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BAIXAS DOSES DE 5-FLUOROURACIL AUMENTAM A ATIVIDADE ANTITUMORAL DE CÉLULAS DENDRÍTICAS TRANSFECTADAS COM RNA DE CÉLULAS DE CÂNCER COLORRETAL

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Palavras-chave: Câncer colorretal; Células dendríticas; Quimiomodulação; Transfecção de RNA.

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Karl Marx

Lista de abreviaturas

5-FU: 5-fluorouracil

MEC: concentração efetiva mínima

NTC: concentração não-tóxica

CRC/CCR: câncer colorretal

CTL: linfócitos T citotóxicos

DCs: células dendríticas

DMT: dose máxima tolerável

O. D: densidade óptica

GM-CSF: fator estimulador de colônias de granulócitos e macrófagos

HSPs: proteínas de choque térmico

HLA-DR: moléculas de histocompatibilidade de classe II

IL:interleucina

MFI: intensidade mediana de fluorescência

MHC I: complexo principal de histocompatibilidade de classe I

MHC II: complexo principal de histocompatibilidade de classe II

MTT:*thiazolyl blue tetrazoliumbromide*

PBMC: células mononucleares de sangue periférico

TLRs: receptores T*oll-like*

Sumário

CAPITULO I

BAIXAS DOSES DE 5-FLUOROURACIL AUMENTAM A ATIVIDADE ANTITUMORAL DE CÉLULAS DENDRÍTICAS TRANSFECTADAS COM RNA DE CÉLULAS DE CÂNCER COLORRETAL

Resumo

O câncer colorretal (CCR) um dos principais tipos de tumor em todo mundo. Em alguns casos observa-se forte influência hereditária em seu desenvolvimento nas formas familiares, enquanto que na maioria dos pacientes (85%) essa doença se apresenta na forma esporádica, cuja origem é multifatorial, com grande influência da alimentação, tabagismo e etilismo, além de processos inflamatórios crônicos. Seu tratamento convencional consiste em intervenção cirúrgica acompanhada de terapias com doses elevadas de drogas citotóxicas. Apesar dessa intervenção radical, recidivas em pacientes com CCR são muito freqüentes, apontando para a necessidade de abordagens mais efetivas e para a possibilidade de combinação de diferentes formas de tratamento. Dada a importância do CCR como problema de saúde pública e da possibilidade de modulação do sistema imunológico dos pacientes, o presente trabalho é apresentado em dois capítulos, sendo o primeiro uma revisão de literatura sobre a doença propriamente dita, abordando os tratamentos empregados, a influência da resposta inflamatória em sua gênese e o papel da resposta imunológica na doença. Essa revisão foi redigida de acordo com as normas da revista *Cancer Science.* O segundo capítulo, redigido seguindo as normas da revista *Cancer Immunology, Immunotherapy*, refere-se ao desenvolvimento do estudo experimental, no qual foi avaliada a eficiência funcional de células dendríticas (DCs) humanas transfectadas com RNA total de células tumorais pré-tratadas com concentrações não tóxicas ou efetiva mínima de 5-fluorouracil (5-FU). Os resultados obtidos indicam que a transfecção das DCs com RNA de células tumorais expostas à droga aumenta sua capacidade de apresentação de antígenos alogênicos aos linfócitos T e de indução de resposta tumor-específica (geração *in vitro* de linfócitos T citotóxicos e produção de INF-γ). Esse achado mostra associação com aumento na expressão de TLR-4 pélas DC e ligeiro aumento da expressão de moléculas de MHC-II. Nossos dados permitem concluir que a exposição de células tumorais a baixas concentrações de 5-FU aumenta a imunogenicidade tumoral, e que a técnica de transfecção de RNA total é eficaz em transferir antígenos tumorais às DCs.

Introdução

O câncer colorretal (CCR) afeta cerca de um milhão de pessoas por todo o mundo a cada ano, e apresenta uma taxa de mortalidade associada à doença de 33% em países desenvolvidos. Em sua maioria, o CCR ocorre esporadicamente, no entanto,

aproximadamente 25% dos pacientes apresentam histórico familial da doença, o que sugere a existência de uma relação entre genes e o ambiente. Apenas 5% dos casos de CCR apresentam mutação herdada de algum dos principais genes relacionados ao desenvolvimento do CCR, e mesmo as formas familiais estão amplamente relacionadas à interação desses genes com o ambiente em que o paciente está inserido [1-3]. Entre os fatores de risco de desenvolvimento dessa doença destacam-se a idade, ser do gênero masculino, pólipos colônicos prévios, e fatores ambientais, tais como dieta rica em carne vermelha, gordura, ingestão inadequada de fibras, obesidade, sedentarismo, diabetes mellitus, tabagismo e alto consumo de álcool [4]. Demais fatores, como doença inflamatória intestinal crônica, como colite ulcerativa e doença de Crohn aumentam significativamente a chance de CCR [5, 6]. Além disso, o CCR pode também estar associado a síndromes hereditárias, tais como Lynch (ou câncer colorretal hereditário não poliposo) e síndrome de pólipos adenomatosos familial. Aproximadamente 3% dos casos de CCR familial estão relacionados com a síndrome de Lynch, uma das síndromes hereditárias associadas ao câncer mais comuns [7], e apenas 1% apresenta polipose adenomatosa familial [8, 9].

Trata-se de uma doença de múltiplos passos, intimamente relacionada ao acumulo de mutações em genes supressores de tumor e oncogenes, o que o torna um dos principais modelos de estudo referentes à compreensão dos processos de múltiplos passos da carcinogênese. O modelo de tumorigênese colorretal inclui diversas modificações genéticas que são usualmente requeridas para iniciação e progressão do câncer [10].

Há quarenta anos os tratamentos convencionais aplicados no combate ao câncer se resumem em cirurgia, radioterapia e quimioterapia citotóxica. Mais de 50% dos casos de pessoas que foram diagnosticadas com câncer recebem quimioterapia, podendo ser utilizada não só no combate a doença, mas também como terapia neo-adjuvante ou adjuvante em tratamentos pré- ou pós-operatório respectivamente [11]. Quimioterápicos citotóxicos são drogas antineoplásicas classificadas de acordo com seu mecanismo de ação, que incluem agentes anti-microtúbulos ou alquilantes, antraciclinas, antimetabólitos, inibidores de topoisomerases, entre outros [12]. Tais drogas podem atuar tanto em células anormais como normais, apresentando em sua maioria, alta toxicidade para um amplo espectro de células que não deveriam ser o alvo da terapia. Um exemplo notório referente aos danos causados às células normais devido à administração de quimioterápicos citotóxicos é a redução da capacidade proliferativa de

células normais da medula óssea, resultando em neutropenia, linfopenia, trombocitopenia e anemia, culminando no aumento da susceptibilidade a infecções bacterianas e favorecendo infecções oportunistas [13]. Muitos dos agentes ativos dessas drogas atuam durante o ciclo celular, obtendo como resultado final a ativação das vias de morte celular programada por apoptose a partir de danos causados ao DNA suficientes que levam à ativação dessa cascata. Assim, o regime convencional de dose máxima tolerável de agentes antineoplásicos pode provocar prejuízo à resposta imunológica, seja de forma direta sobre as células imunocompetentes ou devido à redução da atividade hematopoiética. Em contraste, estudos recentes demonstraram que o uso de baixas doses de drogas citotóxicas, ou seja, 10 a 30% da dose máxima tolerada, em uma frequência maior (quimioterapia metronômica ou quimioterapia de baixa densidade) podem melhorar a eficácia antitumoral devido à inibição da angiogênese no sítio tumoral [14, 15].

Além disso, concentrações ultrabaixas de quimioterápicos podem modular as vias de sinalização e produção de citocinas, tais como IL-12, IL-10, IL-4 e TNF-α em células dendríticas (DCs) devido à secreção da proteína alarmina por células tumorais que estão em processo de apoptose [16]. Estudos prévios do nosso grupo mostraram que agentes quimioterápicos em concentrações não tóxicas aumentam a apresentação de antígenos pelas DCs [16], assim como a imunogenicidade do câncer colorretal humano [17]. As DCs são células apresentadoras de antígenos profissionais, posicionadas estrategicamente para unir a imunidade inata e adaptativa, além de possuírem muitas funções regulatórias, tais como produção de citocinas, apresentação de antígenos para células T *naïve* e polarização e regulação dos subconjuntos de linfócitos T. As DCs também iniciam as respostas das células T contra tumores, associadas a sua capacidade de processar e apresentar antígenos tumorais e estimular as células T *naïve* [18].

As aplicações médicas das células DCs que objetivam a manipulação da resposta imunológica vêm sendo amplamente investigadas, em particular vacinas de DCs, consideradas um poderoso meio para terapia ativa contra o câncer. Para tanto, essas células podem ser sensibilizadas com lisatos de células tumorais [19, 20], peptídeos [18, 21], proteínas de choque térmico (HSPs) [22] ou ácidos nucléiros (DNA ou RNA) [23]. Vale lembrar que a resposta antitumoral é paciente específica, então as vacinas que são produzidas com antígenos de tumores autólogos podem ser mais eficientes [24].

Diante da elevada incidência mundial do CCR, essa doença é considerada um importante problema de saúde pública, não somente pelo impacto na qualidade de vida

do paciente, mas também pelos altos custos gerados aos governos para seu tratamento. Sendo assim, inúmeras pesquisas, referentes à prevenção e tratamento do CCR vêm crescendo ano após ano, cada vez mais direcionadas ao aprimoramento dos tratamentos, visando minimizar os efeitos colaterais e simultaneamente, otimizar a eficiência dos tratamentos quimioterápicos. Dessa maneira, o presente estudo teve por objetivo, avaliar se a transfecção de DCs com RNA total de células tumorais pré-tratadas com 5-FU aumenta a habilidade das DCs em apresentar antígenos. Para tanto, foi avaliada a influência de diferentes doses de 5-FU sobre a atividade antitumoral de DCs transfectadas com RNA de células de câncer colorretal, seguindo o delineamento metodológico exposto a seguir (figura 1).

Com base no exposto, o presente trabalho apresenta uma introdução redigida na forma de uma "review" a ser submetido à revista *Cancer Science* e o estudo, propriamente dito, redigido de acordo com as normas da revista *Cancer Immunology, Immunotherapy*.

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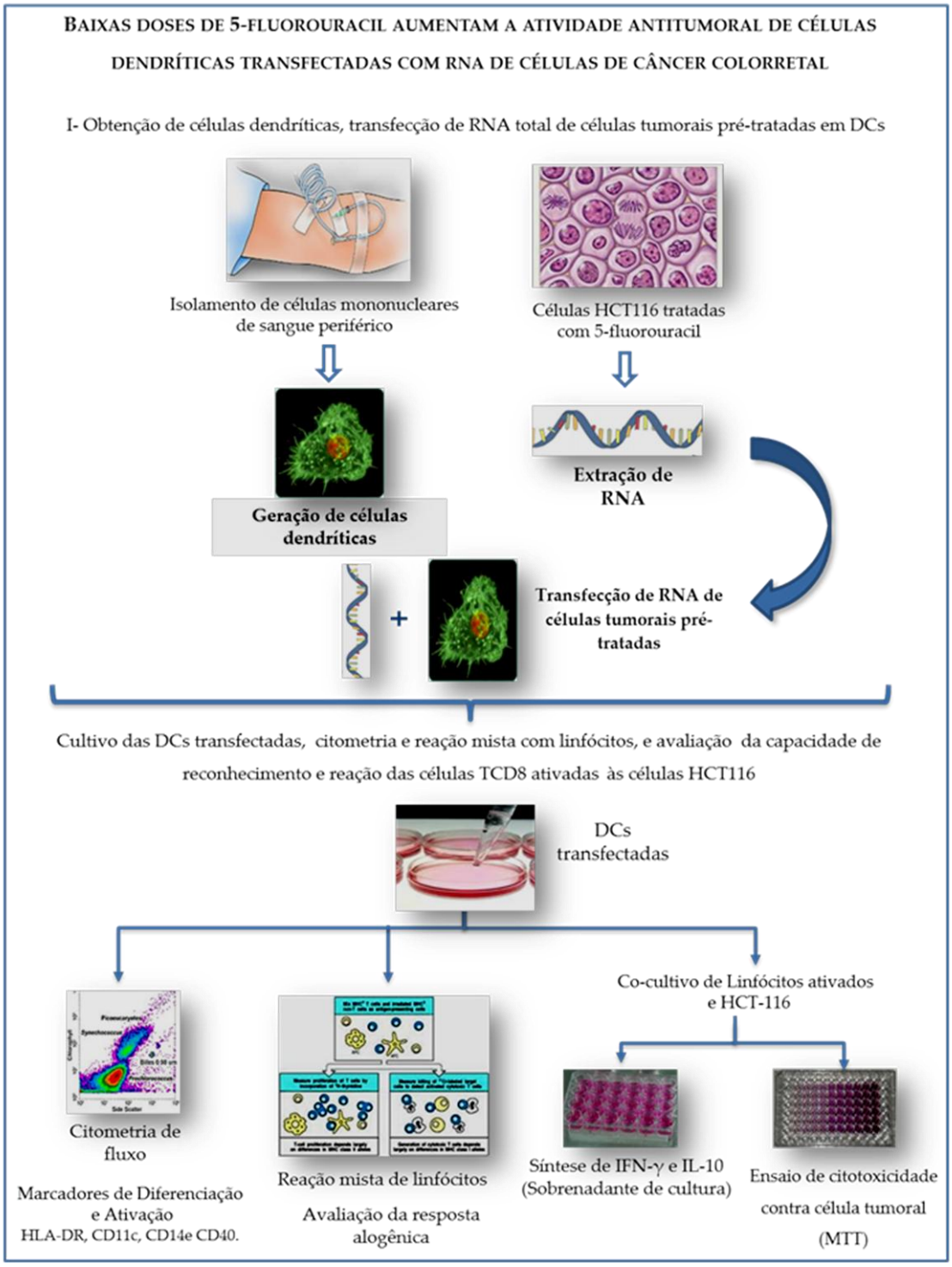
Delineamento experimental



CAPITULO II

REVISÃO BIBLIOGRÁFICA

Artigo redigido com as normas da revista 'Cancer Science'

Colorectal cancer: predisposing factors and immunological aspects

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Abstract

Colorectal cancer (CRC) is one of the most frequent tumor types worldwide, mainly in developed countries, and can be classified as inheritedfamilial (25%) or sporadic (75%) forms, which suggest an important relationship of gene-gene and gene-environment interactions in the development of the disease. CRC is characterized by genetic and epigenetic alterations, and is histologically featured by the infiltration of inflammatory cells among malignant and stromal cells. The most common inflammatory cells in tumor tissue are neutrophils, mast cells, natural killer cells, macrophages and dendritic cells as well as lymphocytes. However, the presence of these cells in CRC can both be associated with cancer inhibition and tumor progression, since the development a chronic inflammatory response can be a predisposal condition for the development of this type of cancer. Considering these multifactorial aspects of CRC development, in this review we present data on the main genetic and epigenetic aspects, and the influence of diet and inflammation on tumor progression. Moreover, we discuss the importance of dendritic cells on CRC immunological responsiveness and their use as therapeutic vaccine for cancer immunotherapy.

Keywords: Chemotherapy; Colorectal cancer; Immunotherapy.

Introduction

Colorectal cancer (CRC) is the third most frequent tumor types worldwide [1], with 40,340 new cases expected in 2013 [2]. Due to it increasing incidence in developing countries, CRC seems to be closely related with changes in the lifestyle [3-5]. In fact, only 25% of patients with this disease have a familial feature, whereas most cases are represented by sporadic forms. Then, it is widely suggested that there is an important contribution of the gene-environment and gene-gene interactions for the development of CRC.

Genetic influence on the development of this disease is evidenced by some molecular profiles, such as chromosomal instability, allelic imbalance at several loci and chromosome amplification and/or translocation, in association with epigenetic alterations, which contribute for tumor aneuploidy [6-9]. Nowadays it is recognized that cancer is not just composed by malignant cells, and includes the stromal and infiltrating immunological or inflammatory cells. Analysis of CRC infiltrating cells demonstrate the presence of the innate immune system cells (macrophages [10], dendritic cells [11], neutrophils [12], mast cells [13] and natural killer cells [14], as well as T and B lymphocytes) [15, 16]. However, the presence of all these cells can be not associated with cancer inhibition; some of them are rather associated with an inflammatory scenario that is closely associated with tumor growth stimulation [17]. In addition, chronic inflammatory diseases are considered a predisposal condition for the development of CRC [18], and inflammation itself, can be influenced by environmental factors such as infections, alcohol consumption, smoking and dietary habits.

Considering such multifactorial influences on CRC, here we reviewed the genetic and epigenetic aspects, the influence of diet and inflammation on it development, the

immunological responsiveness and proposals of immunotherapy based on dendritic cells vaccines.

Genetic and Epigenetic aspects of Colorectal Cancer

The CRC development is a multistep process that involves the accumulation of mutations at both, tumor suppressor genes and oncogenes. In the other words, the tumorigenesis of CRC includes several genetic changes required for initiation and progression of this disease [19]. Nowadays it is know, that it is necessary an association of several intrinsic and extrinsic factories, for the development of CRC such as described by Dong *et al* 2009. In this study, they demonstrated that different alleles of CYP24A1 gene, which is responsible for inactivating vitamin D metabolites, increase the risk of CRC development when vitamin D and calcium are intake together, and associated with UV exposure and estrogen replacement [20]. Thus, it can be stated, that genetic and epigenetic scenario of CRC is very complex, however, there are some classical genetic pathways that are closely involved in the CRC development, which influence on this disease initiation and progress are well established such as the Wnt pathway. Alterations on this pathway could be result from both, inactivation of adenomatous polyposis colitis (*APC*) pathway or activation of β-catenin [21, 22].

Adenomatous polyposis colitis (APC) genes

APC is a tumor suppressor gene on chromosome 5q21, that encodes a large protein with multiple cellular functions and interactions, including an essential regulatory role in the Wnt/Wingless (Wg) signal transduction pathway [23-25]. This pathway is associated with the proteolysis of β-catenin, a multifunctional protein which play a role on genetic regulation and cell-cell adhesion. The APC protein links to β-catenin

preventing the activation of TCF4, a regulatory protein that stimulates the epithelial colon growth when linked to β-catenin. In this way APC is considerate an important contributor for normal growth and differentiation of this epithelium [26]. Loss of this gene is the key initiating trigger for the development of upper and lower gastrointestinal polyps and carcinoma [27]. Mutations in one *APC* allele lead to the intestinal polyp disorder, and familial adenomatous polyposis [28]. Mutations at both alleles are associated with the development of hepatocellular carcinoma [29] and hepatoblastoma [30], being considered an early event in tumourigenesis [31].

More than 95% of known mutations are *frameshift* or nonsense or N-terminal mutations that lead to premature truncation of protein synthesis, whereas just a small fraction of patients presents the silencing of *APC* gene–expression. Germ-line mutations at *APC* are distributed throughout the 5' half of the gene, and the codons 1061 and 1309 are considered hot spots since nearly 35% of *APC* mutations occurs in these two regions [32, 33].

MMR system

Another genetic influence on CRC development is related with the mismatch repair system (MMR), whose genes encode MMR proteins. These proteins are responsible for correcting error on DNA base pairing in newly replicated DNA, particularly prone to slippage and inefficient proofreading by DNA polymerase, such as base mismatches or small insertions/deletions during DNA replication. MMR dysfunction leads to cancer development through the accumulation of unrepaired frameshift mutations in microsatellites, which target genes are involved in cell growth regulation. MMR mutations are associated with tumors in the colorectum, endometrium, stomach and many other organs. About 15% of CRC are caused by mismatch genes repair, and the

most common MMR gene mutations in CRC are at MSH2 (chromosome 2p16) [34] and MLH1 (chromosome 3p21) [35], and the less frequent at MSH6 (chromosome 2p16) [36], PMS1 (chromosome 2q31) and PMS2 (chromosome 7p32) [37]. The most common mutations are the deletion of exon 16 of MLH1, deletion of MSH2 exons 1-6, and A-T transversion in the donor splice site of MSH2 intron 5, but the geographic distribution of these mutations is heterogeneous [38]. Germeline mutations on mismatch repair gene MSH6 are associated with attenuated predisposition to familial CRC [39].

These germeline mutations are the basis of Lynch syndrome or hereditary nonpolyposis colorectal cancer, an autosomal dominant inherited disease, which represents the most common hereditary CRC syndrome (3% of the total CRCs) [33, 40]. Furthermore, almost all CRC associated with hereditary nonpolyposis colorectal cancer present microsatellite instability, but we should to highlight that some patients with microsatellite-stability also present mutations. Therefore, microsatellite instability should not be used as the only basis to select patients for mutational testing to diagnose Lynch syndrome [41, 42].

Microsatellite instability

Microsatellites are short and tandem repeated nucleotides sequences that occur in whole DNA, and are more susceptible to suffer errors during replication due to its repetitive nature [43]. Accumulation of numerous mutations which specifically target these repetitive sequences is another characteristic tumor genetic instability. Frameshift mutations (a germline microsatellite allele has gained or lost repeated units) and base-pair substitutions which undergone a somatic change are commonly found on these regions in some tumors, and they are called microsatellite instability (MSI) [44]. Usually, this phenotype is caused in tumors by loss of the mismatch repair function,

which allows that errors introduced during replication keep unrepaired, leading to MSI [45]. About 15% of sporadic CRC presents MSI, which usually arises because of epigenetic silencing of Human Mut-L Homologue 1 (MSH1) or mutS homolog 2 (MLH2) genes (also referred as *h*MLH1and *h*MSH2) [46] . Aberrations in these caretaker genes, usually do not directly affect cellular functions, such as cell growth, but they can result in mutations in gatekeeper genes such as oncogenes and/or tumor suppressor genes [47]. Aberrant methylation of MLH1 promoter is also associated with BRAF microsatellite instability on CRC [48], one of the RAF kinase family, and an important regulator of the mitogen-activated protein kinase (MAPK) pathway [49] , that are regulated by Ras and mediate cellular responses to growth [50] signals usually mutated. Microsatellite instability in MMR genes is an important genetic alterations, mainly on Hereditary Non Polyposis Colon Cancer (HNPCC), which represents about 5% of the generally inventoried colon cancer cases [51].

Aberrant DNA methylation

Approximately 50% of promoter and transcription start sites of mammalian genome contain CpG islands, a CpG-rich region associated with *de novo* methylation activity [52, 53]. Hypermetilation of these regions is associated with silencing of downstream transcriptional units, being an epigenetic mechanism of silencing [54]. Thus, during the mammalian genome evolution, most of 5'-CpG-3' dinucleotides were lost since these regions are more susceptible to mutations. In fact, it can be observed that as like as the most solid tumors cells, CRC and adenomatous polyp cells show a general hypomethylation in comparison with normal cells. On the other hand, the hypermetilation of regulator genes, which leads to their transcriptional silencing, also

contribute to deregulated expression of important genes associated with cell cycle [55, 56].

Hypermethylation of various genes has been associated with human colorectal tumourigenesis [57, 58], changes that are classified as CIMP (CpG island methylation phenotype), mainly affecting the above mentioned genes. Furthermore, hypomethylation might also decrease the fidelity of chromosomal segregation, which could also contribute for chromosomal instability (CIN) phenotype [59]. Some authors defend that the methylation profile of patient gene could be an important biomarker to early detection of CRC, as well as, that it could be used as prognostic in diagnosis of the disease [60].

Chromosomal instability

In contrast with some other cancers, CRC does not commonly involve amplification of gene copy number or gene rearrangement, but it is usual to observe CIN [7, 61]. It is a phenomenon that is mainly characterized by chromosomal rearrangements and numerical abnormalities due to both gains and losses, and loss of heterozygosity. Several genes have been associated with the CIN pathway, but the molecular bases of this phenomenon are still unclear [62]. One of the most common genes associated with CIN is TP53, which inactivation has been associated with aneuploidy in cancers. Although TP53 are mutated in 29% of CRC, this gene is not specifically associated with CRC development. Actually the loss of it function is a critical event for the development and progression of the most of human carcinomas [63]. The transcription factor encoded by TP53 (p53) is considered a “genome guardian”, once it is involved in fundamental processes such as cellular responses to DNA damage, cell cycle regulation and oxidative stress and apoptosis [64].

KRAS is also considered an important proto-oncogene for tumor with CIN since KRAS somatic mutations are found in 40% of CRC, mainly on codons 12 and 13, and rarely on codon 61 [19, 65]. This gene is involved in growth differentiation, cell survival and proliferation, apoptotic inflammation and cell transformation [66]. Frequency of KRAS mutation closely depends on the size and degree of dysplasia at tumor site, since just 10% of adenomas smaller than 1cm show KRAS mutations, whereas they are found in 40% of larger lesions [67]. However, Tomassi *et al* showed that KRAS gene methylation has no relationship with tumor pathological characteristics such as stage, histological type, grading or nodal status [68].

Other genes associated with CRC development

CDKN2B/p15 mutations and/or methylation were observed in Chinese, North American and Egyptian specimens of CRC [69-71]. Another gene that can be mutated in 15~20% CRC is PTEN [72, 73]. Somatic mutations in AXIN1 and AXIN2 genes have also been observed in some patients, but the significance of these mutations are still unclear [74]. The CDK8 gene, a cyclin-dependent kinase family member, is amplified in approximately 10-15% of CRC [75].

Dietary habits and lifestyle

Nowadays it is estimated that 30-40% of cancer is caused by food, nutrition and other lifestyle factors, making it a somewhat preventable disease. Diet components are the major source of mutagenic compounds that may cause both, initiation and tumor progression [76]. In this way, after reviewed 752 publications, the World Cancer

Research Foundation and the American Institute for Cancer Research concluded that diet is definitely the most important exogenous factor in the etiology of colorectal cancer identified so far [77]. Etiological studies have shown strong correlation between CRC incidence and excessive consumption of fat and protein (mainly of animal sources), processed meat, and substantial consumption of alcohol (more than 30g/day) [78-80].

Furthermore, people with increased intake of heterocyclic amines (HCA) are more susceptible to develop CRC. These heterocyclic amines are formed when meat is cooked for longtime with the internal temperature reaching between 150°C and 200°C, and high external charring (e.g. barbeque). The main heterocyclic amines generated in such conditions are 2-amino-1-methyl-6-phenyl-imidazo [4,5-b]pyridine (PhIP), 2-amino-3,8-dimethylimidazo [4,5-f]quinoxaline (MeIQx), and also benzo[a]pyrene (Bap) a polycyclic aromatic hydrocarbon (PAH), the first reported group of chemical carcinogens for human cells [81].

Some studies reviewed and confirmed by McEvoy*et al* also demonstrated that vegetarian diet helps to prevent cardiovascular diseases, type 2 diabetes and cancer [82]. This theory is supported by the antioxidant hypothesis, which predicts that fruits and vegetables invariably contain antioxidants (vitamins C and E, carotenoids, flavonoids, and others) which scavenge free radicals and prevent DNA damages responsible for mutations and eventually cancer [83]. Furthermore, reactive oxygen species are involved in immune protection and in cell signaling pathways [84]. The vegetarian diet also includes a variety of nuts, which are rich in plant sterols, antioxidant vitamins, minerals and fibers, and all these nutrients are associated with reduction of cancer development risk [85]. All these compounds can protect cells by affecting phases I and II of biotransformation/detoxification pathways, as well as cell

signaling pathways and endogenous antioxidant system [86]. Some micronutrients such as zinc [87] and selenium [88] have been extensively studied and they seems to have important roles on cancer prevention, whereas complexes compounds such as carotenoids [89], flavonoids [90], curcumin and silymarin [91], resveratrol [92], folate [93] and total oligomeric flavonoids [94] show both direct activity against tumor cells and *in vitro*immunomodulatory effects.

Diets can also influence the features of gut microbiota, which is constituted by our own wild microbial community, whose majority of microorganisms (10 to 100 trillion) inhabits our gastrointestinal tract [95-98]. The composition of gut microbiota is influenced by different factors, such as host, age, sex, geography, ethnicity and the diet [99]. In turn, the intestinal microbiota can affect physiological features of the human host such as intestinal epithelial cell proliferation and differentiation, pH, function, and development of the immune system and protection against pathogens [100]. Moreover, the microorganisms of the gut microbiota modulate pathways such as NF- κB [101], and downregulate the enterocyte pro-inflammatory response, producing metabolites which target the NF-κB-dependent pathway [102, 103]. NF- κB is involved with inflammatory gene transcription and with the initiation of a transcriptional response for promoting cell cycle progression and inhibition of apoptosis [104].

Epidemiological studies show that diet-gene interactions are among the leading causes to explain the wide variation of CRC developing risk among different individuals [105]. Considering all these data, the Department of Health and Human Services at National Institutes of Health and The Agency for Healthcare Research and Quality have been trying to implement lifestyle interventions among the population, aiming to alert the importance of diet and healthy lifestyle for preventing diseases including cancer [106, 107]. Not only bad dietary habits are involved with cancer

development and other factors, such as high body mass index (BMI), increased smoking and sedentary lifestyles are strongly associated with increased rates of CRC development and other cancer types [108].

Inflammatory and Immunological response against CRC

Besides the occurrence of genetic and epigenetic abnormalities, the presence of inflammatory microenvironment also plays a crucial role in CRC development. Furthermore, many aspects of malignancy are affected by cancer-associated inflammation, such as proliferation and survival of malignant cells, angiogenesis and tumor metastasis [18, 109,110].

The immune system has also an important role in antitumor resistance, and involves the interaction of several cell types and products secreted during adaptive and innate immune responses [111]. The concept of immune surveillance was first formulated by Mcfarlane Burnet on 1950's, predicting the ability of the immune system to distinguish between self and non-self for eliminating cancer cells [112]. However, nowadays this theory is considered somewhat controversial, since the presence of regulatory T cells (Tregs) is linked to the self-tolerance and immune suppression by producing immune suppressor cytokines [113], and these cytokines can also avoid the immune response against tumor cell. Moreover, various biologically active molecules such as PGE2, TNF-α, interleukins IL-1β and IL-6 are able to promote inflammatory response and enhance the CRC development [97]. In fact, the expression of pro-inflammatory cytokines TNF- α and IL-1β, for example, enhance the expression of adhesion molecules, which is responsible for increased interactions between different inflammatory cells, facilitating local invasion and metastasis [114, 115]. Therefore,

mechanisms of innate barrier can be involved both in antitumor resistance and carcinogenesis promotion, whereas the development of specific responsiveness opens the possibility to handle it for a protective profile.

Genetic control of inflammatory responsiveness and tumor susceptibility has been investigated by some groups. On this subject, it was observed that mice selected for high acute inflammatory reaction (AIRmax mice) show higher susceptibility to the development of chemically induced CRC, than those that show poor responsiveness. Genetic control of inflammatory response and susceptibility to some kinds of tumors seems to be associated with fatty acyl CoA reductase 1 (*Far1*), eukaryotic translation initiation factor 4, γ2 (*Eif4g2*), and protein kinase Cb (*Prkcb*) [116]. The ability for intense or poor inflammatory responsiveness (AIRmax and AIRmin mice) seems to be detached from specific immune responsiveness. However, there is no clear featuring of the role of antigen presenting cells and lymphocytes in these animals [117].

Innate and Adaptive Immunity

In the gastrointestinal system, the innate immunity is performed by mucous, enterocytes and the bowel wall, which represent the physical barrier to pathogens. Under it fail, gut can be infiltrated by phagocytic cells, such as neutrophils and macrophages, followed by activation of inflammatory and complement pathways [118]. The inflammatory process aims to destroy pathogens and abnormal tissues, and is responsible for promoting tissue reconstruction. However, in this disease the inflammation is rather associated with the promotion step of carcinogenesis, since it provides a nutrient-rich microenvironment for cancer cells and promotes neoangiogenesis [119]. Thus, increased microvessel density, as well as maintenance of

the inflammatory response is associated with poor survival and enhanced cancer growth. Examples of procarcinogenic role of inflammation in CRC include it strong association with chronic inflammatory diseases such as Crohn's disease and ulcerative colitis [120-123].

The adaptive response, in turn, is characterized by it highly specific and immunological memory. It is held by B and T lymphocytes, and in CRC tumor, plays both pro- and antitumorigenic roles. Th2, Treg and B lymphocytes are found at the tumor microenvironment, all of them associated with tumor growth stimulation, while infiltration of CD8+ CTL and TH1are associated with tumor growth inhibition [18, 124, 125]. The role of TH17 in cancer is not completely understood since they show pro-tumorigenic behavior in some cases and seems to fight cancer cells in other models [126].

Neutrophils

Neutrophils are the first defense mechanism against pathogens overcoming physical and chemical barriers taking part of acute inflammatory response. Despite neutrophils are commonly found on inflammatory tissues, the levels of this cell are highly increased in CRC as others cancers, reaching up to 15% of the inflammatory infiltrating cells, a proportion that could be higher in necrosis areas. The roles of neutrophils in CRC is largely studied and nowadays they are also being investigated in different cancer populations as prognostic and predictive markers [127]. CXCL1 and CXCL8 are chemokine involved in the recruitment of neutrophils in gastric and colon carcinomas [104, 128-130]. It is already know that they significantly affect tumor angiogenesis, by releasing molecules such as oncostatin M, that in turn stimulates tumor cells to produce VEGF, an important factor for tumor angiogenesis [131, 132]. Angiogenesis can also be

stimulated by pro-inflammatory cytokines themselves. In fact secretion of VEGF and bFGF can be stimulated by IL-1β and TNF-α produced by macrophages.

These cells also produce reactive oxygen species (ROS) and myeloperoxidase that can lead to malignant transformation through oxidative DNA damage and myeloperoxidase related metabolic activation of chemical carcinogenesis, respectively [114, 133].

Macrophages

Besides the intense phagocytic role, macrophages are important antigen presenting cells (*APC*) for the immune system and express molecules required for T cells activation, mainly when associated with an invasive cancer scenario [134]. Macrophages are a significant part of the tumor-infiltrating immune cells and when recruited to the tumor site by growth factors, chemokines and angiogenic factors, they are called tumor associated macrophage (TAMs) [135, 136]. Despite some authors had demonstrate that *in vitro* stimulation of TAM makes them able to kill tumor cells [137, 138], they have no cytotoxic activity when conditioned by the tumor-microenvironment [122]. In this scenario, TAM are rather associated with the growth, angiogenesis, and metastasis of different cancers, such as breast and cervical cancers and transitional cell carcinomas [139]. Furthermore, the secretion of some cytokines such as IL-1, IL-6 and TNF-α activate NF-κα pathway in CRC, a key regulator of innate immunity and inflammation, which in normal conditions is kept in an inactive form. The activation of NF-κB pathway controls the survival of transformed cells and the leukocyte-driven inflammation providing the signaling molecules to sustain the tumor growth [140].

TAMs are the primary source of pro-inflammatory cytokines, such as IL-1β and TNF-α, and are recruited to tumor site by CCL2 and CCL5, VEGF, TGF-β and colony

stimulating factors (GM-CSF and M-CSF) [141, 142]. These cells are classified as type 1 (M1) and 2 (M2) macrophages, whose differentiation depends on the microenvironment stimuli [143, 144]. The classic M1 has high capacity to present antigens, produces high levels of IL-12 and IL-23 production, and activates Th1 response [145]. These cells are also characterized by IL–12^{high}, IL-23^{high}, IL-10^{low} phenotype, and are associated with resistance against intracellular parasites and tumors [146]. In contrast, M2 macrophages show IL-12^{low}, IL-10^{high} phenotype, and are associated with suppression of inflammation, induction of Th2 response,active debris scavenge, wound healing promotion, angiogenesis and tissue remodeling [147, 148].

TAMs, as like as neutrophils, secret VEGF and several other pro-angiogenic factors such as basic fibroblast growth factor (bFGF), TNF-α, IL-1β, IL-8 (CXCL8), COX-2, platelet derived growth factor-β (PDGF-β), hepatocyte growth factor (HGF), matrix metalloproteinases (MMP-7 and MMP12), all of them are associated with metastasis [149-152]. M2-polarized myeloid cells have been shown to influence fundamental aspects of tumor biology, allowing a positive feedback between tumor cells and macrophages since these cells promote tissue remodeling and angiogenesis [123]. These cells release TNF-β and IL-10 that promote the growth of tumor stroma and inhibit the adaptive immunity [97]. However, the role of macrophages which are usually found around necrotic areas at tumor site and advancing tumor margin in CRC [104] is controversial, since at the same time, some authors have shown that an increased density of macrophages in CRC is correlated with a good prognosis [153, 154]. In CRC, the increased secretion of TGF-β by both, tumor cells and macrophages plays a key role in the epithelial-mesenchymaltransition, which enhances the tumor progression and metastasis [155]. Differently of other solid tumor in which peritumoral TAMs prevent tumor development, intratumor TAM concentration was show to correlate with invasion

depth, lymph node metastasis, and CRC staging, suggesting that intratumoral M2 macrophages leads to a more aggressive behavior of cancer cells [156-158].

Natural killer cells

Natural killer cells (NK) has the capacity to eliminate HLA^- tumor cells since they are not MHC restricted effect cells. They express several ligands of the TNF family and can induce apoptosis of malignant target cells, which can be further endocitosized by dendritic cells (DC) and macrophages, and processed for subsequent presentation for T cells [157]. In CRC, high concentrations of NK cells in the inflammatory infiltrate are associated with better prognosis, and this concentration decreases as cancer stage increases [104] confirming their ability of eliminate tumor cells without previous exposure/sensitization or clonal expansion [159]. The use of 5-fluorouracil, the main chemotherapeutic drug for CRC treatment, increases the numbers of NK cells [160, 161].

Mast cells

Mast cells (MCs) are bone marrow derived cells, located around the vessels in the most tissue. They express receptors for several cytokines including, TNF-α, IL-1β, and IL-6, which confirm their intermediate role between innate and adaptive immune response [162].

High numbers of MCs are associated with early stages of CRC [163], and some authors suggested that these inflammatory cell infiltrates lead to an improved survival [164]. Some studies demonstrated that MCs facilitate tumor progression on CRC, since depletion of these cells led to remission of existing polyps [165], however, the role of these cells on CRC progression is not clear. At the same time, since the imbalance

between pro- and anti-inflammatory cytokines is thought to play a pivotal role in modulating colonic inflammation [166], MC should play an import role on CRC development.

Dendritic cells

Dendritic cells (DC) belong to the innate immunity but work as a bridge between innate and adaptive response. These cells are the main antigen presenting cells uptaking and processing exogenous Ags for presentation to T lymphocytes in the context of the major histocompatibility complex (MHC) molecules [167, 168]. Cytolytic CD8+ T cells can also be identified within the tumor infiltrating lymphocytes and both $CD4^+$ and $CD8^+$ T cells are required for effective elimination of HLA class I+ tumor cells [169].

DC are constitutive cells of lamina propria and are involved in every pathological condition. DC obtained by mechanical disaggregation and enzymatic digestion of intestine specimens of patients with different types of colon disease, including colorectal cancer, Chron's disease, ulcerative colitis and non-malignant, non-inflammatory conditions was shown to correspond to 2% of cells from lamina propria [170]. These cells were shown to be low-density, non-phagocytic, non-adherent with oval or pleiomorphic nuclei, and the cytoplasm shows only occasional vesicles. Colon DC express higher density of MHC class II then macrophages and low adherence to fibronectin. Concerning their ability to stimulate lymphocyte activity, DC-rich suspension induces high mixed lymphocyte response (MLR) by T cells. However, tumor infiltrating DC poorly stimulate T lymphocytes in a primary allogeneic culture (MLR) and are not able to induce significant levels of IL-2 and IFN-γ [158].

Immunohistochemical analysis of infiltrating cells showed that mature CD83+ DC are found in almost all primary colon carcinoma samples and in some metastasis. Heterogeneous patterns of infiltration were observed varying since diffused cells in some cases to clustered DC in others. There is a tendency to their accumulation around vascular structures and in marginal zone of lymphoid aggregates [11]. Expression of MHC class II (HLA-DR) was abundant in DC of primary colon cancer but data on activation markers are contradictory. In fact, it was reported that 90% of CD83+ cells were double stained by anti-CD40 or anti-CD86 antibodies, indicating their *in vivo* activation [11], whereas others reported that most of tumor infiltrating DC (64-97%) do not express B-7 molecules [171, 172] even after stimulation with TNF-α, IL-4 and GM-CSF [160]. The data of these two groups on the frequency of tumor infiltrating DC are also conflicting. Schwaab *et al* [11] have found lower number of these cells at the tumor site than in normal colon tissue, mainly in those samples of metastasis, whereas the previous investigations by Chaux *et al* [171, 172] showed higher frequency in the tumor site than in normal lamina propria. DC density at tumor site was higher in patients with high proportion of activation markers (CD86 and CD40), suggesting that mature DC can actively migrate to, or be activated in the tumor microenvironment under exposition to tumor antigen [171]. Under the clinical point of view, it was observed a tendency towards improved survival in those patients with intermediate and high density of DC in tumor site, highlighting the importance of these cells to antitumor resistance.

Being the main professional antigen-presenting cells, DC constitutively express both class I and II major histocompatibility complex (MHC) antigens on their surface. This feature is closely associated with their effective antigen-presenting function, and strategies for improving the expression of these molecules has been proved to enhance

the antitumor response triggered by DC vaccines. In this aspect, it was early observed that increasing the expression of MHC class II molecules on DC by transfecting them with MHC class II transactivator genes (CIITA) induces 4 times more CTL than parental untransfected DC or DC transfected with irrelevant genes [173].

If the expression of MHC class I molecules by DC is important for antigen presentation function of these cells, their expression by tumor cells is also fundamental for the development of an effective immune response, since they are the main target for the specific cytolytic T lymphocytes (CTL). However, many tumor cells fail to express normal levels of MHC class I molecules, which works as one of the main escape mechanism of these cells, since it avoids their recognition by effector CTL [174-176]. Although the low expression of MHC class I allow the tumor cells being recognized by natural killer cells (NK), the latter are not able to completely replace the protective activity of CTL, and MHC class I cells are considered to be more invasive than MHC class I+ tumor cell types [177-179]. Synthesis and expression of MHC class I molecules are dependent on the multistep antigen processing machinery (APM). APM components include those involved in the antigen processing itself and those involved in the assemble of antigen peptides to MHC molecules [180, 181]. Low concentrations of selected chemotherapeutic such as paclitaxel (PAC) and doxorubicin (DOX) upregulate the expression of key APM components including calmodulin, LMP2, LMP7, TAP1 and tapasin of murine DC and it upregulation is associated with increased antigen-presenting function of these cells [169, 182].

Toll-like receptors are also involved with DC functions. Treatment of colon tumor-bearing mice with BCG induced the acquisition of TRAIL (TNF-related apoptosis-inducing ligand) by tumor infiltrating DC and stimulated the T response against colon cancer cells via TRL2, TLR4, and TLR9 on DC. Such a treatment can be combined

with the inoculation of cyclophosphamide to deplete tumor induced Treg [183]. Tlr4-/- and Myd88-/- DC were defective to present tumor antigens, whereas the lack of other receptors did not affect this function [184]. Pulsing wild type DC with TLR4 inhibitory peptide or TLR4-Fc fusion protein also inhibits MHC class I-restricted antigen presentation. Lack of TLR4 affects the ex vivo production of IFN-γ by lymph node cells.

The C-type lectin DC-SIGN (DC-specific intercellular adhesion molecule-3-grabbing non-integrin), is involved in the recognition of colorectal cancer cells by DC [185]. DC SIGN is found in immature, but not mature DC within colon tumor tissue, and interact with tumor cells through the binding to $Lewis_x$ and $Lewis_y$ carbohydrate on CEA. Interestingly, DC-SIGN do not interact with CEA expressed by normal colon epithelium that shows low levels of Lewis epitopes. These findings were reinforced by the observation that DC interact with human colon SW1116 tumor cells that express aberrantly glycosylated Lewis epitopes (Lea/Leb). These epitopes are on CEA and CEA-related cell adhesion molecule 1 (CAECAM1) and such an interaction induces the production of the immunosuppressive cytokines IL-6 and IL-10 [186].

Some tumor cells express the antigen β-galactosidase (β-Gal) and transfection of DC with this antigen confers long-term protection of mice against tumor growth, both in profilaxis and therapeutic models of tumor [187]. Experimental studies have shown that intralesional administration of IL-12-transfected dendritic cells efficiently eradicates established colon tumor mass [184, 188].

Dendritic cell-based vaccine

The central role of DC in antitumor immunity supported the development of DC-based therapeutic vaccines and nowadays there is a variety of approaches to generate

them. In an early report, even before the blooming of proposals for DC-based antitumor vaccines, it was observed that monocyte-derived phagocytic cells could be sensitized by apoptotic bodies obtained by dead tumor cells [189]. Current studies are still using peripheral blood cells to generate human DC and bone marrow cells for murine ones, however, the efficiency of these vaccines appears to be dependent on a number of factors, such as generation of mature DC [174, 190, 191],sustained production of some cytokines [176, 192, 193] and overcoming of suppressive microenvironment provided by regulatory T cells [171, 174, 194] and mieloyd-derived suppressor cells [195]. Many reviews on experimental and clinical data on the feasibility of DC-based anticancer vaccines have been published and it has been observed that each type of tumor has particular features that can hinder the effectiveness of such a vaccine. Then, in this section we focus on the experimental and clinical efforts towards the development of a feasible DC-based vaccine against colorectal cancer.

Experimental and clinical approaches for developing DC-based vaccine for CRC

One of the main issues for generation of clinical graded DC-based vaccines for inducing specific immune response is to choose the technique to load DC with tumor antigens. They range from the easier antigen preparation of tumor cells lysate by quick freeze and thaw cycles until the generation of tumor-DC fusing cells or the DC transfection with tumor DNA or RNA. However, there is no definitive agreement about what strategy is the best. Results with tumor cells lysates loaded DC are controversial since some studies have shown that this approach promotes poor protective role, whereas other authors have been successful with their DC preparation. He *et al* [196] observed that lysate-pulsed DC had poor protective effectagainst the development of CT26 cells, despite of their ability to stimulate CTL activity, as well as INF-γ production during CTL assay. Rather, these DC are likely skew CD4 responses away

from an optimal DC maturation and TH1 responsiveness with limited up-regulation of CD80 and CD86 even after stimulation with LPS [197]. Actually, lysate-pulsed DC showed a more immature phenotype than unpulsed controls, and dramatically reduced the production of IL-12 p70 and TNF-α and increased IL-10. In addition, they observed that whole cell lysate inhibits the TLR-induced activation and IFN-γ production following co-culture with T lymphocytes. Lysate was also unable to induce stimulation of specific CD8 cells against B16.OVA target cells [196]**.**

Conversely, in our own experience, lysate-loaded DC was able to delay and even avoid the s.c. development of MC-38 cells without any supplementary treatment in 60-75% of mice (unpublished data). Moreover, in previous reported results of our group, we demonstrated that human DC loaded with colon cancer cell lysates efficiently stimulated allogeneic responsiveness (MLR) and induces CTL *in vitro* [182, 183]. Our results are in agreement with Larmonier*et al* 2006 that 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 quick freeze-thaw process, induction of syncitia by transfection with fusogenic viral vector, or treatment of HSV-transfected cells with ganciclovir [198]. Results have shown that the way how 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 to induce T cell proliferation. Lysate-pulsed DC are even able to avoid the growth of liver metastasis of colon cancer cells in the murine model of MC-106 cells [199]**.**

In fact, some details can make the difference for the effectiveness of lysate-pulsed DC vaccines. For instanceinhibitory effect of lysate on DC maturation can be reduced

when tumor cells are stressed by heating at 42ºC for 25 min previously to the cell lysate preparation [193]. It is hypothesized that the expression of heat shock proteins by tumor cells can avoid the suppressive effect of cell lysate by increasing DC maturation as also observed by others [200-202]**.**

Sonication of freeze/thawing lysates is another proposed method for preparing tumor antigens. Rossowska *et al* [203] observed that DC loaded with this preparation migrate to tumor draining lymph nodes already at the first day after injection, and the number of DC increased gradually in the tumor site until the 5th day. Most of injected DC survived until the 7th day in the injection site and 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 showed poor effect on the ability of DC to stimulate antitumor activity [204]. Stressed tumor cells were obtained by freeze/thaw cycles or by irradiation at 30Gy, being the irradiation more useful than 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 to stop their proliferative ability and did not affect their usefulness for preparing tumor-DC hybrids. Results have shown that such hybrids had 100% of efficiency for protect mice of tumor development.

Fusion technology was also used by Kao *et al* 2006 [205], who prepared DC vaccine by fusing them with CCCT 26 cells. Although this vaccine was able to prevent growth of lethal inoculum of tumor cells and induced a strong *in vitro* CTL response, it did not showed therapeutic role, failing to inhibit the growth of pre-existing tumor, even after LPS-induced maturation. It was observed a TH-2–dominant response in vaccinated animals, which showed prevalence of IL-5-producing cells over IFN-γ-producing ones

and increased IL-4 and IL-10 producing cells. Since authors did not observed differences in the number of TGF-β-producing cells nor on the number of CD4+/CD25+ cells, they considered that their vaccine induced an aberrant TH2 response rather than increased 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 tested them in clinical in vivo trial [206].

Whether a patient was unable to fight the tumor development, it is probable that his/her own DC was unable to efficiently process and present relevant tumor antigens for generate specific CTLs. Since most of tumor antigens peptides are considered to be self antigens, it makes difficult the generation of an effective *in vitro* CTL response. This point of view has leaded some authors to suggest the use of allogeneic or semi-allogeneic systems to generate DC vaccines. Fusion of allogeneic DC with autologous metastatic colon cancer cells was able to activate both CD4+ and CD8+ T cells in just 24h, in a higher number than controls. These CD8+ cells were significantly able to lysis target cells [206]**.** It also can solve some practical problems such as a) usually it is possible to generate a limited samples of autologous DC for vaccination and a higher number of DC could be generated from health allogeneic or semi-allogeneic donor; b) the cellular reactivity triggered by allogeneic or semi-allogeneic DC for allogeneic MHC antigens could facilitate the elimination of escape tumor variants, as it happens in the recipients of semi-allogeneic bone marrow transplantation [207] and c) autologous tumor cells are sometimes scarce and the use of stable tumor cell lines as the source of allogeneic tumor antigens for pulsing autologous DC could be an alternative to solve this question [208].

Evaluation of the efficiency of syngeneic, allogeneic and semi-allogeneic murine DC have shown that hybrids cells prepared with allogeneic or semi-allogeneic DC

were more effective than syngeneic ones and also worked better as therapeutic vaccines protecting hosts from pulmonary metastasis. Actually, allogeneic and semi-allogeneic DC more effectively induced CTL activity, as well as NK cytotoxicity, and induce higher levels of IFN-γ, increasing the IFN-γ:IL-10 ratio [209].

Combination of T4 phages and tumor Ag-primed T cells increased the percentage of CD8+ IFN-γ producing cells and augmented the expression of differentiation markers on DC. It combination was also able to delay the growth of advanced MC38 colon carcinoma being that T4 phage alone and tumor antigen associated T4 increased the expression of CD80, 86, 40 and CD54 [210].

Transfection of DC for expressing CD40-ligand (CD40L) establish a potent antitumor state in host mice and this effect is sustained even when the number of inoculated DC was 5-fold less than usually inoculated ($2x10^5$). These cells can migrate from the subcutaneous site to spleen, where they activate relevant antitumor T cells [211].

Transfection of DC with known tumor antigens RNA is another strategy for DC loading, being the carcinoembrionic antigen (CEA) one of the preferred target for this purpose in colon cancer [212-216]**.** Ojima *et al* [217] worked with murine MC38 tumor cells transfected with human CEA by adenoviral vector, whereas DC were transfected to express GM-CSF, IL-12 or CEA. *In vivo* experiments showed that 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. Role of these effector cells was confirmed by immunofluorescence microscopy, showing heavy tumor infiltration by both NK and CD8+ surrounding 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 [218]. 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 and it was observed increased expression of memory phenotype ($Ly\text{-}6C^{+}$ and $CD44^{+}$).

Immunization of patients with DC vaccine in a phase I/II clinical trials showed that the vaccine was effective for 16,7% of patients in the phase I study and for 23% of them in the phase II study [209]. Messenger RNA for 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 [219]. Since mRNA represents only 5% of total cell RNA, *in vitro* amplification of mRNA was showed to be feasible to produced immunogenically active CEA-encoding mRNA [210].

Instead of using mRNA for known specific antigens such as CEA and Her2/neu, Nencione A *et al* 2003 [220] transfected DC with total tumor RNA using an EGFP-encoding vector. They observed that DC transfected with colon cancer cells RNA were able to induce CTL response and that effector cells were able to recognize both the original tumor cell line used for RNA preparation (SW480) and other cell lines, HCT-116 (colon cancer) and A498 (renal cancer).Supporting this strategy a clinical trialusing total RNA extracted from metastasis tumor cells for pulsing autologous DC (4

injections, every 4 weeks) showed to be feasible for inducing specific T response against CEA [221].

Analysis of 10 clinical samples of colorectal carcinomas showed that 60% of them overexpressed the antigen EphA2 [222]. Murine DC pulsed with human EphA2 was observed to induce antitumor response against EphA2-transfected MC38 cells. Results have shown that Eph-DC strongly delayed the tumor growth and induced specific CD8+ cells and CD4+ that play critical role in the antitumor response.

In conclusion, despite of the multifactorial rise of CRC immunomodulatory approaches focusing on specific antitumor immune response seems to deserve continuous investigation to achieve effective immunotherapeutical tools. In addition, such immunological approaches can interfere on the development of inflammatory reaction, strongly helping to avoid tumor progression. In this aspect, the development of therapeutic dendritic cells vaccines, specific monoclonal antibodies, cytokine-based interventions, as well as the several gene-therapy proposals should be investigated in association with conventional chemotherapeutic agents.

Conflict of interest statement

Authors declare that there is no conflict of interest.

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CAPITULO III

ARTIGO EXPERIMENTAL

Artigo redigido de acordo com as normas da revista 'Cancer Immunology, Immunotherapy'

Treatment of colon tumor cells with low concentration of 5-fluorouracil enhances the effectiveness of RNA-transfected antitumor dendritic cells vaccine

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Research area: Tumor Immunology

Abstract

INTRODUCTION AND OBJECTIVES: Colorectal cancer (CRC) is the third most common cancer worldwide. Conventional treatment for CRC consists in surgical intervention followed by adjuvant therapy with cytotoxic chemotherapeutic agents, but it systemic effect can induce collateral damage to normal tissues and organs. Lower doses of cytotoxic drugs work mainlyby inhibiting tumor angiogenesis. In addition, low concentrations of selected chemotherapeutics positively modulate dendritic cells (DCs) increasing their ability for antigen presentation. We have previously observed that *in vitro* treatment of colon cancer cells HCT-116 with non-apoptotic concentrations of paclitaxel or doxorubicin increase their immunogenicity. Since this functional alteration is associated with changes in gene expression, in the present study we aimed to evaluate whether DCs transfection with total RNA of 5-fluorouracil (5-FU) treated tumor cell is able to increase the antigen-presenting ability.

MATERIALS AND METHODS: HCT-116 cells were cultured and treated with two different concentrations of 5-FU, and their total RNA was transfected into human monocyte derived DCs. Phenotypic changes were analyzed by flow cytometry. The functional DCs activity was evaluated through their ability to stimulate the proliferation of normal allogeneic T lymphocytes (MLR), and to generate cytolytic T cells. Specific antitumor activity was also evaluated by generation of cytolytic T lymphocytes (CTL), and IFN-γ and IL-10 *in vitro* synthesis by these cells was also analyzed.

RESULTS AND CONCLUSION: Tumor RNA-transfected DCs showed increased ability to induce allogeneic T cell proliferation and generation of specific anti-HCT-116 cytolytic T cells, as well as *in vitro*IFN-γ production. These transfected DC showed a slightly increase of HLA-DR expression, and increased percentage of $TLR4^{+}$ DCs after

transfection. However, no changes were observed on other maturation/activation markers. Our results allow us to conclude that treatment of tumor cells with nontoxic concentration of 5-FU induces immunogenic changes that can be transferred toDCs by transfection of total RNA.

KEYWORDS:Chemomodulation, Colorectal cancer, Dendritic cells, RNA transfection.

Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide, and one of the major causes of morbidity and mortality (after lung and breast cancer) [1]. CRC can be classified as sporadic (about 85%) or familial (15%), and it can be influenced by some genetic factors, such as inherited and somatic gene mutations, as well as inflammatory disease and environmental factors [2]. The incidence of CRC varies among different groups, depending on the country, gender, age and lifestyle, being more frequent in industrialized countries, and more prevalent in men [3, 4].

The primary treatment for CRC is surgical resection of the tumor followed by adjuvant therapy with chemotherapeutic agents [5]. Nevertheless, for patients with metastatic diseases antineoplastic chemotherapy is the first line treatment, aiming to prolong survival and trying to maintain quality of life. In some cases local irradiation is associated with chemotherapy as a preoperative treatment [6]. The main chemotherapeutic agents for CRC are hypomethylating agent 5-azacytidine (5-aza), the antimicrotubuletaxans such as paclitaxel and docetaxel, and the fluoropyrimidine 5-fluorouracil [7].

While surgery and radiotherapy are relatively precise and used to achieve local control, the cytotoxic chemotherapies show systemic effect, which is usually followed by collateral damages in normal tissues. Indeed they induce cell cycle arrest and apoptosis not only in tumor cells, but also in nonmalignant cells, including those of the immune system. The dose-delivery schedule of conventional chemotherapy is based on the maximum tolerated dose (MTD), which is the highest dose of a medication, which associated side effects are tolerable by the patients. Although MTD promotes complete or partial regression in a significant number of patients, it is usually associated with complications such as myelosuppression, neutropenia, trombocytopenia, increased risk

of infection and bleeding, gastrointestinal dysfunctions, arthralgia, liver toxicity, and cardiac and nervous system damage [8-10].

In contrast, the more frequent administration of lower doses of cytotoxic drugs induces weaker adverse effects. This schedule is called metronomic or dose dense chemotherapy and uses 10-33% of the conventional MTD. This therapeutic schedule promotes antiangiogeniceffects [11, 12], as well as activates dendritic cells (DCs) and decreases the activation of Treg cells [13, 14].

We have previously observed that ultralow nontoxic concentrations of selected chemotherapeutics are able to modulate the immune system [15]. We have also reported that treatment of colon cancer cells (HCT-116) with low concentrations of paclitaxel increases their immunogenicity [16]. This enhanced immunogenicity was shown to be associated with increased expression of some gene families that were associated with antigen presentation and tumor immunogenicity such as CALM, PSME, PSMD, HSPA, HSP, and genes KIAA0105, BRCA2 [16].

Since DCs are the main professional antigen-presenting cells and are essential for initiating T-cell responses against tumors, several preparations of DCs-based vaccines have been proposed for active cancer immunotherapy [15]. For instance, DCs have been loaded with total or selected tumor antigens for stimulating tumor-specific cytotoxic T-lymphocytes. DCs vaccines can be loaded with tumor cell lysate [17, 18], peptides [16, 19], heat shock proteins [20], or mRNA [21]. Despite a lot of studies demonstrating the feasibility of DCs vaccines and the ability of peptide-loaded DCs to induce peptide-specific T cell responses in cancer patients is still unclear what is the most efficient strategy for Ag loading. Furthermore, the variability of clinical and immunological responses to the treatments has to be considered, since not all patients

are capable to develop responses that impact the disease stability and the patient survival [22, 23].

Therefore, based on these previous findings, in the present study we aimed to provide evidences that gene-expression changes can be transferred to immature DCs by RNA transfection, inducing them to synthetize tumor proteins or peptides. Since DCs are able to present peptides associated with both class I and II molecules, such a transfection could improve the presentation of tumor antigens for generating specific cytotoxic T cell clones. Since 5-FU-based chemotherapy is the most common chemotherapeutic drug used to treat CRC patients in the adjuvant and metastatic settings, we choose it to perform our experiments.With this propose, tumor cells were pre-treated with noncytotoxic concentration of 5-fluorouracil (5-FU) and their total RNA was transfected to monocyte-derived DCs. Further, these DCs were tested on their ability to induce allogeneic T cell proliferation and generation of tumor-specific T-cells.

Our results demonstrated that DCs transfected with RNA from 5-FU-treated tumor cells became more efficient to stimulate the proliferation of normal allogeneic T lymphocytes as well as autologous T cytotoxic lymphocytes. These results make us believe that chemotherapy with low doses should be better investigate, since in this pre-clinical study they demonstrated that can be an important alternative to conventional chemotherapeutics treatments, but with less adverse effects to the patient.

Materials and Methods

Tumor cell lines and cultures

Human colon cancer cells HCT-116 (10^5cell/ml) were cultured in RMPI-1640 medium supplemented with 10% FBS, 2mM L-glutamine, 1mM sodium pyruvate, 0,1mM nonessencial amino acids, 10mM HEPES and 0,1 mg/ml gentamicin (complete

culture medium) at 37°C under 5%CO2 tension. After 24 hours of culture, cells were treated with 5-fluorouracil (5-FU) (Eurofarma – São Paulo, BRA) 20µM or 1µM for 48 hours. These doses were previously determined by MTT-based cytostatic assay (Fig. 1). The concentration of 20µM showed cytotoxic activity and was referred as minimum effective concentration (MEC). The lower concentration (1µM) that did not affect the cell growth was referred as nontoxic concentration (NTC).

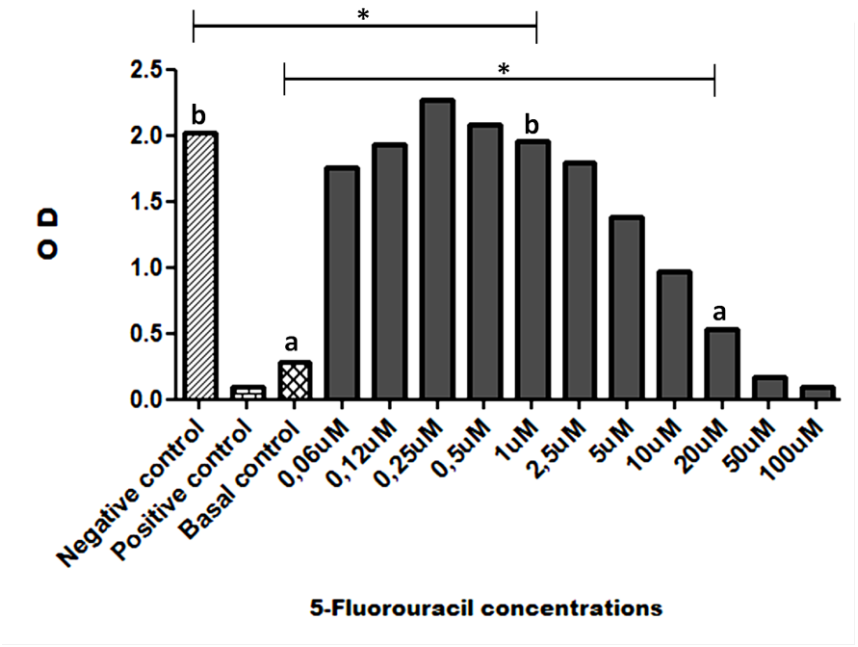

Fig. 1: Determination of MEC and NTC of 5-FU on HCT-116 cell line. The cell monolayer was treated for 48 hours with decreased concentrations of 5-FU varying from 100µM to 0.06µM. After this period the viable cells were quantified by MTT colorimetric assay. 20µM was referred as Minimum Effective Concentration (MEC) since it was the minor dose that demonstrated cytostatic activity, while 1µM was nominated as Non Cytotoxic Concentration, once it was the highest dose that did not affect tumor growth. Cellular viability was analyzed by trypan blue dying. The columns represent media and standard deviation of four independent assays. Positive control: maximal lysis control; negative control: spontaneous lysis; basal control: spontaneous growth in 24 hours; a≠b. $p<0.0001$; ANOVA, followed by Tukey Test.

Tumor cell RNA

Drug treated tumor cells were washed with PBS, and the total RNA was immediately extracted using Total RNA Purification Kit (NorgenBiotek Corp – Ontario, CAN). The purity and concentration of extracted RNA were checked by spectrophotometry 260/280nm (Nanodrop Technologies, Inc – Wilmington, USA). The integrity of RNA was confirmed with cDNA transcription of EpCAM gene (Fig. 2).

Human monocyte-derived dendritic cells

Human DCs were generated from peripheral blood adherent mononuclear cells of healthy donors. After separation on Ficoll-isopaque gradient and lysis of red blood cells, PBMCs were suspended in AIM-V culture medium (Invitrogen - Carlsbad, Ca) and seeded in 6-well plates ($5x10^6$/ml). After 1h at 37°C, non-adherent cells were removed and adherent monocytes were cultured in complete culture medium with 80ng/ml recombinant human GM-CSF and 80ng/ml (rh)IL-4 (PeproTech -Rocky Hill, NJ - USA) for 7 days [24]. On day 7, they were transfected with total tumor RNA. Cell cultures were divided into four groups: DCs (non-transfected DCs); WT (DCs transfected with RNA from untreated tumor cells); MEC (DCs transfected with RNA of tumor cells pre-treated with minimum effective concentration of 5-FU), and NTC (DCs transfected with RNA of tumor cells pre-treated with non-toxic concentrations of 5-FU).

DCs transfection with tumor total RNA

DCs were seeded in 6-well plates ($2x10^5$cell/well) with antibiotic-free and serum-free fresh RPMI-1640 medium, and cultured for 18 hours at 37°C. After 18h, cells were transfected using 7µl of DMRIE-C reagent (Invitrogen - Carlsbad, Ca) + 5µg of total RNA/well, following the manufacturer instructions. The transfected cells were maintained in culture for 24h and further harvested for the assay.

Expression of epithelial cell adhesion molecule (EpCAM) by transfected cells.

In order to evaluate the efficiency of the transfection reaction, total RNA was extracted from transfected DCs, and cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen - Carlsbad, Ca), following the manufacturer instructions. For RT-PCR we used GoTaq Green Mix Promega, according to the manufacturer instructions, and EpCAM specific primer [(Sense: ATCGTCAATGCCAGTGTACTTCA) and (Antisense: TTTGCTCTTCTCCCAAGTTTTGAG)]. Electrophoresis of RT-PCR products were made in agarosis gel 0.8%.

Flow cytometry

For the phenotypic analysis, transfected DCs were incubated with mAbs for 30 minutes and washed with PBS containing 0.1% BSA and 0.1% sodium azide. DCs were labeled with mAbs for MHC-Class II, CD11c, CD14 and CD40. Since we have previously observed that treatment of tumor cells with dose concentration of drugs increases the expression of MYD88 gene [16], we also analyzed the expression of TLR-4. The samples were read in a *FACS Calibur cytometer* (Becton Dickinson, San Jose, CA, USA) and analyzed by *FlowJosoftware* version 7.2.4 (Three Star).

Mixed Lymphocyte Reaction (MLR)

The functional activity of transfected DCs was evaluated through their ability to stimulate the proliferation of normal allogeneic T lymphocytes. Transfected DCs from different donors were co-cultured with T lymphocytes of a single donor in flat-bottomed 96-well plates in different DCs:T proportions (1:1, 1:3, 1:10 e 1:30). The lymphocyte proliferation was evaluated 4 days later by the ability to transform MTT in formazan crystals, which were further solubilized with 2-mercaptoethanol and measured by

spectrophotometry at 540nm. The results were expressed as proliferation index, calculated by the equation (experimental OD – lymphocytes OD)/lymphocytes OD, where lymphocytes OD refers to formazan reduction by lymphocytes themselves and experimental OD was obtained by wells containing lymphocytes plus DCs.

Cytolytic T lymphocytes generation

For the generation of T cells, transfected DCs were co-cultured with autologous T lymphocyte-rich suspension in complete culture medium added with IL-7 and IL-2. The culture was pulsed with IL-2 every three days for 14 days. On day 14, the lymphocytes were harvested, washed with complete culture medium and evaluated for cytotoxic activity against HCT-116 target cells. Lymphocytotoxic assay was performed according to Yu *et al* [25] and Kaneno *et al* [16] with modifications. Briefly, HCT-116 was previously cultured in a flat-bottomed 96-well plate, and dyied with MTT solution. In vitro generated lymphocytes were put on HCT-116 monolayer (5:1/Ly:HCT-116), and then, these cells were co-cultured for 18h at 37°C under 5% CO2; then wells were washed for disposing suspended lymphocytes and dead cells-debris. Percentage of surviving target cells were counted by measuring residual formazan crystals dissolved with DMSO. The results were expressed as proliferation index, and calculated by the equation: ((Ly OD –experimental OD)/ Ly OD) x 100.

IFN-γ and IL-10 detection

The supernatant of cytotoxicity assay was collected and preserved at -80°C. Then, they were analyzed on the in vitro synthesis of IFN-y and IL-10 by ELISA, according to the manufacturer's instruction (R&D Systems).

Statistical analysis

The homogeneity of variance was accessed by the Bartlett test [26], and data was analyzed by analysis of variance (ANOVA) followed by multiple comparison Tukey-Kramer test. The differences were considered significant when the error probability was less than 5% ($p<0, 05$). All experiments were repeated at least five times.

Results

Transfected DCs show higher levels of EpCAM expression

Epithelial cell adhesion molecule (EpCAM) is a transmembrane protein with dual function: cell adhesion and mitogenic signaling. It is normally expressed in the basal membrane layers and is overexpressed in various carcinomas and tumor initiating cells (i.e cancer stem cells) [27]. As it can be observed in figure 2, EpCAM expression was increased in all RNA transfected cells. The transfection process itself was not able to increase it expression (data not shown).

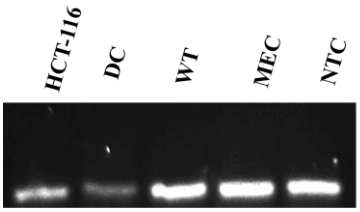

Fig. 2: RT-PCR electrophoresis shows increased expression of EpCAM gene after transfection with total RNA of tumor cells treated with different doses of 5-FU. 1) HCT-116 cDNA; 2) Non-transfected DCs cDNA; 3) cDNA from DCs transfected with wild type HCT-116 RNA; 4) cDNA from DCs transfected with HCT-116 pre-treated with MEC RNA; 5) cDNA from transfected DCs with HCT-116 pre-treated with NTC RNA.

Effect of transfection of DCsdifferentiation and maturation

The next step was to analyze the influence of transfection of the expression of DC differentiation and maturation markersby flow cytometry. Fig. 3 and table 1 show that DC transfected with RNA from tumor cells have a slightly increased expression of HLA-DR (DC = 233,1±70,95; HCT= 339,4±98,85MEC= 296,4±132,7; NCT = 308,7±93,24). No significant alteration was observed on the other analyzed markers (Table 1). However, on fig. 4 it could be observed a tendency of increasing percentage of TLR-4^{+} cells following transfection.

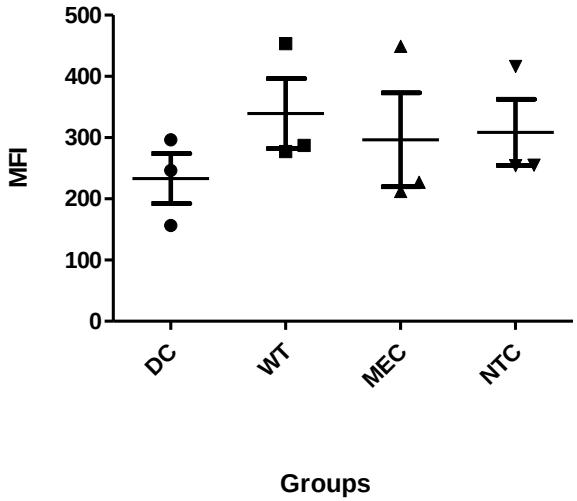

Figure 3: Tumor RNA transfection slightly increases HLA-DR expression in DCs. DCs from healthy donor were generated from monocytes treated with GM-CSF and IL-4 for 7 days. On day 7 tumor RNA from different cultures was transfected or not to immature DCs culture. Graphs show the analysis of DCs from 7 donors, which surface molecules expression were compared to DC to the control group (No transfected DCs) with DCs submitted to tumor RNA transfection: RNA of non-pre-treated tumor cells; RNA of pre-treated tumor cell with 20μM (MEC) and RNA of pre-treated tumor cell with 1μM (NTC), respectively. DC = non-transfected dendritic cells; WT = DC transfected with RNA from non-pre-treated HCT-116; MEC = DC transfected with RNA of HCT-116 treated with minimum effective concentration; NTC = DC transfected with RNA of HCT-116 treated with non-toxic concentration.

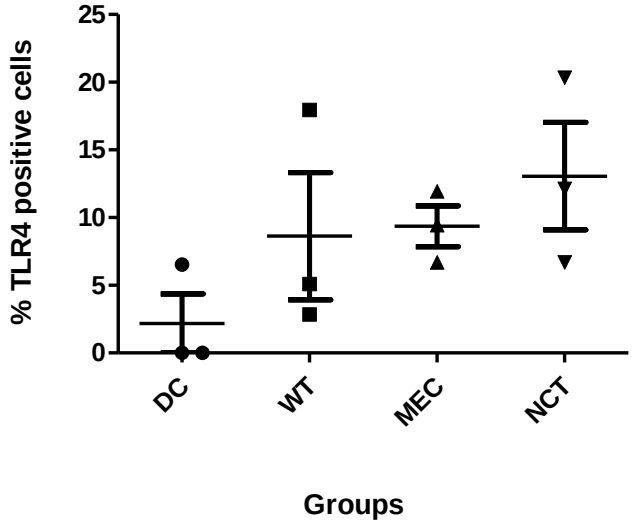

Figure 4: Effect of tumor RNA transfection on the TLR-4 expression by DCs. DCs from healthy donor were generated from monocytes treated with GM-CSF and IL-4 for 7 days. On day 7 tumor RNA from different cultures was transfected or not to immature DCs culture. Graphs show the analysis of DCs from 7 donors, which surface molecules expression were compared to DC to the control group (No transfected DCs) with DCs submitted to tumor RNA transfection: RNA of non-pre-treated tumor cells; RNA of pre-treated tumor cell with 20μM (MEC) and RNA of pre-treated tumor cell with 1μM (NTC), respectively. DC = non-transfected dendritic cells; WT = DC transfected with RNA from non-pre-treated HCT-116; MEC = DC transfected with RNA of HCT-116 treated with minimum effective concentration; NTC = DC transfected with RNA of HCT-116 treated with non-toxic concentration.

Table 1.Effects of 5-FU in minimum effective concentration and non-toxic concentration on the phenotypic maturation of DC.

Donor	Treatment	CD11c	CD14	CD40	HLA-DR
A	DC	32	29	34	122
	WT	31	27	32	195
	MEC	41	27	34	**341**
	NTC	38	25	32	**328**
B	DC	30	17	27	216
	WT	30	15	29	268
	MEC	28	15	24	**322**
	NTC	23	16	25	**265**

DCs transfection with RNA of 5-FU treated tumor cells increases allogeneic Ag presentation

In order to analyze the effect of transfection on the antigen presenting function of DCs, we performed the MLR assay. Fig. 5 shows that DCs transfected with tumor RNA induces higher levels of lymphocyte proliferation than control untransfected DCs. Those DCs transfected with RNA from 5-FU treated tumor cells (both MEC and NTC) showed higher effectivity than those receiving the wild type RNA (DC = 4,014±0,6201; MEC = 8,206±0,9514; NCT = 8,861±1,132) Despite there was no significant difference between MEC and NTC groups, it could be observed a slightly increased activity at the NTC.

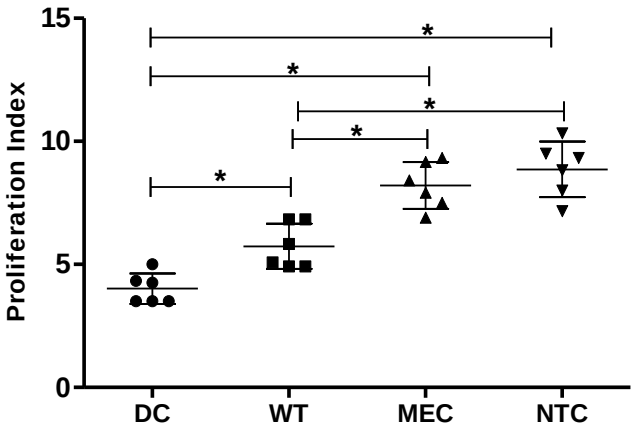

Fig. 5: DCs transfection with tumor RNA enhances the in vitro allogeneic response by T-cells. DCs were transfected with tumor RNA and co-cultured with heterologous lymphocyte in order to analyze the capacity of transfected DCs to activate lymphocytes by Ag presentation. The result represents six independent assays from different donors. All assays were made using lymphocytes from the same donor. The figure illustrates the dilution 1:10 (DCs/Ly). DCs = non-transfected dendritic cells; WT = DCs transfected with RNA from non-pretreated HCT-116; MEC = DCs transfected with RNA of HCT-116 treated with minimum effective concentration; NTC = DCs transfected with RNA of HCT-116 treated with non-toxic concentration (* $p \leq 0{,}001$).

DCs transfection with tumor RNA enhances the in vitro generation of CTL as well as the cytotoxicity of lymphocytes

The cell death mediated by cytotoxic T lymphocytes is considered the main mechanism of class I+ target cells killing. Thus, we tested the efficiency of transfected DCs for the generation of autologous tumor specific T-lymphocytes. We observed that cultures of lymphocytes that were previously exposed to transfected DCs yielded cytotoxic cells with higher activity than control cultures to HCT-116 cells. The results at Fig.6 show that DCs transfected with RNA of 5-FU tumor cells (both MEC and NTC) are able to induce higher levels of anti HCT-116 activity (DC = 15,87±10,31; MEC = 37,60±9,168; NCT = 47,64±9,917).

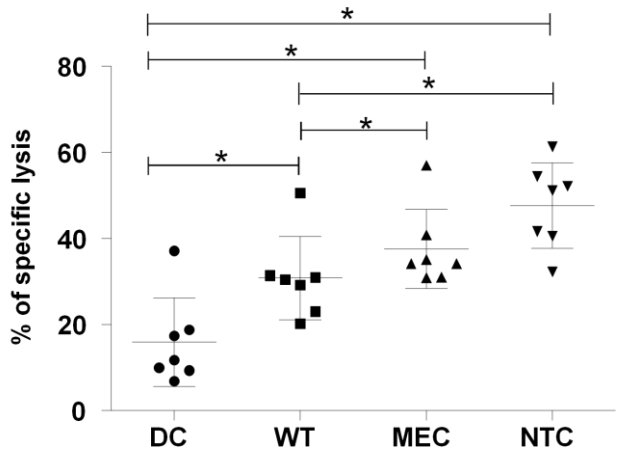

Fig. 6: DCs transfection with 5-FU treated tumor RNA improves the generation of CTL. Monocytes derived DCs were transfected with tumor RNA and co-cultured with autologous lymphocytes for 14 days. In vitro generated CTL were then put on pre-marked with MTT HCT-116 in a dilution of 50:1 (Ly: HCT-116) target monolayer and co-cultured by 18 hours. Cytotoxic activity was calculated based on the density of living target cells keeping formazan crystals. DCs = non-transfected dendritic cells; WT = DCs transfected with RNA from non-pre-treated HCT-116; MEC = DCs transfected with RNA of HCT-116 treated with minimum effective concentration; NTC = DCs transfected with RNA of HCT-116 treated with non-toxic concentration. (* p≤0,001).

In vitro cytokine synthesis

IFN-γ is one of the main cytokines involved in the generation of an effective antitumor immune response. Since it is produced by both Th1 and activated CTLs, it measurement at the CTL-assay supernatant is a strong tool for evaluating specific responses. It can be observed inFig.7A that lymphocytes generated by co-cultures with transfected cells produce more IFN-γ than control DCs group(DC= 103,3±6,417; MEC= 129,5±9,020; NCT= 133,1±9,135). Since IL-10 is one of the main regulatory cytokines, we also analyzed its secretion at the co-cultured supernatant (DC= 32,10±1,484; MEC= 31,02±0,8931; NCT= 31,71,1±1,560). Fig.7B shows that DCs transfection did not induce significant changes on IL-10 secretion cells.

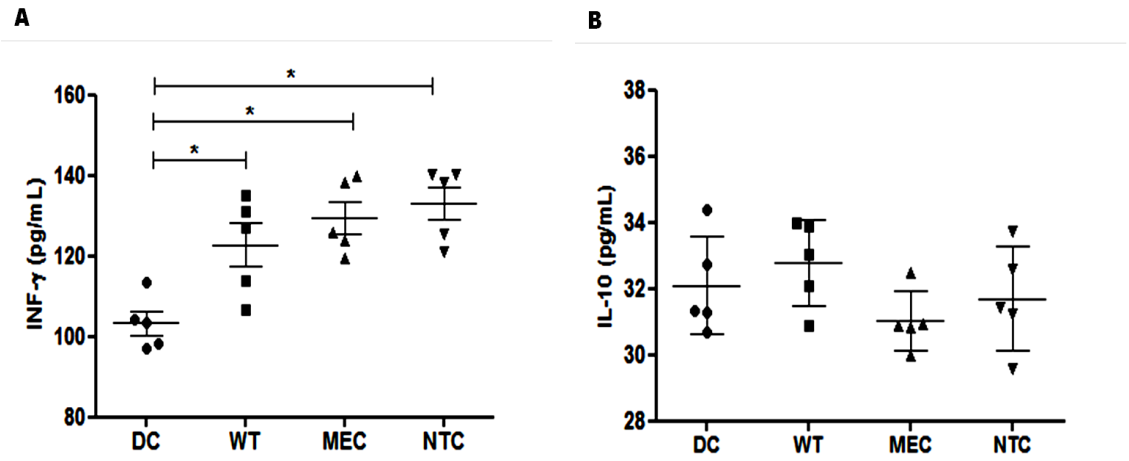

Fig. 7: IFN-γ and IL-10 synthesis by lymphocytes co-cultured with autologous transfected or not PBMC-DCs. PBMC-DCs were cultured during 7 days in complete medium added with GMC-SF and IL-4, then they were transfected with tumor RNA and co-cultured with autologous lymphocyte, and cultured for 14 days. After that, the lymphocytes were co-cultured with HCT-116 for 72h. Then the supernatant was collected in order to quantify the concentrations of INF-γ and IL-10 cytokines synthesis. The results were compared with non-transfected DCs (control DCs), were also co-cultured with autologous lymphocytes. A) IFN-γ synthesis increase from transfected DCs compared with control. B) Secretion of IL-10 after DCs transfection was not altered by RNA transfection. The data represent 5 independent assays from different donors, using the 1:50 DCs:Lypropostion.

Discussion and conclusion

The anti-metabolic agent 5-fluorouracil [28, 29], in the first line drug for colorectal cancer. This agent interferes with DNA and RNA synthesis of in both, normal and tumor cells [30]. Its metabolite fluorouridine triphosphate is extensively incorporated into RNA, disrupting the normal processing and function [31, 32]. Furthermore, since it incorporation into tumor RNA, the structural modification could increase its immunogenicity. It leaded us to evaluate the whether RNA changes induced by 5-FU can be transferred to monocyte-derived DCs, enhancing their feasibility for using as therapeutic vaccine against CRC.

First, we tested the best strategy for RNA transfection into DCs, comparing electroporation and liposomal approaches. We observed that electroporation method was very aggressive for DCs and induced a high level cell mortality (data not shown). Then, we tested different liposome reagents, since lipid carriers are required to stabilize RNAs against nucleases [33-35]. Among severalprotocols we have tested, best results were achieved with DMRIE-C Reagent (Invitrogen - Carlsbad, Ca). In order to show the ability of DMRIE-C to transfect total tumor RNA into DCs, we performed RT-PCR assay using specific primer for epithelial cell adhesion molecule (EpCAM) gene. EpCAM is a carcinoma-associated antigen, and is expressed on most normal epithelial cells and gastrointestinal carcinomas [27]. We observed that EpCAM expression by DCs is not equal among different donors, since it can be constitutively expressed in some healthy people [36], being absent in others (data not shown). Nevertheless, after tumor RNA transfection, negative EpCAMDCs began to express this gene while the expression on positive DCs was increased.

In order to investigate the capacity of RNA-transfected DCs to activate lymphocytes we first performed the MLR assay, which analyzes alloreactive T cells

response against foreigner MHC molecules. Our data shown that tumor RNA is effective for loading DCs, since transfected DCs were more efficient to promote proliferation ofallogeneic lymphocytes than non-transfected cells. Furthermore, transfection of 5-FU-treated tumor RNA showed higher efficiency than RNA from wild type tumor cells to modulate this functions.

It has been reported that RNA-transfected DCs can stimulate tumor antigen specific CTLs in different cancer systems, such as prostate [37], cervical [38], renal [39], and colon [40] cancers. Then, we also analyzed whether the proposed DC-loading approach would improve this DC function and observed that induction of CTL by those DCs prepared with nontoxic concentration of 5-FU was much more efficient than untransfected control and even those transfected with wild type tumor RNA. Therefore, results showed in figure 4 it is suggest that low concentrations of 5-FU are able to promote changes on tumor cells gene expression, that can be transferred to normal DCs by RNA transfection.

Another useful parameter to verify the generation of specific lymphocyte activity is the *in vitro* production of IFN-γ, co-culturing CD8+T cells (CTLs) with target cells. Our results have shown that autologous T lymphocytes generated by culturing with DCs of both MEC and NTC groups, showed increased IFN-γ production, reinforcing the findings on the activity of cytotoxic lymphocytes. Interleukin-10 levels were not influenced by different DCs preparations, although we expected reduced production of this cytokine in those groups showing increased IFN-γ.

In order to analyze the effects of the transfection onDCs phenotype differentiation and maturation markers were analyzed by flow cytometry. The results show that transfection slightlyincreases the percentage of HLA-DR+ DCs. HLA-DR protein belongs to the MHC class II system, which is constitutively expressed in antigen

presenting cells - APCs (monocytes, macrophages, dendritic cells, B lymphocytes). It presents the processed antigen to T lymphocytes, forming the immune synapse with the T lymphocyte receptors. Once HLA-DR demonstrated a tendency to increase, we judged that DCs were more activated after transfection. It is important to notice that DCs transfected only with DMRIE-C (RNA-free) have no alterations on their phenotype (data not shown). Since stimulation of MHC-I and –II expression is one of the main role of IFN-γ, increased HLA-DR expression is in accordance with our data on the production of this cytokine. Maybe the changes were not so intense because IL-10 was still being produced in all groups, modulating the activity of IFN-γ. No significant differences were observed on other differentiation and maturation markers, such as CD11c, CD14 and CD40.

We have previously observed that treatment of tumor cells with nontoxic concentrations of paclitaxel and doxorubicin promotes increased expression of MyD88 (myeloid differentiation primary response 88) [16], a cytosolic adapter protein that works as signal transducer in both interleukin-1 and *Toll*-like receptors signaling pathways[27]. Since this protein is linked with the cytoplasmic portion of both TLR-4 and TLR-2, we analyzed the TLR-4 expression on DCs. Our results show an increased percentage of TLR-4^{+} DCs following transfection, mainly in those transfected with NTC-treated tumor RNA. These results are important since TLR-4 interact with heat shock proteins (HSP), mainly HSP70 that up-regulate the expression of TH1-cytokines, such as IFN [41, 42], potentiating the stimulatory activity of DCs on the immune system. In a parallel study developed by our group, it was observed that treatment of colorectal tumor cell line with low concentrations of paclitaxel induces the expression of HSP40, HSP70 and HSP90. DC loading with lysates from such cells also showed increased ability to induce allogeneic lymphoproliferative response (unpublished data).

It is possible that tumor exposure to 5-FU also increases the expression of these proteins. If so, HSPs on target cells could preferentially bind to TLR-4 expressing DC (RNA-transfected cells), increasing the antigen uptake by immature DC. Being a damage-associated molecular patterns (DAMPs) [43], HSPs can also work as targets for CTL [44] and NK cells [45].

Taken together, our results corroborate our previous observation that exposition of tumor cells to low concentrations of cytotoxic agents increases their immunogenicity [16]. Although we have analyzed just a small number of DC donors *(n=7)*, our observation is in agreement with our hypothesis that changes induced by this treatment can be successfully transferred to DCs by transfection of total tumor RNA, thus priming them for triggering specific antitumor response. Thus, we believe that our protocol deserves a wider investigation in order to be improved for clinical use, reinforcing the feasibility of administration of low doses of chemotherapeutic agents in combination with DC therapeutic vaccines.

Conflict of interest statement

Authors declare that there is no conflict of interest.

Acknowledgements

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ANEXOS

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Registrado no Ministério da Saúde
em 30 de abril de 1997

Botucatu, 01 de março de 2.010 OF. 043/2010-CEP

Ilustríssimo Senhor
Prof. Dr. Ramon Kaneno
Departamento de Microbiologia e Imunologia do
Instituto de Biociência de Botucatu

Prezado Dr. Ramon,

De ordem do Senhor Coordenador deste CEP, informo que Projeto de Pesquisa **(Protocolo CEP 3437-2010)** **"Quimioimunomodulação com agentes antineoplásticos e geração de vacinas de células dentríticas contra o câncer colorretal"**, a ser conduzido por Vossa Senhoria e pelos Pesquisadores Colaboradores: Denise da Silva Reis, Marcela Rodrigues de Camargo, Mariana de Souza Nars e Marianna Bartelega Magalhães, contendo os seguintes **sub-projetos**: 1) **"Efeitos de doses efetivas mínimas de paclitaxel e 5-aza-2-dioxicitidina sobre a função das células dendríticas humnas e imunogenicidade das células de câncer colorretal"**; 2) **"Efeitos de doses efetivas mínima e doses não-tóxicas de 5-fluorouracil e leucovorina sobre a atividade de células dentríticas humanas e imunogenicidade de células de câncer colorretal"**; 3) **"Geração de vacinas antitumoriais de células dendríticas humanas sensibilizadas com RNA de células tumoriais pré-tratadas com agentes antineoplásticos"**, que serão conduzidos por todos os pesquisadores acima, recebeu do relator parecer favorável, aprovado em reunião de 01 de março de 2.010.

Situação do Projeto: APROVADO. Ao final da execução deste Projeto, apresentar ao CEP **"Relatório Final de Atividades"** do projeto mãe e de seus sub-projetos.

Atenciosamente,

Alberto Santos Capelluppi
Secretário do CEP

CONSENTIMENTO LIVRE E ESCLARECIDO

Eu,_____,
RG nº_____, telefone (opcional): _____,
residente à_____
_____,
sou doador voluntário de sangue ao Hemocentro de Botucatu, FMB, UNESP e fui informado(a) sobre a realização da pesquisa **"Quimioimunomodulação com agentes antineoplásicos e geração de vacinas de células dendríticas contra o câncer colorretal".** Entendi que os pesquisadores querem testar o efeito de algumas drogas antitumorais sobre células de defesa do organismo, com o objetivo de desenvolver um tipo de vacina para melhorar a resposta imunológica contra o câncer de cólon e reto. Entendi também que para o desenvolvimento desse estudo é necessário usar as células brancas (leucócitos), uma parte do sangue que pode ser removida, sem prejuízo para o receptor da transfusão.

Consentindo em participar, estou ciente de que apenas as células brancas do meu sangue serão usadas para a realização de culturas celulares e que tanto as hemácias (células vermelhas) quanto o plasma, que contém plaquetas e outros componentes importantes para o Banco de Sangue, serão preservados. Compreendi que essa pesquisa não me trará nenhum risco, que não haverá necessidade de alterar a quantidade de sangue a ser doado e que haverá sigilo em relação aos resultados obtidos. Embora eu não vá ser diretamente beneficiado pelos resultados, os mesmos poderão fornecer informações importantes para o desenvolvimento das vacinas antitumorais, beneficiando outras pessoas no futuro.

O trabalho será desenvolvido pelo **Prof. Dr. Ramon Kaneno**, do Departamento de Microbiologia e Imunologia do Instituto de Biociências de Botucatu, e pela aluna **Carolina de Almeida Araujo,** CPF: 326.178.258-76.

Assim considero-me esclarecido(a) e concordo em colaborar com o desenvolvimento do projeto, permitindo o uso dos meus leucócitos. Afirmo não ter sido pressionado(a) física ou psicologicamente para colaborar com a pesquisa ou assinar o presente termo, estando ciente de que os responsáveis por este trabalho estarão disponíveis para responder a quaisquer perguntas ou dúvidas no Departamento de Microbiologia e Imunologia. O não consentimento da minha parte não interferirá na qualidade do atendimento de minha saúde em qualquer setor do HC-FMB/UNESP.

Botucatu, _____ de _____ de _____.

_____ **Doador**

_____ **Prof. Dr. Ramon Kaneno**
Depto. Microbiologia e Imunologia
Instituto de Biociências de Botucatu
UNESP

_____ **Colaboradora**