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*World J Gastroenterol* 2007 November 28; 13(44): 5822-5831
***World Journal of Gastroenterology*** ISSN 1007-9327


*TOPIC HIGHLIGHT*

**Ignacio Gil-Bazo, MD, PhD,** *Series Editor*

# Immunotherapy and immunoescape in colorectal cancer

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Received: June 5, 2007 Revised: June 30, 2007

## Abstract

Immunotherapy encompasses a variety of interventions and techniques with the common goal of eliciting tumor cell destructive immune responses. Colorectal carcinoma often presents as metastatic disease that impedes curative surgery. Novel strategies such as active immunization with dendritic cells (DCs), gene transfer of cytokines into tumor cells or administration of immunostimulatory monoclonal antibodies (such as anti-CD137 or anti-CTLA-4) have been assessed in preclinical studies and are at an early clinical development stage. Importantly, there is accumulating evidence that chemotherapy and immunotherapy can be combined in the treatment of some cases with colorectal cancer, with synergistic potentiation as a result of antigens cross-presented by dendritic cells and/or elimination of competitor or suppressive T lymphocyte populations (regulatory T-cells). However, genetic and epigenetic unstable carcinoma cells frequently evolve mechanisms of immunoevasion that are the result of either loss of antigen presentation, or an active expression of immunosuppressive substances. Some of these actively immunosuppressive mechanisms are inducible by cytokines that signify the arrival of an effector immune response. For example, induction of 2, 3 indoleamine dioxygenase (IDO) by IFNγ in colorectal carcinoma cells. Combinational and balanced strategies fostering antigen presentation, T-cell costimulation and interference with immune regulatory mechanisms will probably take the stage in translational research in the treatment of colorectal carcinoma.



**Key words:** Colorectal carcinoma; Immunotherapy; Gene therapy; Interleukin-12; Dendritic cells; CD137; Indoleamine 2, 3 dioxygenase

Mazzolini G, Murillo O, Atorrasagasti C, Dubrot J, Tirapu I, Rizzo M, Arina A, Alfaro C, Azpilicueta A, Berasain C, Perez-Gracia JL, Gonzalez A, Melero I. Immunotherapy and immunoescape in colorectal cancer. *World J Gastroenterol* 2007; 13(44): 5822-5831

http://www.wjgnet.com/1007-9327/13/5822.asp

## INTRODUCTION

Conventional therapy for cancer is based on surgical resection, chemotherapy with drugs with selective toxic effects against dividing cancer cells, and localized gamma irradiation. Biological therapy has only recently been introduced[1]. This includes the use of agents that interfere with growth factors for malignant cells, and block tumor neovascularization[2]. Among the monoclonal antibodies (mAbs) that have been approved for cancer treatment, most operate *via* indirect mechanisms, and only a minority target natural or artificial mechanisms of cell destruction.

Colorectal carcinoma (CRC) is one of the leading causes of cancer-related deaths worldwide[3]. Unfortunately, more than 20% of patients with CRC have metastatic disease at the time of diagnosis (http://www.seer.cancer.gov). Although the most common indication for liver resection in developed countries is metastatic CRC, surgery can only be performed in 20% patients, with the 5-year survival rate of 25%-40% despite adjuvant chemotherapy[4]. Regardless of this depressing scenario, a better understanding of tumor biology, combined with advances in molecular and cell biology, have opened up novel avenues of treating advanced CRC using immunotherapeutic strategies.

### *Tumor escape: Perverted local and systemic immune regulation by tumors*

The cellular immune system has been endowed with powerful and at the same time toxic mechanisms designed to induce inflammation and cell destruction, which should be kept under tight control and guided precisely to the target tissues. Cytotoxic mechanisms are designed to recognize and destroy cells that are infected with viruses or other intracellular pathogens, whereas inflammation

is a vascular and leukocyte mediated local response that selectively directs the cellular and macromolecular elements of the innate and adaptive immune systems to the infected site. If properly aimed and enhanced, both immune functions can be therapeutically exploited to control and even eradicate malignant lesions[5]. Genetic and epigenetic changes involved in carcinogenesis generate antigens that are recognized by T lymphocytes in analogous fashion to microbial antigens[6]. Unfortunately, tumor cells in spite of being antigenic are very poorly immunogenic by themselves. Therefore, advanced cancer disease can impede any effort to induce antitumor immunity.

Genetically unstable cells can undergo genetic or epigenetic changes in order to escape a tumoricidal immune response in a "survival of the fittest" type of selection. The escape mechanisms may result from loss of antigen or antigen presentation as well as from active biosynthesis of immunosuppressive molecules[7,8]. These factors include TGF-β, VEGF, IL-8 and IL-10 which are known to cause significant inhibition of both innate and adaptive mechanisms of tumor immunity. Recent evidence points to activation of the transcription factor *Stat3* as a master switch in the control of various immunoevasive substances in tumor cells[9]. Moreover, intrinsic *Stat3* signaling in hemopoietic cells hindered their performance in tumor immunity including dysfunction of NK cells, granulocytes, and conventional DCs which become tolerogenic. Infiltration of tumors by effector T cells seems largely an inefficient process that may be related to poor expression of chemokines and vascular adhesion molecules in the malignant lesions[10]. Besides, the myeloid and lymphoid cells present in tumor stroma appear to be related more to the mechanisms of inhibition than to the activation of tumor immunity.

Indoleamine 2, 3 dioxygenase (IDO) catalyses the degradation of the essential amino acid tryptophan and synthesizes immunosuppressive metabolites[11]. Local up-regulation of the expression and activity of IDO in tumors and the draining lymph nodes can suppress T cell activation and is thought to facilitate the escape of tumor cells from the immune system[12]. Indeed, this enzyme depletes tryptophan and produces kynurenines locally in such a way that both mechanisms impair the function of T cells[13]. IFNs are the key factors upregulating IDO, thus generating a clever mechanism that becomes operational when tumors sense an active immune response in their neighborhood. There is recent evidence indicating that upregulation of IDO by colorectal cancer cells provides an immunosuppressive microenvironment created by tumors to promote cancer growth and spread[14]. We have observed in *in vitro* studies that the addition of IFN-γ to CT26 murine colorectal carcinoma cells induces IDO mRNA expression as well as IDO enzymatic activity, detected as kynurenine production (Figure 1).

Co-signaling molecules are cell-surface glycoproteins that can direct, modulate and fine tune T-cell receptor (TCR) signals[15]. The functional outcome of T cell activity upon its binding to a ligand on an adjacent cell membrane classifies co-signaling molecules as co-stimulators and co-inhibitors. Tumors can express co-inhibitory B7 family

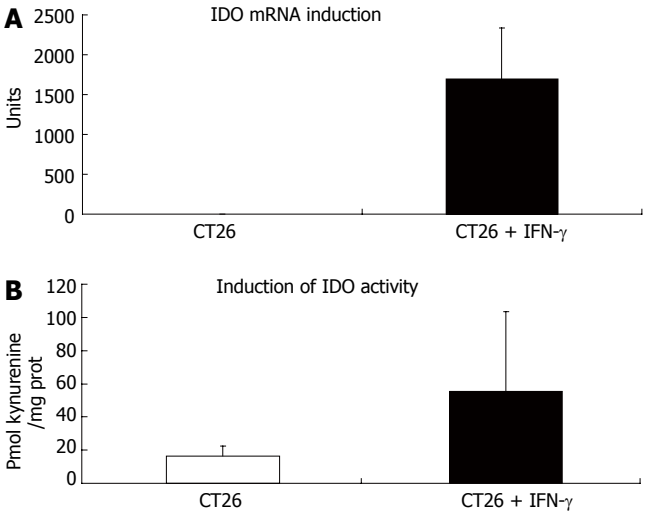


**Figure 1** IFN-γ induces IDO mRNA and enzymatic activity in colon cancer cells. **A**: IDO mRNA was induced after 48 h stimulation with 1000 IU/mL of IFN-γ, as assessed by real time-PCR; **B**: In the same culture conditions, IDO activity was measured in CT26 cellular extracts as previously described by Takikawa *et al*[83].

members, such as B7-H1, B7-H4, and B7-1 (CD80) at a low density, which downregulates T cell activation and/or cytolytic activity[16,17]. Tumors can also induce B7-H1 and B7-H4 expression on tumor-associated macrophages (TAM)[18]. Myeloid suppressor cells can further inhibit anti tumor T cells *via* the production of nitric oxide by the enzyme arginase[19].

Regulatory T cells (T-reg) are important inhibitors of anti tumor immunity[20]. T-reg, characterized by the FoxP3 transcription factor, up-regulate a number of cell membrane molecules, including LAG-3, CTLA-4, GITR, and neuropilin. T-reg can inhibit effector T cell activation and function *via* T-T inhibition or inhibition of antigen presenting cells. There is experimental evidence to support a grim scenario in which T cells in tumor tissue or draining lymph nodes can be perverted into regulatory T cells[21]. Local production of TGF-β may be a key factor in transforming effector T cells locally into suppressive T-reg. Convincing data concerning the role of $CD4^+$ $CD25^+$ regulatory T cells in human cancer comes from the work of Curiel *et al*[22], who showed that the presence of such T-reg in advanced ovarian cancer correlated with reduced survival. Considering the role of T-regs as inhibitors of anti tumor immunity, it has been observed in murine models and in patients that prior host immunosuppression with chemotherapeutic agents (such as cyclophosphamide) can increase the efficacy of adoptive cell therapy as well as other kinds of immunotherapy[23]. The reason for this immunomodulatory effects is based, at least partially, on the elimination of $CD4^+$ $CD25^+$ T cells and the engraftment of specific cytotoxic T lymphocytes[24].

Experimental evidence with TCR transgenic mice clearly shows that tumor-reactive T cells can be tolerized to the point where there is no response to the surrogate tumor antigen. Tolerance results from presentation in the context of a DC that is not expressing high levels of costimulatory molecules and does not secrete cytokines such as IL-12, IL-15 and IFNs.

Chronic exposure to high levels of antigen drives T lymphocytes to a state of non-responsiveness termed "exhaustion". This phenomenon may play a role in impaired CD8 T-cell activity in response to persistent tumor antigens. In a way, the phenomenon of CD8 T-cell exhaustion is actually encouraging from the perspective of immunotherapy, since tumor-specific CD8 T-cells may be present and partially primed in a tumor-bearing host. The B7H1 and PD1 ligand receptor pair is a clear candidate to mediate and sustain exhaustion and offers an opportunity for therapeutic intervention.

In many cases however, a responsive TCR repertoire and tumor antigens coexist without signs of immunization or tolerization. Such a situation is termed immunological ignorance or indifference[25]. Ignorance can conceivably take place in two different ways. First, the quantity of antigen presented to the lymphoid tissue may be too small to induce immunity or tolerance. That would be ignorance/indifference at the priming phase of the immune response[26]. Second, studies in mice show that an expanded effector cell population respects tissues that are not inflamed[27,28]. This can be termed ignorance at the peripheral level that can occur in peripheral solid tumors[28,29].

### The possibility of overcoming immunoescape

Immunotherapy, which is an intervention designed to increase anti-cancer immunity, remains an experimental discipline[30]. However several approaches including inducing and redirecting immunity to either the malignant cells or to critical components of the tumor stroma, such as the vasculature or the connective tissue, have been shown to profoundly impact disease progression in mouse models of cancer[31,32].

Therapeutic vaccination has been attempted in several ways. The immunogenic source can be autologous or allogenic malignant cells that are modified to increase their immunogenicity[33]. *Ex-vivo* or *in vivo* gene transfer of cytokines and other immune-potentiating molecules is a promising strategy. Alternatively, many experimental protocols rely on *in vitro* culture/differentiation of DCs manipulated in such a way that they artificially present tumor antigens[34]. However, the promising results in mouse models have not been replicated in clinical trials. In spite of this drawback there is ample biological evidence in humans that there is an increase in the numbers and activity of lymphocytes against the vaccinating antigen, although such increases fail to reach by 1-2 logs the levels of T cell immunity observed in viral infections.

Adoptive T cell therapy with activated T lymphocytes reaches higher levels of circulating antitumor T cells[35]. These techniques are based on *ex-vivo* reactivation and expansion of cloned or polyclonal cultures of tumor reactive T cells. After culture, T cells are reinfused into the patient along with IL-2. Three important concepts have gained experimental support: (1) polyclonal cultures that recognize several antigen specificities improves the outcome, and the development of tumor-escape antigen loss variants are less likely to occur, (2) co-infusion of both CD4 and CD8 tumor reactive T cells improves antitumor activity, and (3) treatment with lymphodepleting chemotherapy before reinfusion increases the duration and *in vivo* re-expansion of the infused T cells. This is due to both depletion of regulatory T cells and decrease in the competition for T cell homeostatic survival factors such as IL-15 and IL-7. Adoptive T cell therapy probably will benefit much more from the availability of clinical grade IL-15, which can condition the infused cells and sustain their function on administration to the patient.

The sense that chemotherapy and immunotherapy are incompatible is a fading paradigm in tumor immunotherapy. It used to be reasoned that if T cell responses require cell expansion, active or adoptive immunotherapy could not be used in combination with chemotherapy drugs that are selectively toxic for dividing cells. Several lines of experimental evidence suggest otherwise. In fact, there are a number of mechanisms that define additive and synergistic effects: (1) tumor cell destruction makes tumor antigens available for cross presentation by DCs, (2) there is decrease in regulatory T cells, and (3) there is reduced competition for T-cell homeostatic growth factors during/after active immunization. Local destruction of tumors followed by injection of proinflammatory substances holds much promise according to preclinical data and probably represents the simplest method of converting tumors into tumor vaccine.

### Immunostimulatory monoclonal antibodies for the treatment of colorectal carcinoma

Immunostimulatory mAbs directed to immune receptors have emerged as a new and promising strategy to fight cancer[36]. In general, mAbs can be designed to bind molecules on the surface of lymphocytes or antigen presenting cells to provide activating signals (e.g., CD28, CD137, CD40 and OX40)[36]. On the other hand, mAbs can also be used to block the action of surface receptors that normally downregulate immune responses (CTLA-4 and PD-1/B7-H1). In combined regimes of immunotherapy, these mAbs are expected to improve therapeutic immunizations against tumors as observed in preclinical studies.

Anti-4-1BB (agonistic anti-CD137) is one of the most interesting mAbs tested as anti-cancer molecules in preclinical studies[36]. 4-1BB is a member of the tumor necrosis factor/nerve growth factor family of receptors and has a natural ligand (4-1BBL) that is expressed on activated T lymphocytes as well as on NK cells and dendritic cells[37]. This mAb, which acts against CD137, has the ability to stimulate potent antitumor responses[38] and, paradoxically, ameliorates autoimmune manifestations in mice[36]. On the other hand, therapy with mAbs against CTLA-4, which block the inhibitory action of CTLA-4 on T-cells, is capable of inducing antitumor responses in mice as well as in humans but is accompanied with adverse events in the form of autoimmune reactions[39].

Kocak *et al*[40] took advantage of both the mAbs and showed that the combination of CTLA-4 and 4-1BB acts synergistically in the eradication of MC38 colorectal carcinoma after stimulation of a potent antitumor immune response. It was observed that this antitumoral effect

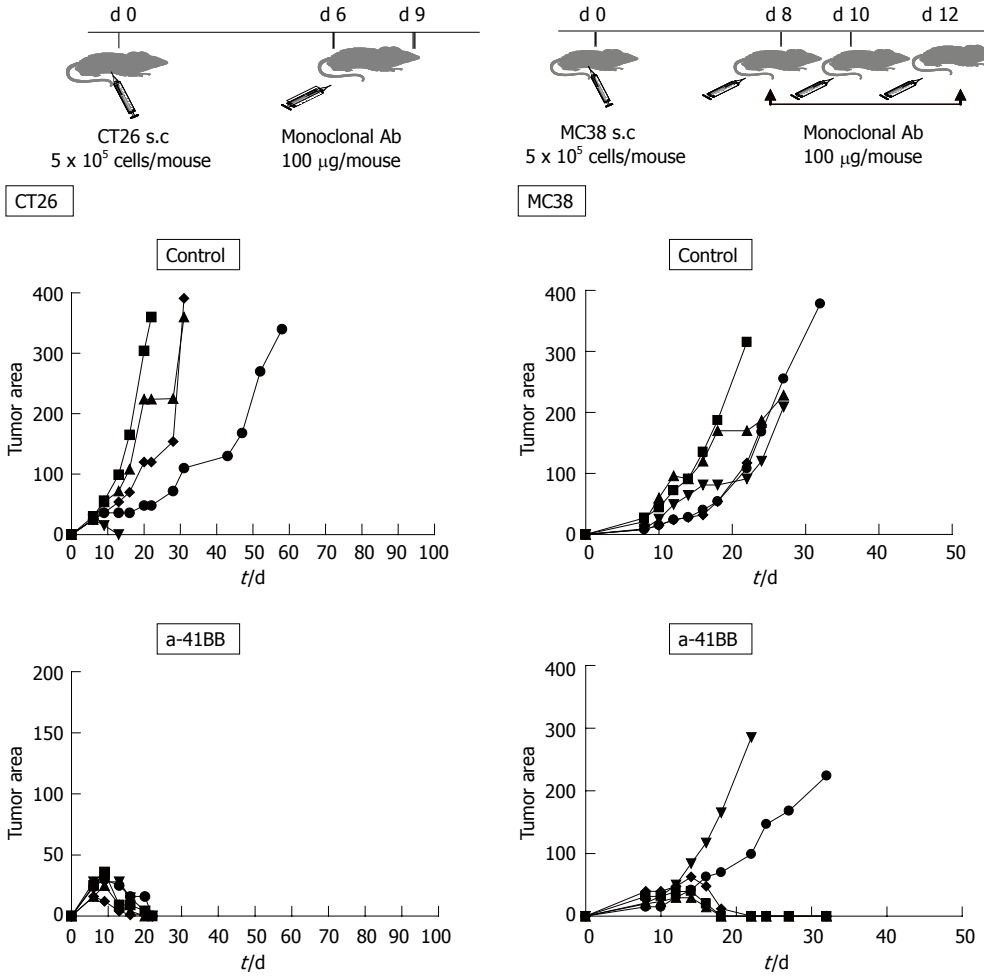


**Figure 2** Systemic treatment with agonist anti-CD137 monoclonal antibodies eradicates transplanted murine colon cancers. Mice subcutaneously grafted with 5 x $10^5$ CT26 or MC38 cells were treated with anti-CD137 (2A) mAb or polyclonal rat IgG as a control. Sequential follow up of tumor size (mean diameter) is depicted for individual mice.

is critically dependant on the presence of $CD8^+$ T-cells induced after treatment[40]. However, we did not observe such a synergy in the same experimental model (A Arina *et al*, unpublished observations).

In our studies in mice, we used the MC38- and CT26-derived tumor model (colorectal carcinoma cell lines) to explore the antitumor effect of repeated systemic injections of agonistic anti-CD137 (anti-4-1BB) mAbs. As a result of the amplification properties of anti-CD137 antibodies on CTL immune response, this treatment was able to induce tumor eradication in 3 out of 5 mice bearing CT-26 tumors and in 3 out of 5 animals with MC38 nodules (Figure 2).

CD137 stimulation can be achieved not only by direct administration of mAbs in monotherapy, but also in the context of different combinations usually including immunostimulatory cytokines. For example, simultaneous gene transfer of local-membrane bound 4-1BB ligand and IL-12 results in successful eradication of advanced colorectal liver metastasis induced in mice[41]. In a similar line of work, Martinet *et al*[41] demonstrated that the combination of 4-1BB costimulation using an adenovirus expressing membrane-bound 4-1BB-L with another adenovirus expressing IL-12 genes induced a potent antitumor response in mice with colorectal carcinoma. Systemic administration of soluble Ig-4-1BB ligand gave rise to a stronger T-cell immune response compared to local gene transfer[42]. It appears that anti-4-1BB can upregulate a formerly weak immune response, but it fails to initiate an immune response if it was nonexistent initially[43].

Systemic treatment with anti CTLA-4 mAb increased the number of CTLs and caused complete tumor regression in established colorectal carcinoma in mice[44]. Another attractive immunostimulatory combination was recently examined by Tirapu *et al*. These workers searched for strategies to enhance the efficacy previously achieved by intratumoral injection of DCs engineered to secrete IL-12 in a mouse model of colorectal carcinoma (using MC38 cell line). They were able to induce a systemic immune response (measured by IFN-γ ELISPOT assay) that eradicated large and metastatic tumor lesions using a combination of systemic anti-CD137 mAb and IL-12 producing semiallogeneic DCs injected intratumorally[45]. This study offers a promising technique of enhancing the efficacy of DC-based strategies currently been tested in clinical studies[46].

## GENE TRANSFER OF IMMUNOSTIMULATORY MOLECULES AND GENETIC VACCINATION

Several cytokines (e.g., IL-2, IL-4, IL-6, IL-7, IL-10, IL-12, IFN-γ, TNF-α and GM-CSF) demonstrate an ability to increase anti-tumor immunity when expressed by cancer

**Table 1 Gene transfer of immunostimulatory molecules and genetic vaccination**

| Cytokine | Vector | Clinical application | Mechanism | Ref. |
|---|---|---|---|---|
| IL-2 + IL-12 | Ad | No | CTLs | 10 |
| IL-10 | Retrovirus | No | $CD8^+$ | 54 |
| TNF-alpha | Ad | Yes | Antiangiogenic, bystander effect | 75 |
| HLA-B7/b2 microglobulin | DNA | Yes | CTLs | 76 |
| IL-12 | Ad | Yes | NK, $CD4^+$, $CD8^+$ | 46 |
| IL-12 + IL-10 | Retrovirus | No | $CD8^+$, $CD4^+$, NK, Macrophages, Neutrophils | 55 |
| IL-2 | Ad, retrovirus | Yes | CTLs | 59 |
| CCL21/LIGTH | Ad | No | DC, $CD8^+$, Macrophages | 61 |

Ad: Adenovirus; DNA: Plasmid DNA; CTLs: Cytotoxic T lymphocytes; NK: Natural killers.

cells[47]. However, systemic administration of recombinant cytokines has limitations because of their short half-life, production difficulty and toxicity. Gene therapy appears to be a novel strategy that may help in delivering therapeutic genes locally, as well as the possibility of controlling transgene expression using specific and regulatable promoters[48] (Table 1).

Currently, we consider two principal approaches to the transfer of immunostimulating molecules inside tumors in order to facilitate immunity against colorectal cancer[47]: (1) *in vivo* injection of vectors expressing cytokines/costimulatory molecule genes into the tumor milieu (may be the most straightforward technique), and (2) tumor cells, DC and lymphocytes can be transduced *ex vivo* with vectors encoding cytokines/costimulatory molecules and re-administered into the host. One of the aims of these strategies is to induce high tumoral or peritumoral production of transferred cytokines, to promote localized regional inflammation (to stimulate innate anti-tumor response), and to induce systemic immunity capable of eliminating disseminated disease.

One of the most extensively studied cytokines in cancer treatment is interleukin-12 (IL-12), which has been shown to have significant antitumor activity against a wide panel of experimental malignancies. IL-12 promotes antitumor immunity because of its ability to activate cytotoxic T lymphocytes (CTLs), natural killer (NK cells) and Th1 response[49,50]. Moreover, IL-12 has antiangiogenic effect, dependent on Interferon gamma (IFN-γ) Inducible Protein 10 (IP10) that facilitates its anticancer effect through different mechanisms[51,52]. It is well known that systemic therapy with rIL-12 protein carries the risk of severe toxicity because of the stimulation of large quantities of IFN-γ, with the potential for individually heterogeneous susceptibility[53].

It has been observed that a combination of immunostimulatory genes may achieve superior therapeutic effects. Narvaiza *et al*[51] demonstrated that intratumoral administration of an adenovirus encoding IL-12 (AdIL-12) together with another adenovirus encoding the chemokine IP-10 (AdIP-10) results in marked antitumoral synergy leading to eradication of metastatic colorectal carcinomas. In this study, the authors used vectors in doses that were not effective when given separately. Moreover, this strategy allowed reduction in the dose of AdIL-12 without losing its anti-tumor efficacy and with less risk of IL-12-related toxicity[51]. The underlying principle of combining AdIL-12 and AdIP-10 is based on the prospect of attracting lymphocytes to tumors expressing IP-10 and to activate them by simultaneous infection of the tumor with AdIL-12.

It is well known that IL-12 has the ability to induce a Th1 type of immune response. By contrast, IL-10 is mainly expressed by Th2 cells and downregulates the production of IL-12 by antigen presenting cells, thus decreasing Th1 activity[51]. However, it has been observed that IL-10 enhances IL-2-induced proliferation and differentiation of $CD8^+$ T-cells[29]. Adris *et al*[54] showed that inoculation of mice with tumor cells expressing IL-10 inhibits the establishment of colorectal carcinoma cells and induces a T cell-mediated tumor suppression in the context of a systemic Th2 response. In an effort to treat colorectal carcinomas using both cytokines, Lopez *et al*[55] have shown that tumor cell vaccines producing both IL-10 and IL-12 act synergistically to eradicate established colorectal cancer (CT26 cell line) and, surprisingly, mammary carcinomas as well. The authors also observed that the antitumor effect of the combined immunotherapy was mainly dependent on $CD8^+$ cells.

In addition to IL-12, heat shock proteins (HSPs) also have the ability to stimulate antigen-presenting cells and induce a Th1-type response. HSP have been employed as an adjuvant to facilitate the induction of specific immunity. Moreover, HSPs have been evaluated in clinical studies as an adjuvant in combination with BCG (Bacille Calmette-Guerin) and HPV16E7 in patients with papillomavirus-related carcinoma[56]. Wu *et al*[57] demonstrated that vaccination of transgenic mice with HSP70-like protein (Hsp70L1) fused with a fragment of carcinoembryonic antigen (CEA576-669) induced the maturation of DCs, with a strong specific CD8 T cell response and *in vivo* antitumor activity in mice.

Systemic administration of recombinant IL-2 has been used in clinical practice in patients with metastatic renal carcinoma and malignant melanoma, although with low efficacy and high toxicity[58]. Among other functions, IL-2 is necessary for the survival of activated T cells and is employed in large doses in protocols were immune cells are adoptively transferred to cancer patients. Adenovirus containing mouse IL-2 cDNA can be injected into tumors, and in combination with a suicide gene (herpes simplex virus thymidine kinase vector) can be a powerful tool in the treatment of metastatic colon carcinoma of the liver[59].

One of the synergistic combinations include a chemokine plus a T-cell-activating cytokine designed to promote the attraction and activation of infiltrating immune cells (attraction theory). Macrophage inflammatory protein 3 (MIP-3) is a chemokine mainly secreted by activated macrophages, which attracts leukocytes to inflammatory foci with selectivity for tisular

DCs. The combination of two adenoviruses, one encoding MIP-3 (Ad MIP-3) and the other IL-12 genes (AdIL-12) given intratumorally in mice with colorectal carcinoma eradicates nearly 90% of subcutaneously implanted tumors[60]. Similarly, co-expression of the chemokine CCL21/secondary lymphoid tissue chemokine and a costimulatory molecule LIGHT in colon carcinoma cells (CT26) resulted in significantly reduced tumor growth in mice. A markedly increased infiltration of mature DCs and $CD8^+$ T cells was observed in the tumor mass, and the splenocytes showed a potent CTL activity against CT26 tumor and IFN-γ production. These results suggest that combined treatment with CCL21 and LIGHT is capable of inducing a synergistic antitumor effect[61].

### *Dendritic cell-based immunotherapy*

Dendritic cells (DCs) are leukocyte populations that present antigens captured in peripheral tissues to T cells *via* both MHC class Ⅱ and I antigen presentation pathways[62]. DC maturation is referred to as the status of DC activation at which such antigen-presenting DCs leads to T-cell priming, while its presentation by immature DCs results in tolerance[63]. DC maturation is chiefly caused by biomolecules with microbial features detected by innate receptors (bacterial DNA, viral RNA, endotoxin, *etc*), pro-inflammatory cytokines (TNF, IL-1, IFNs), ligation of CD40 on the DC surface by CD40L, and substances released from cells undergoing stressful cell death.

It is well known that DCs are potent inducers of immune responses and the activation of these cells is a critical step for the induction of antitumoral immunity. We successfully tested a technique designed to take advantage of the therapeutic effect of IL-12 infecting DCs *ex vivo* with an adenovirus that expresses IL-12 genes (AdIL-12), and injecting the engineered cells into colorectal carcinomas in mice[64]. This strategy has proved to be exceptionally effective in eliminating neoplastic nodules and in eliciting anti-tumor immunity. This strategy is also effective in mouse models when DCs are transfected to express IL-7[65] and IL-15[66].

Transfection of DCs with mRNA is a promising antigen-loading technique of stimulating strong antitumor immunity. Chu *et al*[67] transfected RNA from CT26 colorectal adenocarcinoma to the bone marrow-derived monocytes and obtained strong specific CTL activity *in vivo*. Saha *et al* [68] showed that immunization of CEA transgenic mice with bone marrow-derived mature dendritic cells loaded with the antidote antibody 3H1 (which mimics CEA) resulted in a CEA-specific immune response and suppression of colon carcinoma cells (expressing CEA) in nearly 100% of mice, whereas only 40% of experimental mice immunized with dendritic cells loaded with CEA were protected from tumor growth.

Furumoto *et al*[69] injected MIP-3 chemokine together with CpGs into colorectal carcinomas in order to activate *in vivo* dendritic cells without *ex vivo* manipulation. These workers observed an increase in the number of activated DCs in tumors that were eradicated through specific T cell-mediated antitumor response.

CD40L, a costimulatory molecule expressed on activated $CD4^+$ T cells, acts on B cells and DCs, and plays a key role both for maturation of antibody responses and for CTL induction. Investigators from Crystal's group demonstrated in studies on mice, synergy in the eradication of subcutaneously implanted CT26 when treated with a combination of intratumor injection of an adenovirus expressing CD40-L with DCs or when each treatment was applied sequentially[70,71].

Morse *et al* reported a phase I clinical trial in which autologous dendritic cells loaded with carcinoembrionic antigen RNA (peptide CAP-1) were administered to patients with resected liver metastases from colorectal carcinoma. The procedure was well tolerated, and one patient had a minor response, and one showed stable disease[72]. With the aim to expand the presence of circulating DCs (DC mobilization), Fong *et al*[73] in a phase I study used the hematopoietic growth factor Flt3 ligand prior to the injection of CEA-derived peptide loaded DCs in 12 patients with colon or non-small cell lung cancer. One patient had a mixed response while two showed stable disease.

DCs engineered to produce IL-12 have been shown to induce potent anti-tumor responses. We have recently completed a phase I clinical trial which involved intratumor injection of monocyte-derived autologous dendritic cells transfected *in vitro* with an adenovirus encoding human IL-12 in patients with metastatic gastrointestinal carcinomas[46]. The main objectives of the trial were to assess feasibility and safety, and secondarily to determine biologic and clinical responses. We observed that this strategy was safe and well tolerated, with injection of up to $50 \times 10^6$ dendritic cells. Five patients showed increased NK activity and 4 showed augmented intratumor $CD8^+$ T-cell infiltrate. One partial response and two stabilizations were observed. The reasons for the weak antitumor response were explored. It appears that DCs can be retained inside malignant tissue by means of high intratumor concentrations of IL-8. Besides, scintigraphic tracking of intratumorally injected DCs labelled with $^{111}$In indicated the retention of DCs inside malignant lesions in patients with digestive carcinomas[74].

## CYTOKINE GENE TRANSFER FOR COLORECTAL CARCINOMA IN CLINICAL SETTING

Over 1100 gene therapy clinical trials have been carried out around the world and almost 70% of them were directed at the treatment of advanced or metastatic cancer. In clinical trials, cytokine and tumor antigen genes represent 42% of the genetic material that is transferred (for details see: www.wiley.co.uk/genmed/clinical). In the following section, we focus on some of the most important cytokines currently under clinical investigation in immunogene therapy of colorectal carcinoma.

The encouraging results obtained with the administration of non-replicative adenovirus encoding for IL-12 genes in several experimental models of gastrointestinal cancers (for review see reference[1])

prompted us to initiate a clinical trial at the University of Navarra in patients with advanced gastrointestinal carcinomas[46]. Patients with hepatic tumors (either primary or secondary colorectal carcinomas) were treated intratumorally in a dose-scale fashion with an adenovirus encoding human IL-12 genes. This strategy was safe and well tolerated with only minor side effects. Biological activity was observed in some patients (e.g., rise in serum levels of IFN-γ, infiltration of tumors by $CD8^+$ T cells and induction of neutralizing anti-adenovirus antibodies). Partial tumor regression was observed in one patient and stable disease in 30% patients. Reduction in the gap between doses in the same patient, or application of the vector as neoadjuvant therapy before tumor resection are some of the potential approaches to increase the efficacy of this treatment strategy.

The dose-limiting toxicity of large systemic concentrations of TNF- has led to a decline in its use in cancer patients. By contrast, local gene transfer of this cytokine using an adenovirus (TNFerade®) may reduce the systemic effects. TNF- gene under the control of an early growth response 1 (EGR-1) promoter followed by external beam radiation allows the control of TNF- release. Promising antitumor activity without any significant toxicity was observed in patients with solid tumors[75]. TNFerade® in combination with capecitabine and radiation therapy is now being tested in a phase Ⅱ clinical trial on patients with rectal cancer, before surgical resection.

Rubin *et al*[76] showed that direct gene transfer of HLA-B7 and 2-microglobulin, which together form a MHC-I complex, into the liver of patients with metastatic colorectal carcinoma is a feasible and safe procedure. These workers used a single plasmid construct that encodes for both genes in a formulation containing the lipid complex DMRIE-DOPE (Allovectin-7®). Genes transfected into tumors were detected by PCR in 14 out of 15 patients, however. the clinical results have not published. It should be noted that better results have been obtained in patient with melanoma.

With the advent of agents such as irinotecan and oxaliplatin, chemotherapy has made some progress in the treatment of colorectal carcinoma. The use of biological therapy with monoclonal antibodies against VEGF and EGFR has been shown to benefit a small proportion of patients[77]. Immunotherapy in different forms should be tested in addition to the conventional treatment regimens which improve patient survival.

### *Concluding remarks and future directions*

There is a striking correlation between lymphocyte infiltration in colorectal cancer and the overall outcome of the disease[78,79]. Indeed, the density of T cells close to the tumor cells in the primary tumor is a better predictor of survival in these patients than traditional staging based on tumor size and spread[80]. According to this study, patients whose tumors contained large numbers of CD3-positive T cells, had a 5-year survival rate of 73%, compared with 30% in patients with low density of these cells.

There are important conclusions to be drawn from this study: (1) There is much natural immune pressure on colon cancer that may control the disease successfully in many patients, (2) The immune pressure possibly selects tumor variants that eventually escape immune control, (3) Artificial augmentation of the immune response may tilt the balance towards a curative response at least in some cases.

Immunotherapy intervention requires tumor-debulking and therefore should be combined with surgery and chemotherapy. To make the most of immunotherapy, this technique should be tested on patients whose tumors have been completely resected but are at high risk of relapse. For instance, our current efforts are focused on patients whose liver metastases have been resected surgically and are receiving adjuvant chemotherapy. In these patients, measures to induce/enhance cellular antitumor immune responses may confer a clinically significant delay in tumor relapse. Moreover, the complete removal of any detectable disease greatly diminishes the immunosuppressive mechanisms that may otherwise be induced by the cancer, while the surgical samples provide a rich antigenic source for immunization. Interference with the immunosuppressive mechanisms is clinically feasible with the use of low doses of cyclophosphamide[81] and other such mechanisms may become clinically available in the near future.

In our opinion, it is at the stage of minimal residual disease when immunotherapy should be fully deployed with a combination of strategies comprising of immunization with different tumor antigens and amplification techniques using cytokines or/and immunostimulatory monoclonal antibodies[82].

## ACKNOWLEDGMENTS

The authors acknowledge their long term collaboration with Drs. Prieto, Qian, Sangro, Hernandez-Alcoceba, Berraondo, Bendandi and Peñuelas. Financial support was obtained from Ministerio de Educación y Ciencia (MEC-SAF2005-03131), Departamento de Educación del Gobierno de Navarra, Redes temáticas de investigación cooperativa and "UTE for project FIMA. OM and AAr are recipients of scholarships from Ministerio de Educación y Ciencia and Fondo de Investigación Sanitaria (FIS). C.A is a Jose Estensoro-YPF Foundation student awardee. SH-S was supported by Asociación Española Contra el Cancer (AECC). GM work is supported in part by grants from Agencia Nacional de Promocion Cientifica y Tecnologia (PICT-2005 and PICTO-CRUP 2005 to G.M.), Ines Bemberg and Programa Bicentenario-Banco Mundial, Conicyt, Chile CTE-06 (to G.M.).

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