[Frontiers in Bioscience 13, 369-381, January 1, 2008]

Combination of radiofrequency ablation and immunotherapy

Francesco F. Fagnoni[1], Alessandro Zerbini[2], Guido Pelosi[2], Gabriele Missale[2]

[1]Laboratory of Experimental Oncology, Fondazione Salvatore Maugeri-Clinica del Lavoro e della Riabilitazione, IRCCS, Pavia, Italy, [2]Unit of Infectious Diseases and Hepatology, Azienda Ospedaliero-Universitaria di Parma, Parma, Italy

TABLE OF CONTENTS

1. ABSTRACT

The enhancement of immune response against tumor antigens has shown some efficacy when used as a single mode of systemic treatment in patients with late stage disease. Novel strategies of active immunotherapy could be more effective in patients with less advanced disease who receive standard therapies supporting concomitant stimulation of the immune system. Radio-Frequency Ablation (RFA) is a minimally invasive technique which is used as standard local therapy of primary and metastatic liver tumors. Tumor ablation by RFA induces effects important for boosting anti-tumor immune responses. Tumor cell necrosis generates a permanent immunogenic source of tumor antigens. These antigens can be uptaken, processed and presented by dendritic cells for effective immunization without requirement for ex vivo antigen loading. Further immune activation can be originated by RFA through induction of heat shock proteins on tumor cells, acute phase response which causes the release of pro-inflammatory cytokines, and mobilization of antigen presenting cells and effector lymphocytes. Thus, RFA can facilitate immune responses to tumor antigens driven by active immunotherapy. On the other hand, immunotherapy is expected to eradicate residual disease after RFA and prevent tumor recurrences. The combination of RFA and active immunotherapy may well have synergistic effects for cancer treatment.

2. INTRODUCTION

Cancer disease ranks as one of the highest among all causes of mortality. Established therapies, including surgery, radiation, and antitumor drugs, are constantly being improved; however, cancer-related death rates still do not appear to be declining. Immunotherapy is one of the promising new approaches for cancer treatment. Nowadays there is evidence that the immune system is able to recognize Tumor Associated Antigens (TAAs) as targets and to subsequently destroy the tumor cells. There are, however, challenges in translating this knowledge into clinical applications: TAAs are generally self antigens, and even if these antigens may be over-expressed, *in vivo* enhancement of a tumor-specific immune response can be difficult to achieve because of immunological tolerance. Moreover, tumor genetic diversity and complexity does not allow an easy identification of molecular targets common to several cancers. These challenges has until now made the pharmaceutical industry hesitant to invest efforts in the design and development of such applications.

During the last 10 years several trials of active immunotherapy have been conducted with results that in most of the cases did not meet the expectations. Today, however a lot more is known on the optimal ways of activating a patient's immune system and directing it towards the cancer target. Improvement of biotechniques in

genomics, proteomics, safe recombinant vectors engineering provide increasingly sophisticated tools for designing new treatments with active immunotherapies. Apart from technical aspects, clinical results from previous trials were unsatisfactory probably also because immunotherapy has been so far employed as a monotherapy in patients bearing tumor masses no longer controlled by standard care therapies and with limited chances of clinical responses. Therefore, the next wave of clinical trials based on immunotherapy should include the opportunity to evaluate the potential antitumor effects of such therapies in earlier disease phases. In this perspective, the most promising approaches of active immunotherapy showing safety, feasibility and some clinical efficacy should be considered for clinical testing in association with therapies commonly used for front line treatment. However, the search for synergic combinations between active immunotherapy and standard care therapies is just beginning.

The identification of synergies between different treatment modalities is a primary objective for clinical oncology. This is well known for standard therapeutical approaches such as surgery and chemotherapy, or percutaneous ablative treatments and chemotherapy for metastatic liver disease. Recent results have shown the favorable effect of passive immunotherapy combined with chemotherapy, like the combination of trastuzumab and chemotherapy (1), or the synergistic use of anti-EGFR antibodies with tyrosine-kinase inhibitors. New targeted therapies have even shown the ability to overcome chemotherapy resistance in some patients without increasing drug toxicity (2). Not only monoclonals may be used in association with standard chemotherapy or biological agents; indeed, recent results are opening the way to future combinatory approaches of active immunotherapy with radiotherapy, chemotherapy and biological drugs. Proteasome inhibitors can pharmacologically sensitize tumor cells to TRAIL (3) or to the lytic effects of DC-activated immune effector cells (4); radiotherapy may not only kill cancer but may also modify tumor microenvironment and induce inflammation thereby favoring spontaneous anti-tumor T-cell responses (5). Also, even if apparently in contrast, chemotherapy and active immunotherapy may be used in association enhancing the effect each other (6, 7).

Some standard therapies for loco-regional treatment of cancer such as RFA and cryoablation have shown immunomodulatory potential. Other techniques that are in part still experimental, such as microwave, laser, high-frequency ultrasonic hyperthermia and magnetic embolization hyperthermia, are also of great interest in this perspective. Radiofrequency thermal ablation (RFA) represents a minimally invasive procedure that compete with surgery for treatment of solid malignancy and other ablative procedures. RFA, however, not only destroys the tumor mass but also releases antigenic material, induces inflammation, and generates “danger signals” including heat shock proteins and inflammatory cytokines. For all these reasons, there has been attention to this technique also for its immunomodulatory potential and its possible exploitation in different protocols of cancer vaccination or active immunotherapy. We will discuss on the use of RFA as a well established ablative technique for loco-regional treatment of tumor masses and on the potential advantages of using the thermally ablated necrotic tumor tissue as a permanent source of tumor antigen to elicit systemic antitumor immunity. The immunological effects observed after RFA in animal models, as well as in human cells *in vitro* and in patients *in vivo*, suggest that this ablative technique may be appropriate for combination with strategies aimed at boosting antitumor immune responses with the final goal of eliminating residual disease and prevention of late recurrences. Some early phase clinical trials based on such combination have been already inaugurated whereas other promising strategies including RFA and immunotherapy could be tested in the near future.

3. THE EVOLUTION OF ANTI-TUMOR IMMUNOTHERAPIES

The general definition of anti-tumor immunotherapy includes various approaches sharing the unique property of antigen-specific recognition by either B-cell derived antibodies, or T cells (8). Various forms of immunotherapy comprehend passive humoral immunotherapy with monoclonal antibodies (mAbs), passive cell therapy with adoptive transfer of immune effector cells, and active immunotherapy (9,10). Passive immunotherapy has already met with broad clinical application since bioengineered mAbs are used in an increasing list of malignancies (11), and adoptive transfer of T cells associated to allogeneic bone marrow transplantation constitutes the first choice therapy for several hematological malignancies (12). By contrast, the active form of immunotherapy represented by the induction and/or intensification of immune responses against the autologous tumor remains the most ambitious but still not widely applied design for immunotherapy. The conditions making active immunotherapy such a difficult task are many; however, experimental and clinical evidences indicate that specific, potent, systemic and long-lasting antitumor effect can be realized in some instances (13, 14) and therefore this effort may be considered worth being pursued.

The question as to whether the immune system can recognize and eliminate malignant tumors has been widely debated for decades (15). Long ago it was provocatively claimed that immunization against cancer might be as difficult as to reject one ear while leaving intact the other (16). More recently, however, there has been renewed enthusiasm for several reasons. First of all, tumor-specific recognition has been formally proved with the successful characterization of relatively immunogenic TAA and the demonstration that cancer patients' T cells can recognize TAA more frequently than expected (17). In addition, the possible lack of specific recognition should no longer be seen as a critical issue when considering that malignant tumor cells are genetically very unstable (18). This genetic instability, indeed, can generate thousands of new mutations producing large numbers of novel individual unknown TAA that are also potentially immunogenic (19).

Thus, the current common view is that the immune system can recognize tumor cells, but either it does not react spontaneously or it reacts ineffectively against them.

The inability of the immune system to react against cancers has been attributed both to absent delivery of "danger signals" (20) and to active immune suppression by the growing tumors (21). In either case, immune effectors are neither activated nor directed to target tumor cells because mechanisms controlling immune responses are indifferent. In the last two decades, it has been revealed that initiation and regulation of immune responses is under the key control of dendritic cells (DC) (22). Consistently, a huge number of experimental preclinical studies in animal models and in human *in vitro* has indicated that DC, loaded with TAA and appropriately activated, are competent for antigen presentation to TAA-specific T cells and can boost effective tumor- specific T cell responses (23). Thereafter, a long list of early phase clinical trials has been completed and many others are still underway with the use of DC prepared *ex vivo* and loaded with TAA to immunize cancer patients against their own malignancies (24). Several of these trials have demonstrated the induction or amplification of antitumor immune responses with cases of successful partial or even complete clinical responses (25). Ten years after the first pilot clinical trial with DC prepared *ex vivo* (26), the aggregated results from over a thousand of patients treated in over a hundred different protocols (27), offer the proof of principle of some clinical efficacy. Such evidence should be considered relevant when considering that clinical results were obtained from protocols including patients at advanced disease stages with high tumor burden, resistant to standard therapies, and often immunosuppressed by previous chemotherapies and the high tumor burden itself (28). Perhaps the most relevant final result deriving from these early trials may just concern the definitive answer to the initial debated question, and the conclusion is that the immune system not only can recognize, but can also eliminate malignant tumor cells in a clinically effective manner. Moreover, another important aspect is that immunotherapy alone may not be sufficient to eliminate bulky tumor masses as a monotherapy. Therefore, elimination of minimal residual disease after standard therapies and prevention of disease recurrence in patients meeting criteria of complete clinical response is likely the most appropriate use of active immunotherapy (29). Alternatively, it should at least be used in the context of limited tumor load, possibly in disease stages not too advanced, and most importantly in combination with existing standard care therapies. In any event, the design of future strategies should take into account whether certain standard care therapies may act synergistically with immunotherapy, for example by enhancing immune responses. Eventually, the combination with standard care therapies would provide the required answer as to whether active immunotherapy may find effective, efficient, and wide application in clinical oncology across distinct clinical settings including different cancer types and stages.

To resolve the critical issue concerning the design of optimal combination strategies and clinical settings there are several aspects that remain to be addressed in the near future. Assuming that DC may be essential for initiating most immune responses, and that DC may be regularly altered by tumor immune suppressive mechanisms, the role of these cells should not be underestimated when considering active immunotherapy strategies. But current protocols for preparing DC *ex vivo* for clinical use in active immunotherapy are far from satisfactory for several reasons. *In vitro* manipulations are dictated under stringent regulations because of contamination risks and therefore require both specific facilities and personnel with adequate expertise, impose relevant costs for clinical grade reagents to be used, and, most important of all, are not yet standardized to produce consistent cell therapy products. These limitations are not absolute and still leave open the opportunity to proceed in the case of a favorable risks/costs to benefits ratio, but make this approach too cumbersome for large-scale clinical application. The ultimate protocol for large scale clinical use should be short and simple (possibly without requirement for extensive *ex vivo* manipulation), and should provide DC loaded with TAA and fully matured in order to exert unequivocal immunostimulation (30, 31). The need for this improvement is indeed concurring to gradually shift the attention focus of experiments in animal models from the *ex vivo* to *in vivo* manipulation of DC (32), with the final goal of using the tumor as the source of TAA to generate an immune response against itself.

4. USING THE TUMOR AGAINST ITSELF

The tumor itself is conceivable as a permanent source of tumor antigen to be uptaken, processed and presented by DC for effective immunization (33). The amount of intratumoral DC is commonly low and their function is depressed by the suppressive milieu present within established growing tumors that actively produce inhibitory factors such as TGF-b or IL-6 (34, 35). One possibility to increase the number of DC is the direct intratumoral injection of cells isolated *ex vivo*, an approach already proven to be safe and feasible in one clinical study (36). Alternatively, *in vivo* manipulation would require not only DC mobilization with the use of agents such Flt3-L, (37, 38), but also concomitant chemoattraction by local administration of DC-directing chemokines (39-41). The increase of DC number within tumors however, either through direct intratumoral injection or soluble factors, does not allow significant breaking of tumor tolerance since only modest effects are seen even with optimized conditions in animal tumor models (40, 42, 43). Thus, the abundance of DC within malignant tissue is probably necessary, but not sufficient to break the tolerance to high tumor cells load to which the immune system is already exposed. Other additional factors must be involved especially when considering the active immune suppression exerted by growing tumors.

To overcome the suppressive effects of tumors and tumor-derived factors on DC function it may be considered to either reinforce DC function, or cut down tumor immune suppression, or both. A number of studies in animal models have recently shown that many agents reinforcing immune stimulation by intratumoral DC may

indeed have additional effects on the induction of systemic antitumor immunity. Such agents include IL-12 (42, 44), CD40L (45-47), IL-7 (48), IL-18 (49), IL-23 (50), IFN-alpha (51), TNF- alpha (52, 53), CpG motifs (32, 40, 54-56), and as CCL21/SLC (57) and Fractalkine (58). Notably, a clinical trial based on intratumoral injection of DC engineered to secrete IL-12 by recombinant adenovirus has been conducted in patients with metastatic gastrointestinal carcinomas (59). The approach revealed to be safe and well tolerated, however only one case of partial response was reported. The use of (111) Indium-labeled DC showed that most DC remained inside tumor tissue instead of migrating out to afferent lymph nodes (60). This trapping effect probably due to IL-8 produced by tumor tissues sustains the hypothesis that exposure of DC to the suppressive local microenvironment might be significantly involved in cancer immune evasion.

An alternative approach to avoid the exposure of DC to this negative effect is to induce preliminary tumor cells destruction by current standard therapies. Systemic chemotherapy by various agents followed by intratumoral injection of naive singeneic DC demonstrated synergy for the treatment of established experimental tumors (61-64). Remarkably, this effect was associated with induction of systemic antitumor immune response and resulted in long-term memory with resistance to tumor rechallenge (64). Likewise, tumor tissue destruction by disparate loco-regional approaches such as radiotherapy (65-67), ethanol intratumoral injection (68), cryotherapy (69), photodynamic therapy (70, 71), followed by *in situ* delivery of DC, provided consistent data. Taken together, these findings obtained in preclinical experimental models suggest that synchronized tumor cell death along with recruitment of DC can induce and expand systemic antitumor responses. Nevertheless, translation of this combined approach in clinical trials still requires the definition of the essential and accessory elements possibly providing the best opportunities for obtaining clinical responses. To this end, the use of the tumor as a permanent antigen source following standard therapies would require that: (i) tumor cells may be not only dead, but also available in an immunogenic formulation for antigen uptake; (ii) antigen uptake, presentation and cross-priming may be exerted by robust naive DC; (iii) tumor cell killing agent(s) may not interfere negatively with DC function and with immune activation; (iv) immature DC loaded with tumor antigen might be matured/activated before cross-priming in order to avoid tolerance induction. Under these circumstances, the choice of the approach for inducing tumor cell death before DC injection may reveal to be critical for several reasons. On a theoretical basis, the apoptosis of tumor cells induced by chemotherapy is expected to be accompanied with low immune reactivity, although recent reports uncovered that there might be exceptions to this tenet (72, 73). Similarly, chemotherapy and radiotherapy are known to exert suppressive effects on immune activation (74, 75). Overall, the ultimate combination should include an approach that may cause necrotic immunogenic tumor cell death, induce immune activation without preventing T cell clonal expansion, and possibly stimulate DC maturation and activation. Most of these requirements are likely provided by radiofrequency ablation that therefore constitute an attractive candidate for combination with active immunotherapy.

5. THERMAL TISSUE NECROSIS AND THE IMMUNE SYSTEM

When a thermal ablation needle is placed within a tumor lesion and high frequency alternating current is applied, temperature rises above 60-65 °C and cellular proteins coagulate, cell membranes fuse, and cellular coagulative necrosis is induced. Actually, the distinct type of cell death, apoptotic or necrotic cell death, is one of the key factors in Matzinger's "danger" model of immunity (76, 77). According to this theory, apoptotic cell death is a physiologic phenomenon not recognized as harmful by the immune system, while necrotic cell death is recognized as a result of dangerous events. Consequently, necrosis is accompanied by release of "danger signals" competent for triggering adaptive T cell responses if an adequate antigen presenting cells activation would occur and non-self or modified-self peptides can be presented to T cells.

Just surrounding the necrotic area temperature drops below 60 °C. At this site tumor cells undergo thermal stress with release of "danger signals" including acute phase proteins, pro-inflammatory cytokines such as IL-1, IL-6 and TNF-alpha, and heat shock proteins (HSPs). In particular, an increased synthesis and cell surface expression of HSPs have been experimentally demonstrated after radiofrequency thermal ablation of HCC (78, 79) and after laser ablation of colorectal liver metastasis (80). HSPs are cell chaperones involved in mechanisms of cell repair by refolding or elimination of nonnative proteins playing an important anti-apoptotic effect exploited by tumor cells for the neoplasm development. They play also important functions for the immune system. HSPs bind to DC by pattern recognition receptors (PRRs) and may be internalized by receptor-mediated endocytosis via CD91 (81, 82), scavenger receptor A (83), CD40 (84), lectin-like oxidized low-density lipoprotein receptor 1 (85), and TLR2/4 (86). In turn, DCs activated by HSPs can cross-present antigenic peptides generating a cytotoxic T lymphocyte response against cells producing these peptides. Therefore, the HSPs–peptide complexes released into the extracellular environment upon cell necrosis and lysis may not only represent the "danger signals" for the immune system but they may be carriers of immunogenic peptides released by the stressed cells. Moreover, large amounts of tumor debris derived from tumor cells necrosis may undergo phagocytosis by immature dendritic cells surrounding the area. These same DC can be activated by stress-proteins and other inflammatory mediators released at the same site, thus favoring maturation and migration to the afferent lymph nodes. As a whole, radiofrequency thermal ablation may not only induce immunogenic tumor cells necrosis, but it may also activate the immune system and modify the local area around tumor necrosis in favor of DC tumor antigen loading and activation (Figure 1).

The effects of thermal ablation on immune system activation and anti-tumor T-cell responses have

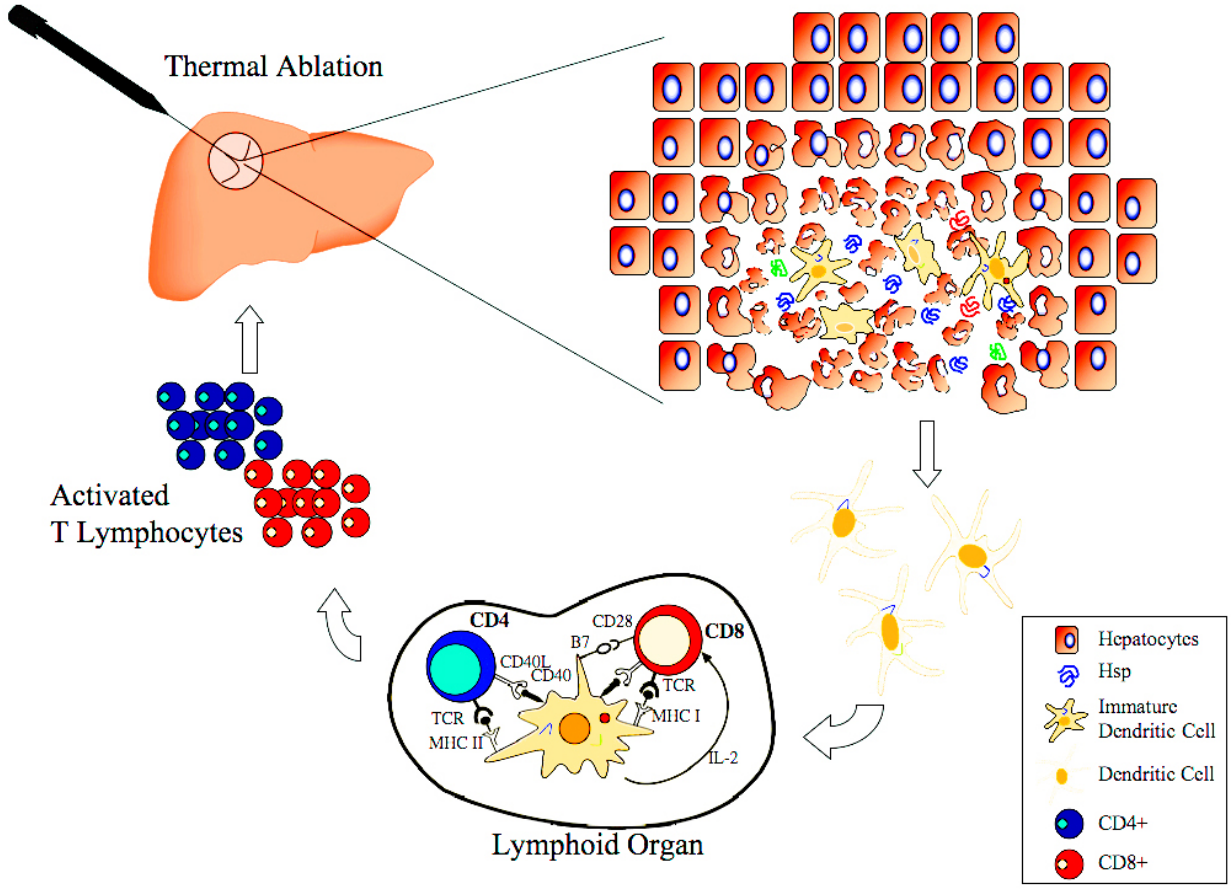

Figure 1. Activation of the immune response after radiofrequency thermal ablation. Thermal ablation of a neoplastic liver nodule may provide antigenic material and local conditions for DCs uptake of cellular debris, maturation and migration to the afferent lymphnode, where tumor-specific CD4 and CD8 T-cells are primed.

been studied in animal models and in patients. The first results were generated in rabbits undergoing RFA after previous liver implantation of experimental epithelial tumor. As early as 24 hours after thermal ablation a T-cell infiltrate could be observed at the periphery of the coagulative necrotic area. Two weeks later such T-cell infiltrate extended into the necrotic area of the treated lesion. Concomitantly, a tumor-specific proliferative T cell response could be detected in the peripheral blood of RFA treated animals (87). In another animal model, mice previously injected subcutaneously with a OVA-transfected murine melanoma cell line were also treated with RF. Antigen-specific anti-tumor CD8+ T cell reactivity was demonstrated after treatment and, perhaps even more importantly, revealed to be partially protective against tumor implant as shown by adoptive transfer of splenocytes in syngeneic mice (88). More recently, always in mice models, it has been shown that 7 to 13% of DC migrating to the draining lymph nodes are loaded with antigens present in the ablated tumoral lesions by thermal and cryoablation, respectively (89). Moreover, DCs loaded with tumor derived antigen up-regulate costimulatory molecules such as CD80 or CD86, showing that local tumor ablation can lead to efficient DC antigen loading, migration and maturation (89).

As far as human studies are concerned, the effects of thermal ablation on innate and adaptive immune system have been mainly studied in patients bearing HCC. Regarding the innate immune system, it has been demonstrated that thermal ablation of HCC can induce a functional transient activation of the myeloid DC arm circulating in peripheral blood associated with increased serum levels of pro-inflammatory cytokines TNF- alpha and IL-1beta (90). Regarding the adaptive immune system, it has been demonstrated that effector responses of circulating T cells stimulated with whole tumor lysates obtained from autologous HCC, either before or immediately after RFA treatment, were increased by RFA treatment. Interestingly, this boosting effect directed against undefined antigens and still persisting at one month after RFA treatment, revealed to be specific for HCC-associated antigens since responses to whole lysates deriving from the autologous non tumoral liver tissue counterpart were unchanged. These results indicate that thermal ablation can activate and enhance systemic T-cell responses against HCC-associated antigens and suggest that this effect may derive from generation of local conditions prone to induce activation of T-cell responses (91). These data were confirmed and extended by another study

showing similar results obtained after treating not only HCC liver nodules, but also colorectal liver metastasis (92).

Finally, we also studied the effect that necrotic debris recovered just at the end of the RFA procedure in patients with HCC may exert on monocytes or DCs. Patients derived monocytes and *in vitro* differentiated immature dendritic cells were exposed to protein lysates either derived from the post-RF necrotic debris and from HCC tumor cells obtained before treatment. In agreement with animal studies, monocyte and DCs matured with the RF-treated tumor tissue, showed higher up-regulation of costimulatory molecules, enhanced capacity of proinflammatory cytokine secretion and better antigen presentation function (our unpublished observations). The data confirm that radiofrequency thermal ablation can provide adequate local conditions for activation and maturation of antigen presenting cells. The overall picture emerging from animal and human studies is that locally released antigens may be picked up, processed and presented by DCs to tumor-specific T-cells. Even though RF-activated T-cells may be partially protective in experimental tumor models, the effect of RF seems not to be sufficient per se to control tumor recurrence. This could be due to several reasons: insufficient DC recruitment, the tolerant state of tumor-specific T-cells that may be only partially stimulated by RF treatment, previous selection of tumor escape antigens i.e. tumor editing, or activation of regulatory T-cells counteracting tumor-specific T-cell response.

6. THE USE OF RADIO FREQUENCY ABLATION IN CANCER

Radiofrequency thermal ablation is based on delivery to the target tissue of alternating current with ionics agitation and friction generation within tissues producing heat that leads to cell death by coagulative necrosis. Local delivery of radiofrequency is achieved through the use of different needles of different size guided to the focal lesion by ultrasounds or CT. Therefore, at the practical level, RFA may provide a feasible strategy to allow access of various immunomodulatory agents in the neighborhood (intratumoral, peritumoral) of the ablated lesion.

The other relevant advantage of RFA when considering the potential combination with immunotherapy is its wide application for various cancer types. The main application of RFA is for treatment of HCC nodules. In particular, RFA has become the standard of care for HCC not only when surgical treatment cannot be performed because of the site of the liver nodule or functional liver impairment, but it has recently been proposed as an alternative to surgery because of similar results in terms of disease free survival have been reported (93-95).

Beside its undisputable role in HCC management, RFA also represents the second line of intervention in other oncological settings. In patients with colon cancer, the first choice treatment for liver metastasis is represented by surgical resection, either with or without adjuvant chemotherapy, independently from size and number of liver lesions. Indeed, for cancer metastases surgery can provide higher assurance of free resection margins over RFA. However, the actual benefit in terms of disease free and overall survival of one technique over the other has not been defined. Two retrospective studies suggested comparable results between the two techniques (96, 97), but results from randomized prospective comparisons are still to come. In addition, RFA is likely to be increasingly applied for the treatment of liver metastasis from colon cancer. In a recent study, patients that were diagnosed to have colorectal liver metastasis underwent RFA ablation during the waiting time between diagnosis and surgical resection (98). Such indication is related to standard clinical protocols requiring a period of time before liver resection in order to avoid the risk of surgery ablation in patients shortly developing multiple metastatic lesions. In this setting, RFA obtained complete ablation in 53 of the 88 enrolled patients and avoided metastasectomy in 52 cases: in 23 cases because disease did not recur and in 29 cases because too rapid progressive disease occurred during the follow-up. Lastly, another highly relevant clinical setting for treatment of colorectal liver metastases is in patients with inoperable colorectal liver metastasis. In this regard, a broad multicentric randomized phase II study sponsored by the European Organisation for Research and Treatment of Cancer (EORTC) is currently ongoing in order to compare chemotherapy combined with RFA versus chemotherapy alone. Not surprisingly, unpublished preliminary results from this study suggest a better cumulative survival for the combined treatment group (99).

RFA is also used for loco-regional treatment of several other tumors such as unresectable intrahepatic cholangiocellular carcinoma (100), prostate cancer (101), head and neck cancers (102), primitive and metastatic tumors of the lung (103), renal cell carcinoma (104), lymphomas (105), breast cancer (106), and symptom palliation of bone metastasis (107). Among different types of metastatic liver tumors, major benefits are found in neuroendocrine tumors (108) because of the natural history of this disease that is characterized by small liver metastasis over a long period of time that allow repeated treatments sparing functional liver tissue differently from classic segmental liver resection. Even medicine specialists outside oncology make use of RFA for non-surgical ablative technology: cardiologists ablate abnormal cardiac cells that can cause cardiac arrhythmias (109), gynecologists use ablation to destroy uterine fibroids thus avoiding hysterectomies in some cases (110), and anesthesiologists use these techniques for pain palliation (111). In conclusion, RFA is a minimally invasive technique applied not only for primary and secondary liver tumors, but also increasingly tested for many other cancer types. On this basis, its indications as monotherapy or in combination with other standard and experimental therapies are expected to increase in the near future.

In theory, the general applicability of immunotherapy against autologous whole tumor cells harmonize well with the wide use of loco-regional ablative treatment by RFA. Although, it should be noted that the

systemic immunomodulatory effects of RFA have been observed so far only in patients with liver tumors (90-92). Therefore, it remains to be seen whether these effects are just limited to ablation of liver tumors or constitute a general outcome of RFA possibly favoring an extensive applicability of the synergic combination with immunotherapy beyond tumors seeded in liver tissue.

7. COMBINATION OF RADIOFREQUENCY ABLATION AND IMMUNOTHERAPY: ONGOING STUDIES AND FUTURE PERSPECTIVES

As discussed previously, RFA may induce a weak anti-tumor immunization that is far from effective not only at the clinical level, but even in immunogenic experimental tumor models. The combination with other approaches aimed at boosting anti-tumor immunization is expected to provide the opportunity to improve results in distinct experimental settings, and pave the way to reach the clinical application. The first possibility to combine immunotherapy with RFA would be to reinforce the antigen presenting activity within tumors by injecting unloaded naive DC prepared *ex vivo* in the area of the ablated tumor tissue. According to such hypothesis, a phase I clinical trial led by Edgar Engleman at Stanford is currently evaluating the feasibility, the safety and the immunogenicity of intratumoral DC immunotherapy in patients with thermally ablated liver metastases (112). Similarly, the intratumoral injection of DC is being tested also by other groups after ablation obtained by various other locally ablative techniques. The effects of direct injection of autologous immature DC into tumor in fourteen patients with advanced HCC after conformal radiotherapy has been already reported (113). Also, small amounts of DC have been injected into cancer nodules of four patients with HCC after ablation by 100% ethanol (114). Moreover, a phase II trial of external beam radiation with intratumoral injection of DC as neo-adjuvant treatment for high-risk soft tissue sarcoma is currently open at the H. Lee Moffitt Cancer Center and Research Institute at University of South Florida in Tampa (FL) (115). Finally, a trial of immunotherapy based on intratumoral DC after cryoablation of prostate cancer is planned to be started in the near future at the Prostate Institute of America in Ventura, CA (116). This latter trial will be the continuation of an identical setting already underway at the Asian Hospital and Medical Center in Manila (Philippines). As a result, the clinical testing of the combination of loco-regional ablative approaches including RFA, is still in its earliest phase. Therefore, it will probably take at least few more years to know whether combining RFA with intratumoral administration of DC may confer synergistic advantage useful for clinical purposes.

In the case *ex vivo* prepared DC can increase anti-tumor immunization till a level near to reach clinical efficacy, there are still issues deserving attention for future research and technical developments. First of all, *ex vivo* manipulation of DC should be simplified and minimized in order to enable preparation of minimally manipulated cell therapy products at the blood transfusion level (24). To this end, the intratumoral administration of lineage specific DC precursors, followed by *in vivo* induction of differentiation toward immature DC and final maturation after antigen loading, could be considered for clinical testing. Soluble factors such as GM-CSF and IFN-alfa that drive the differentiation of monocytes into DC (117) and are routinely used for other clinical purposes, could be introduced along with monocytes to induce their differentiation locally *in vivo*. This approach would represent a sort of combined *ex vivo* and *in vivo* manipulation of DC. Alternatively, complete *in vivo* manipulation of DC could be pursued by using chemokines attracting DC precursors at the tumor site, as already shown in animal models (39-41). The second most important issue to optimize anti-tumor immunization based on the use of DC relates to their maturation and activation. Currently, there is enormous excitement about various ligands of TLRs, including CpG ODN motifs, and others (118, 119). In experimental tumor models some of these molecules have already demonstrated significant efficacy after local ablation by either RFA or cryotherapy (120), and their testing in clinical setting is eagerly awaited. Thus, the elements currently to be considered for the design of future early phase clinical trials to test the combination of RFA and active immunization would probably include chemoattracting, differentiating and maturating agents for DC. Assuming that these biological agents may be rendered available as clinical grade GMP reagents ready for clinical use, they might finally allow to turn from *ex vivo* DC manipulation into *in vivo*.

Future therapeutic approaches combining RFA and immunotherapy will not only be aimed at improving tumor-antigen processing and presentation, but they could also focus on the regulatory mechanisms interfering with the anti-tumor T cell response. To this regard, the therapeutic effect of regulatory T-cells depletion or CTLA-4 blocking by monoclonal antibodies has also been explored in mice undergoing RFA or cryoablation for experimental murine tumors (88, 89). Administration of monoclonal antibodies against CTLA-4 after local tumor ablation resulted in higher frequency of tumor-specific CD8+ T cells, higher fraction of tumor-specific T cells able to secrete IFN-γ upon antigen stimulation, and increased protection against challenge with the same tumor cell line. Likewise, depletion of regulatory T cells by anti-CD25 antibodies yielded similar results in terms of protection and frequency of functional anti-tumor CD8 cells. These results suggest that when suppressive regulation is switched off, the immune activation that follows tumor destruction may lead to anti-tumor immune responses that could be exploited for clinical purposes (121). Indeed, a humanized anti-CTLA-4 monoclonal antibody has been recently synthesized and tested in patients in association with therapeutic vaccination with two modified HLA-A*0201-restricted peptides from the gp100 melanoma-associated antigen (122). In this pilot trial, clinical results were promising, although accompanied by relevant autoimmunity phenomena. A recent update on the effects of this anti-CTLA-4 monoclonal antibody in a broad cohort of patients with metastatic melanoma and renal cell cancer confirmed that most patients showing objective tumor responses experienced also induction of several immune

mediated corticosteroid-responsive toxicities including enterocolitis, dermatitis, hypophysitis, uveitis, hepatitis, and nephritis (123). Thus, the provisional view is that CTLA-4 plays a significant double edged role in tolerance to tumors and in protection against autoimmunity, and its blockade by mAbs in cancer patients should be considered with caution (124). Nonetheless, the potency of effects observed after CTLA-4 blockade confirms the notion that activation of T cells for anti-tumor immunotherapy can be achieved not only by targeting stimulatory pathways but also by blocking inhibitory pathways. The potential synergy between targeting stimulatory pathways and CTLA-4 blockade could be tested by tapering the dose of anti-CTLA-4 monoclonal antibody in association with active immunotherapy against antigens more extensively immunogenic than short peptides. Under this perspective, the design of clinical trials combining RFA treatment, active immunotherapy, and CTLA-4 blockade, could be also considered in the future.

Another possibility would be to combine simply RFA with passive immunotherapy by mAbs. Several mAbs currently used as standard therapy, including trastuzumab, rituximab, and cetuximab, are directed to specific TAA expressed on the surface of specific tumor cell types. The mechanism of action of these mAbs is complex, although a dominant part of their anti-tumor activity against experimental tumors *in vivo* depends on engagement of Fcgamma receptors on host's immune effector cells (125). Engagement of Fcgamma receptors can trigger effector mechanisms such as antibody dependent cell cytotoxicity (ADCC) by effector cells such as NK lymphocytes and others (126). Consistently, in patients with breast cancer overexpressing Her2 the capability to mediate *in vitro* antibody-dependent cellular cytotoxicity activity appeared highest in those showing complete remission or partial responses following preoperative therapy with trastuzumab (127). Interestingly, in a totally different clinical setting we also found that RFA of HCC liver nodules is followed by an increase of circulating NK cells and T lymphocytes expressing low affinity Fcgamma receptors (CD16) (91, 92) potentially useful to engage the constant fragment (Fc) of humanized IgG mAbs bound to tumor cells, and to mediate antibody-dependent antineoplastic activity. Consequently, it would be worth to evaluate whether ADCC activity may increase after RFA in other clinical settings, and whether the clinical efficacy of mAbs targeted to surface molecules may also increase when used in combination with RFA.

Similarly, therapy with mAbs targeted against EGF receptors (cetuximab) or VEGF (bevacizumab) could be easily tested in association with RFA of colorectal liver metastasis. In this context, it would be interesting to evaluate whether preoperative treatment with antiangiogenic factors such as VEGF inhibitors before thermal ablation could render colorectal liver metastasis more sensitive to thermal ablation. Indeed, ischemia seems to increase the efficacy of thermal ablation as huge HCC nodules (over 5 cm in diameter) have been treated successfully especially when RFA was preceded by trans arterial chemoembolization (TACE) inducing extensive tumor tissue ischemic embolization (128).

In conclusion, RFA constitute a minimally invasive procedure increasingly used for loco-regional treatment of tumor nodules in various clinical settings. The flexibility of its use and some peculiar effects including the capability to induce immunogenic necrosis, to increase the expression of HSPs by tumor cells, to induce acute phase response accompanied by production of pro-inflammatory cytokines, and to induce mobilization of antigen presenting cells and immune effector cells, render RFA a standard therapeutic modality attractive for combination with immunotherapy.

8. REFERENCES

1. M. Gonzalez-Angulo, G. N. Hortobagyi and F. J. Esteva: Adjuvant therapy with trastuzumab for HER-2/neu-positive breast cancer. *Oncologist* 11(8), 857-67 (2006)
2. E. E. Vokes and E. Chu: Anti-EGFR therapies: clinical experience in colorectal, lung, and head and neck cancers. *Oncology* 20(5 Suppl 2), 15-25 (2006)
3. A. Lundqvist, S. I. Abrams, D. S. Schrump, G. Alvarez, D. Suffredini, M. Berg and R. Childs: Bortezomib and depsipeptide sensitize tumors to tumor necrosis factor-related apoptosis-inducing ligand: a novel method to potentiate natural killer cell tumor cytotoxicity. *Cancer Res* 66(14), 7317-25 (2006)
4. L. Y. Schumacher, D. D. Vo, H. J. Garban, B. Comin-Anduix, S. K. Owens, V. B. Dissette, J. A. Glaspy, W. H. McBride, B. Bonavida, J. S. Economou and A. Ribas: Immunosensitization of tumor cells to dendritic cell-activated immune responses with the proteasome inhibitor bortezomib (PS-341, Velcade). *J Immunol* 176(8), 4757-65 (2006)
5. S. Demaria, N. Bhardwaj, W. H. McBride and S. C. Formenti: Combining radiotherapy and immunotherapy: a revived partnership. *Int J Radiat Oncol Biol Phys* 63, 655-666 (2005)
6. G. Chong and M. A. Morse: Combining cancer vaccines with chemotherapy. *Expert Opin Pharmacother* 6(16), 2813-2820 (2005)
7. M. S. Mitchell: Combinations of anticancer drugs and immunotherapy. *Cancer Immunol Immunother* 52(11), 686-92 (2003)
8. T. A. Waldmann: Immunotherapy: past, present and future. *Nat Med* 9(3), 269-277 (2003)
9. J. A: Sogn: Tumor Immunology: the glass is half full. *Immunity* 9, 757-763 (1998)
10. F. F: Fagnoni and G. Robustelli della Cuna: Immunotherapy: on the edge between experimental and clinical oncology. *J Chemother* 13(1), 15-23 (2001)
11. D. O'Mahony and M. R. Bisho: Monoclonal antibody therapy. *Front Biosci* 11, 1620-1635 (2006)
12. D. L. Porter, M. S. Roth, C. McGarigle, J. L. M. Ferrara and J. H. Antin: Induction of graft-versus-host disease as immunotherapy for relapsed chronic myelogenous leukemia. *N Engl J Med* 330, 100-106 (1994)
13. D. L. Longo: Idiotype vaccination in follicular lymphoma: knocking on the doorway to cure. *J Natl Cancer Inst* 98(18), 1263-65 (2006)

14. S. Inoges, M. Rodriguez-Calvillo, N. Zabalegui, A. Lopez-Diaz de Cerio, H. Villanueva, E. Soria, L. Suarez, A. Rodriguez-Caballero, F. Pastor, R. Garcia-Munoz, C. Panizo, J. Perez-Calvo, I. Melero, E. Rocha, A. Orfao and M. Bendandi: Clinical benefit associated with idiotypic vaccination in patients with follicular lymphoma. *J Natl Cancer Inst* 98(18), 1292-301 (2006)
15. R. Parish: Cancer immunotherapy: the past, the present and the future. *Immunol Cell Biol* 81(2), 106-113 (2003)
16. W. H. Woglom: Immunity to transplantable tumours. *Cancer Rev* 4, 129-214 (1929)
17. N. Renkvist, C. Castelli, P. F. Robbins and G. Parmiani: A listing of human tumor antigens recognized by T cells. *Cancer Immunol Immunother* 50(1), 3-15 (2001)
18. C. Lengauer, K. W. Kinzler and B. Vogelstein: Genetic instabilities in human cancers. *Nature* 396, 643-649 (1998)
19. T. Sjoblom, S. Jones, L.D. Wood, D. W. Parsons, J. Lin, T.D. Barber, D. Mandelker, R. J. Leary, J. Ptak, N. Silliman, S. Szabo, P. Buckhaults, C. Farrell, P. Meeh, S. D. Markowitz, J. Willis, D. Dawson, J. K. Willson, A. F. Gazdar, J. Hartigan, L. Wu, C. Liu, G. Parmigiani, B. H. Park, K. E. Bachman, N. Papadopoulos, B. Vogelstein, K. W. Kinzler and V. E. Velculescu: The consensus coding sequences of human breast and colorectal cancers. *Science* 314(5797), 268-74 (2006)
20. P. Matzinger: The danger model: a renewed sense of self. *Science* 296(5566), 301-5 (2002)
21. S. Kusmartsev and D. I. Gabrilovich: Effect of tumor-derived cytokines and growth factors on differentiation and immune suppressive features of myeloid cells in cancer. Cancer Metastasis Rev [Epub ahead of print] (2006)
22. J. Banchereau and R. M. Steinman: Dendritic cells and the control of immunity. *Nature* 392(6673), 245-52 (1998)
23. W. O'Neill, S. Adams and N. Bhardwaj: Manipulating dendritic cell biology for the active immunotherapy of cancer. *Blood* 104(8), 2235-46 (2004)
24. C. G. Figdor, I. J. de Vries, W. J. Lesterhuis and C. J. Melief: Dendritic cell immunotherapy: mapping the way. *Nat Med* 10(5), 475-80 (2004)
25. A. Nencioni and P.Brossart: Cellular immunotherapy with dendritic cells in cancer: current status. *Stem Cells* 22(4), 501-13 (2004)
26. F.J. Hsu, C. Benike, F. F. Fagnoni, T. M. Liles, D. Czerwinski, B. Taidi, E. G. Engleman and R. Levy: Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. *Nat Med* 2(1), 52-58 (1996)
27. D. Ridgway: The first 1000 dendritic cell vaccinees. *Cancer Invest* 21(6), 873-886 (2003)
28. S. Mocellin, S. Mandruzzato, V. Bronte and F. M. Marincola: Cancer vaccines: pessimism in check. *Nat Med* 10(12), 1278-1279 (2004)
29. P.L. Lollini, F. Cavallo, P. Nanni, G. Forni: Vaccines for tumour prevention. *Nat Rev Cancer* 6(3), 204-216 (2006).
30. M. V. Dhodapkar, R. M. Steinman, J. Krasovsky, C. Munz and N. Bhardwaj: Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. *J Exp Med* 193(2), 233-238 (2001)
31. J. de Vries, W. J. Lesterhuis, N. M. Scharenborg, L. P. Engelen, D. J. Ruiter, M. J. Gerritsen, S. Croockewit, C. M. Britten, R. Torensma, G. J. Adema, C. G. Figdor and C. J. Punt: Maturation of dendritic cells is a prerequisite for inducing immune responses in advanced melanoma patients. *Clin Cancer Res* 9(14):5091-5100 (2003)
32. M. Merad, T. Sugie, E. G. Engleman and L. Fong: *In vivo* manipulation of dendritic cells to induce therapeutic immunity. *Blood* 99(5), 1676-1682 (2002)
33. M. R. Crittenden, U. Thanarajasingam, R. G. Vile, M. J. Gough: Intratumoral immunotherapy: using the tumour against itself. *Immunology* 114(1), 11-22 (2005)
34. M. R. Shurin, G. V. Shurin, A. Lokshin, Z. R. Yurkovetsky, D. W. Gutkin, G. Chatta, H. Zhong, B. Han and R. L. Ferris: Intratumoral cytokines/chemokines/growth factors and tumor infiltrating dendritic cells: friends or enemies? *Cancer Metastasis Rev* Sep 23; 333-56 (2006)
35. M. Ratta, F. F. Fagnoni, A. Curti, R. Vescovini, P. Sansoni, B. Oliviero, M. Fogli, E. Ferri, G. R. Della Cuna, S. Tura, M. Baccarani and R. M. Lemoli: Dendritic cells are functionally defective in multiple myeloma: the role of interleukin-6. *Blood* 1(100), 230-237 (2002)
36. P. L. Triozzi, R. Khurram, W. A. Aldrich, M. J. Walker, J. A. Kim and S. Jaynes: Intratumoral injection of dendritic cells derived *in vitro* in patients with metastatic cancer. *Cancer* 89(12), 2646-2654 (2000)
37. E. Maraskovsky, K. Brasel, M. Teepe, E. R. Roux, S. D. Lyman, K. Shortman and H. J. McKenna: Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified. *J Exp Med* 184(5), 1953-1962 (1996)
38. L. Fong, Y. Hou, A. Rivas A, C. Benike, A. Yuen, G. A. Fisher, M. M. Davis and E. G. Engleman: Altered peptide ligand vaccination with Flt3 ligand expanded dendritic cells for tumor immunotherapy. *Proc Natl Acad Sci U S A* 98(15), 8809-8814 (2001)
39. T. Fushimi, A. Kojima, M. A. Moore and R. G. Crystal: Macrophage inflammatory protein 3alpha transgene attracts dendritic cells to established murine tumors and suppresses tumor growth. *J Clin Invest* 10(105), 1383-1393 (2000)
40. K. Furumoto, L. Soares, E. G. Engleman and M. Merad: Induction of potent antitumor immunity by *in situ* targeting of intratumoral DCs. *J Clin Invest* 113(5), 774-783 (2004)
41. C. Guiducci, E. Di Carlo, M. Parenza, M. Hitt, M. Giovarelli, P. Musiani and M. P. Colombo: Intralesional injection of adenovirus encoding CC chemokine ligand 16 inhibits mammary tumor growth and prevents metastatic-induced death after surgical removal of the treated primary tumor. *J Immunol* 172(7), 4026-4036 (2004)
42. Y. Nishioka, M. Hirao, P. D. Robbins, M. T. Lotze and H. Tahara: Induction of systemic and therapeutic antitumor immunity using intratumoral injection of dendritic cells genetically modified to express interleukin 12. *Cancer Res* 59(16), 4035-4041 (1999)
43. I. Melero, M. Duarte, J. Ruiz, B. Sangro, J. Galofre, G. Mazzolini, M. Bustos, C. Qian, J. Prieto: Intratumoral injection of bone-marrow derived dendritic cells engineered to produce interleukin-12 induces complete regression of established murine transplantable colon adenocarcinomas. *Gene Ther* 6, 1779-1784 (1999)

44. T. Saika, T. Satoh, N. Kusaka, S. Ebara, V. B. Mouraviev, T. L. Timme and T. C. Thompson: Route of administration influences the antitumor effects of bone marrow-derived dendritic cells engineered to produce interleukin-12 in a metastatic mouse prostate cancer model. *Cancer Gene Ther* 5(11), 317-324 (2004)
45. T. Kikuchi, M. A. Moore and R. G. Crystal: Dendritic cells modified to express CD40 ligand elicit therapeutic immunity against preexisting murine tumors. *Blood*. 96 (1), 91-99 (2000)
46. Y. Liu, D. Xia, F. Li, C. Zheng and J. Xiang: Intratumoral administration of immature dendritic cells following the adenovirus vector encoding CD40 ligand elicits significant regression of established myeloma. *Cancer Gene Ther* 12(2), 122-132 (2005)
47. Z. R. Yurkovetsky, G. V. Shurin, D. A. Barry, A. C. Schuh, M. R. Shurin and P. D. Robbins: Comparative analysis of antitumor activity of CD40L, RANKL, and 4-1BBL *in vivo* following intratumoral administration of viral vectors or transduced dendritic cells. *J Gene Med* 8, 129-37. (2006)
48. P. W. Miller, S. Sharma, M. Stolina, L. H. Butterfield, J. Luo, Y. Lin, M. Dohadwala, R. K. Batra, L. Wu, J. S. Economou and S. M. Dubinett: Intratumoral administration of adenoviral interleukin 7 gene-modified dendritic cells augments specific antitumor immunity and achieves tumor eradication. *Hum Gene Ther* 11(1), 53-65 (2000)
49. T. Tatsumi, J. Huang, W. E. Gooding, A. Gambotto, P. D. Robbins, N. L. Vujanovic, S. M. Alber, S. C. Watkins, H. Okada and W. J. Storkus: Intratumoral delivery of dendritic cells engineered to secrete both interleukin (IL)-12 and IL-18 effectively treats local and distant disease in association with broadly reactive Tc1-type immunity. *Cancer Res* 63(19), 6378-6386 (2003)
50. J. Hu, X. Yuan, M. L. Belladonna, J. M. Ong, S. Wachsmann-Hogiu, D. L. Farkas, K. L. Black and J. S. Yu: Induction of potent antitumor immunity by intratumoral injection of interleukin 23-transduced dendritic cells. *Cancer Res* 66(17), 8887-8896 (2006)
51. N. Kuwashima, F. Nishimura, J. Eguchi, H. Sato, M. Hatano, T. Tsugawa, T. Sakaida, J. E. Dusak, W. K. Fellows-Mayle, G. D. Papworth, S. C. Watkins, A. Gambotto, I. F. Pollack, W. J. Storkus and H. Okada: Delivery of dendritic cells engineered to secrete IFN-alpha into central nervous system tumors enhances the efficacy of peripheral tumor cell vaccines: dependence on apoptotic pathways. *J Immunol* 175(4), 2730-2740 (2005)
52. K.A. Candido, K. Shimizu, J.C. McLaughlin, R. Kunkel, J.A. Fuller, B. G. Redman, E. K. Thomas, B. J. Nickoloff, J. J. Mulé. Local administration of dendritic cells inhibits established breast tumor growth: implications for apoptosis-inducing agents. *Cancer Res* 61, 228-236 (2001)
53. A. Kianmanesh, N. R. Hackett, J. M. Lee, T. Kikuchi, R. J. Korst and R. G. Crystal: Intratumoral administration of low doses of an adenovirus vector encoding tumor necrosis factor alpha together with naive dendritic cells elicits significant suppression of tumor growth without toxicity. *Hum Gene Ther* 12(17), 2035-2049 (2001)
54. K. Heckelsmiller, S. Beck, K. Rall, B. Sipos, A. Schlamp, E. Tuma, S. Rothenfusser, S. Endres and G. Hartmann: Combined dendritic cell- and CpG oligonucleotide-based immune therapy cures large murine tumors that resist chemotherapy. *Eur J Immunol* 32(11), 3235-3245 (2002)
55. C. Guiducci, A. P. Vicari, S. Sangaletti, G. Trinchieri and M P. Colombo: Redirecting *in vivo* elicited tumor infiltrating macrophages and dendritic cells towards tumor rejection. *Cancer Res* 65(8) 3437-3446 (2005)
56. Okano, M. Merad, K. Furumoto and E. G. Engleman: *In vivo* manipulation of dendritic cells overcomes tolerance to unmodified tumor-associated self antigens and induces potent antitumor immunity. *J Immunol* 174(5), 2645-2652 (2005)
57. S. C. Yang, R. K. Batra, S. Hillinger, K. L. Reckamp, R. M. Strieter, S. M. Dubinett and S. Sharma: Intrapulmonary administration of CCL21 gene-modified dendritic cells reduces tumor burden in spontaneous murine bronchoalveolar cell carcinoma. *Cancer Res* 66(6), 3205-3213 (2006)
58. M. Nukiwa, S. Andarini, J. Zaini, H. Xin, M. Kanehira, T. Suzuki, T. Fukuhara, H. Mizuguchi, T. Hayakawa, Y. Saijo, T. Nukiwa and T. Kikuchi. Dendritic cells modified to express fractalkine/CX3CL1 in the treatment of preexisting tumors. *Eur J Immunol* 36(4), 1019-1027 (2006)
59. G. Mazzolini, C. Alfaro, B. Sangro, E. Feijoo, J. Ruiz, A. Benito, I. Tirapu, A. Arina, J. Sola, M. Herraiz, F. Lucena, C. Olague, J. Subtil, J. Quiroga, I. Herrero, B. Sadaba, M. Bendandi, C. Qian, J. Prieto and I. Meleto: Intratumoral injection of dendritic cells engineered to secrete interleukin-12 by recombinant adenovirus in patients with metastatic gastrointestinal carcinomas. *J Clin Oncol* 23(5), 999-1010 (2005)
60. E. Feijoo, C. Alfaro, G. Mazzolini, P. Serra, I. Penuelas, A. Arina, E. Huarte, I. Tirapu, B. Palencia, O. Murillo, J. Ruiz, B. Sangro, J. A. Richter, J. Prieto and I. Melero: Dendritic cells delivered inside human carcinomas are sequestered by interleukin-8. *Int J Cancer* 116(2), 275-281 (2005)
61. Y Tong, W. Song and R. G. Crystal: Combined intratumoral injection of bone marrow-derived dendritic cells and systemic chemotherapy to treat pre-existing murine tumors. *Cancer Res* 61(20), 7530-7535 (2001)
62. F. Tanaka, H. Yamaguchi, M. Ohta, K. Mashino, H. Sonoda, N. Sadanaga, H. Inoue and M. Mori: Intratumoral injection of dendritic cells after treatment of anticancer drugs induces tumor-specific antitumor effect *in vivo*. *Int J Cancer* 101(3), 265-269 (2002)
63. A. K. Nowak, B. W. Robinson and R. A. Lake: Synergy between chemotherapy and immunotherapy in the treatment of established murine solid tumors. *Cancer Res* 63(15), 4490-4496 (2003)
64. W. Song and R. Levy: Therapeutic vaccination against murine lymphoma by intratumoral injection of naive dendritic cells. *Cancer Res* 65(13), 5958-5964 (2005)
65. E. Y. Nikitina and D. I. Gabrilovich: Combination of gamma-irradiation and dendritic cell administration induces a potent antitumor response in tumor-bearing mice: approach to treatment of advanced stage cancer. *Int J Cancer* 94(6), 825-833 (2001)
66. M. Ehtesham, P. Kabos, M. A. Gutierrez, K. Samoto, K. L. Black and J. S. Yu: Intratumoral dendritic cell vaccination elicits potent tumoricidal immunity against

malignant glioma in rats. *J Immunother* 26(2), 107-116 2003
67. S. Teitz-Tennenbaum, Q. Li, S. Rynkiewicz, F. Ito, M. A. Davis, C. J. McGinn and A. E. Chang: Radiotherapy potentiates the therapeutic efficacy of intratumoral dendritic cell administration. *Cancer Res* 63(23), 8466-8475 2003
68. T. Kumagi, S. M. Akbar, N. Horiike and M. Onji: Increased survival and decreased tumor size due to intratumoral injection of ethanol followed by administration of immature dendritic cells. *Int J Oncol* 23(4), 949-955 (2003)
69. A. Machlenkin, O. Goldberger, B. Tirosh, A. Paz, I. Volovitz, E. Bar-Haim, S. H. Lee, E. Vadai, E. Tzehoval and L. Eisenbach: Combined dendritic cell cryotherapy of tumor induces systemic antimetastatic immunity. *Clin Cancer Res* 11(13), 4955-4961 (2005)
70. B. Jalili, M. Makowski, T. Switaj, D. Nowis, G. M. Wilczynski, E. Wilczek, M. Chorazy-Massalska, A. Radzikowska, W. Maslinski, L. Bialy, J. Sienko, A. Sieron, M. Adamek, G. Basak, P. Mroz, I. W. Krasnodebski, M. Jakobisiak and J. Golab: Effective photoimmunotherapy of murine colon carcinoma induced by the combination of photodynamic therapy and dendritic cells. *Clin Cancer Res* 10(13), 4498-4508 (2004)
71. H. Saji, W. Song, K. Furumoto, H. Kato and E. G. Engleman: Systemic antitumor effect of intratumoral injection of dendritic cells in combination with local photodynamic therapy. *Clin Cancer Res* 12(8), 2568-2574 (2006)
72. N. Casares, M. O. Pequignot, A. Tesniere, F. Ghiringhelli, S. Roux, N. Chaput, E. Schmitt, A. Hamai, S. Hervas-Stubbs, M. Obeid, F. Coutant, D. Metivier, E. Pichard, P. Aucouturier, G. Pierron, C. Garrido, L. Zitvogel and G. Kroemer: Caspase-dependent immunogenicity of doxorubicin-induced tumor cell death. *J Exp Med* 202(12), 1691-1701 (2005)
73. R. A. Lake and B. W. Robinson: Immunotherapy and chemotherapy-a practical partnership. *Nat Rev Cancer* 5, 397-405 (2005)
74. F. T. Hakim, R. Cepeda, S. Kaimei, C. L. Mackall, N. McAtee, J. Zujewski, K. Cowan, R. E. Gress: Constraints on CD4 recovery postchemotherapy in adults: thymic insufficiency and apoptotic decline of expanded peripheral CD4 cells. *Blood* 90(9), 3789-3798 (1997)
75. M. W. Wichmann, G. Meyer, M. Adam, W. Hochtlen-Vollmar, M. K. Angele, A. Schalhorn, R. Wilkowski, C. Muller and F. W. Schildberg: Detrimental immunologic effects of preoperative chemoradiotherapy in advanced rectal cancer. *Dis Colon Rectum* 46(7), 875-887 (2003)
76. E. J. Fuchs and P. Matzinger: Is cancer dangerous to the immune system? *Semin Immunol* 8(5), 271-280 (1996)
77. S. Gallucci, M. Lolkema and P. Matzinger: Natural adjuvants: endogenous activators of dendritic cells. *Nat Med* 5(11), 1249-1255 (1999)
78. G. Schueller, J. Kettenbach, R. Sedivy, H. Bergmeister, A. Stift, J. Fried, M. Gnant and J. Lammer: Expression of heat shock proteins in human hepatocellular carcinoma after radiofrequency ablation in an animal model. *Oncol Rep* 12(3), 495-499 (2004)
79. G. Schueller, J. Kettenbach, R. Sedivy, A. Stift, J. Friedl, M. Gnant, J. Lammer: Heat shock protein expression induced by percutaneous radiofrequency ablation of hepatocellular carcinoma *in vivo*. *Int J Oncol* 24(3), 609-13 (2004)
80. M. Nikfarjam, V. Muralidharan, K Su, C. Malcontenti-Wilson and C. Christophi: Patterns of heat shock protein (HSP70) expression and Kupffer cell activity following thermal ablation of liver and colorectal liver metastases. *Int J Hyperthermia* 21, 319–332 (2005)
81. S. Basu, R. J. Binder, T. Ramalingam, and P. K. Srivastava: CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin. *Immunity* 14, 303–313 (2001)
82. R. J. Binder and P. K. Srivastava: Essential role of CD91 in re-presentation of gp96-chaperoned peptides. *Proc Natl Acad Sci USA* 101, 6128–6133 (2004)
83. A. Berwin, J. P. Hart, S. Rice, C. Gass, S. V. Pizzo, S. R. Post, and C. V. Nicchitta: Scavenger receptor-A mediates gp96/GRP94 and calreticulin internalization by antigen-presenting cells. *EMBO J* 22 6127–6136 (2003)
84. T. Becker, F. U. Hartl, and F. Wieland: CD40, an extracellular receptor for binding and uptake of Hsp70-peptide complexes. *J Cell Biol* 158, 1277–1285 (2002)
85. Y. Delneste, G. Magistrelli, J. Gauchat, J. Haeuw, J. Aubry, K. Nakamura, N. Kawakami Honda, L. Goetsch, T. Sawamura, J. Bonnefoyand and P. Jeannin: Involvement of LOX-1 in dendritic cell mediated antigen cross-presentation. *Immunity* 17, 353–362 (2002)
86. A. Asea, M. Rehli, E. Kabingu, J. A. Boch, O. Bare, P. E. Auron, M. A. Stevenson, and S. K. Calderwood: Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. *J Biol Chem* 277, 15028–15034 (2002)
87. T. T. Wissniowski, J. Hunsler, D. Neureiter, M. Frieser, S. Schaber, B. Esslinger, R. Voll, D. Strobel, E. G. Hahn and D. Schuppan: Activation of tumor-specific T lymphocytes by radio-frequency ablation of the VX2 hepatoma in rabbits. *Cancer Res* 63, 6496–6500 (2003)
88. M. H. den Brok, R. P. Sutmuller, R. van der Voort, E. J. Bennink, C. G. Figdor, T. J. Ruers and G. J. Adema: *In situ* tumor ablation creates an antigen source for the generation of antitumor immunity. *Cancer Res* 64(11), 4024-4029 (2004)
89. M. H. den Brok, R. P. Sutmuller, S. Nierkens, E. J. Bennink, C. Frielink, L. W. Toonen, O. C. Boerman, C. G. Figdor, T. J. Ruers and G. J. Adema: Efficient loading of dendritic cells following cryo and radiofrequency ablation in combination with immune modulation induces anti-tumour immunity. *Br J Cancer* 95(7), 896-905 (2006)
90. M. Y. Ali, C. F. Grimm, M. Ritter, L. Mohr, H. P. Allgaier, R. Weth, W. O. Bocher, K. Endrulat, H. E. Blum and M. Geissler: Activation of dendritic cells by local ablation of hepatocellular carcinoma. *J Hepatol* 43(5), 817-822 (2005)
91. A. Zerbini, M. Pilli, A. Penna, G. Pelosi, C. Schianchi, A. Molinari, S. Schivazappa, C. Zibera, F. F. Fagnoni, C. Ferrari, G. Missale: Radiofrequency thermal ablation of hepatocellular carcinoma liver nodules can activate and enhance tumor-specific T-cell responses. *Cancer Res* 66(2), 1139-46 (2006)
92. J. Hansler, T. T. Wissniowski, D. Schuppan, A. Witte, T. Bernatik, E. G. Hahn, D. Strobel: Activation and dramatically increased cytolytic activity of tumor specific T

lymphocytes after radio-frequency ablation in patients with hepatocellular carcinoma and colorectal liver metastases. *World J Gastroenterol* 12(23),3716-3721 (2006)
93. M. Vivarelli, A. Guglielmi, A. Ruzzenente, A. Cucchetti, R. Bellusci, C. Cordiano and A. Cavallari: Surgical resection versus percutaneous radiofrequency ablation in the treatment of hepatocellular carcinoma on cirrhotic liver. *Ann Surg* 240, 102-107 (2004)
94. A. M. Cho, W. Y. Tak, Y. O. Kweon, S. K. Kim, Y. H. Choi, Y. J. Hwang and Y. I. Kim: The comparative results of radiofrequency ablation versus surgical resection for the treatment of hepatocellular carcinoma. *Korean J Hepatol* 11(1), 59-71 (2005)
95. M. D. Lu, M. Kuang, L. J. Liang, X. Y. Xie, B. G. Peng, G. J. Liu, D. M. Li, J. M. Lai and S. Q. Li: Surgical resection versus percutaneous thermal ablation for early-stage hepatocellular carcinoma: a randomized clinical trial. *Zhonghua Yi Xue Za Zhi* 12(86), 801-805(2006)
96. A. Elias, T. De Baere, T. Smayra, J. F. Ouellet, A. Roche and P. Lasser: Percutaneous radiofrequency thermoablation as an alternative to surgery for treatment of liver tumour recurrence after hepatectomy. *Br J Surg* 89(6), 752-756 (2002)
97. A. Oshowo, A. Gillams, E. Harrison, W. R. Lees and I. Taylor. Comparison of resection and radiofrequency ablation for treatment of solitary colorectal liver metastases. *Br J Surg* 90(10), 1240-1243 (2003)
98. T. Livraghi, L. Solbiati, F. Meloni, T. Ierace, S. N. Goldberg and G. S. Gazelle: Percutaneous radiofrequency ablation of liver metastases in potential candidates for resection: the "test-of-time approach". *Cancer* 97(12), 3027-3035 (2003)
99. A. R. Gillams: The use of radiofrequency in cancer. *Br J Cancer* 92(10), 1825-1829 (2005)
100. W. Zgodzinski, N.J. Espat: Radiofrequency ablation for incidentally identified primary intrahepatic cholangiocarcinoma. *World J Gastroenterol* 11(33), 5239-40 (2005)
101. A.R. Zlotta, B. Djavan, C. Matos, J.C. Noel, M.O. Peny, D.E. Silverman, M. Marberger, C.C. Schulman: Percutaneous transperineal radiofrequency ablation of prostate tumour: safety,feasibility and pathological effects on human prostate cancer. *Br J Urol* 81(2), 265-75 (1998)
102. R.P. Owen, T.S. Ravikumar, C.E. Silver, J. Beitler, S. Wadler, J. Bello: Radiofrequency ablation of head and neck tumors: dramatic results from application of a new technology. *Head Neck* 24(8), 754-8(2002)
103. L. Thanos, S. Mylona, M. Pomoni, K. Athanassiadi, N. Theakos, L. Zoganas, N. Batakis: Percutaneous radiofrequency thermal ablation of primary and metastatic lung tumors. *Eur J Cardiothorac Surg* 30(5), 797-800 (2006)
104. V. Mouraviev., S. Joniau, H. Van Poppel, T.J. Polascik: Current Status of Minimally Invasive Ablative Techniques in the Treatment of Small Renal Tumours. *Eur Urol* 51(2), 328-36 (2006)
105. Sudheendra D., M.M. Barth, U. Hegde, W.H. Wilson, B.J. Wood: Radiofrequency ablation of lymphoma. *Blood* 107(4), 1624-6 (2006)
106. T. Susini , J. Nori, S. Olivieri, L. Livi, S. Bianchi, G. Mangialavori, F. Branconi, G. Scarselli: Radiofrequency ablation for minimally invasive treatment of breast carcinoma. A pilot study in elderly inoperable patients. *Gynecol Oncol* 104(2),304-10 (2007)
107. G. Poggi, C. Gatti, M. Melazzini, G. Bernardo, M. Strada, C. Teragni, A. Delmonte, C. Tagliaferri, C. Bonezzi, M. Barbieri, A. Bernardo, P. Fratino: Percutaneous ultrasound-guided radiofrequency thermal ablation of malignant osteolyses. *Anticancer Res* 23(6D), 4977-83 (2003)
108. A.R. Henn, E.A. Levine, W. McNulty, R.J. Zagoria: Percutaneous radiofrequency ablation of hepatic metastases for symptomatic relief of neuroendocrine syndromes. *AJR Am J Roentgenol* 181, 1005-10 (2003)
109. D. Gupta , R.K. Al-Lamee, M.J. Earley, P. Kistler, S.J. Harris, A.W. Nathan, S.C. Sporton, R.J. Schilling: Cryoablation compared with radiofrequency ablation for atrioventricular nodal re-entrant tachycardia: analysis of factors contributing to acute and follow-up outcome. *Europace* 12,1022-6 (2003)
110. V. Bergamini., F. Ghezzi, A. Cromi, G. Bellini, G. Zanconato, S. Scarperi, M. Franchi: Laparoscopic radiofrequency thermal ablation: a new approach to symptomatic uterine myomas. *Am J Obstet Gynecol* 192, 768-73 (2005)
111. R.V. Shah, J.J. Ericksen, M. Lacerte: Interventions in chronic pain management. 2. New frontiers: invasive nonsurgical interventions. *Arch Phys Med Rehabil* 84, S39-44 (2003)
112. http://clinicaltrials.gov/ct/show/NCT00185874
113. H. Chi, S. J. Liu, C. P. Li, H. P. Kuo, Y. S. Wang, Y. Chao and S. L. Hsieh: Combination of conformal radiotherapy and intratumoral injection of adoptive dendritic cell immunotherapy in refractory hepatoma. *J Immunother* 28(2), 129-35 (2005)
114. T. Kumagi, S. M. Akbar, N. Horiike, K. Kurose, M. Hirooka, A. Hiraoka, Y. Hiasa, K. Michitaka and M. Onji: Administration of dendritic cells in cancer nodules in hepatocellular carcinoma. *Oncol Rep* 14(4), 969-73 (2005)
115. http://clinicaltrials.gov/ct/show/NCT00365872
116.http://www.prostate-cancer.org/education/novelthr/Ragde_Bahn_Cryo-Immunotherapy.html
117. S. M. Santini, T. Di Pucchio, C. Lapenta, S. Parlato, M. Logozzi and F. Belardelli: A new type I IFN-mediated pathway for the rapid differentiation of monocytes into highly active dendritic cells. *Stem Cells* 21(3), 357-62 (2003)
118. G.M. Barton, R. Medzhitov: Toll-like receptors and their ligands. *Curr Top Microbiol Immunol* 270, 81-92 (2002)
119. R.R. Koganty: Vaccines: exploiting the role of toll-like receptors. *Expert Rev Vaccines* 2, 123-4 (2002)
120. M.H. den Brok, R.P. Sutmuller, S. Nierkens, E.J. Bennink, L.W. Toonen, C.G. Figdor, T.J. Ruers, G.J. Adema: Synergy between *in situ* cryoablation and TLR9 stimulation results in a highly effective *in vivo* dendritic cell vaccine. *Cancer Res* 66, 7285-92 (2006)
121. A.J. Korman, K.S. Peggs, J.P. Allison: Checkpoint blockade in cancer immunotherapy. *Adv Immunol* 90, 297-339 (2006)
122. G.Q. Phan, J.C. Yang, R.M. Sherry, P. Hwu, S.L. Topalian, D.J. Schwartzentruber, N.P. Restifo, L.R. Haworth, C.A. Seipp, L.J. Freezer, K.E. Morton, S.A.

Mavroukakis, P.H. Duray, S.M. Steinberg, J.P. Allison, T.A. Davis, S.A. Rosenberg: Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. *Proc Natl Acad Sci U S A* 100, 8372-7 (2003)
123. K.E. Beck, J.A. Blansfield, K.Q. Tran, A.L. Feldman, M.S. Hughes, R.E. Royal, U.S. Kammula, S.L. Topalian, R.M. Sherry, D. Kleiner, M. Quezado, I. Lowy, M. Yellin, S.A. Rosenberg, J.C. Yang: Enterocolitis in patients with cancer after antibody blockade of cytotoxic T-lymphocyte-associated antigen 4. *J Clin Oncol* 24, 2283-9 (2006)
124. H.L. Kaufman, J.D. Wolchok: Is tumor immunity the same thing as autoimmunity? Implications for cancer immunotherapy. *J Clin Oncol* 24, 2230-2 (2006)
125. R.A. Clynes, T.L. Towers, L.G. Presta, J.V. Ravetch: Inhibitory Fc receptors modulate *in vivo* cytoxicity against tumor targets. *Nat Med* 6, 443-6 (2006)
126. A. Iannello, A. Ahmad: Role of antibody-dependent cell-mediated cytotoxicity in the efficacy of therapeutic anti-cancer monoclonal antibodies. *Cancer Metastasis Rev* 24, 487-99 (2005)
127. R. Gennari, S. Menard, F. Fagnoni, L. Ponchio, M. Scelsi, E. Tagliabue, F. Castiglioni, L. Villani, C. Magalotti, N. Gibelli, B. Oliviero, B. Ballardini, G. Da Prada, A. Zambelli, A.Costa: Pilot study of the mechanism of action of preoperative trastuzumab in patients with primary operable breast tumors overexpressing HER2. *Clin Cancer Res* 10, 5650-5 (2004)
128. A. Veltri, P. Moretto, A. Doriguzzi, E. Pagano, G. Carrara, G. Gandini: Radiofrequency thermal ablation (RFA) after transarterial chemoembolization (TACE) as a combined therapy for unresectable non-early hepatocellular carcinoma (HCC). *Eur Radiol* 16, 661-9 (2006)

Abbreviations: TNF: tumor necrosis factor, IFN: interferon, DC: dendritic cell, TAA: tumor associated antigens, RFA: radiofrequency ablation, HCC: hepatocellular carcinoma, HSP: heat shock protein, IL: interleukine, PPR: pattern recognition receptor, mAb: monoclonal antibody, VEGF: vascular endotelial growth factor

Key Words: RFA, Immunotherapy,TNF, IFN, VEGF, Tumor Necrosis Factor, Interferon,Vascular Endotelial Growth Factor, HCC, TAA, DCs, Review

Send correspondance to: Dr Gabriele Missale, Unit of Infectious Diseases and Hepatology, Azienda Ospedaliero-Universitaria di Parma, Via Gramsci 14, 43100 Parma, Italy, Tel: 39-0521-702310, Fax: 39-0521-703857, E-mail: missale@tin.it

http://www.bioscience.org/current/vol13.htm