Abstract. Transformation of normal cells into malignant cancer involves a number of changes in the genome. These changes include chromosomal translocations, exon deletions and gene mutations, to name a few, that result in deregulation of the regulatory circuits of the cell and consequently in profound changes in their antigenic composition, including expression of mutated proteins, overexpression of proteins that are produced at much lesser levels in normal tissue and expression of aberrantly glycosylated proteins. It is well established that these new antigenic entities, referred to as tumor-associated antigens (TAA), are recognized by the immune system and elicit immune reactivity. However, the immune reaction is overpowered by the cancer potential to grow and to metastasize. Cancer immunotherapy aims at boosting the naturally occurring immune response at a level that will prevail over the ability of cancer cells to escape immune attack. After more than 20 years of intense research and a growing number of clinical trials, most of which gave marginal results, it is now clear that the task is not an easy one. However, during the same period of time, we have gained a much better understanding of the mechanisms of tumor growth, the mechanisms by which the immune system is activated or becomes tolerant and of the natural relationship between cancers and the immune system. Also, even though few, some immunotherapy strategies have demonstrated positive clinical responses and are already used as standard therapies. These developments give the impetus to explore and devise new strategies that will by more effective in strengthening the immune reactivity at a level that will counteract the tumor potential. In this paper we review the knowledge gained from basic research related to cancer immunity and from clinical trials. We propose that, based on this knowledge, improved clinical protocols can be elaborated.

Successful immunotherapy of cancer requires understanding of tumor biology and of its natural relationship with the immune system. Tumors have several characteristics that distinguish them from normal tissues, among them genetic instability, ability to invade across natural barriers and to metastasize into distant organs. All these properties of tumors are potentially immunogenic.

The genetic instability of cancer cells results in the production of mutated proteins with an antigenic profile that differs from that of their normal counterparts. Also, due to the aberrant functioning of the regulatory machinery of the cell, tumor cells produce proteins that are expressed normally at earlier ontogenetic stages, express proteins of the normal tissue at dramatically higher levels, or proteins with aberrant protein glycosylation (1,2). All these changes in the protein profile of the tumor cells generate potential antigenic targets that are referred to as tumor-associated antigens (TAA).

The existence of TAA was indirectly shown in the 1950’s in transplantation experiments demonstrating that mice are able to specifically recognize and reject tumors (3). Next, indirect evidence was provided by cloning T cells from melanoma patients that specifically responded to melanoma antigens (4). Currently, a significant number of tumor-specific or tumor-associated antigens for multiple types of cancer have been identified and cloned, and the ability of the immune system to recognize them and to mount an immune response has been proved (5-7).

Immune cells play an important role in cancer immunity (8,9). Especially it is well documented that T cell-mediated immunity significantly contributes to the rejection of virus- and chemically-induced tumors in experimental animals. Also, T cells that specifically recognize TAA have been isolated from the blood of cancer patients (10,11), and
cytotoxic T lymphocytes (CTL) can be generated against TAA. These observations, in combination with advances in understanding of the mechanisms of T cell activation, provided the ground for clinical protocols involving the recruitment of CTLs with effector function against tumors. NK cells can also kill cancer cells in an MHC unrestricted way (12). NK cells represent approximately 90% of lymphokine-activated killer cells (LAK). LAK cells are generated in vitro after activation of peripheral blood lymphocytes with IL-2 and are able to kill most cancer cells, while most normal cells are resistant to killing by LAK cells (13).

In contrast to cellular immune response, humoral responses do not seem to contribute to tumor immunity. Even though antibody responses against tumor antigens have been detected or can be induced in experimental animals and in humans, and anti-tumor antibodies are able to lyse tumor cells in vitro, their ability to control tumor growth has not been demonstrated in vivo (14). However, use of monoclonal antibodies targeting selective antigens have shown promise for certain type of cancers (15).

Although the ingredients of an anti-tumor immune response are present, the magnitude of this response is limited and insufficient to restrain tumor growth. Also unexplained is the ability of tumors to metastasize without inducing an immune response. In fact, under normal conditions disruption of tissue architecture initiates the production of proinflammatory cytokines that trigger a vigorous innate and adaptive immune response. Why then do tumor metastasis and invasion of normal tissue not activate the effector mechanisms of the immune system? Many studies have shown that, in the tumor microenvironment, a complicated network of cytokines and growth factors is produced. However, these factors do not promote the immune response but instead regulate tumor growth directly or indirectly. Some evidence indicates that tumors take advantage of those properties of cytokines that are beneficial to their growth while the functions that are potentially harmful are neutralized. Often this occurs after mutational inactivation of the cytokine receptors on tumor cells, inactivation of their downstream effector molecules (16,17), and/or modulation of their activity by other cytokines that are produced in the tumor microenvironment (18). On the other hand, several experiments strongly suggest that tumors grow not only because they are able to regulate their own growth, but also because the tumor microenvironment has the capacity to suppress the immune response. This property of tumors may indeed explain why most clinical trials come up with poor clinical responses. Therefore, immunotherapy should not only aim at increasing the immune reactivity, but also at blocking the immune suppressive activity of the tumor.

**Anti-cancer immunotherapy strategies**

Many strategies for cancer immunotherapy aiming to boost the naturally occurring immune response have been assessed or are presently in clinical trials, including: a) cytokine-based therapies, b) vaccination with tumor-associated antigens, using different vehicles or reinfusing autologous dendritic cells (DC) loaded with immunogenic peptides, c) adoptive transfer of TAA-specific cytotoxic T cells, and d) mAb-based treatments. Also many protocols combine more than one of these treatments, aiming either to enhance immune reactivity or to suppress negative regulatory mechanisms.

**Cytokine-based therapies.** Systemic administration of cytokines was the first treatment aimed at boosting the effector arm of the immune response. IFN-γ treatment showed some promise by enhancing NK activity and T cell cytotoxicity, activating apoptotic genes in tumor cells and improving antigen presentation by increasing MHC expression (19,20). Because IL-2 increases the cytotoxic activity of NK and CD8+ T cells, its anti-tumor activity was assayed in several mouse models with encouraging results (21-23). However, high doses of IL-2 were necessary for cancer patients, a treatment that was not well tolerated (24).

GM-CSF, because of its ability to promote DC development and migration and to stimulate macrophage activity and TNF-α release, was also thought to increase anti-tumor activity. GM-CSF was first used in the B16 melanoma mouse tumor model. B16 cells were engineered to secrete GM-CSF or 10 other cytokines then injected in mice (25). B16 cells expressing GM-CSF induced a durable and specific immunity against cancer superior to other cytokines (25,26). Next to these results, autologous tumor cells were engineered to secrete high levels of GM-CSF then reinfected to the patients. This treatment resulted in increased anti-tumor immunity characterized by elevated anti-tumor antibody levels and infiltration of tumors with DCs, macrophages, eosinophils and T cells. GM-CSF treatment was assessed in several types of tumors including melanomas, multiple myeloma, leukemia, prostate, pancreas and kidney carcinomas (27-31) with, however, limited clinical improvements.

The efficacy of IL-12 was evaluated in preclinical trials using different strategies to express IL-12 in the tumor site. Therapy with IL-12 alone demonstrated limited results in most cases, however, when IL-12 was combined with B7.1 costimulation, enhancement of the immune response was observed and complete regression of tumors was observed in 80% of a pancreatic cancer murine model (32). Furthermore, the beneficial effect of combined IL-12/B7 therapy was shown in prostate cancer models. In one study 1/3 of the animals had complete regression of the tumor.
while only 1/38 animals that were treated with IL-12 alone was tumor-free (33). In addition, to its enhancing effect on immune effector mechanisms, IL-12 was also shown to affect tumor growth by suppressing VGEF production, blocking the release and activity of matrix metalloproteinases (34) and by altering the expression of adhesion molecules preventing tumor attachment (35). The efficacy of IL-12 treatments in human cancer has not yet been established.

In general, despite the fact that some positive results have been reported, the use of cytokines as a sole agent to boost immune reactivity against tumors is not effective. Systemic administration of high doses of cytokines is not well tolerated, while achieving limited results without clinical improvement. However, cytokines, especially GM-CSF and IL-2, in combination with other treatments could eventually enhance the immune effector mechanisms and therefore should be further evaluated (36).

**T cell-based therapies.** T cell-based immunotherapy is considered to have a greater potential than antibody-based therapies. This assumption relies on two facts; first, the greater role of T cells in natural anti-tumor immunity and, second, on their ability to recognize and to become activated by intracellular antigens that are presented in the context of MHC by APCs. Therefore, T cell therapies, in contrast to antibody therapies, may not only target membrane antigens but also intracellular TAA. Tumor antigen-specific T cells are present in the blood and also infiltrate tumors, however, their frequency and their avidity is low. T cell immunotherapy aims at expanding the already existing pool of tumor-specific cytotoxic T lymphocytes. To this end different approaches have been employed, including: a) vaccination with TAA epitopes, b) in vitro activation of autologous tumor infiltrating lymphocytes (TIL) without respect to their specificity, which after expansion are reinfused to the patients, and c) the same as above but T lymphocytes specific for a TAA are isolated from the patient, expanded in vitro, then reinfused back to the host. The two latter approaches have more often been used in clinical trials with limited improvement in overall survival rates in patients with melanoma, kidney carcinomas and lung cancer (37,38). The major reason for the failure of the reinfused cytotoxic T cells to kill or even to contain tumor growth is that they are quickly eliminated, most probably because negative regulatory mechanisms block their activation and, as a consequence, the cells die. Indeed, to support the in vivo persistence of infused T cells, high doses of IL-2 have been required, a treatment with serious side-effects, such as vascular leak syndrome (24).

However, two recent studies, both in patients with metastatic melanoma, gave grounds for optimism. In the first study (39), specific CD8+ CTLs from the peripheral blood of patients, recognizing the M27 peptide of the MART-1 antigen or the G154 of the gp100 protein, were expanded in vitro. Both gp100 and MART-1 are proteins expressed by normal melanocytes and overexpressed in melanoma cells. The novelty of the study consisted of the fact that the patients received a first administration of CTLs without any additives, while the following infusions of CTLs were done in association with systemic daily administration of low doses of IL-2. The use of low doses of IL-2 avoided toxic side-effects and increased the half-life of reinfused T cells from 7 to 17 days. Furthermore, the study showed that CD8+ MART-1 specific T cells selectively infiltrated tumor biopsies, consisting of up to 38% of infiltrating CD8+ cells. Most important, 5 out of 10 patients showed disease stabilization, and 3 more patients showed minor or mixed disease responses. The median duration of the clinical response was 11 months up to 21 months. Historically survival of refractory metastatic melanoma patients is only 4 months. In the second study (40), TILs from refractory metastatic melanoma patients were expanded in vitro, without regard to specificity, then reinfused into the patients after lymphoablative treatment in association with high doses of IL-2. Lymphoablation has been shown to have a remarkable positive effect in preclinical mouse studies. Probably, this positive effect is due to the destruction of regulatory cells and, in general, in the abrogation of tolerogenic mechanisms that neutralize anti-tumor reactivity. Four patients out of 13 who participated in this study achieved a duration of clinical response from 9 up to 24 months. In 2 patients, infused T cells represented more than 60% of the total circulating CD8+ cells 5 months after the initiation of the therapy. In both patients tumor regression was greater than 95%. Autoimmune reactivity was observed in 4 patients. However since melanocytes are dispensable for life this treatment is considered tolerable. Similar approaches could be applied in patients with cancers affecting organs that are not necessary for survival.

**Vaccination protocols.** Vaccination with TAA or peptides aims at increasing both the humoral and the cell-mediated immune response. Vaccination strategies included immunization with selected peptides in incomplete Freund's adjuvant, delivery of the antigen on liposomes, in combination with cytokines (IL-2, IL-12, GM-CSF) or subcloning of the TTA gene in different viral or bacterial vectors, then delivering these constructs to the patients. DCs loaded with cancer antigens or peptides have also been used to improve antigen presentation. Recently DCs transfected with recombinant retrovirus or adenovirus encoding tumor antigens have also been assessed in animal models with promising results (41,42). In melanoma patients, vaccination with MART-1, tyrosinase and gp100 alone generated specific CD8+ T cells responses (43-45). Use of adjuvants or GM-CSF, IL-2 or IL-12 have been reported
to increase the immunogenicity of the vaccines. In a different approach, PBMCs from patients with androgen-independent prostate cancer were cultured in the presence of IL-4 and GM-CSF and inactivated BCG then pulsed with recombinant PSMA and reinfused to the patients. No disease progression was observed in 21% of the participants for a period of 6 months (46).

Regression or stabilization of metastatic cancers has been reported in a few patients in different clinical trials after vaccination. Tumor regression in these patients was usually associated with measurable immune responses to the vaccinating antigen. However, often strong immune responses did not correlate with objective clinical benefits (47). As a general conclusion the vaccination strategies used up to now did not result in objective and durable clinical improvements except in single patients. Vaccination is able to induce anti-tumor immune reactivity, which however does not rise above a certain threshold necessary to counteract the tumor potential.

**Monoclonal antibody-based therapy.** Monoclonal antibodies (mAb) used in tumor immunotherapy may target proteins that are directly critical to tumor development, such as growth factor receptors, or they may target components of the tumor microenvironment, such as receptors involved in the formation of the tumor vasculature (48). In both cases the mAb blocks receptor-ligand binding and consequently signalling necessary for tumor growth or survival. Alternatively the antibody may target a tumor membrane antigen to induce complement-mediated cytotoxicity or antibody-dependent cellular toxicity (ADCC). The mAb may be unlabeled or conjugated with radioisotopes or other toxic substances to create a cytotoxic agent (15). Despite the fact that targeting tumors using the "magic bullet" as Ehrlich called it seems promising in the first place, several hurdles have been encountered in practice. First, mAb raised in mice generated human anti-mouse antibodies (HAMA) that neutralized the xenogenic mAb and therefore limited the number of injections. Engineering humanized antibodies, that contain only the CDR of the mouse mAb and thereby are unable to induce a xenogenic response, have overcome this obstacle of HAMA generation. An additional problem is that some targeted antigens are secreted and bind the antibody in the circulation thereby limiting the antibody that is available to target the tumor (49). Yet, the most important obstacle to effective mAb therapy is the reduced ability of the mAb to penetrate the tumor. Due to increased hydrostatic pressure inside the tumor and the disordered tumor vasculature, it has been estimated that an IgG molecule needs 7 months to reach 1cm into a tumor and a Fab fragment 2 months (50).

These problems, along with the unproven role of the naturally occurring antibodies in tumor immunity and the initial failure of mAb therapies, raised scepticism as to whether this type of approach could lead to positive outcomes. In spite of these reservations, recent clinical trials prove the value of this strategy. Indeed, impressive clinical responses were demonstrated with a chimeric mAb directed against the CD20 molecule, an antigen expressed on B lymphocytes. This antibody, also known as rituximab, was evaluated in clinical trials in patients with refractory or relapsing non-Hodgkin's lymphomas (51). An overall 31% response rate was observed with 9% complete and 22% partial responses. Similar results were observed in two other studies with response rates of 46% and 48% (52,53). Even more impressive results were obtained when rituximab was given in conjunction with chemotherapy (CHOP) to treat patients with low-grade B cell lymphoma. The overall response observed was 95%, with 55% complete and 40% partial responses (54,55). Furthermore, median response duration and time to progression has not been reached after more than 29 months of follow-up. The improved therapeutic value of the combination therapy is most probably due to the reported property of rituximab to increase the sensitivity of tumor cells to the toxic effects of chemotherapy (56). Rituximab is the first mAb to receive approval from the FDA for use in human cancer. The mechanisms that lead to cell death by rituximab involve ADCC, complement-mediated lysis and apoptosis (57).

Labelling anti-CD20 mAb with cytotoxic radioisotopes is an additional approach that has been tested for hematological malignancies. Two antibodies, the first targeting the B-1 epitope of the CD20 molecule (Tositumomab) and the second targeting an epitope distinct from the B-1 (Ibritumomab tiuxetan), have been tested in chemotherapy refractory lymphomas. Tositumomab labelled with $^{131}$I has been used in combination with autologous bone marrow transplantation with very good results; 62% of the patients exhibited progression-free survival after 2 years follow-up (117). Also non-transplanted patient treatment with tositumomab demonstrated a 50% response rate with a median duration of 16 months (58,59). Ibritumomab labelled with $^{90}$Y has also demonstrated significant results and is the first radiolabelled mAb approved by the FDA (60). In fact, in a randomised phase III trial, ibritumomab demonstrated superior efficacy in comparison to rituximab (61).

Mab therapy met with less success when used to treat solid tumors. The only meaningful results up to now have been reported for breast cancer using trastuzumab, a mAb that targets the Her-2/neu antigen, a receptor tyrosine kinase that belongs to the EGF receptor family (62). Overexpression of Her-2/neu is observed in 25% of breast cancer and correlates with bad prognosis. A phase II trial in patients with metastatic breast cancer reported a 16% objective response rate and median overall survival of 13
months, which is clearly better than most results reported with second-line chemotherapy (63). A combination of trastuzumab with chemotherapy demonstrated higher efficacy than chemotherapy alone (64,65). The addition of trastuzumab to a chemotherapy regimen improved survival by 16% at one year and by 25% at 29 months (66). Her-2/neu is also overexpressed in other adenocarcinomas. However, clinical trials with other types of cancer do not document good results (67).

**Tumor escape mechanisms**

Even though all the ingredients for an immune response against tumors are present, the naturally occurring immune response is weaker than the growth potential of the tumors. In addition, enhancement of the naturally occurring immune reactivity against cancer cells by means of vaccination or passive transfer of cultured and expanded *in vitro* anti-tumor CTLs in most cases does not correlate with tumor regression. When limited benefits are observed, tumor growth is suppressed for only a short period of time. A rational explanation for the ability of tumors to overpower the immune attack is that the tumor itself, through different mechanisms, is able to contain the immune reactivity. Current concepts propose either that tumors are resistant to killing by immune cells or that tumors are able to induce a state of immune tolerance. Evidence supporting both possibilities has been provided by several studies. However, conclusive evidence is still lacking for either one of these two hypotheses and it is likely that both mechanisms are in operation to maximize the immune escape potential of tumors.

*TAAb loss and mutational variation.* Observations supporting the tumor resistance hypothesis include TAA loss or mutations, down-regulation of MHC class I expression on tumor cells, defective processing of intracellular antigens and expression of Fas ligand on tumor and stromal cells.

Tumor antigen loss is well documented in murine models of tumor after vaccination (68,69). In humans, loss of the cognate antigen was observed in melanoma patients after passive transfer of CD8+ T cells specific for MART-1 or for gp100 (39). Also specific antigen loss after treatment of melanoma patients with peptide vaccine has been reported in several studies (70,71). Since CTL recognize discrete epitopes, not only antigen loss but even mutations on the antigenic epitope may lead to loss of antigenicity. An example is that of SV40 transformed cancer cells, which could escape CTL killing *in vitro* due to point mutations in the immunodominant epitope (72). However, antigen loss in unmanipulated hosts has not been documented. In contrast, TAA-associated antigens demonstrate persistent expression throughout the different disease stages in many cancers. For example, in prostate cancer several TAA, *i.e.* PSA, PSMA, PSCA and PTH-rP, are expressed or even up-regulate their expression during disease progression (73,74). Therefore, even though under external pressure, for example vaccination, selection of antigen-loss variants may occur, it is very unlikely that, in the natural course of the disease, selection of antigen-loss clones is a major mechanism for tumor escape.

Down-modulation of class I MHC expression and defective antigen peptide processing has been reported in many types of tumors; an observation suggesting that tumor antigens are not efficiently presented to stimulate immune effector mechanisms. Decreased expression of class I MHC has more often been observed in breast, lung and prostate cancer in humans (75). Complete loss of MHC class I has also been reported more often as a result of deletion of the β2-microglobulin gene (76). Also, loss of individual HLA alleles has been found in many cases (75). This observation caused speculation that these alleles are lost because they present immunodominant peptides. Down-regulation of the transporter associated with the antigen presentation (TAP) gene has also been reported in different tumors types including prostate cancer (77,78). However, the importance of diminished MHC expression in the evasion of immune response by tumors has never been established. Attempts to correlate tumor progression with levels of expression of MHC did not provide consistent results, supporting the notion that MHC down-modulation or loss is a major mechanism in tumor immune escape. In addition, in most renal cancers, increased MHC expression was observed (75,79). On the other hand, MHC loss is not necessarily a good strategy for tumor escape since NK cells recognize and kill cells that do not express MHC antigens. Indeed in some studies low MHC expression was associated with diminished cell growth consequent to NK cell killing (80,81).

FasL expression on tumor cells has been proposed as another strategy employed by tumors to evade immune attack by inducing apoptosis of Fas-expressing T cells. Increased expression of FasL was initially found on melanoma, glioblastoma cells and in human colon cancer and was associated with Fas-mediated T cell killing (82,83). However, later reports did not reproduce these results in melanoma cells (84,85). Furthermore, anti-tumor effects of FasL expression have been described. Thus, transfer of the FasL gene in CT26 colon carcinoma augmented inflammatory responses and inhibited tumor growth *in vivo* (86).

In conclusion, while all of the above mechanisms may contribute to tumor resistance to cytotoxic attack by T cells, they do not seem to be the major and universal mechanism for tumor immune escape.
**Immune tolerance.** Several distinct tolerogenic mechanisms including deletion, anergy, ignorance and the newly described regulatory T cells (Treg) operate to safeguard the immune system from attacking self-tissues, and to down-modulate the immune effector mechanisms when infectious agents have been successfully eliminated (87,88). Observations in human cancer, but mainly experiments with transgenic mice, provide strong evidence that tumors are able to induce immune tolerance, a strategy employed to escape from immune attack.

Experiments with TCR-transgenic mouse T cells, specific for an Ig expressed in a murine myeloma, showed induction of T cell tolerance towards the myeloma cells. In this study, both central and peripheral tolerance was observed (89,90). In another set of experiments, both CD4+ and CD8+ T cells were rendered tolerant when influenza hemagglutinin (HA)-specific TCR transgenic T cells were adoptively transferred in hosts bearing HA-expressing lymphomas or HA-expressing carcinomas (91,92). In a similar approach, investigators examined tolerance induction in a prostate tumor model expressing HA (93). Increased recognition of HA-expressing prostate cells by HA-specific T cells was observed upon transformation of prostate epithelium to prostate cancer. However, this did not lead to activation of effector functions as assayed by defective production of IFN-γ by the HA-specific T cells. Also, using a model of spontaneously arising insulinoma expressing a defined antigen, investigators showed that presentation of TAA to high avidity T cells does take place. Unlike previous reports, in this tumor model specific T cells did not become tolerant and were capable of proliferating upon antigen presentation. Nonetheless, these T cells demonstrated limited effector functions in vivo (94).

In humans, Cardoso et al. showed that human pre-B acute lymphoblastic leukemia cells lack B7-1 (95). Antigen presentation in the absence of B7-1 induced alloantigen-specific T cell tolerance. In addition to classical tolerance induction, tumors also use other mechanisms not yet well understood to block immune effector mechanisms. Thus a recent study in melanoma patients showed that development of lymph node metastases induced T cell-mediated immunity as assessed by increased frequency of MART-1-specific T cells. However, only a minority of tumor-invading CD8+ T cells were perforin-positive, while most of them demonstrated a precursor phenotype (96). Also, in a study by Lee and colleagues, using peptide/HLA-A0201 tetramers, the authors were able to demonstrate the presence of circulating CD8+ T cells specific for MART-1 and tyrosinase in metastatic melanoma patients (7). While these cells had the phenotypic markers of effector cells, they were unable to lyse melanoma cells or to produce cytokines in response to mitogens. No such defect was observed in CD8+ T cells specific for EBV from the same patient (7). These results demonstrate that TAA-specific T cell responses can develop in cancer patients, but antigen-specific unresponsiveness is also established resulting in the defective control of tumor growth. In agreement with these findings are observations in mice showing that while immunization induced T cells with a classical effector phenotypic profile, these cells were of small size and did not express perforin (97,98). Therefore these cells, characterized as cytotoxic T cells by phenotypic analysis, may be unable to kill in the tumor microenvironment.

It should be emphasized that, regardless of the mechanisms that are implicated, a common conclusion of all these studies is that TAA-specific T cells become tolerant or hyporeactive. This observation provides a strong argument that induction of immune tolerance is a basic mechanism through which tumors evade the immune response. The mechanisms that tumors use to induce tolerance have not yet been elucidated, and this task is of paramount importance in our quest for better immunotherapy protocols. Recent findings with Stat-3 provide some insights in that respect, evoking the possibility that at least some of the molecules and the pathways that are involved in oncogenesis may also act as suppressors of the immune system. Stat-3 is a molecule that is constitutively activated in most tumors, affecting expression of cell-cycle regulatory genes such as cyclin D1 and antiapoptotic genes such as BCL-XL (99). In a recent study, increased Stat-3 activity in mouse APCs resulted in impaired antigen-specific T cell responses. Conversely, APCs devoid of Stat-3 break antigen-specific T cell anergy (100). It is also intriguing that blocking of Stat-3 signaling in tumor cells results in up-regulation of production of several proinflammatory cytokines and chemokines. On the contrary, expression of constitutively activated Stat-3 into fibroblasts blocks the production of proinflammatory cytokines in response to activation with LPS or other stimuli (101).

Very little is also known about the role of the newly discovered CD4+CD25+ Treg cells in cancer immunity. Indirect evidence, however, suggests that these cells play a significant role in blocking the immune response against tumors. In one of these studies, treatment of mice with anti-IL2 receptor-α antibodies prior to vaccination with a GM-CSF-producing B16 melanoma cell line was much more effective in eliminating tumors than vaccination alone (102). These findings suggest that disruption of more than one of the negative regulatory mechanisms may be necessary for effective tumor immunotherapy.

**Is there a future for immunotherapy?**

Both the failures and the few successes in tumor immunotherapy teach us important lessons. Some experimental evidence strongly points out that cancers create a microenvironment that blocks immune effector
mechanisms and induces immune tolerance towards tumor antigens. This is probably the main mechanism employed by tumors to escape from immune surveillance. Indeed current concepts propose that that two events are a prerequisite for tumor establishment. First, de-regulation of several cellular check-point mechanisms inactivates suppressor and pro-apoptotic genes and, in parallel, the second event suppresses the expression of proinflammatory signals and impairs T cell activation. Whether the same molecule(s) or signaling pathways control(s) these two events, as the experiments with Stat-3 point out, has to be further evaluated. Yet, in either case, in the absence of adequate proinflammatory signals, APCs present antigen to T cells in a microenvironment that, instead of promoting immune reactivity and subsequent tumor elimination, induces immune tolerance. This hypothesis explains why even when large numbers of exogenously-activated TAA-specific T cells are administered to patients, the number of these cells wanes rapidly. Active tolerance induced by cancers also explains another common finding of almost all experimental models and clinical trials, that is the extent of the immune response after vaccination of cancer patients with tumor antigens is always weaker than the response against viral antigens.

Therefore, since tumors are able to induce immune tolerance, boosting the immune reactivity against cancer either by vaccination or by infusion of ex vivo expanded T cells alone, most probably will not be sufficient to control tumor expansion. Combination therapies, that in parallel to increasing immune reactivity will also reverse the tolerant state or will block inhibitory signals, could result in improved clinical responses. Several experiments using animal models of cancer clearly support this view. These experiments show that vaccination with parallel blocking of inhibitory signals for T cell costimulatory pathways such as CTLA-4 provided better results than vaccination alone (103). CTLA-4 is expressed subsequent to T cell activation and binds with higher affinity than the costimulatory molecule CD28 to B7.1 and B7.2 molecules. After its binding to B7 receptors, CTLA-4 delivers inhibitory signals to T cells that oppose the costimulatory signals delivered by CD28. Both in mouse models of melanoma and prostate cancer, administration of CTLA-4 blocking antibodies in conjunction with GM-CSF or B-7, transducing vaccines were able to eliminate residual metastatic cancers or to significantly reduce the incidence of spontaneously arising tumors in the transgenic animals, whereas either treatment alone failed to achieve these results (104,105). One important consideration in this type of treatment is that blocking of inhibitory signals for costimulatory pathways may induce autoimmune reactions. Indeed, mice receiving the melanoma-GM-CSF+anti-CTLA-4 treatment developed vitiligo, an autoimmune response restricted to melanocytes (105). Similarly, in the prostate cancer model, mice treated with anti-CTLA-4 blocking antibodies developed prostatitis (104). However, in both experimental models no other signs of autoimmunity were diagnosed. Therefore, this type of approach can be used in the treatment of cancers that affect organs dispensable for life.

Even more effective may be therapies that, in addition to blocking the inhibitory signal delivered by CTLA-4, would also directly target the breaking of the established immune tolerance by eliminating the suppressor T cells. For example, the combination of vaccination with anti-CTLA-4 treatment plus depletion of Treg cells was much more effective than vaccination plus anti-CTLA-4 treatment alone (102). Even though this type of combination therapy is clearly more promising, further studies are necessary before going to clinical trials, since breaking of the immune tolerance may promote autoimmune reactions against vital organs.

Since cancers produce a number of growth factors and cytokines that form a network which is beneficial for tumor growth, blocking the signals that are central in this network is an additional strategy aimed at diminishing the tumor replicative potential. The effectiveness of this approach is underlined by the success of blocking the Her-2/neu receptor using antireceptor mAbs in patients with breast cancer. The choice of the receptor(s) to be targeted with mAbs is an issue that has to be addressed in a rational way and should be studied individually for each type of cancer. For example, in prostate cancer several indications exist supporting that IL-6, IL-8 and EGF positively regulate tumor growth. TGF-β seems also to participate in the network of these factors and to promote tumor growth indirectly, especially at advanced stages of tumor development. However, the exact role and the importance of each of these factors in tumor development has not been fully established in vivo. With today's technological capabilities, i.e. the existence of transgenic mice that spontaneously develop different types of cancer and the possibility to inactivate selected genes in a tissue-specific and conditional way, it is feasible and necessary to better determine the role of cytokines and growth factors during the different stages of tumor development. The next step involves the development of the appropriate reagents (mAbs or antagonistic molecules) that block receptor signaling and impede cancer growth potential.

However, since cancers have accumulated a number of growth advantages and they evolve in a way analogous to Darwinian evolution, it is hard to imagine that depriving them of one growth factor will result in durable disease improvement. On the other hand, if the hypothesis that tumors grow not only because they are self-sufficient in growth factors, but also because they block the immune effector mechanisms is correct –as many experiments indicate– then simultaneously targeting both of these tumor capabilities certainly would provide better results in cancer therapy. Based on these considerations and assumptions, we propose a two-hit model for cancer immunotherapy (Figure 1). This involves: a)
boosting the immune response either through vaccination or T cell-based therapies in combination with treatments that will break the tolerogenic mechanisms, and b) reducing tumor growth potential by means of mAbs that block essential growth signals. This strategy, by simultaneously reducing tumor growth potential and increasing the immune system capability to kill cancer cells, may provide durable clinical disease improvement.

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Thyphronitis and Koutsilieris: Tumor Immunotherapy


Received February 11, 2004
Accepted May 6, 2004