following contact with tumor target cells. Then, spleen cells were co-cultured with MC-38 cells and supernatant were collected 24 h later and frozen for further analysis. Levels of IFN-ɣ and IL-10 were measured by ELISA MAX Deluxe Set, BioLegend according to the instructions of the manufacturer.

2.9 Histological Analysis

The tumors mass were excised, fixed in formalin (10% formaldehyde) for 48 h, washed in running water for 24 h and kept in ethanol 70% until their inclusion in paraffin. Samples were dyied by H&E for morphological observation.

2.10 Statistical Analysis

Groups were tested for homogeneity of variances (Bartlett test) and analyzed by ANOVA statistical test using Graph pad Instat statistical program. Differences among the groups were considered significant when $p < 0.05$.

3. RESULTS

3.1 Effect of transfection on DC phenotype

Flow citometry analyses of lipotransfected cells revelated an increase of 63% for CD 40^{+}, 35% for $CD86^{+}$ and 47% for $MHCII^{+}$ in the expression of 5-FU group cells compared to DC group (Fig 1). This positive effect shows that previous exposure of the tumor cells to 5-FU, associated with the DCs lipotransfection RNA for construction of the vaccine is able to increase the expression of all markers analyzed in most of the groups and transfection with RNA of wild type tumor cells (WT) did not induce the same phenomenon.

Table 1: Flow citometry analysis of transfected (WT and FU) and no-transfected dendritic cells

Cells	% $CD11c^{+}/CD40^{+}$	MFI of $CD11c^{+}/CD40^{+}$	% $CD11c^{+}/CD86^{+}$	MFI of $CD11c^{+}/CD86^{+}$	% $CD11c^{+}/MHCII^{+}$	MFI of $CD11c^{+}/MHCII^{+}$
DC	37,665	48,1	39,825	215,67	51,29	312,60
WT	49,71	34,2	46,8	109,18	75,11	201,76
FU	61,395	35,71	53,44	85,65	75,345	210,79

Phenotype of transfected (WT and FU) and no-transfected dendritic cells (DC) after 6 days of culture. Results expressed as percentage of total cells and mean of fluorescence intensity (MFI). Mean ± SD of two independent experiments.

3.2 Mixed Lymphocyte Reaction

When the ability of DCs to stimulate allogeneic lymphocytes in culture was evaluated, DC transfected with tumor RNA showed a harmed antigen presenting function compared with control DC. This effect seems to be not due to the pretreatment of the tumor cells with 5-FU, since the same cells transfected with RNA from untreated tumor (WT) was also effective for promoting lymphocyte proliferation (Fig 1). Non transfected DC promoted the highest allogeneic proliferative response, indicating that transfection of tumor RNA into DC was not able to improve their ability to promote alloresponse by lymphocytes.

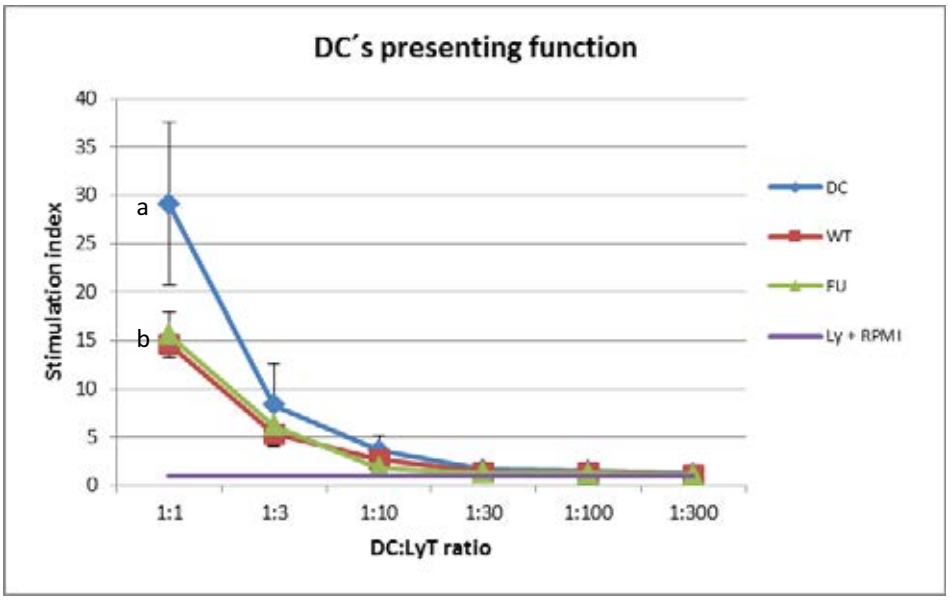


Fig 1: Ability of transfected (WT and FU) and no-transfected dendritic cells (DC) after 6 days of culture on T cells proliferation. Cells co-cultured with spleen cells from allogeneic mice. Average of two experiments performed independently. Data expressed as stimulation index and analyzed by ANOVA and Tukey-Kramer Multiple Comparisons Test, $a \neq b$, $p < 0.05$. $N = 8$.

3.3 *In vitro* production of IFN-ɣ and IL-10

Quantification of cytokines in the supernatant of spleen cells co-cultured with MC-38 cells, showed that the FU vaccine is more effective *in vivo* since it generate an specific anti tumor T lymphocyte response, as evidenced by IFN-g production (Fig 2).

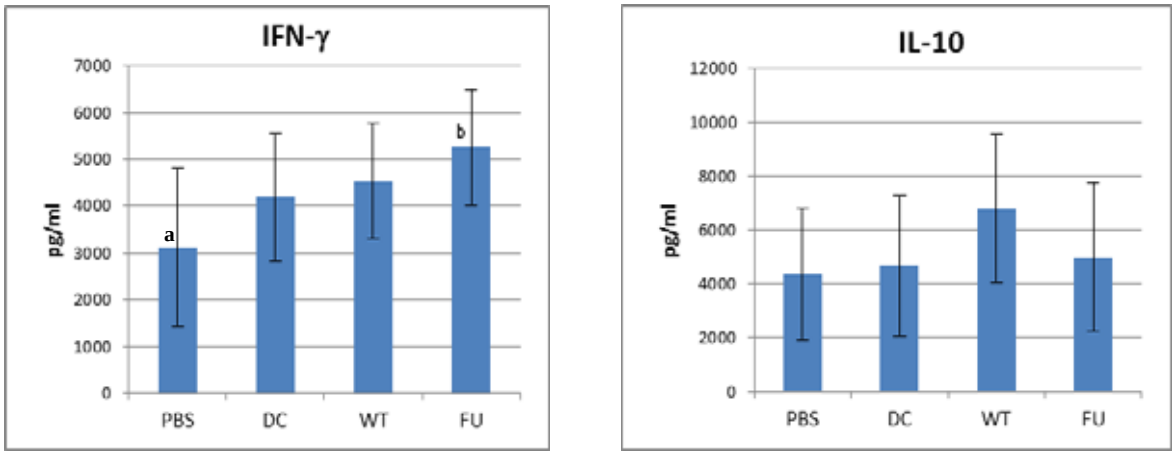


Fig 2: *In vitro* IFN-ɣ and IL-10 levels at the supernatants of spleen cells co-cultured with MC-38 by 24h and analyzed by ELISA. Mean of two experiments performed independently. Statistical analysis by ANOVA and Tukey-Kramer Multiple Comparisons Test, $a \neq b$, $p < 0.05$. $N = 8$.

3.4 Tumor growth

Measurement of the tumor area of each animal was performed once a week for 30 days to calculate the tumor growth, that was significantly decreased by inoculation of DC, independently of it sensibilization with tumor RNA. In fact, tumor growth in DC, WT and FU groups was lower than in PBS control, but there was no difference among the experimental groups (Fig.3).

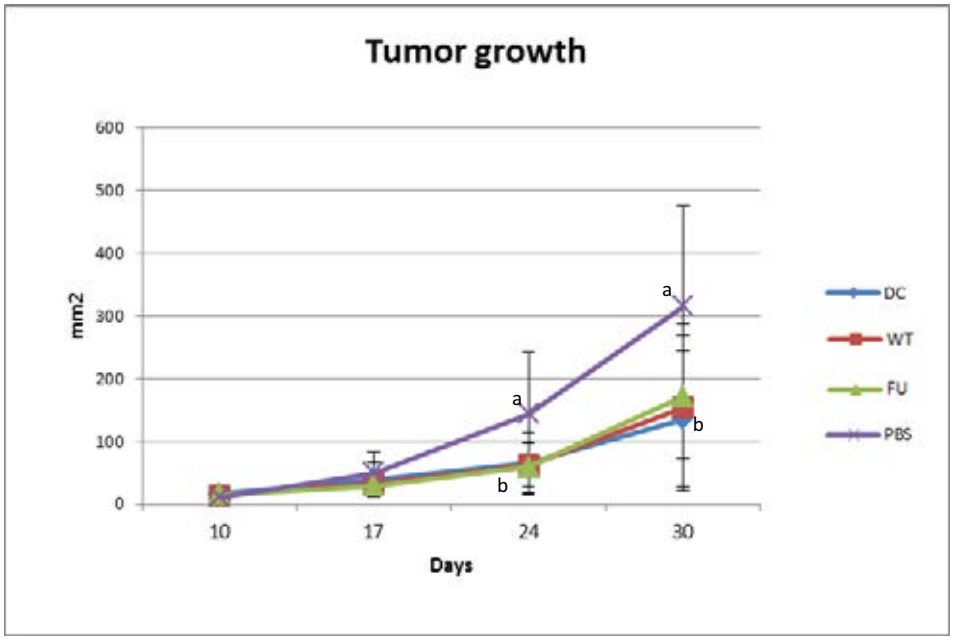


Fig 3: Effect of dendritic cell vaccine on tumor growth. Once a week measurement of tumor area during 30 days. Mean ± SD of two experiments performed independently. Statistical analysis by ANOVA and Tukey-Kramer Multiple Comparisons Test, $a \neq b$, $p < 0.05$. $N = 8$.

3.5 Histopatological Analysis of tumor growth

Histopatological analysis showed extensive areas of necrosis, ulceration and diffuse inflammatory infiltration in tumor tissue of PBS group. FU group showed rare mitotic figures and high amount of cells with morphology of PMN cells. Inflammatory infiltration was predominantly around the vessels and diffusely spread over necrotic areas (Fig.4). Despite the rich PMN infiltration the animals of this group showed less frequency of tumor ulceration.

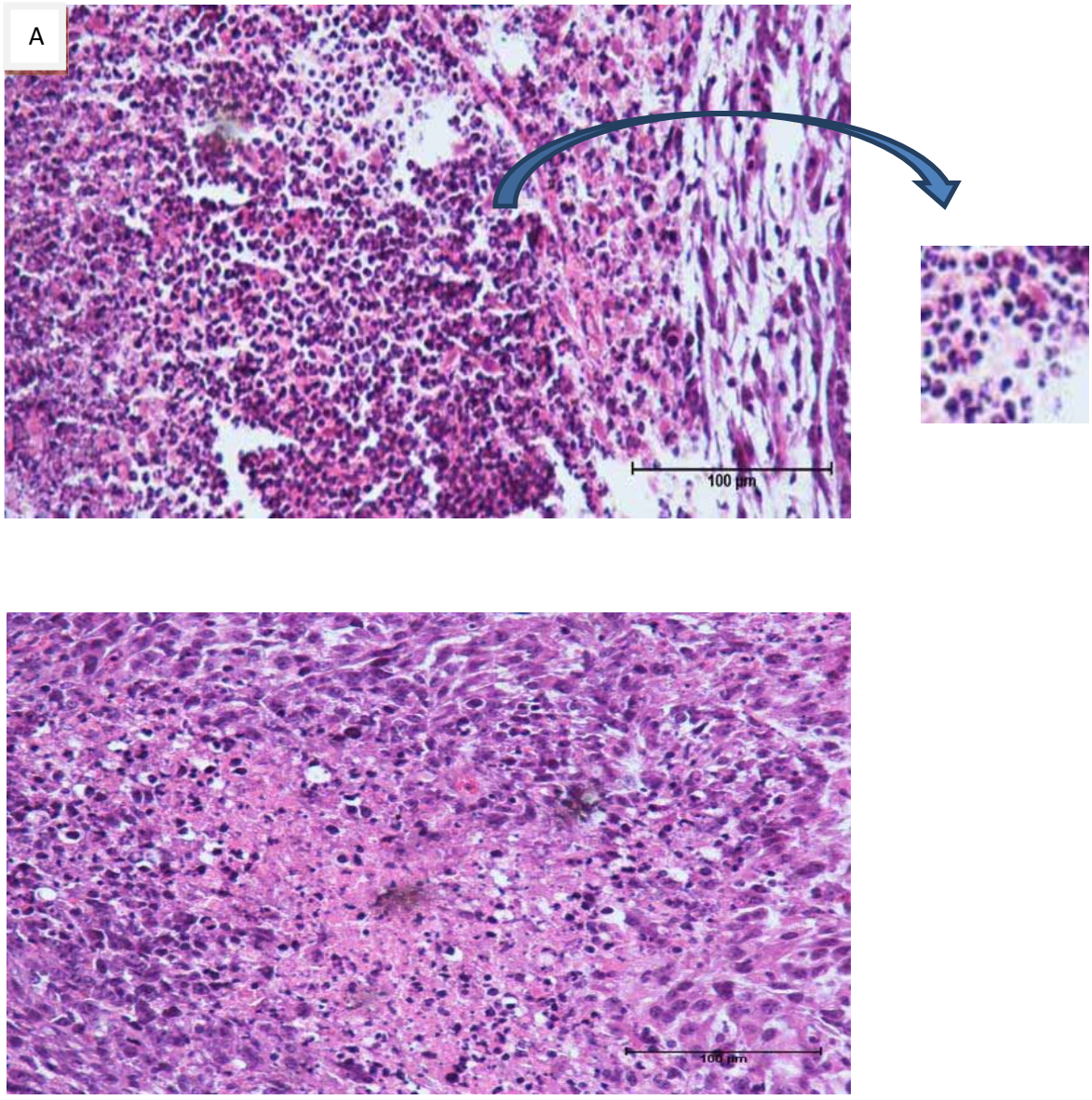


Fig 4: **A:** Inflammatory infiltration at the periphery of necrotic area of the tumor, growing in animals treated with DC transfected with MC-38 RNA pre-treated with 5-FU. **B:** Necrotic area of the tumor, growing in animals treated with PBS.

4. DISCUSSION

Previous studies of our group showing that low concentration of paclitaxel (PAC), is able to increase the immunogenicity of colon cancer cells (17), and increase maturation and functions of human DCs, (14, 16), stimulated us to investigate the feasibility of *in vivo* modulation of antitumor resistance. In the present study we observed that RNA of MC-38 cells pretreated with minimum effective concentration of 5-FU, increases the expression of CD40, CD86 and MHCII expression when transfected in DCs. Increased expression of MHC molecules, is one of the main features of DC maturation (19, 20), as well as upregulation of co-stimulatory molecules such as CD40 and CD80 on the cell surface. This upregulation is necessary for activation of T-cells at draining lymph nodes and initiate immune responses (21-23). Mature DCs are able to migrate more rapidly to secondary lymphoid organs, where they can rapidly initiate anti-tumor T-cell responses (24, 25). On the other hand, splenocytes from animals treated with vaccine showed higher production of IFN-γ, a cytokine that promote cell-mediated responses, which play a key role against tumor (26, 27). The increase in expression of cell surface molecules and a significative pro-inflamatory cytokine production, suggests that our transfection facilitate the maturation and activation of DC, thus improving the antigen presenting ability. It supports our concept that treating tumor cells with drugs before extracting RNA for transfection becomes the vaccine more efficient to fight colorectal cancer cells. However, significant increase was observed in the MLR activities of non transfected group. We observed an effective lymphoproliferative response to control untransfected DC, but not at RNA transfected groups (WT or Fu-MEC). At the same time, observing intensity of fluoresce mean of the results, we found that our control group DC presented more fluorescence intensity for CD40, CD86 and MHCII than transfected groups demonstrating that an upregulation on cell surface receptors enhance the ability of DCs to activate T cells (28-30). According to

(31). In this line, our *in vivo* results showed no difference on antitumor activity among transfected and no transfected DC preparations, suggesting that the transfection methodology may affect vaccine efficiency. Furthermore, we found the presence of PMNs morphologically-like cells around necrosis area of vaccine treated animals. Activity of neutrophils is classically considered a tumor enhancing factor, especially when these cells are associated with chronical inflammatory responses (32). Tumor immunosuppression can be directed by many factors, but specially throw myeloid cells, including myeloid derived suppressor cells (MDSC), and tumor-associated neutrophils (TAN) (33, 34). These myeloid cells have been shown to promote tumor progression by direct immune suppression and by production of angiogenic factors, matrix-degrading enzymes and growth factors (32). However, neutrophils are morphologically similar to myeloid-derived suppressor cells (MDSC), a group of cells with potent immunosuppressive activity (35). These cells are generated from bone marrow precursor cells during the inflammatory activity of tumor microenvironment and have the ability to alter the antigen-specific responses of effector lymphocytes (36, 37). If these infiltrating polymorphonuclear cells are MDSC, instead of neutrophils, we hypothesize that these tumor antigen loaded DC are modulated *in situ*, even showing stimulatory features *in vitro.* Therefore, further studies are required to clear the actual role of chemomodulated DC on the development of MDSC.

5. CONCLUSION

Our results indicate that transfection induces phenotypic and *in vitro* functional changes in DCs used for vaccination, but were not able to *in vivo* significantly decrease the tumor growth.

6. REFERENCES

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA: a cancer journal for clinicians. 2011;61(2):69-90.

2. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. CA: a cancer journal for clinicians. 2012;62(1):10-29.

3. Lynch HT, Lynch JF, Lynch PM. Toward a consensus in molecular diagnosis of hereditary nonpolyposis colorectal cancer (Lynch syndrome). J Natl Cancer Inst. 2007;99(4):261-3.

4. Li L, Zhang S, Zhang Y, Yu B, Xu Y, Guan Z. Paracrine action mediate the antifibrotic effect of transplanted mesenchymal stem cells in a rat model of global heart failure. Molecular biology reports. 2009;36(4):725-31.

5. Harpaz N, Talbot IC. Colorectal cancer in idiopathic inflammatory bowel disease. Seminars in diagnostic pathology. 1996;13(4):339-57.

6. Brackmann S AS, Aamodt G, Langmark F, Clausen OP, Aadland E, Fausa O, Rydning A, Vatn MH. Relationship between clinical parameters and the colitiscolorectal cancer interval in a cohort of patients with colorectal cancer in inflammatory bowel disease. 2009;44:46-55.

7. Yu P, Fu YX. Tumor-infiltrating T lymphocytes: friends or foes? Lab Invest. 2006;86(3):231-45.

8. Novellino L, Castelli C, Parmiani G. A listing of human tumor antigens recognized by T cells: March 2004 update. Cancer Immunol Immunother. 2005;54(3):187-207.

9. Nencioni A, Muller MR, Grunebach F, Garuti A, Mingari MC, Patrone F, et al. Dendritic cells transfected with tumor RNA for the induction of antitumor CTL in colorectal cancer. Cancer Gene Ther. 2003;10(3):209-14.

10. Schwaab T, Weiss JE, Schned AR, Barth RJ, Jr. Dendritic cell infiltration in colon cancer. J Immunother. 2001;24(2):130-7.

11. Dadabayev AR, Sandel MH, Menon AG, Morreau H, Melief CJ, Offringa R, et al. Dendritic cells in colorectal cancer correlate with other tumor-infiltrating immune cells. Cancer Immunol Immunother. 2004;53(11):978-86.

12. Takagi S, Miyagawa S, Ichikawa E, Soeda J, Miwa S, Miyagawa Y, et al. Dendritic cells, T-cell infiltration, and Grp94 expression in cholangiocellular carcinoma. Human pathology. 2004;35(7):881-6.

13. Boczkowski D, Nair SK, Nam JH, Lyerly HK, Gilboa E. Induction of tumor immunity and cytotoxic T lymphocyte responses using dendritic cells transfected with messenger RNA amplified from tumor cells. Cancer Res. 2000;60(4):1028-34.

14. Kaneno R, Shurin GV, Tourkova IL, Shurin MR. Chemomodulation of human dendritic cell function by antineoplastic agents in low noncytotoxic concentrations. J Transl Med. 2009;7:58.

15. Zhong H, Han B, Tourkova IL, Lokshin A, Rosenbloom A, Shurin MR, et al. Low-dose paclitaxel prior to intratumoral dendritic cell vaccine modulates intratumoral cytokine network and lung cancer growth. Clin Cancer Res. 2007;13(18 Pt 1):5455-62.

16. Shurin GV, Tourkova IL, Kaneno R, Shurin MR. Chemotherapeutic agents in noncytotoxic concentrations increase antigen presentation by dendritic cells via an IL-12-dependent mechanism. J Immunol. 2009;183(1):137-44.

17. Kaneno R, Shurin GV, Kaneno FM, Naiditch H, Luo J, Shurin MR. Chemotherapeutic agents in low noncytotoxic concentrations increase immunogenicity of human colon cancer cells. Cellular oncology (Dordrecht). 2011

34(2):97-106.

18. Valentini AM, Armentano R, Pirrelli M, Caruso ML. Chemotherapeutic agents for colorectal cancer with a defective mismatch repair system: the state of the art. Cancer treatment reviews. 2006;32(8):607-18.

19. Gilboa E. The makings of a tumor rejection antigen. Immunity. 1999;11(3):263-70.

20. Wang RF, Wang X, Atwood AC, Topalian SL, Rosenberg SA. Cloning genes encoding MHC class II-restricted antigens: mutated CDC27 as a tumor antigen. Science. 1999;284(5418):1351-4.

21. Sallusto F CM, Danieli C, Lanzavecchia Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. J Exp Med. 2005;182:389-400.

22. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature. 1998;392(6673):245-52.

23. Pollara G, Kwan A, Newton PJ, Handley ME, Chain BM, Katz DR. Dendritic cells in viral pathogenesis: protective or defective? International journal of experimental pathology. 2005;86(4):187-204.

24. Steinman RM. The dendritic cell system and its role in immunogenicity. Annual review of immunology. 1991;9:271-96.

25. Herr W, Ranieri E, Olson W, Zarour H, Gesualdo L, Storkus WJ. Mature dendritic cells pulsed with freeze-thaw cell lysates define an effective in vitro vaccine designed to elicit EBV-specific CD4(+) and CD8(+) T lymphocyte responses. Blood. 2000;96(5):1857-64.

26. Sato T, Terai M, Yasuda R, Watanabe R, Berd D, Mastrangelo MJ, et al. Combination of monocyte-derived dendritic cells and activated T cells which express CD40 ligand: a new approach to cancer immunotherapy. Cancer Immunol Immunother. 2004;53(1):53-61.

27. Wu RS, Kobie JJ, Besselsen DG, Fong TC, Mack VD, McEarchern JA, et al. Comparative analysis of IFN-gamma B7.1 and antisense TGF-beta gene transfer on the tumorigenicity of a poorly immunogenic metastatic mammary carcinoma. Cancer Immunol Immunother. 2001;50(5):229-40.

28. Lin CL, Suri RM, Rahdon RA, Austyn JM, Roake JA. Dendritic cell chemotaxis and transendothelial migration are induced by distinct chemokines and are regulated on maturation. Eur J Immunol. 1998;28(12):4114-22.

29. Sallusto F, Schaerli P, Loetscher P, Schaniel C, Lenig D, Mackay CR, et al. Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. Eur J Immunol. 1998;28(9):2760-9.

30. Sozzani S. Dendritic cell trafficking: more than just chemokines. Cytokine & growth factor reviews. 2005;16(6):581-92.

31. Grunebach F, Muller MR, Brossart P. New developments in dendritic cell-based vaccinations: RNA translated into clinics. Cancer Immunol Immunother. 2005;54(6):517-25.

32. Balkwill F, Coussens LM. Cancer: an inflammatory link. Nature. 2004;431(7007):405-6.

33. Fridlender ZG, Albelda SM. Tumor-associated neutrophils: friend or foe? Carcinogenesis. 2012;33(5):949-55.

34. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. Nature reviews. 2012;12(4):253-68.

35. Youn JI, Collazo M, Shalova IN, Biswas SK, Gabrilovich DI. Characterization of the nature of granulocytic myeloid-derived suppressor cells in tumor-bearing mice. Journal of leukocyte biology. 2012;91(1):167-81.

36. Marigo I, Dolcetti L, Serafini P, Zanovello P, Bronte V. Tumor-induced tolerance and immune suppression by myeloid derived suppressor cells. Immunol Rev. 2008;222:162-79.

37. Bronte V. Myeloid-derived suppressor cells in inflammation: uncovering cell subsets with enhanced immunosuppressive functions. Eur J Immunol. 2009;39(10):2670-2.

Capítulo 3

Artigo - Chemoimmunomodulation by low concentration of 5-fluorouracil associated with DC vaccine against murine colorectal cancer

Chemoimmunomodulation by low concentration of 5-fluorouracil associated with DC vaccine against murine colorectal cancer

Marcela R de Camargo[1,2], Suzanne Ostrand-Rosemberg[3], Pedro Vannini, Ramon Kaneno[1,2].

[1]Department of Pathology, School of Medicine of Botucatu, UNESP – Univ Estadual Paulista; [2]Departament of Microbiology and Immunology, Institute of Biosciences of Botucatu, UNESP – Univ Estadual Paulista, Botucatu, SP, Brasil; University of Maryland Baltimore County, MD, United States.

Address for correspondence: Departamento de Microbiologia e Imunologia, Instituto de Biociências - UNESP, Cx. Postal 510, Distr. Rubião Júnior s/n, 18618-970 - Botucatu, São Paulo, Brazil, Fax: +55-1438153744; phone: +55-1438800432 e-mail: rskaneno@yahoo.com.br

Research area: Tumor Immunology

ABSTRACT

We have recently observed that human dendritic cells (DCs) treated with ultra low doses of antitumor chemotherapeutic agents gain the phenotype of mature and activated cells, and increases the antigen presentation activity. Thus, the present project was designed to evaluate whether the *in vitro* modulatory effects of chemotherapeutics also occur *in vivo* using the experimental model of murine colorectal cancer MC-38 cells. Considering that one of the schedules for antitumor chemotherapy is based on metronomic administration of antitumor agents, we *in vivo* evaluated the association of DC vaccines with the minimum effective dose of the chemotherapeutic drug 5-fluorouracil (5-FU). Then, C57BL/6 mice were subcutaneously inoculated with syngeneic MC-38 cells. Ten days later they were treated with DC vaccines associated with chemotherapeutic treatment twice a week. Thirty days after tumor implantation, animals were evaluated for the infiltration of MDSC, macrophages, and T cell subsets in the tumor site. The results showed that combination of DC vaccine with low dose 5-FU induced tumor regression in 77% of the animals of this group and induced the lowest number of circulating MDSC. Our results suggest that low dose 5-FU enhances the effectivity of DC vaccine to fight tumor development.

Keywords: chemotherapy; colorrectal cancer; dendritic cell; immunomodulation; vaccine.

1. INTRODUCTION

The incidence and mortality by colon cancer reach the whole world and the highest incidence rates are found in Australia and New Zealand, Europe, and North America (1). The standard treatment has been 5-fluorouracil (5-FU) chemotherapy alone, but additional treatment options, including combination chemotherapies and immunotherapies have been used (2, 3).

Therapeutic dendritic cell (DC) vaccination against cancer is a strategy for activating the immune system to recognize and destroy tumor cells (4). Since DCs are able to activate naive $CD4^+$ T helper cells and $CD8^+$ cytotoxic T lymphocytes (5). Patients with colorectal cancer (CRC), who have high numbers of NK cells, $CD4^+$ and $CD8^+$ T cells in their peripheral blood or tumor tissue, have higher survival rate compared with patients with lower numbers of these cells (6, 7). Similar results were observed on CRC-bearing mice with stronger anti-tumor responsiveness in animals with high numbers of CD8, CD11 and NK cells at tumor site (8). Special attention is paid to cytotoxic T cells, which specifically recognize and kill tumor cells in a MHC-restricted fashion (9, 10). In breast carcinoma, for example, T cells are the predominant infiltrating population, found in 81% of the tumor mass, while B cells are found in only 50% (11). L-selectin (CD62L) also helps the immune system by playing an important role in the lymphocytes extravasations from blood and lymphatic vessels for homing lymph nodes and tumor sites (12-14). In addition, low levels of this molecule trigger an inefficient immune response against tumor (15).

Generation of a T cell antitumor response is highly dependent on the role of DCs and we have observed that sensitization of human DCs with tumor lysate is enhanced by previous incubation with low concentration of paclitaxel (16). Similarly, the pre-treatment

of tumor cells with low concentrations of paclitaxel, increases their immunogenicity, facilitating the generation of specific cytotoxic T lymphocytes (17).

Mature DCs increase IFN-γ production and modulate Th1 responses (18), while DC pulsed with allogeneic tumor antigen increases the *in vivo* anti-tumor response (19, 20). However, DC vaccines can be hindered by tumor-induced regulatory cytokines and immune suppressive cells that create an anti-inflammatory microenvironment (21). This scenario can be due to the activity of regulatory T cells (Treg) or myeloid-derived suppressor cells (MDSC) that strongly suppress $CD4^+$ and $CD8^+$ T cells (22-24). In fact, $CD4^+/CD25^+/FOXP3^+$ Tregs account for 5-15% tumor infiltrating $CD4^+$ T cells that are able to suppress the function of other immune cells by producing IL-10 and TGF-β. Toomey *et al*. 2008 (25), observed that the down regulation of Treg cells, decreases tumor burden and increases the survival of tumor-bearing mice.

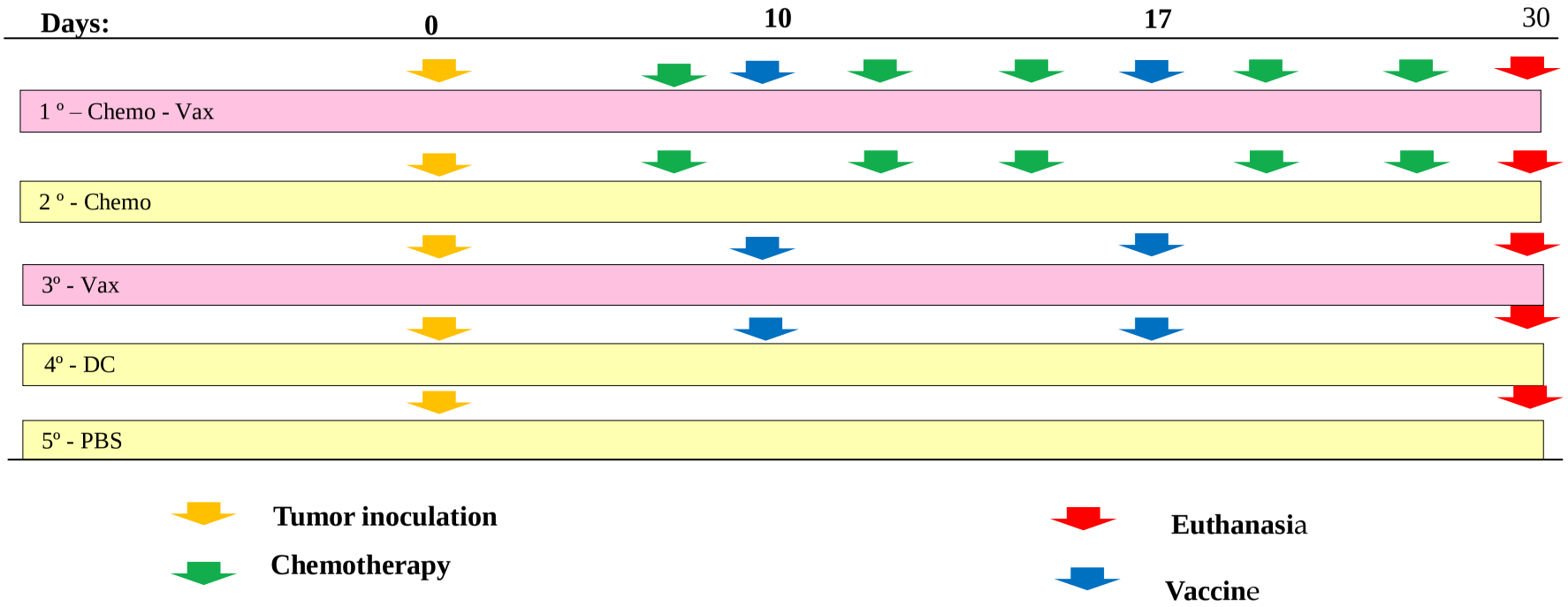
Myeloid derived suppressor cells (MDSC) have also been associated with decreased antitumor response in different tumor models. In the bone marrow, these cells are the precursors of macrophages, granulocytes and dendritic cells and directly suppress T cell activation (26) and produce IL-10, which promotes development of Tregs (27, 28), and prevent T cells homing into the lymph nodes (15). In the C26 murine adenocarcinoma model increased numbers of MDSC inhibit immune cell responsiveness to IFNs, hindering the development of an effective antitumor activity (29). The antibody-mediated depletion of MDSC increases the antitumor activity against lung cancer enhancing APC activity and increasing the frequency and activity of both NK and T cells in the tumor (30). According to Srivastava *et al*, 2012, (31), solid tumors are infiltrated by MDSCs that maintain an immune-suppressive network in the tumor microenvironment. Splenic MDSC isolated from MC-38-bearing mice inhibit IFN-γ production by specific lymphocytes mesuared by

anti-OVA responsiveness (32), whereas depletion of those cells delays tumor progression and facilitates its rejection (26). Considering that targeting MDSCs may have a long-term benefit for anticancer therapies, this study aimed to evaluate if combinatory therapy of low dose 5-FU with DC-vaccine decreases the level of tumor infiltrating MDSC and Tregs. Our results showed that low dose of chemotherapy agents associated with DC vaccine may help to prevent immunossupression and delay the tumor growth.

2. EXPERIMENTAL DESIGN

As designed at the Fig. 1, C57BL/6 mice were s.c. inoculated with MC-38 cells. Mice were treated twice a week with minimum effective concentration of 5-FU plus folic acid, and received two doses of DC vaccine during the experiment (once a week). Peripheral blood was analyzed for MDSC and T cells at day zero, 15 and 30, when the animals were euthanized to characterize the spleen populations and tumor infiltrating cells.

Fig. 1 - Experimental Design



- **Chemo – Vax:** animals receiving 5-FU (minimum effective concentration) plus folic acid and DC vaccine pulsed with MC-38 lysate.
- **Chemo:** animals receiving 5-FU (minimum effective concentration) plus folic acid.
- **Vax:** animals receiving only DC vaccine pulsed with MC-38 lysate.
- **DC:** non pulsed dendritic cells.
- **PBS:** animals receiving PBS only

3. MATERIAL AND METHODS

3.1 DC vaccine

Bone marrow was collected from the tibiae and femurs of healthy mice for generation of myeloid DC. Erythrocytes were lysed with hemolytic Gey´s solution and T and B lymphocytes were depleted with anti-CD4, CD8 and B220 magnetic beads (Miltenyi, Inc.). Cell suspension was transferred to 6-well culture plates, and cultured with 80 ng/ml of GM-CSF and IL-4 at 37ºC in a 5% CO_2 chamber. After 3 days, non-adherent and loosely adherent cells were harvested, splitted into 6 well culture plates and cytokines concentration was adjusted again. Three days later MC-38 cell lysate was added (100 µg protein/10^6 cells, that is equivalent to 5:1 tumor:DC proportion), and maintained by additional 24h. The resulting lysate loaded DCs were inoculated s.c. into the peritumoral area (10^6 cells/100µl per animal).

3.2 MC-38 lysate

A murine colorectal cancer MC-38 tumor cells were detached with trypsin solution and frozen at -80^{o}C. The cells were lysed by 5 cycles of fast freezing and thawing and the resulting lysate centrifuged at 13000 rpm for 15 min. The supernatant were collected, quantified for protein concentration by Bradford´s technique, and cryopreserved at -80^{0}C.

3.3 Isolation of tumor infiltrating cells and spleen cell suspensions

MC-38 tumor masses from 3 animals/group were excised, dissociated, and digested with collagenase type IV (1%), hyaluronidase type IV (0,1%) and deoxyribonuclease I (300u/ml) for 1 h at 37^{0}C .Cell suspensions were washed once with complete culture

medium and twice with PBS. Resulting cells were phenotyped and quantified for MDSC, macrophages and T cell subsets by flow citometry.

Spleen cells of tumor-bearing mice were dissociated in order to obtain single-cell suspensions. Erythrocytes were depleted by treatment with hemolytic Gay´s solution and cell suspensions were analyzed by flow citometry and co-cultured with MC-38 for 24h. The supernatants were frozen for cytokines quantification.

3.4 Antibodies and flow citometry

Tumor-infiltrating, spleen and blood cells were labeled with mAbs for flow cytometry analysis. MDSC were characterized as $CD11b^+/Gr1^+$ cells (FITC/PE fluorocromes), macrophages as $F4/80^+$ (Pac Blue) cells and T lymphocytes subsets were analyzed using anti-CD3(Pac Blue), CD4 (APC-Cy7), CD8 (PERCP-Cy5), CD25 (FITC) and FOXp3 (ALEXA 647) mAb. Effector cytolytic T cells expressing L-selectin were evaluated with anti-CD62L (PE), purchased from BD Pharmingen or Biolegend, Inc. All samples were labeled with isotype matched mAbs as a negative control and data aquired at a CyAn ADP flow cytometer (Beckman Counter) using the Summit software.

3.5 Confocal microscopy

Frozen tumor tissue (18 µm) were air-dried, fixed in cold acetone for 2 min at room temperature, rehydrated in PBS, stained with anti-mouse Abs to CD11b and Gr-1 (BD Pharmingen), followed by Alexa fluor 647 or Alexa fluor 700 -conjugated secondary Abs (Invitrogen), cover-slipped with VectaShield DAPI mounting medium (Vector Laboratories), and examined using the inverted Leica SP5 ATOF microscope and

photographed with CCD SPOT RT Camera (Diagnostic Instruments). Intensity of fluorescence was analyzed in three power fields by LAS AF Leica program.

3.6 Statistical analysis

Statistical analysis made by Dunn and Kruskal-Wallis Test were performed after Bartlett´s Test, using Graph pad Instat and Prism 5 statistical programs. Differences among the groups were considered significant when $p \leq 0.05$.

4. RESULTS

4.1 Combination of chemotherapy and DC vaccine delays the tumor growth

Tumor growth was weekly evaluated during 30 days. Combination of low dose of 5-Fu + folic acid and DC vaccine (Chemo-Vax) resulted in a tumor growth 77% lower than untreated mice (PBS) (Fig 2). Animals treated with just 5-FU + folic acid (Chemo), vaccine (Vax) or unloaded DC (DC) also slightly delayed the tumor growth but no significant differences were found among these groups.

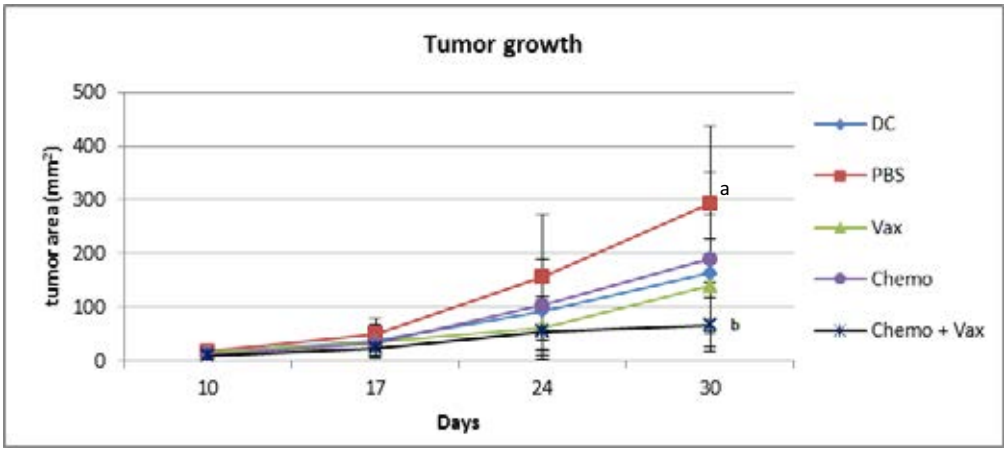


Fig 1: Tumor growth in animals receiving DC vaccine combined or not with 5-fluorouracil. **Chemo-Vax**: animals receiving 5-FU plus folic acid and DC vaccine. **Chemo**: animals receiving 5-FU plus folic acid. **Vax:** animals receiving only DC vaccine pulsed with MC-38 lysate. **DC**: unloaded dendritic cells. **PBS**: animals receiving PBS. Data analyzed by ANOVA and Tukey-Kramer Multiple Comparisons Test. $a \neq b$; $p<0.01$. N=6.

4.2 Cell surface phenotype of dendritic cells and vaccine

The culture conditions for generation of tumor lysate loaded DC yielded cells with slightly increased CD40 expression (3%), compared with unloaded DC. The percentage of contaminating macrophages was just 10% in the culture conditions, assessed by measuring macrophage-related surface molecule F4/80$^+$ cells. No relevant changes were observed on the other analyzed markers (Table 1).

Flow citometry analysis of transfected (WT and FU) and no-transfected dendritic cells

	Cells	% of $CD11c^+/CD40^+$	MFI of $CD11c^+/CD40^+$	% of $CD11c^+/CD86^+$	MFI of $CD11c^+/CD86^+$	% of $CD11c^+/MHCII^+$	MFI of $CD11c^+/MHCII^+$
1st experiment	DC	10,62	14,48	9,03	39,81	44,23	1371,69
	Vaccine	13,22	17,35	8,84	51,26	33,76	1898,59
2nd experiment	DC	19,31	46	16,56	101,82	21,26	3036,36
	Vaccine	23,43	42,79	17,38	76,27	27,5	1643,19

Table 1: Flow citometry of dendritic cells after 6 days of culture and vaccine after lysate stimulation. Results expressed as percentage of total cells and mean of fluorescence intensity (MFI). Mean ± SD of two independent experiments. Acquisition of 10.000 events at flow cytometer.

4.3 White blood cells screening

In order to evaluate if administration of 5-FU or in combination with Vax would be able to promote systemic immunological changes, white blood cells were evaluated 3 times during the experiment. Cells were stained and analyzed by flow citometry for MDSC, lymphocytes subsets and L-selectin expressing cytotoxic T cells (CD62L).

Fig. 3A shows that tumor implantation increased the number of circulating MDSC (CD11b+/Gr1+ cells) at the 30th day. It is interesting to note that the highest number of circulating MDSC was observed in the animals treated with 5-FU + folic acid (Chemo), whereas it combination with vaccine (Chemo-Vax) slightly modulated this response.

On the other hand,the number of both CD4+ and CD8+ cells decreased at the 30th day (Fig. 3B and C). This apparent downmodulation of T cell responsiveness was not followed by increased number of Treg (CD3+/CD4+/CD25+/FOXP3+ cells), that showed some variations at the 15th day, but reached normal levels at the 30th day (Fig. 3D).

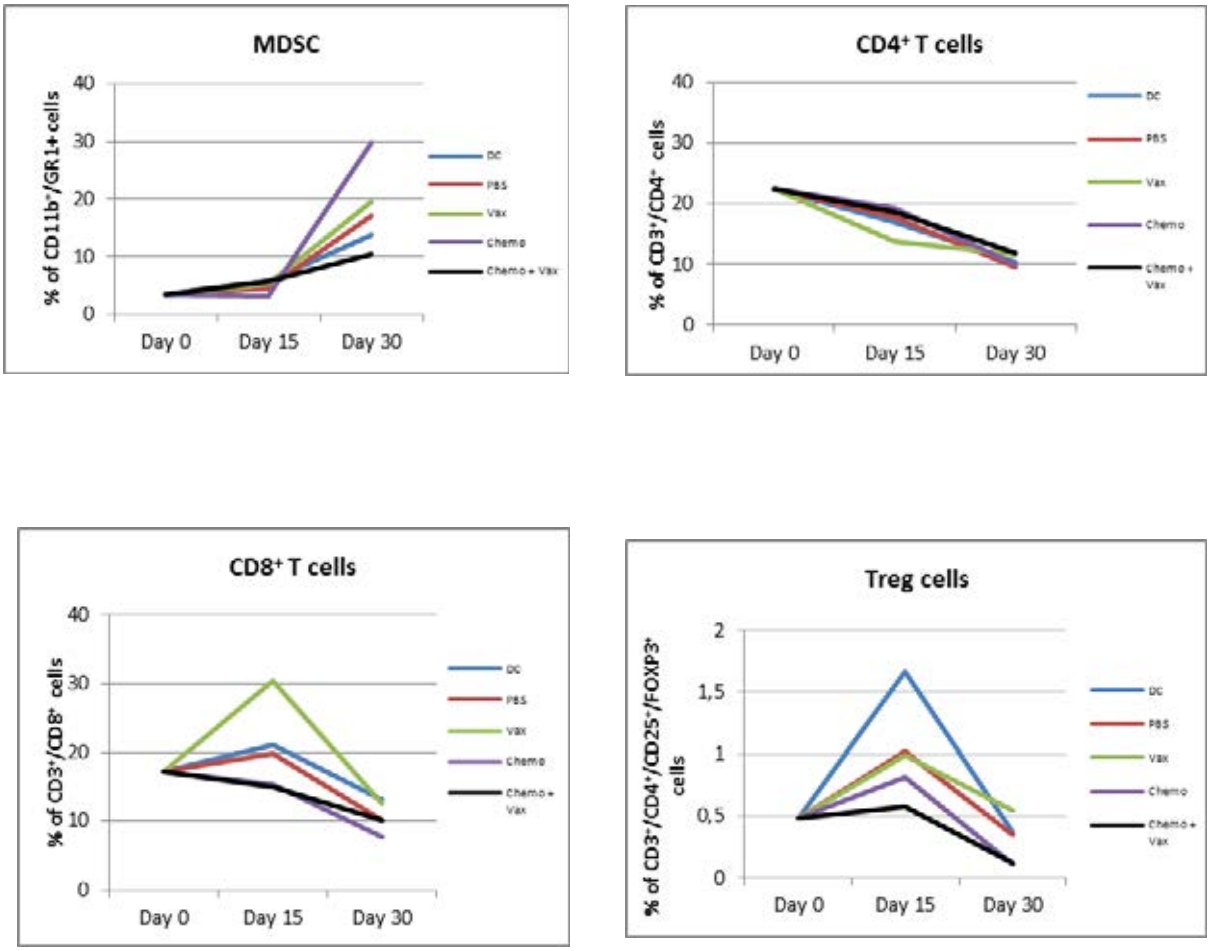


Fig. 3: Percentage of circulating MDSC ($CD11b^+/Gr1^+$) (A), Th ($CD3^+/CD4^+$) (B), Tc ($CD3^+/CD8^+$) (C) and Treg ($CD3^+/CD4^+/CD25^+/FOXP3^+$) cells at the peripheral blood, analyzed by flow cytometry. Statistical analysis made by Dunn and Kruskal-Wallis Test. Median of two independent experiments; $a \neq b$; $p<0.05$; N=3-6.

Considering that L-selectin actively take part in the T cell homing at lymphoid organs and infiltration into the tumor site (12) and that effector and memory cells differentially express this molecule (CD62L) (33) we asked if Chemo or Chemo-Vax treatment would enhance the generation of effector $CD8^+/CD62L^+$ lymphocytes. Although the differences were not significant, results at the Fig. 4 suggest that PBS group induce high numbers of activated effector $CD8^+$ cells, while both Chemo and Chemo-Vax

showed the lowest numbers at this time. However, at the 30th day all the groups restored the normal numbers.

The numbers of monocytes tended to increase in the PBS and Vax groups by the 15th day, but unfortunately we were not able to evaluate their numbers at the 30th day (data not shown).

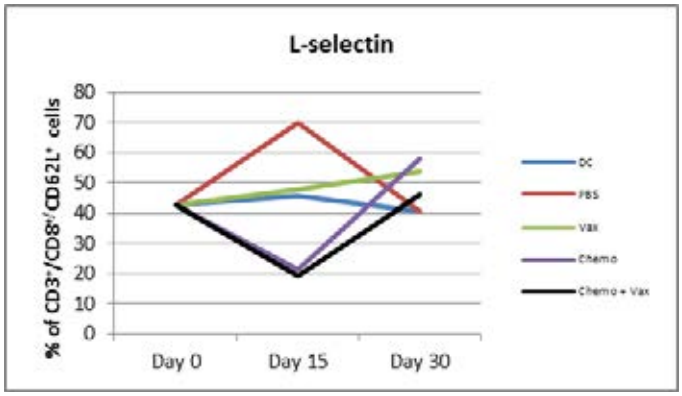


Fig. 4: L-selectin expressing lymphocytes were quantified in the peripheral blood at the days 0, 15 and 30 by flow cytometry. Data was expressed as median percentage of $CD3^{+}/CD8^{+/}CD62L^{+}$ cells. Statistical analysis made by Dunn and Kruskal-Wallis Test. Median of two independent experiments, N=3-6.

4.4 Production of Arginase, ROS and iNOS by MDSC

Activation of MDSC promotes upregulated expression of immune suppressive factors such as arginase and iNOS and increases the production of ROS. In order to obtain additional data of *in vivo* effects of combinatory therapy on the functions of these cells, arginase, ROS and iNOS expression were analyzed in circulating MDSC at the 30th day. Fig 5A shows that the administration of 5-FU plus Vax in tumor-bearing mice increases iNOS production (Chemo and Chemo-Vax groups), arginase (Fig. 5B) and ROS (Fig. 5C). Suggesting an upregulation of MDSC. Althought 5-FU seems to induce overexpression of

iNOS (Fig. 5A) itself, the combination with Vax shows more intense effect on all of these parameters.

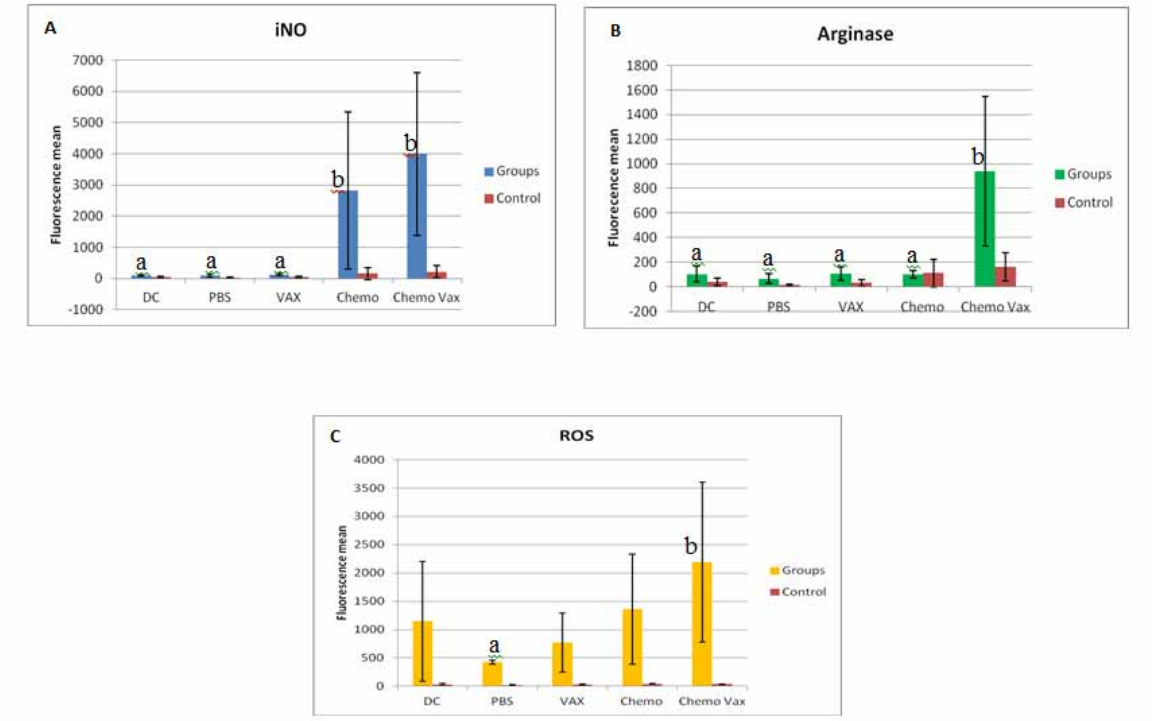


Fig. 5: Production of iNOS (A), arginase (B) and ROS (C) by $CD11b^{+}/Gr1^{+}$ peripheral blood cellsof tumor bearing mice. Data analyzed by flow cytometry at day 30 after tumor implantation and expressed as fluorescence mean. Control of each group refers to non stimulated cells. Statistical analysis made by ANOVA and Tukey-Kramer Multiple Comparisons Test, $a \neq b$; $p<0.05$. N=6.

In order to have a wider view on the systemic effect of the treatments mice, spleens of tumor-bearing mice were also analyzed on immunocompetent cells. As can be observed at the Fig. 6A, none of the treatments induced relevant changes in the lymphocyte subsets.

Interestingly, we observed a higher number of CD62L-expressing lymphocytes in the Chemo-Vax than in DC group (Fig. 6B). The number of these cells in Chemo group was comparable to Chemo-Vax suggesting that 5-FU is able to increase the CD62L expression itself, but no statistical significance was found.

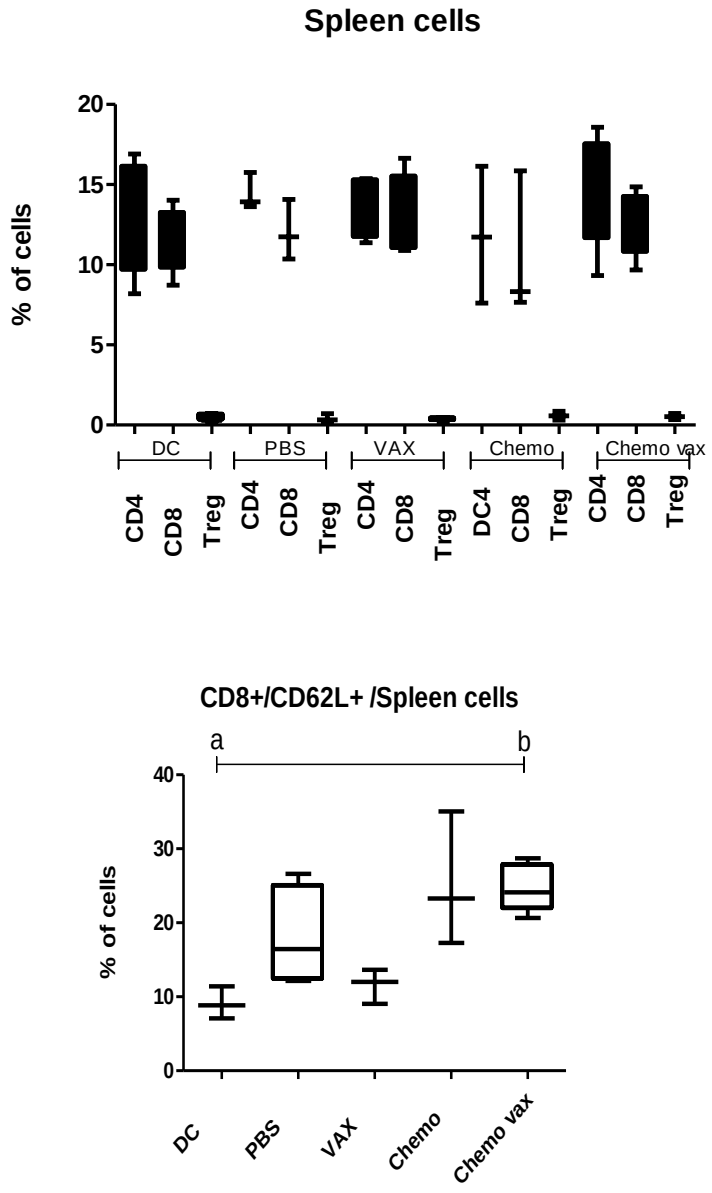


Fig. 6: Phenotypic analysis of spleen cells performed from tumor bearing mice by flow cytometry. **A:** Analysis for T cells, $CD4^+$, $CD8^+$, $CD3^+$, Treg. **B:** $CD8^+$ T cells were analyzed for L-selectin (CD62L) marker. Comparison between all groups on 30^{th} day of experiment. Statistical analysis made by Dunn and Kruskal-Wallis Test, , $a \neq b$; $p<0.05$.N=3-6.

4.5 Cytokines

Since higher number of CD8+/CD62L+ cells were observed in Chemo-Vax group, we decided to evaluate if spleen cells of these animals were able to preferencially produce IFN-γ. Then, spleen cells were co-cultured with MC-38 target cells for 24h and supernatants were analyzed for IL-10, IL-12 and IFN-ɣ, and compared with spontaneous cytokine production. Results at Fig. 7 shows that neither chemotherapy nor it combination with vaccine was able to modulate the IFN-ɣ and IL-10 production. No measurable quantities of IL-12 were found in the supernatants (data not shown).

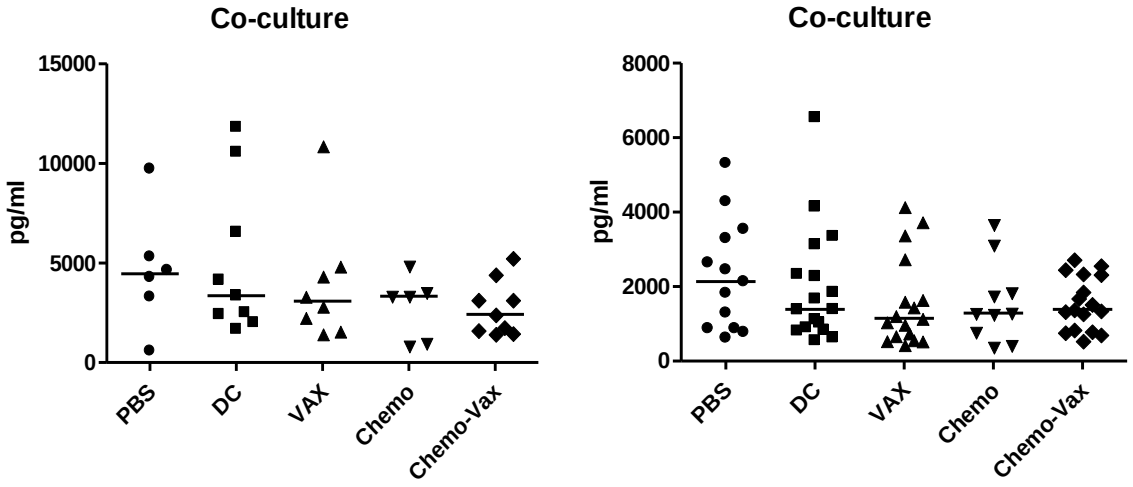


Fig. 7: **A:** *In vitro* production of IFN-ɣ (A) and IL-10 (B) by spleen cells of tumor-bearing mice, co-cultured with MC-38 target cells for 24h. Culture supernatants were analyzed by ELISA. Statistical analysis made by Dunn and Kruskal-Wallis Test, N=3-6.

4.6 Analysis of tumor infiltrating cells

Analysing tumor infiltrating cells we found CD4+ and CD8+ cells, as well as a small proportion of Treg (Fig 8A). High percentages of MDSC and macrophages were also observed at the tumor site (Fig. 8C). Chemo and Chemo-Vax treated animals showed a slightly higher number of both CD4 and CD8 lymphocytes, MDSC and macrophages (non significant). Just a low number of L-selectin expressing lymphocytes infiltrated the tumor tissue (Fig. 8B).

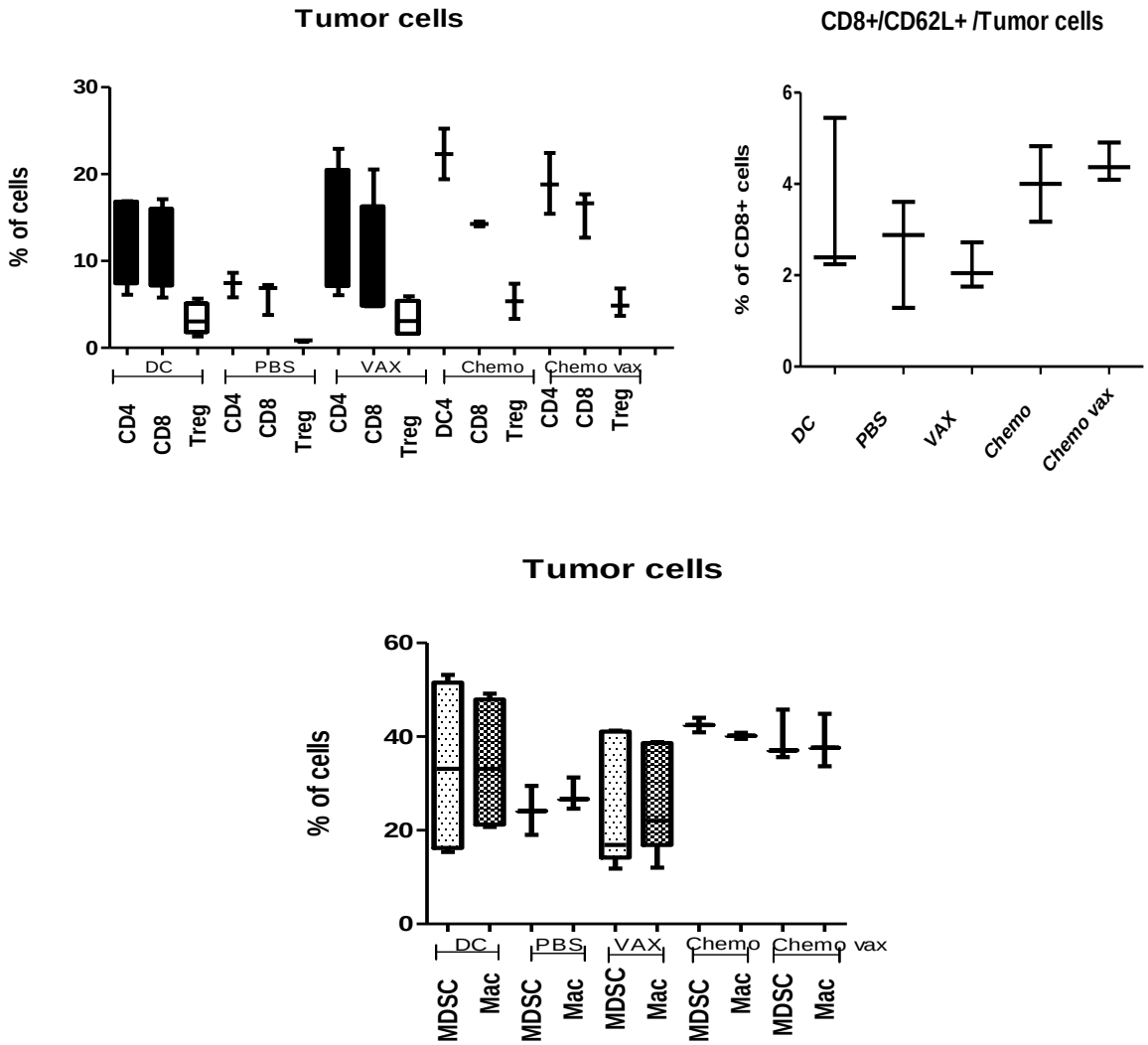


Fig 8: Phenotypic analysis of tumor infiltrating cells showing the T lymphocytes subsets **A:** Analysis for Th, Tc and Treg cells; **B:**.$CD8^+$ T cells expressing L-selectin (CD62L)**; C:** Analysis for MDSC and macrophages. Comparison between all groups on 30^{th} day of experiment. Statistical analysis made by Dunn and Kruskal-Wallis Test, N=3-6.

4.7 Identification of CD11b+/Gr1+tumor-infiltrating cells by confocal microscopy

Tumor infiltration by MDSC was also analyzed by confocal microscopy, usinf the anti-CD11b and anti-Gr1 antibodies. Analysis of different regions of tumor tissue indicated higher frequency of colocalization at the peripheral area of all samples (data not shown).

Medians of collocalization spots – considering the peripheral, median and deeper areas of each tumor sample, showed that all the groups have similar amounts of CD11b+/Gr1+ cells infiltrating the tumor microenvironment (Fig. 9A). Analysis of just the peripheral region (more intensely labeled) shows that vaccine (Vax and Chemo-Vax gropus) was able to decrease the intensity of CD11b+/Gr1+ labelling (Figs. 9B and 10).

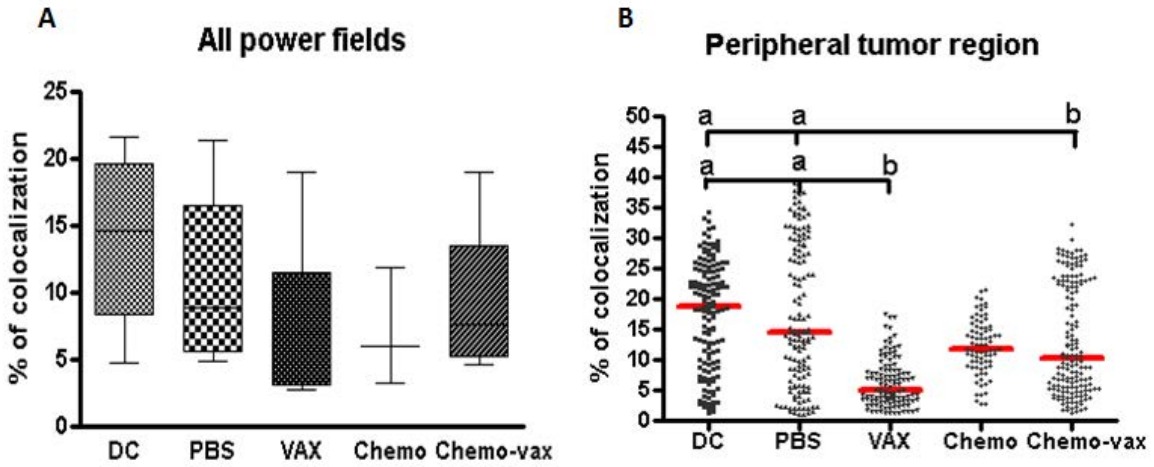


Fig 9: Percentage of $CD11b^{+}/Gr1^{+}$ by tumor infiltrating cells. **A:** Analysis of three power fields per group. **B:** Analysis of peripheral power field per animal. Data analyzed by Dunn and Kruskal-Wallis Test. a≠b, $p<0,05$. N=6.

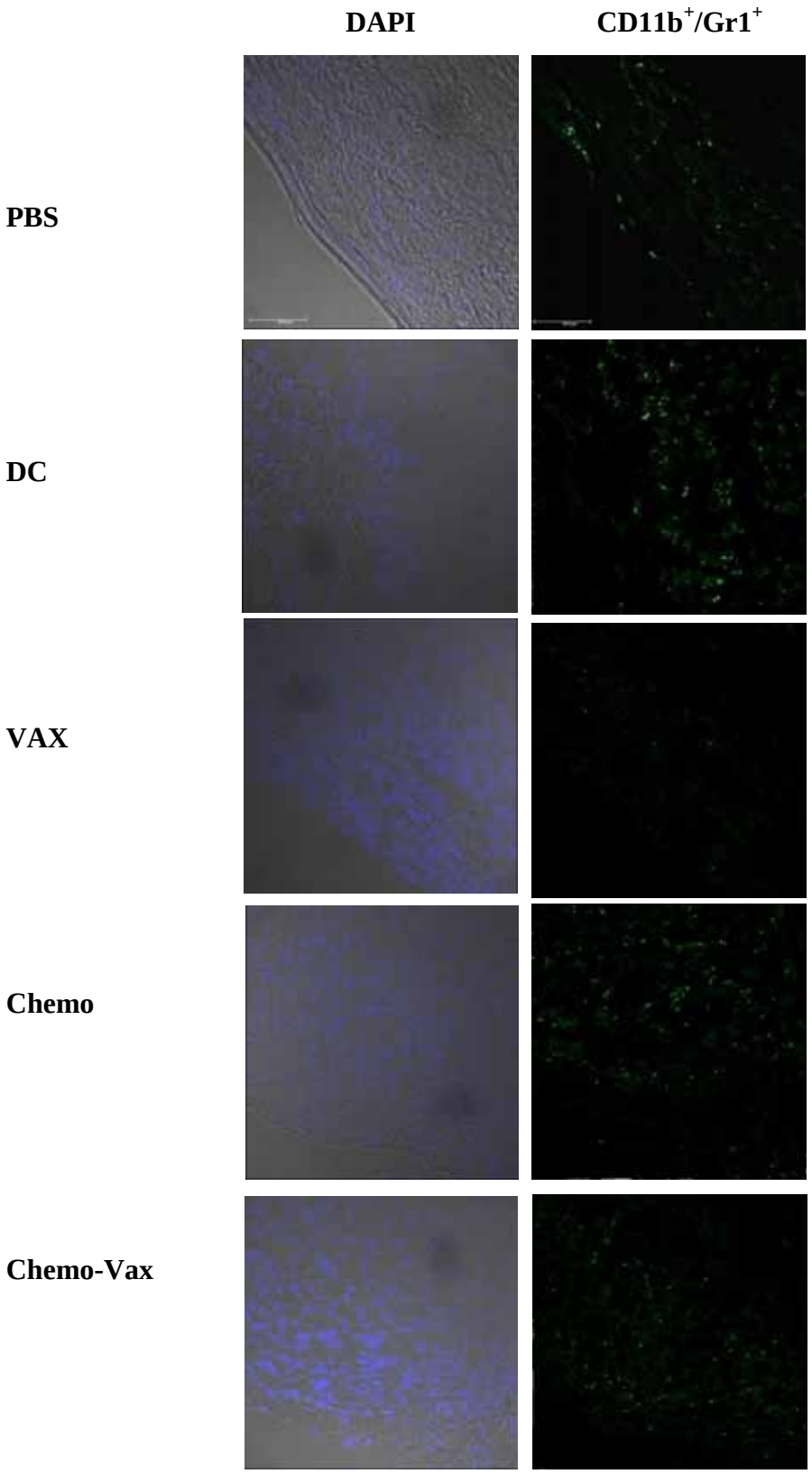


Fig. 10: Confocal microscopic analysis shows staining of CD11b + (green) with Gr1 + (white dots) cells at the peripheral area of the tumor mass. Lens 64x (numeric open 1.30) oil immersion, allowing a resolution of up to ~ 200nm on x, y and ~ 400nm on z axis.

5. DISCUSSION

Previous studies of our group showed that low concentrations of chemotherapeutic agents increase the immunogenicity of tumor cells (17), and stimulate maturation and fuctions of human DCs (16), stimulating us to investigate the feasibility of *in vivo* modulate antitumor resistance combining low dose chemotherapy with DC vaccine. In the present study we firstly observed that non-cytotoxic dose of 5-FU plus conventional concentration of folic acid, enhanced the protective effect of DC-based vaccination decreasing in 70% the tumor mass as compared with PBS-treated tumor-bearing control.

We have used tumor-lisate pulsed DCs because this antigen loading process can activate both MHC class I-restricted $CD8^+$ T cells and MHC class II-restricted $CD4^+$ T cells (34, 35) since proteins processed through endocytic route can be cross-presented by MHC class I molecules (36). Lysate loaded DC did not show intense changes on the expression o maturation marker CD40, and even the expression of MHC class II^+ cells was similar to unloaded DC. It is interesting that despite this lack of maturation, combined administration of 5-FU and vaccine delayed the tumor growth in 70% of the tumor mass. This results suggest that administration of low dose of 5-FU is able to cause immunogenic death of tumor cells, which antigens could be endocitosized and *in vivo* processed by those immature DC inoculated at the peritumoral site. Immunogenic death seems to be an important aspect for the generation of an effective antitumor response, involving the expression of danger signals such as heat shock family proteins (HSPs) (37) and high mobility group box protein 1 (HMGB1) (38). We have previously observed that exposition of human colon cancer cells to low concentration of paclitaxel and doxorubicin increased the levels of transcription of HSPs genes (17), that induced respective HSP 70 and 90 protein synthesis (unpublished data). We have also observed that 5-FU is able to increase

the immunogenicity of mRNA of human colon cancer cell line HCT-116 (unpublished data). Therefore, it is possible that similar phenomenon is inducible *in vivo*, enhancing the protectrive role of DC vaccine.

We observed that the numbers of $CD4^+$ and $CD8^+$ circulating cells decreased as the tumor grow, while MDSC increased. Murine MDSC are describe as CD11b+/Gr1+ cells (39), that are hypothesized to originate in the bone marrow of tumor bearing mice (26, 40). These cells populate the blood and peripheral lymphoid organs during the tumor growth and enter the tumor site where they play an immunomodulatory / immunosuppressive role (26,40). Therefore, the rise of MDSC we observed in the blood of tumor-bearing mice would be expected as well as in the spleen. Although there was no statistically significant difference, administration of 5-FU seems to increase the number of circulating MDSC, whereas combination with Vax would be able to prevent this rising.

Tongu *et al*. (41) observed that combination of low doses of cyclophosphamide and gemcitabine suppressed growing of CT26 colon cancer cells in Balb/c mice. This treatment decreased the percentages of tumor-infiltrating $Gr\text{-}1^{high}/CD11b^+$ MDSCs. In accordance with these results, it was observed that depletion of this population prevents tumor growth in murine models (42). In our case, treatment with just 5-FU was not enough for decrease the tumor growth, nor to modulate the number of blood MDSC, however, both the model (CT-26 in Balb/c vs MC-38 in C57Bl/6) are different and we worked with a different drug and, probably with different dose/animal body relation.

Contrasting with the findings in peripheral blood, analysis of spleen cells showed that both Chemo and Chenmo-Vax groups have similarly high levels of MDSC. This increased frequency suggests they would have infiltrated the tumor site to induce local modulation and enhancement of it growth. However, we found just a small number of CD11b+/Gr1+ cells in the tumor infiltrate (1-5.5%) that seems to be not enough to affect

the tumor growth in Chemo-Vax group, despite the apparent higher frequency they have shown.

Activated MDSC upregulate the expression of suppressive factors such as arginase (Arg) and iNOS and increase the production of ROS (43), that are able to modify the peptide recognition by T-cell receptors, inhibit JAK – STAT, and the expression of MHC-II (44-46). In addition, production of peroxynitrite (a chemical reaction between NO and superoxide anion O^{2-}) by MDSC induces nitration of the T cells receptor (TCR) that modifies the specific peptide binding sites and prevent cell responsiveness (47). In our study all these factors are upregulated in Chemo-Vax group, indicating that MDSC are activated. However, since there was not an expansion of this population and just a small number infiltrated the tumor site, the activity of circulating cells seems to be not enough to *in situ* suppress the antitumor response. This view is supported by the observation that the treatments did not change the profile of lymphocyte subsets.

Our next question was whether the increased number of MDSC in the spleen would influence the type of lymphocyte subsets populating this organ or their migration to the tumor site. Our results showed no relevant alterations in the Th, Tc and Treg populations in the spleen or at the tumor mass. Effective antitumor responsiveness involves migration of immunocompetents cells for the lymphoid organs and further migration of effector memory T lymphocytes to the tumor site. Such migration and cell homing is dependent on the expression of L- selectin (CD62L) by leukocytes that promotes extravasion from blood to lymphoid organs through the high endodothelial vascular cells (HEV). (12-14, 48). On the other hand, L selectin also take part in the infiltration of effector memory cells into the tumor site.

It is estimated that the density of; L-selectin ranges from 50,000 to 70,000, molecules per cell, indicating their strong contribution to signalling during leukocyte recruitment (49). L-selectin-dependent leukocytes have been used as a therapeutic target in many inflammatory diseases (33, 50, 51) while a low level impairs the immune response against tumor (15). In the present study, we observed that expression of L-selectin on $CD8^+$ cells from Chemo-Vax group is higher than in DC group suggesting that the combination of 5-FU chemotherapy and our vaccine enhance the homing of T cells to lymphoid organs and tumor sites. According to Chao *et al*, 1997 and Raffler, 2005 (33, 52) murine $CD8^+$ cells stimulated with anti-CD3 antibody presented initial loss of surface L-selectin due to cleavage and all cells became L-$selectin^{low/negative}$ some days later. Activated cells rapidly divide and differentiate into L-$selectin^{low/positive}$ effector memory cells that migrate to inflammatory sites. After that, most T cells undergo apoptosis but some of them evolve into memory cells.

MDSC can be downregulate, L-selectin on naïve T cells inhibiting their activation by interfering with the traffic pattern (53). In this study we observed that Chemo-Vax schedule downregulates peripheral blood MDSC while upregulated them at the spleen. Despite this rising, we found increased levels of $CD8^+/CD62L^+$ cells in this organ suggesting that MDSCs were not able to induce systemic immunosupression.

Besides L-selectin regulation, other factors such as prostaglandins, GM-CSF, TGF-β, S100A8/A9 and several other molecules involved in STAT 3 signaling pathway regulate the expansion of MDSC at the tumor microenvironment (27, 54-56). Immunossupressive activity of MDSCs at tumor sites can be decreased by combinatory immunotherapy as demonstrated that administration of noncytotoxic dose of paclitaxel in murine melanoma model, significantly decreased accumulation and immunosuppressive activities of tumor-

infiltrating MDSC restoring $CD8^+$ T cell effectors functions associated with the inhibition of p38 MAPK activity, TNF-α and production, and S100A9 expression in MDSCs (57). Other study (58) reported that combinatory treatment of 5-FU as adjuvant to CD40L immunogene therapy was able to shift myeloid-derived suppressor cell phenotype into a less suppressive population achieving a survival rate of 68 % and specific MB49 bladder tumor immunity. In addition, Vincent *et al* 2010, (59) showed that 5-FU can selectively target MDSCs by triggering their apoptotic cell death. Depletion of these cells triggers increased production of IFN-γ by tumor-specific $CD8^+$ infiltrating T cells and promoted a T-dependent antitumor effect. Moreover, Sinha *et al* 2011, (60) showed that once T cells are activated, they are able to lead MDSC to apoptosis via caspase 3.

In summary, our results show that combinatory therapy in C57Bl/6 mice was able to delay the tumor growth in 70% compared with animals that did not receive this treatment. Combinatory therapy activated MDSC but did not promote cell expansion, allowing increased CD62L expression on $CD8^+$ cells and maintenance of their traffic. Our data suggest that chemoimmunomodulation can induce a tumor-specific immune response against MC-38 response *in vivo.* Chemoimmunemodulation is a growing concept that deserves to be better exploited, in order to pave a new direction for tumor therapy.Low dose chemotherapy associated with DC vaccine is able to improve the communication between cells and system against tumor growth by recruiting and activating a T cell-mediated antitumor response and inhibiting immunossupressive cells.

6. REFERENCES

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA: a cancer journal for clinicians. 2011;61(2):69-90.

2. Hafid SR, Radhakrishnan AK, Nesaretnam K. Tocotrienols are good adjuvants for developing cancer vaccines. BMC cancer. 2010;10:5.

3. Gill S, Blackstock AW, Goldberg RM. Colorectal cancer. Mayo Clinic proceedings. 2007;82(1):114-29.

4. Radhakrishnan AK, Sim GC, Cheong SK. Comparing the ability of freshly generated and cryopreserved dendritic cell vaccines to inhibit growth of breast cancer in a mouse model. BioResearch open access. 2012;1(5):239-46.

5. Gilboa E. DC-based cancer vaccines. J Clin Invest. 2007;117(5):1195-203.

6. Qiu H, Xiao-Jun W, Zhi-Wei Z, Gong C, Guo-Qiang W, Li-Yi Z, et al. The prognostic significance of peripheral T-lymphocyte subsets and natural killer cells in patients with colorectal cancer. Hepato-gastroenterology. 2009;56(94-95):1310-5.

7. Pages F, Kirilovsky A, Mlecnik B, Asslaber M, Tosolini M, Bindea G, et al. In situ cytotoxic and memory T cells predict outcome in patients with early-stage colorectal cancer. J Clin Oncol. 2009;27(35):5944-51.

8. Ishii S, Hiroishi K, Eguchi J, Hiraide A, Imawari M. Dendritic cell therapy with interferon-alpha synergistically suppresses outgrowth of established tumors in a murine colorectal cancer model. Gene Ther. 2006;13(1):78-87.

9. Yu P, Fu YX. Tumor-infiltrating T lymphocytes: friends or foes? Lab Invest. 2006;86(3):231-45.

10. Nencioni A, Muller MR, Grunebach F, Garuti A, Mingari MC, Patrone F, et al. Dendritic cells transfected with tumor RNA for the induction of antitumor CTL in colorectal cancer. Cancer Gene Ther. 2003;10(3):209-14.

11. Helal TE, Ibrahim EA, Alloub AI. Immunohistochemical analysis of tumor-infiltrating lymphocytes in breast carcinoma: relation to prognostic variables. Indian journal of pathology & microbiology. 2013;56(2):89-93.

12. Rosen SD. Ligands for L-selectin: homing, inflammation, and beyond. Annual review of immunology. 2004;22:129-56.

13. Khan AI, Landis RC, Malhotra R. L-Selectin ligands in lymphoid tissues and models of inflammation. Inflammation. 2003;27(5):265-80.

14. Gallatin WM, Weissman IL, Butcher EC. A cell-surface molecule involved in organ-specific homing of lymphocytes. Nature. 1983;304(5921):30-4.

15. Hanson EM, Clements VK, Sinha P, Ilkovitch D, Ostrand-Rosenberg S. Myeloid-derived suppressor cells down-regulate L-selectin expression on CD4+ and CD8+ T cells. J Immunol. 2009;183(2):937-44.

16. Kaneno R, Shurin GV, Tourkova IL, Shurin MR. Chemomodulation of human dendritic cell function by antineoplastic agents in low noncytotoxic concentrations. J Transl Med. 2009;7:58.

17. Kaneno R, Shurin GV, Kaneno FM, Naiditch H, Luo J, Shurin MR. Chemotherapeutic agents in low noncytotoxic concentrations increase immunogenicity of human colon cancer cells. Cellular oncology (Dordrecht). 2011

34(2):97-106.

18. Schuler-Thurner B, Schultz ES, Berger TG, Weinlich G, Ebner S, Woerl P, et al. Rapid induction of tumor-specific type 1 T helper cells in metastatic melanoma patients by vaccination with mature, cryopreserved, peptide-loaded monocyte-derived dendritic cells. J Exp Med. 2002;195(10):1279-88.

19. Palucka AK, Ueno H, Connolly J, Kerneis-Norvell F, Blanck JP, Johnston DA, et al. Dendritic cells loaded with killed allogeneic melanoma cells can induce objective clinical responses and MART-1 specific CD8+ T-cell immunity. J Immunother. 2006;29(5):545-57.

20. Lee H, Park HJ, Sohn HJ, Kim JM, Kim SJ. Combinatorial therapy for liver metastatic colon cancer: dendritic cell vaccine and low-dose agonistic anti-4-1BB antibody co-stimulatory signal. J Surg Res. 2011;169(1):e43-50.

21. Palucka K, Banchereau J. Cancer immunotherapy via dendritic cells. Nature reviews Cancer. 2012;12(4):265-77.

22. Maloy KJ, Powrie F. Regulatory T cells in the control of immune pathology. Nature immunology. 2001;2(9):816-22.

23. Ostrand-Rosenberg S, Sinha P. Myeloid-derived suppressor cells: linking inflammation and cancer. J Immunol. 2009;182(8):4499-506.

24. Talmadge JE. Pathways mediating the expansion and immunosuppressive activity of myeloid-derived suppressor cells and their relevance to cancer therapy. Clin Cancer Res. 2007;13(18 Pt 1):5243-8.

25. Toomey D, Conroy H, Jarnicki AG, Higgins SC, Sutton C, Mills KH. Therapeutic vaccination with dendritic cells pulsed with tumor-derived Hsp70 and a COX-2 inhibitor induces protective immunity against B16 melanoma. Vaccine. 2008;26(27-28):3540-9.

26. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. Nature reviews. 2012;12(4):253-68.

27. Sinha P. Proinflammatory s100 proteins regulate the accumulation of myeloid-derived suppressor cells. J Immunol. 2008;181:4666–75.

28. Sinha P, Clements VK, Ostrand-Rosenberg S. Interleukin-13-regulated M2 macrophages in combination with myeloid suppressor cells block immune surveillance against metastasis. Cancer Res. 2005;65(24):11743-51.

29. Mundy-Bosse BL, Lesinski GB, Jaime-Ramirez AC, Benninger K, Khan M, Kuppusamy P, et al. Myeloid-derived suppressor cell inhibition of the IFN response in tumor-bearing mice. Cancer Res. 2011;71(15):5101-10.

30. Srivastava MK, Zhu L, Harris-White M, Kar UK, Huang M, Johnson MF, et al. Myeloid suppressor cell depletion augments antitumor activity in lung cancer. PloS one. 2012;7(7):e40677.

31. Srivastava MK, Andersson A, Zhu L, Harris-White M, Lee JM, Dubinett S, et al. Myeloid suppressor cells and immune modulation in lung cancer. Immunotherapy. 2012;4(3):291-304.

32. Nagaraj S, Gabrilovich DI. Regulation of suppressive function of myeloid-derived suppressor cells by CD4+ T cells. Seminars in cancer biology. 2012;22(4):282-8.

33. Raffler NA R-NJ, Ley K. L-selectin in inflammation, infection and immunity. Drug Discovery Today: Therapeutic Strategies. 2005;2:213-20.

34. Brossart P, Bevan MJ. Presentation of exogenous protein antigens on major histocompatibility complex class I molecules by dendritic cells: pathway of presentation and regulation by cytokines. Blood. 1997;90(4):1594-9.

35. Sen G, Chakraborty M, Foon KA, Reisfeld RA, Bhattacharya-Chatterjee M. Preclinical evaluation in nonhuman primates of murine monoclonal anti-idiotype antibody that mimics the disialoganglioside GD2. Clin Cancer Res. 1997;3(11):1969-76.

36. Arina A, Tirapu I, Alfaro C, Rodriguez-Calvillo M, Mazzolini G, Inoges S, et al. Clinical implications of antigen transfer mechanisms from malignant to dendritic cells. exploiting cross-priming. Experimental hematology. 2002;30(12):1355-64.

37. Theriault JR, Mambula SS, Sawamura T, Stevenson MA, Calderwood SK. Extracellular HSP70 binding to surface receptors present on antigen presenting cells and endothelial/epithelial cells. FEBS letters. 2005;579(9):1951-60.

38. Apetoh L, Ghiringhelli F, Tesniere A, Criollo A, Ortiz C, Lidereau R, et al. The interaction between HMGB1 and TLR4 dictates the outcome of anticancer chemotherapy and radiotherapy. Immunol Rev. 2007;220:47-59.

39. Youn JI, Nagaraj S, Collazo M, Gabrilovich DI. Subsets of myeloid-derived suppressor cells in tumor-bearing mice. J Immunol. 2008;181(8):5791-802.

40. Younos IH, Dafferner AJ, Gulen D, Britton HC, Talmadge JE. Tumor regulation of myeloid-derived suppressor cell proliferation and trafficking. Int Immunopharmacol. 2012;13(3):245-56.

41. Tongu M, Harashima N, Monma H, Inao T, Yamada T, Kawauchi H, et al. Metronomic chemotherapy with low-dose cyclophosphamide plus gemcitabine can induce anti-tumor T cell immunity in vivo. Cancer Immunol Immunother. 2013;62(2):383-91.

42. Medina-Echeverz J FJ, Zabala M, Ardaiz N, Prieto J, Berraondo P. Successful colon cancer eradication after chemoimmunotherapy is associated with profound phenotypic change of intratumoral myeloid cells. J Immunol. 2011;2:807-15.

43. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. Nature reviews. 2009;9(3):162-74.

44. Nagaraj S, Gupta K, Pisarev V, Kinarsky L, Sherman S, Kang L, et al. Altered recognition of antigen is a mechanism of CD8+ T cell tolerance in cancer. Nature medicine. 2007;13(7):828-35.

45. Bingisser RM, Tilbrook PA, Holt PG, Kees UR. Macrophage-derived nitric oxide regulates T cell activation via reversible disruption of the Jak3/STAT5 signaling pathway. J Immunol. 1998;160(12):5729-34.

46. Harari O, Liao JK. Inhibition of MHC II gene transcription by nitric oxide and antioxidants. Current pharmaceutical design. 2004;10(8):893-8.

47. Kusmartsev S, Nagaraj S, Gabrilovich DI. Tumor-associated CD8+ T cell tolerance induced by bone marrow-derived immature myeloid cells. J Immunol. 2005;175(7):4583-92.

48. Warnock RA, Askari S, Butcher EC, von Andrian UH. Molecular mechanisms of lymphocyte homing to peripheral lymph nodes. J Exp Med. 1998;187(2):205-16.

49. Ivetic A. Signals regulating L-selectin-dependent leucocyte adhesion and transmigration. The international journal of biochemistry & cell biology. 2013;45(3):550-5.

50. E EE. No detectable endothelial- or leukocyte-derived L-selectin ligand activity on the endothelium in inflamed cremaster muscle venules. Journal of leukocyte biology. 2008;84:93–103.

51. Eriksson EE. Intravital microscopy on atherosclerosis in apolipoprotein e-deficient mice establishes microvessels as major entry pathways for leukocytes to advanced lesions. Circulation. 2011;124(19):2129-38.

52. Chao CC, Jensen R, Dailey MO. Mechanisms of L-selectin regulation by activated T cells. J Immunol. 1997;159(4):1686-94.

53. Ostrand-Rosenberg S. Myeloid-derived suppressor cells: more mechanisms for inhibiting antitumor immunity. Cancer Immunol Immunother. 2010;59(10):1593-600.

54. Sinha P, Clements VK, Fulton AM, Ostrand-Rosenberg S. Prostaglandin E2 promotes tumor progression by inducing myeloid-derived suppressor cells. Cancer Res. 2007;67(9):4507-13.

55. Bromberg J. Stat proteins and oncogenesis. J Clin Invest. 2002;109(9):1139-42.

56. Serafini P. High-Dose GM-CSF-Producing Vaccines Impair The Immune Response Through The Recruitment Of Myeloid Suppressor Cells. Cancer Res. 2004;64:6337–43.

57. Sevko A, Michels T, Vrohlings M, Umansky L, Beckhove P, Kato M, et al. Antitumor effect of Paclitaxel is mediated by inhibition of myeloid-derived suppressor cells and chronic inflammation in the spontaneous melanoma model. J Immunol. 2013;190(5):2464-71.

58. Liljenfeldt L, Gkirtzimanaki K, Vyrla D, Svensson E, Loskog AS, Eliopoulos AG. Enhanced therapeutic anti-tumor immunity induced by co-administration of 5-fluorouracil and adenovirus expressing CD40 ligand. Cancer Immunol Immunother. 2013.

59. Vincent J, Mignot G, Chalmin F, Ladoire S, Bruchard M, Chevriaux A, et al. 5-Fluorouracil selectively kills tumor-associated myeloid-derived suppressor cells resulting in enhanced T cell-dependent antitumor immunity. Cancer Res. 2010;70(8):3052-61.

60. Sinha P, Chornoguz O, Clements VK, Artemenko KA, Zubarev RA, Ostrand-Rosenberg S. Myeloid-derived suppressor cells express the death receptor Fas and apoptose in response to T cell-expressed FasL. Blood. 2011;117(20):5381-90.