Immune Manipulation of Advanced Breast Cancer: An Interpretative Model of the Relationship Between Immune System and Tumor Cell Biology

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Abstract: This review summarizes some recent clinical immunological approaches with cytokines and/or antibodies for therapy of advanced breast cancer. It considers the recent advances in genetics and molecular tumor biology related to impaired immunosurveillance involving cytokines and growth factors to explain clinical results. Evasion of the host immune attack might be induced by the following groups of mechanisms: (a) tumor dependent (genomic instability, HLA class I antigen abnormalities, upregulation of fetal type nonclassical HLA class I molecules, epitope immunodominance, apoptosis inhibition by defective death receptor signaling, apoptosis of activated T cells, tumor cannibalism and constitutive activation of signal transducer, and activator of transcription-3 (Stat 3) and nuclear factor-κB (NF-κB) signaling); (b) host dependent (CD4⁺CD25⁺ regulatory T cells (T reg), CD4⁺ T cells anergy, Th2 antitumor immunity diversion and myeloid suppressor cells); (c) tumor and host dependent (lack of co-stimulation molecules, immunosuppressive cytokines (vascular endothelial growth factor (VEGF), interleukin (IL)-10, prostaglandin (PG)E2, transforming growth factor (TGF)-β). Cytokines and growth factors are involved in virtually all three types of mechanisms. These mechanisms are integrated with the current knowledge of tumor growth and inhibited apoptosis primarily mediated by cytokines and growth factors to propose an interpretation of the relationships among tumor cells, tumor stroma, and tumor-infiltrating lymphocytes. Tumor growth, defective immunorecognition and immunosuppression are the three principal effects considered responsible for immune evasion.

Key words: advanced breast cancer; immunotherapy; tumor biology
1. INTRODUCTION

Despite widespread use of screening mammography that has decreased mortality by 30%, breast cancer remains the most common cancer and the principal cause of death in women between the ages of 35 and 55 in Western countries. Much effort has been devoted to improve the poor prognosis of advanced breast cancer, where the course of the disease is often fatal. In this setting, anthracyclines, taxanes (paclitaxel, docetaxel), 5-fluorouracil (5-FU), and/or vinorelbine-based regimens are the conventional treatments for chemosensitive patients, while high dose chemotherapy (HDC) continues to be investigational.

As breast cancer growth and metastasis are angiogenesis dependent and tumor cells can express hormone or HER2 receptors, so-called targeted therapies have become additional options. Treatment with antiestrogens was the first among these therapies, and for a long time, tamoxifen has been the standard first-line salvage therapy in estrogen receptor-positive postmenopausal advanced breast cancer. However, as a few prospective randomized trials have recently confirmed a superior therapeutic index of a new, third, and more potent highly selective generation of drugs targeted against the aromatase enzyme, these drugs have replaced tamoxifen as the standard first-line salvage therapy in these patients. Breast cancer has been broadly investigated in in vitro and in vivo experimental models and different immunological approaches have also been introduced recently to improve the outcome in advanced patients. Many among these new immunological approaches have used cytokines or antibodies against the HER2 receptor (trastuzumab) or vascular endothelial growth factor (VEGF) (bevacizumab) to fight the tumor. However, in spite of the introduction of chemotherapeutic agents (taxanes) and new targeted therapies (aromatase inhibitors, trastuzumab, bevacizumab) into clinical practice, the median overall survival (OS) of metastatic breast cancer has not been significantly improved and still ranges from 20 to 30 months.

This review describes the recent approaches of immune manipulation in advanced breast cancer and summarizes the recent advances in genetics and molecular tumor biology related to cancer immune manipulation, reporting in details the role of cytokines and growth factors. Finally, an interpretation of the immune system–tumor relationship will be proposed and used to better explain the presented clinical data.

2. RECENT IMMUNOTHERAPEUTIC APPROACHES TO ADVANCED BREAST CANCER WITH CYTOKINES OR MONOCLONAL ANTIBODIES AGAINST GROWTH FACTOR RECEPTORS

The principal immunological approaches recently used for medical therapy in advanced breast cancer can be divided into two main categories. The former includes the use of cytokines to stimulate the cellular immune system and/or to induce synthesis of hormone receptors, and the latter includes the use of monoclonal antibodies against tumor growth factor receptors.

A. Immunostimulation With Cytokines

1. IL-2 or IL-6 Alone, IL-2 Given With Other Cytokines (IFNα, G-CFS) or Trastuzumab or Epirubicin, and IL-12 and Trastuzumab

In locally advanced or metastatic breast cancer, IL-2 given at low dose, alone or in association with other cytokines and/or other drugs in an outpatient setting, is well tolerated. In most studies, the expected laboratory immunological effects of the evaluated cytokines have been reported. When IL-2 or IL-6 was given alone, no clear antitumor response was
observed although the sample size was small. In addition, when IL-2 was combined with IFNα, no significant improvement in 2-year disease-free survival (DFS) or OS was observed.9 IL-2 plus trastuzumab was evaluated in a phase I trial where most of the enrolled patients (33 out of 45) had metastatic breast cancer that overexpressed HER-2 and were therefore not suitable for or had previously failed effective standard therapy.10 Clinical benefit (two complete (CR), two partial responses (PR), and nine stable diseases (SD)) occurred in 56% of the evaluated patients. However, IL-2 did not increase the activity of trastuzumab. In 43 advanced breast cancer patients, IL-2 and granulocyte-colony stimulating factor (G-CSF) were subcutaneously administered in combination to develop a graft versus host disease (GVHD) and a graft versus tumor (GVT) effect;11 no clinical antitumor effect was reported. In 100 randomized hormone refractory patients receiving IL-2 and epirubicin or epirubicin alone encouraging results were obtained in favor of the IL-2-epirubicin arm. However, no data on survival were reported, rendering the findings inconclusive.12 In a phase I trial conducted in 15 patients, including 12 with HER2 ++ (seven patients) or HER2 +++ (five patients) metastatic breast cancer, IL-12 did not enhance clinical efficacy of trastuzumab.13

2. Single (α, β, γ) or Combined IFNs (α and γ) Administration
In three studies IFNα, IFNβ, or IFNγ were given alone or combined.14–16 In one of the studies,15 IFNα and IFNγ given locally, alone, or combined were effective with 53% CR in the treatment of cutaneous recurrences associated with a well-documented enhancement of intralesional cell-mediated immunological response. In the two remaining studies, immunological effects were not reported and clinical outcome compared with conventional treatment was not significantly ameliorated.

3. IFNs and Hormone Therapy With or Without Retinyl Palmitate
A few pilot and nonrandomized phase II studies have been conducted with IFNs given in an outpatient setting with tamoxifen or medroxyprogesterone acetate (MAP).17–26 IFNs were responsible for relatively limited side effects and in most studies17–24 immunological effects were not reported. The overall response rate (from 13 to 59%) was not much different from that seen in estrogen-resistant and estrogen-sensitive metastatic patients, respectively, treated with hormone therapy only. The median OS reported in only three studies ranged from 32 to 38 months.20,24,25 The median OS of 32 and 38 months found in two20,24 of these three studies was compatible27,28 with the studied population, including patients with locally advanced disease without distant metastases. The median OS of 36 months in the other trial25 was similar to that expected in a similar population of patients with distant metastases.29–31 A relatively prolonged survival was reported in only one study and in the subgroup of 11 patients on maintenance therapy with IFNβ, retinyl palmitate, and tamoxifen following complete remission due to chemotherapy.20 In fact, in these 11 patients the median OS reached a relatively long duration of 66 months.20 Finally, in one study,32,33 metastatic patients with responsive or stable disease during antiestrogen first-line salvage therapy were recruited for immunotherapy with IL-2, IFNβ, and melatonin and subjected to prolonged follow-up. True 5–10 year OS rates from first-line antiestrogen treatment and from diagnosis of distant metastases were 65 or 73% and 22 or 20%, respectively, i.e., about three times more than in historical controls and other comparable series treated with antiestrogens only.32,33

B. Monoclonal Antibodies Against Growth Factor Receptor (Trastuzumab) or Growth Factor (Bevacizumab)
Recently, a humanized monoclonal antibody, trastuzumab, directed against the extracellular domain of the 185 KDa HER2 receptor was constructed for therapy. HER2/neu activity was
evaluated either by measurement of gene amplification or overexpression of its product, the 185 KDa glycoprotein. The ErbB2 overall rate of positivity in 22,616 cases examined was 21.4%. ErbB2 positivity is inversely related to ERs and PRs and positively related to a high proliferation rate as well as with a shorter DFS and OS. Women with metastatic breast cancer and HER2 overexpression (+3 by IHC) and/or gene amplification (by FISH) tumors are candidates for trastuzumab. In these patients, trastuzumab as a single agent first-line therapy achieved response rates of 20–25% and among those previously treated with chemotherapy, response rates of 10–15% occurred. Retrospective analyses of these trials showed higher response rates (31% as first line and 18% as second and third lines) among those tumors having more pronounced overexpression of HER2 (+3 by immunohistochemistry). In metastatic disease, compared with chemotherapy alone, chemotherapy plus trastuzumab showed improved clinical outcomes, including response rate, time to disease progression, duration of response, and OS (median survival 25.1 versus 20.3 months, \( p = 0.046 \)). Angiogenesis inhibitors, which specifically inhibit new blood vessel growth, represent a relatively promising new type of cancer treatment. Breast tumor growth and metastasis are both hormone sensitive and angiogenesis dependent; VEGF is the most potent regulator of angiogenesis in human carcinogenesis. Bevacizumab is a recombinant humanized monoclonal antibody against the VEGF-A ligand, with a long half-life that permits intravenous administration once every 2 to 3 weeks. In one of two large phase III studies, for patients with refractory metastatic breast cancer, bevacizumab doubled the response rate of capecitabine (Xeloda®, Roche, Welwyn Garden City, UK) given alone, but it did not affect survival. In the other study, the combination of bevacizumab and paclitaxel showed a significant increase in overall response rate and progression-free survival compared with the single agent paclitaxel, but again OS was not statistically significant. These positive, although limited, results suggest that in advanced cancer patients, it is possible to obtain a clinical advantage by intervening with the immune system. As any manipulation of the immune system involves immunosurveillance against cancer, we took the opportunity to review old and new data on cancer immunosurveillance, giving particular consideration to the role of cytokines and growth factors.

3. IMMUNOSURVEILLANCE THEORY

A. Historical Background

The theory of immunological surveillance against cancer suggests that a mutant cell potentially developing an overt tumor has at least one abnormal antigen, which induces a clone of immunologically competent cells such that thymus-derived (T) lymphocytes can destroy newly appearing tumor cells \textit{in situ}. Burnet also predicted a higher incidence of malignant disease in conditions associated with depression of the immune system. Tumor antigens were also the backbone of the theory from diagnostic and immunotherapeutic perspectives. Various types of tumor antigens were defined: tumor-specific antigens (TSA), tumor-associated antigens (TAA), tumor-associated transplantation antigens (TATA), and antigens of virus-induced tumors (oncodnaviruses and oncornaviruses). Research on tumor antigens and the discussion about their implications for immunotherapy continues today. A systematic survey of the humoral and cellular immune responses of patients to their own tumors was initiated in the 1970s by an approach termed autologous typing. The characterization of the molecular targets recognized by autologous typing was made possible using different techniques and a milestone was the molecular characterization of the first human melanoma antigen recognized by T cells. Subsequently, many shared
self-differentiation and unique human antigens have been discovered. They can be divided into four classes: differentiation antigens (e.g., melanocyte differentiation antigens, Melan-A/Mart-1), mutational antigens (e.g., abnormal forms of p53), overexpressed/amplified antigens (e.g., HER2/neu), viral antigens (e.g., EBV and HPV), and cancer-testis (CT) antigens. CT antigens, due to their unique characteristics, are of particular interest. In fact, in adult normal tissues their expression is limited to germ cells in the testis, whereas in cancer a variable proportion of a wide range of different tumor types expresses CT antigens. Recently, seven gene pairs as putative cancer biomarkers overexpressed by malignant lesions independent of tissue origin and with high predictive power (87%) in segregating malignant from benign lesions have been identified. They were found to be associated with aggressiveness, uncontrolled proliferation, and metastatic potential. From the beginning to the middle of 20th century, many in vitro and in vivo experiments were conducted; these suggested an immunological enhancement of tumor growth by an inappropriate type of immune response. An autoimmune origin of cancer by an extension of a physiological growth-facilitating mechanism of the immune system has also been postulated. However, the theory of immunosurveillance as stated by Burnet still persisted prominently until the mid-1970s, when it became clear that it was only partially confirmed by clinical and experimental studies. In particular, in spite of the predicted increased frequency of polyclonal tumors, patients rendered immunodeficient by immunosuppressant drugs after kidney transplantation had an increased cancer incidence of almost exclusively lymphoid neoplasms. Furthermore, in several diseases (leprosy, sarcoidosis) with pronounced immunosuppression, there was no increased tumor evidence and immunosuppressed mice did not develop spontaneous tumors. These results were better explained by the work on the cellular origin of tumors, which undoubtedly pointed to the monoclonal origin of most of them. Finally, it was clarified that athymic nude mice did not form more chemically induced tumors compared with their wild-type counterparts, nor did they show a shortened tumor latency period after carcinogen injection. This led to the abandonment of the original immunosurveillance theory.

B. Renaissance of the Immunosurveillance Theory: The Role of Cytokines

Between 1994 and 1998 relevant studies renewed the interest in this theory. Endogenously produced interferon-gamma (IFNγ) was shown to protect the host against the growth of transplanted tumors and the formation of primary chemically induced and spontaneous tumors. In addition, it was observed that mice lacking perforin (perforin −/−) were more prone to MCA-induced tumor formation compared with their wild-type counterparts. Perforin is a component of the cytolytic granules of cytotoxic T cells and NK cells, which are important in mediating lymphocyte-dependent killing of many different target cells including tumor cells. These observations showed that components of the immune system were involved in controlling primary tumor development. However, the fundamental suggestion that a cancer immunosurveillance process depends on both IFNγ and lymphocytes again resulted from the use of gene-targeted mice. Mice deficient in either recombination activating gene 1 (RAG-1) or RAG-2 fail to rearrange lymphocyte antigen receptors and thus completely lack natural killer T (NKT), T and B cells. Specifically, RAG-2−/− mice developed significantly more MCA-induced sarcomas and spontaneous histologically different type cancers than wild-type mice. RkSk mice, lacking both RAG-2 and the signal transducer and activator of transcription 1 (Stat 1) that is important in mediating the IFNγ receptor signal, additionally developed spontaneous breast tumors, which were very rarely observed in wild-type or RAG-2−/− mice and very late in Stat 1−/− mice. This demonstrated that there

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was not a complete overlap between RAG-2 and Stat 1. Similar findings were made in carcinogenesis experiments with mice that lacked perforin, IFNγ, or both. Further studies have shown that genetic, immunochemical, or functional ablations of NKT, γδ T cells, NK cells, αβ T cells, IFNγ, or interleukin-12 (IL-12) lead to increased susceptibility of the host to tumors. Data have also been collected showing that like IFNγ, endogenously produced IFN αβ is required for the prevention of the growth of primary carcinogen-induced and transplantable tumors. In the late 1960s, it was shown that mice transplanted with syngeneic tumors survived significantly longer when treated with crude preparations of type I IFNs. However, only recently studies of endogenously produced type I IFNs assessed its involvement in the cancer elimination phase. Gresser and colleagues were the first to show that neutralizing polyclonal goat antisera specific for mouse type I IFNs increased the growth and metastasis in mice of several different tumor cells xenografts. More recently it has been shown that mice that had been treated with blocking monoclonal antibody specific for the type I IFN receptor failed to reject a panel of highly immunogenic, MCA-induced sarcomas that were rejected by wild-type mice treated with a control monoclonal antibody. This result shows that type I IFNs have an important role in the elimination of immunogenic syngeneic tumors and that they participate in naturally occurring protective immune response to primary tumors. In particular, IFNAR1-deficient 129/Sv mice challenged with MCA were significantly more susceptible to tumor formation and formed tumors with faster kinetics than their wild-type counterparts. Moreover, it has been found that host hematopoietic cells are critically targeted during the IFNαβ development of protective antitumor responses provided that immunosurveillance does exist in mice, the question was whether it also occurs in humans. Long-term observations of many transplant patients who were immunosuppressed and of individuals with primary immunodeficiencies showed a significantly higher relative risk for cancer development, although some tumors were of viral origin (non-Hodgkin’s lymphoma, Kaposis’s sarcoma, and carcinomas of the genitourinary and anogenital regions). These malignancies are now occurring with increasing frequency in AIDS patients. With spontaneous tumors, clearly a confounding factor in immunodeficient humans was the shortened life spans and other intercurrent medical problems of these patients. Nevertheless, greater relative risk ratios were observed for a broad subset of tumors with no apparent viral etiology as malignant melanomas and non-Kaposi’s sarcomas. A highly standardized prevalence of lung, colon, bladder, kidney, ureter, and endocrine tumors as well as malignant melanomas were reported in the assessment of 5,692 renal transplant patients in Nordic countries as compared with the general population. In addition, a correlation between the presence of lymphocytes in the tumor tissue, in particular CD8+ T cells, and increased patient survival has been clearly shown.

1. Innate and Adaptive Immunities

Based on the previously summarized studies in mice and observations in humans, a new theory termed “cancer immunoediting” has been proposed. While cancer immunosurveillance was thought to be a host-protective function carried out by the adaptive immune system, only at the earliest stages of cellular transformation, the new theory recognized that both the innate and adaptive immune compartments participate in the process and not only protect the host but also sculpt, or edit, the immunogenicity of tumors that may eventually form. Innate immune cells are represented by natural killer (NK) cells, NKT cells, dendritic cells (DCs), macrophages, neutrophils, basophils, eosinophils, mast cells and the more recently defined human intraepithelial lymphocytes (IELs), and γδ T cells. NKT cells are a T lymphocyte sub-lineage that also express markers for the NK population. Contrary to T lymphocytes, NKT cells recognize glycol–lipid antigens presented by the class-I-like antigen presenting molecule CD1d via their invariant TCR-α chain rearrangement.
NKT cells can up- or down-regulate immune responses by promoting either the secretion of the Th1 and Th2-like cytokines or by recruiting other suppressive cell populations. Macrophages and mast cells serve as sentinel cells that are prestationed in tissue and continuously monitor their microenvironment. IELs are T cell receptor $\alpha\beta^+$ CD8$^+$ T cells that likely act as a first-line innate host defence against dysplastic or malignant epithelial cells between which they are located. DCs take up foreign antigens and migrate to lymphoid organs where they present their antigens to adaptive immune cells. They are, therefore, key players in the interface between innate and adaptive immunity. Human $\gamma\delta$ T cells are a subset of lymphocytes carrying the $\gamma\delta$ TCR, i.e., T cell antigen receptors (TCRs) are composed of V (variable)$\gamma$ and V$\delta$ chains and $\gamma\delta$ T lymphocytes first identified in 1986, appear early in the thymus and only represent 1–5% of the thymocytes and lymphocytes present in secondary organs. Unlike $\alpha\beta$, the main histocompatibility system in these cells is not involved in antigen recognition and they have unique selectivity for small nonpeptide antigens of mostly microbial origin. Therefore, these activated human $\gamma\delta$ T cells have antigen presentation features similar in potency and efficacy to those seen in DCs and, as DCs, seem to be a bridge between innate and adaptive immunity. These lymphocytes have been ascribed an antitumor activity that has been demonstrated in vitro in Daudi lymphoma cell lines and subsequently confirmed in numerous other tumor cell lines. It is noteworthy that tumor cells differentially express MHC-class-I-chain-related proteins A and B (MICA/B) that function as ligands for two receptors, NKG2D and $\gamma\delta$ TCR. In normal tissues, MICA expression was found only in the gastrointestinal epithelium of the stomach and large and small intestine. In contrast, constitutive MICA/B expression has been documented in many different primary carcinomas. The NKG2D receptor is expressed on IELs, $\gamma\delta$ T cells, and NK cells. Thus, $\gamma\delta$ T cells have two mechanisms for recognizing the MIC markers on tumors: one involves a direct interaction with the $\gamma\delta$ TCR and the other is mediated by a more globally expressed NKG2D activating receptor. Two data sets mainly link MICA/B recognition to immunosurveillance. First, it has been demonstrated that MIC-expressing cells are recognized and killed by the V$\delta$1 $\gamma\delta$ T cell subset and a strong in vivo correlation ($p < 0.0001$) between MICA/B expression on tumors and tumor infiltration by V$\delta$1 $\gamma\delta$ T cells has been observed. Second, recent data have demonstrated a correlation between downregulation of NKG2D on tumor infiltrating lymphocytes (TILs) and the expression of MICA/B in the tumor. Adaptive immune cells include B lymphocytes, CD4+ helper T lymphocytes, and CD8+ cytotoxic T lymphocytes (CTLs). They are distinguished from innate leukocytes by the expression of somatically generated diverse antigen-specific receptors, which are formed as a consequence of random gene rearrangements and allow a flexible and broader repertoire of response than innate immune cells that express germ-line-encoded receptors.

2. Immunoediting
Cancer immunoediting should be composed of three processes, termed the “three Es of cancer immunoediting”: elimination, equilibrium, and escape. Elimination corresponds to immunosurveillance and when it is successful in deleting the developing tumor, it represents the complete editing process. During elimination once solid tumors reach a certain size they require an enhanced blood supply that arises due to the production of stromagenic and angiogenic proteins. Invasive growth causes minor disruption within the surrounding tissue with inflammatory signals and recruitment of cells of the innate immune system (NKT, NK, $\gamma\delta$T cells, macrophages, and DCs) into the site. Structures on the transformed cells are recognized by infiltrating lymphocytes, such as NKT, NK, or $\gamma\delta$T cells, which are then stimulated to produce IFN-γ that may induce death of some tumoral cells by antiproliferative and apoptotic mechanisms. IFN-γ also induces the production of...
chemokines CXCL10 (interferon-inducible protein-10, IP-10), CXCL9 (monokine-induced by IFNγ, MIG), and CXCL11 (interferon-inducible T cell chemoattractant, I-TAC) from the tumor cells themselves and from surrounding normal host tissues. Some chemokines have potent angiostatic capacities and recruit more NK cells and macrophages to the site. The tumor infiltrating NK cells and macrophages transactivate one another by reciprocal production of IFNγ and IL-12 and kill more of the tumor by mechanisms involving TNF-related apoptosis inducing ligand, perforin, and reactive oxygen and nitrogen intermediates. Events of innate immunity charge the adaptive response: tumor cells killed due to the increased cytocidal activities of NK cells and activated macrophages are ingested by DCs that migrate to the draining lymph node and present antigen to naïve CD4+ and CD8+ T cells. The induced tumor-specific CD4+ T helper cells expressing IFNγ (TH1 cells) in turn make the development of tumor-specific CD8+ T cells easier. Tumor-specific CD4+ and CD8+ T cells home to the tumor site where cytolytic T lymphocytes destroy the remaining antigen-bearing tumor cells, whose immunogenicities have been enhanced by exposure to locally produced IFNγ. Equilibrium represents the process by which the immune system iteratively selects and/or promotes the generation of tumor cell variants with increasing capacities to survive immune attack. Escape is the process wherein the immunologically sculpted tumor expands in an uncontrolled manner in the immunocompetent host. Recently, a paradoxical dual role of the cellular immune system and some constituents or mediators, including macrophages and IL-2 with induced tolerance to the tumor and suppression of the antitumor immune response during cancer development has been hypothesized. Moreover, different subpopulations of regulatory T cells (Treg), which include CD4+/CD25+/Foxp3+ T lymphocytes of thymic origin, IL-10-releasing Tr1 lymphocytes, and TGF-β-producing Th3 lymphocytes, and their suppressor activity, have been better defined. Tregs accumulate in the body during tumor growth, taking the part of TILs and inhibit the proliferation and activity of CD4+ and CD8+ effector T lymphocytes. Thus, the data collected in recent decades from both animal and human studies again have provided support, although with new insights and extension, to the immune surveillance theory originally conceived by Burnet.

4. MECHANISMS OF IMPAIRED IMMUNE SURVEILLANCE: RECENT ADVANCES

Progress in the past decade in molecular biology and genetics has permitted deeper investigation of the possible mechanisms of the immune escape. A simple way to describe studies and hypotheses on immune evasion is to group them according to whether the prevailing mechanism depends on the tumor, the host, or both. Table I lists these mechanisms and indicates the involved cytokines and growth factors.

A. Tumor-Dependent Mechanisms

Tumor evasion from host immune attack are induced by genomic instability, HLA class I antigen abnormalities, upregulation of the fetal type nonclassical HLA class I molecules, or epitope immunodominance.

Slightly different explanations have been formulated to express the main concept that cancer cells can avoid immune recognition. One hypothesis is that cell tumor variants not recognized by the host cellular immune system derive from a natural selection based upon genomic instability. This passively develops in the tumor environment. Another hypothesis is that the tumor is actively imprinted by the immunologic environment in which it forms. This “cancer immunoediting” hypothesis supposes that this imprinting process eliminates high immunogenicity tumor cells and leaves behind tumor variance of reduced
immunogenicity. This process is thought to occur continuously, although more probably in early stages, and that it targets some genes encoding the major histocompatibility complex (MHC) antigens or components of the IFNγ receptor signaling pathway. In particular, HLA class I antigen abnormalities in malignant cells have certainly been well documented. These antigens may be lost or down-regulated by different mechanisms: (a) structural defects in a β2microglobulin (β2m) gene associated with the loss of one copy of the β2m gene; (b) structural defects in the transporter associated with antigen processing (TAP); (c) defects in components of the antigen processing machinery; (d) loss of one copy of chromosome 6 or of a DNA fragment containing HLA-A, -B, and -C genes; (e) defects in the regulatory mechanisms controlling HLA class I antigen expression; and (f) mutations in HLA class I heavy chains. The fetal-type nonclassical HLA class I molecules (HLA-G, HLA-E, and HLA-I) differ from the classical HLA class molecules due to their restricted distribution to immune privileged sites and may prevent tumor immune rejection by inhibiting the activity of tumor-infiltrating NK, CTLs, and antigen presenting cells (APC) through binding with inhibitory receptors present on immune cells. The upregulation of these molecules on the tumor cell surface and the presence of a subpopulation of stem cells within the tumor cells are two other possible reasons for tumor escape. Different studies reported that antigen levels presented to the immune system by an edited tumor remain above the threshold required for recognition and suggest that cancer progression follows tumor-cell-mediated immunity interactions that reduce both the antigenic profile of the

<table>
<thead>
<tr>
<th>Prevailing mechanism</th>
<th>Cytokine and/or growth factor</th>
<th>Reference no.</th>
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<tbody>
<tr>
<td><strong>Tumor dependent</strong></td>
<td></td>
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<tr>
<td>Genomic instability, HLA class I antigen abnormalities, fetal type nonclassical HLA class I molecules upregulation, epitope immunodominance</td>
<td>–</td>
<td>111–121</td>
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<tr>
<td>Tumor apoptosis inhibition by defective death receptor signaling</td>
<td>–</td>
<td>122–126</td>
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<td>Apoptosis of activated T cells induced by tumor cells (a) or by microvesicles release (b) and tumor cannibalism (c)</td>
<td>(a) IL-2 α(s)</td>
<td>127–140</td>
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<tr>
<td>Tumor constitutive activation of Stat 3 and NF-κB signaling</td>
<td>IFNβ-RANTES-IL-6-IL-12-TNFα (the “danger signal”) decreased α(s); IL-10-VEGF-others increased α(ss); EGF unchanged α(ss)</td>
<td>141–157,161–166</td>
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<tr>
<td><strong>Host dependent</strong></td>
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<td></td>
</tr>
<tr>
<td>CD4+CD25+ regulatory T cells (Treg)</td>
<td>IL-2 decreased α(d), IL-10 increased α(ss)</td>
<td>120,167–182</td>
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<tr>
<td>CD4+ T cells anergy (a) and Th2 antitumor immunity diversion (b)</td>
<td>(b) IL-4-IL-5 increased α(ss)</td>
<td>155,182–194</td>
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<tr>
<td>Myeloid suppressor cells</td>
<td>IL-10-VEGF-GM-CSF-IL-6-TGFβ increased α(ss)</td>
<td>195–199,201–214</td>
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<tr>
<td><strong>Tumor and host dependent</strong></td>
<td>IL-2 decreased α(s)</td>
<td>215–221</td>
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<tr>
<td>Lack of co-stimulation molecules</td>
<td>IL-10-TGFβ-VEGF-PGE2 increased α(ss)</td>
<td>155,198,223–228,234–249</td>
</tr>
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Also see text.

αImmunological action: s, stimulatory; ss, suppressive; d, dual.
evolving tumor and the functional capacity of the tumor antigen-specific T cells. These changes resulted in a homeostasis in which a less immunogenic tumor emerges in the face of an immune system less capable of responding to it.\textsuperscript{120} A different hypothesis suggested that some immunodominant tumoral epitopes should be preferentially immunodetected. Hence, the parental tumor cells carrying the immunodominant epitope serve as a red flag for immune attack thereby diverting attention from the tumor variant. Once the parental cell is eliminated, a new hierarchy is established among the variant subpopulations and formerly immunorecessive epitopes become dominant.\textsuperscript{111,121}

1. Tumor Apoptosis Inhibition by Defective Death Receptor Signaling

In tumor cells, there may be defects at multiple sites in the death receptor pathways. Down regulation or loss of Fas expression through missense mutations and loss of the gene encoding Fas have been identified in different cancer types.\textsuperscript{122–124} Different mutations of the genes in the proximal pathways downstream of Fas signaling including inactivating mutations of Fas-associated death domain (FADD) and caspase 10 have been found.\textsuperscript{111} Defective death receptor signaling by overexpressing PI-9 (also known as SPI-G) or serine protease inhibitor\textsuperscript{125} and low expression of death receptors with tumor resistance to TRAIL-mediated apoptosis and other reasons\textsuperscript{111,126} have also been reported.

2. Apoptosis of Activated T Cells Induced by Tumor Cells Themselves or by Microvesicles Release and Tumor Cannibalism

A possible role of Fas–FasL interactions in the tumor setting may be the induction of activation-induced cell death (AICD) of antitumor T cells. In fact, a variety of cancer cells express functional FasL that induces apoptosis of Fas$^+$ susceptible target cells. These include lung, melanoma, colon, and hepatocellular carcinomas.\textsuperscript{127–130} Other data suggest that FasL is expressed by CD8$^+$ T cells displaying MHC-class-I-restricted cytotoxicity.\textsuperscript{131} It is well known that upon repeated stimulation with Ag (particularly in the presence of IL-2), T cells become susceptible to the induction of apoptotic cell death mediated by FasL binding to the Fas receptor. Significant levels of AICD have been found in tumor reactive T cell lines when they were activated by either cognate tumors or zβ T cell receptor (TCR) ligation with anti-CD3 mAb. As T cell lysis was inhibited by blocking the Fas pathway with anti-Fas mAbs or peptide inhibitors of caspase proteases, it was suggested that T cells themselves expressing FasL undergo suicidal or fratricidal apoptosis.\textsuperscript{131} Hence, this form of AICD has also been dubbed “propriocidal regulation” and is postulated to be one of the control mechanisms by which T cell responses are turned off during and after antigenic stimulation.\textsuperscript{132} Recently, it has been reported that human tumors constitutively release through an exocytosis pathway endosome-derived organelles of 50–100 nm size, named microvesicles, or exosomes transporting a broad array of biologically active molecules from their originating cell with detrimental effects on different immune cells. These effects range from induction of fas-mediated apoptosis in activated antitumor T cells\textsuperscript{133,134} to impairment of monocyte differentiation into DCs and induction of myeloid suppressive cells.\textsuperscript{135} Immunosuppressive exosomes of tumor origin have been found in neoplastic lesions and sera from cancer patients suggesting their potential role in \textit{in vivo} tumor progression.\textsuperscript{136} Cell cannibalism that is the ability of a cell to phagocytize another cell in humans has been recognized as an exclusive property of malignant tumor cells. Tumor cannibalism has been clearly detected \textit{in vivo} in metastatic lesions of melanoma and in other tumors\textsuperscript{137–139} and often involved T cells that were found in a degraded state within tumor cells. This cannibalistic activity increases tumor cell survival and shows that the tumor cell may use the consumption of live lymphocytes as a way to “feed” in condition of low nutrient supply. As the ingested live T cells are instead programmed to kill tumor cells, this suggests a novel mechanism of tumor immune escape aimed at sustaining survival and progression of malignant tumor cells in an unfavorable microenvironment.\textsuperscript{140}
3. Tumor Constitutive Activation of Stat 3 and NF-κB Signaling is Responsible for Host Immunosuppression

One of the most recently recognized oncogenic signaling pathways involves the Stat proteins. This family of proteins comprises seven members, Stat 1 to Stat 4, Stat 5a and Stat 5b, and Stat 6. They act as cytoplasmic signaling proteins and as nuclear transcription factors that activate a diverse set of genes including some that are implicated in malignant progression. In normal cells, Stat proteins transmit cytoplasmic signals to the nucleus from growth factors that have intrinsic tyrosine kinase activity or from polypeptide cytokines that upon ligand engagement activate receptor-associated tyrosine kinases, which are often members of the Janus kinase (Jak) family and in some cases are src family kinases. In tumor cells the tyrosine kinase activity of growth factor receptors can become constitutively activated as a result of mutations that affect their structure, simple overexpression, or continuous engagement of ligands that come from autocrine or paracrine sources. Nonreceptor cytoplasmic tyrosine kinases that signal through Stats include ABL (Abelson leukaemia protein) and src-related kinases. These may become constitutively activated by direct genetic alteration as frequently occurs in the case of the BCR–ABL (a fusion of the breakpoint cluster region and ABL proteins) or by association with overactive receptor kinases as in the case of src and EGFR. Recent studies have indicated that constitutive Stat 3 signaling inhibits the production of proinflammatory mediators as interferon β (IFNβ), tumor necrosis factor α (TNFα), IL-6, RANTES, and IP-10, which usually activate innate immune cells to produce many immunological danger signals. These danger signals include nitric oxide and are part of a cascade of inflammatory events that stimulate immune responses against tumor cells. The inhibited inflammatory mediators cannot also activate DCs, which usually activate antigen-specific T cells. Constitutive Stat 3 activity in tumor cells induces production of factors, namely VEGF and IL-10, which through Stat 3 activation in progenitor cells inhibit differentiation and maturation of DCs resulting in T cell tolerance. Consistently, blocking Stat 3 signaling in macrophages induces the expression of the cytokines IL-12 and RANTES, leading to loss of immune tolerance and restoration of T cell responsiveness. In the danger model of immunity, appropriate danger signals at the tumor site are required to activate the efferent arm of the immune system to undertake its functions. However, most cancers do not have the requisite danger signals and antigen exposure in the absence of co-stimulatory molecules is not enough to activate and sustain effective immune responses. Both Stat 3 and Stat 5 have also been shown to directly or indirectly up-regulate the expression of genes including c-myc, cyclin D-1, and cyclin D-2, which are required for uncontrolled proliferation or as BCL-XL, MCL-1, and survivin, which are required for uncontrolled survival. In addition, Stat 3 negatively regulates the expression of p 53, as a key regulator of VEGF expression, also stimulates tumor angiogenesis. NF-κB is a transcription factor that has been found constitutively activated in most types of cancer by activated receptors of the toll-like receptor (TLR) family and inflammatory cytokines. Most of these genes are antiapoptotic genes that contribute to tumor promotion and cell cycle progression, such as cyclin D-1 or angiogenesis promoting genes as IL-8 and COX-2.

B. Host Dependent

1. CD4+CD25+ Regulatory T cells (Treg) Responsible for Immune Tolerance

Recently, suppressor T cells have been phenotypically identified in naïve mice as a small subset of CD4+ T cells with high levels of CD25. Due to their established regulatory nature, now CD4+CD25+ are defined as CD4+CD25+ T regulatory cells. It has been
demonstrated that the equivalent of CD4+CD25+ T reg identified in mice also exists in the blood of healthy human adults.167 These CD4+CD25+ T cells inhibit antitumor immunity in mice and humans. The prevalence of Treg has been found to be increased in peripheral blood and the tumor microenvironment of patients with pancreas and breast carcinomas.168 CD4+CD25+ T cells possessing regulatory properties (also known as Th3 and Tr1 cells) have been reported to be among the TILs in different types of human cancers.169,170 It is possible that the tumor microenvironment preferentially recruits naturally occurring Treg cells or converts CD4+ T cells to CD4+CD25+ Treg or expands naturally occurring CD4+CD25+ T cells. T reg cells seem to have a key role in the maintenance of immune tolerance to both self and foreign antigens. It has been shown that upon antigen stimulation this CD4+CD25+ cell population potently suppresses the activation/proliferation of other CD4+ or CD8+ cells in vitro.171,172 Moreover, repetitive stimulation of naïve CD4+ T reg with immature DCs resulted in differentiation into CD4+CD25+ T cells that produced high levels of IL-10. These IL-10-producing Treg cells could act directly on activated Th1 cells and inhibited their antigen-specific proliferation and cytokine production in a cell contact-dependent manner.173 The inhibition of IL-2 transcription in the effector population has been hypothesized to be the mechanism of suppression. In fact, suppression can be abrogated by the addition of large amounts of exogenous IL-2174 or by enhancing endogenous IL-2 production by means of anti-CD28 Ab.175 However, recent studies have also suggested that Treg cells suppress IL-2 mRNA induction in responder cells even in the presence of large amounts of IL-2176 and that T cell suppressive activity in vitro is dependent on IL-2 production by nonregulatory T cells, as it is abrogated in the presence of IL-2 neutralizing antibodies.176,177 Taken together, the available data show that the maintenance of CD25 expression on Treg cells depends on IL-2.178 Furthermore, they suggest that increased amounts of IL-2 resulting from immune activation may stimulate the expansion of the Treg population.109,179–181 There is evidence that tumor-specific CD4+ T cells change their phenotype from effectors to suppressors during cancer progression.120 Cancer development is generally associated with an inflammatory response. It has been proposed that CD4+ cells predominantly play an enhancing immune helper role during the initial stages of tumor progression, but once tumors become chronically persistent, an increased accumulation of CD4+ T reg occurs to down-regulate anti-inflammatory mechanisms together with cytokines like TGFβ and IL-10. This anti-inflammatory reaction may inhibit antitumor immunity. Thus, although elevated levels of TILs have been associated with a better prognosis, recently it has been suggested that the proportion of infiltrating Treg type cells may be a more critical determinant for prognosis.182

2. CD4+ T Cells Anergy and Th2 Antitumor Immunity Diversion
CD4+ T cells are a component of the antitumor immune response and are equally critical as CD8+ CTLs. APCs, mainly DCs, macrophages, B lymphocytes, and also γδ T lymphocytes that serve as APCs in peripheral tissue and act as potential antitumor elements,183,184 play a key role with regard to the effector mechanisms of the immune system. APCs process protein antigens and express co-stimulatory molecules necessary for a complete response by T lymphocytes.185 It is well documented that IL-12 and also IL-4 are produced by APCs and acting via Stat 4 and Stat 6, respectively, are major determinants of the TH commitment process.186,187 Particularly, IL-12 and IL-4 induce the stimulation of Th1 and Th2 subsets by naïve CD4+ T cells, which are defined both by their functions and cytokine profiles.188,189 Therefore, APCs are commonly considered the driving force of cancer-related immunity. The Th1 response is characterized by the production of interferon γ (IFNγ) and preferential induction of cellular immunity, whereas the Th2 response occurs by the production of IL-4, IL-5, and other cytokines and stimulation of humoral immunity.182 It is likely that tumor antigens are ingested, processed, and presented to CD4+ T cells by macrophages. Primed

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tumor-specific CD4+ T cell secrete lymphokines in response to antigen presentation by macrophages. Successively, both Th1 and Th2 effector pathways, respectively, activate either macrophages to produce reactive oxygen intermediates or eosinophils to release their granule contents. Recently, it has been shown that CD4+ T cells specific for tumor antigen can become actively tolerized during progression of tumors. It has been hypothesized that cross presentation by tolerizing bone-marrow-derived APCs occurs. VEGF and other tumor-secreted cytokines or substances can suppress DCs in the tumor microenvironment and induce tolerance. Particularly, IL-6 skews monocyte differentiation into tumor-associated macrophages at the expense of DCs. Mucin-1 glycoproteins are endocytosed by DCs and retained in endosomes leading to inefficient processing and presentation to T cells. Another proposed mechanism of CD4+ T cells unresponsiveness to tumors is that tumor antigens, presented to CD4+ T cells by B cells, divert antitumor immunity toward a “nonproductive” Th2 type humoral immune response and away from a “productive” Th1 type cellular response.

3. Myeloid Suppressor Cells (MSCs)
MSCs represent a heterogeneous population that can be identified in mice by expression of CD11b and Gr-1. CD11b+/Gr-1+ cells comprise myeloid precursors that can generate mature granulocytes, macrophages, and DCs when cultured in vitro with cytokines or followings in vivo adoptive transfer. More recently, human MSCs were characterized in the peripheral blood of patients with different cancer histotypes including breast cancer. The MSCs showed a prevalent immature phenotype CD34+, CD33+, CD15−, and CD13+. Within this population, two subsets were defined according to the expression of HLA-DR and CD11c molecules: (1) immature monocyte/DCs and (2) earlier myeloid differentiation stages. The immune system has evolved feedback mechanisms to prevent damage caused by excessive or prolonged inflammation in the host during anaphylactic shock, autoimmune diseases, or other immuno-pathologic situations. These mechanisms encompass the generation and/or the expansion of cell populations that negatively regulate T-cell functions. MSC is a potent inactivator of both CD4+ and CD8+ T cells and is responsible for significant immune dysfunction seen in tumor-related and unrelated settings. MSCs can be detected in the blood, bone marrow, spleen, and lymph nodes as well as in both the tumor microenvironment and immune-activated environment where they suppress immunity through complex molecular pathways. MSC accumulation in secondary lymphoid organs has long been known to correlate with immune suppression. However, only recently MSC biology, functional properties, and cellular interactions have begun to be understood. Numerous findings indicate that tumor-derived factors (TDFs) promote not only MSC recruitment, but also their maturation toward an immunosuppressive phenotype. For more then 10 years, efforts have been made to identify these TDFs. Tumor secrete a large panel of cytokines, chemokines, or other diffusible molecules that, alone or in combination, can induce MSC recruitment and increase their maturation into fully suppressive cells. To date, a number of candidate proteins have been identified that include colony stimulating factor 1 (CSF-1), IL-6, IL-10, VEGF, and GM-CSF. Within the tumor microenvironment, MSCs inhibit tumor-specific T cell function via liver arginase (ARG1) and/or inducible nitric oxide synthase (iNOS) activation. Tumor cell conditioned medium was shown to induce Jak2 and Stat 3 activation in the tumor-associated MSCs. Stat 3 activation via IL-10 up-regulated the α chain of the IL-4 receptor, leading to an increased IL-4-dependent expression of ARG1. Furthermore, IL-10 synergises with LPS in producing iNOS, which can also be induced in myeloid cells by different tumor-secreted factors, such as VEGF, GM-CSF, and IL-4. It is also becoming evident that the different suppressive cell populations do not act alone but instead interconnect to control the immune responses.
responses. The suggestion of a relationship between NKT and MSCs derives from experiments in which NKT were activated in vivo by one of their ligands, the α-galactosylceramide. NKT promptly responded by producing large amounts of GM-CSF that resulted in a rapid MSC mobilization from the bone marrow into the spleen and blood. Furthermore, it was demonstrated that tumor-activated NKT were able to prime MSCs to suppress tumor-specific CTL. Functional studies revealed that the CD8+ T cell suppression was mediated by IL-13 through the IL-4 R α Stat 6 pathway. As CTLs lack IL-13R expression, the authors showed that IL-13 triggered MSC secretion of TGF-β, which directly suppressed CD8+ CTLs.

C. Tumor- and Host-Dependent Mechanisms

1. Lack of Co-Stimulation Molecules

One explanation for the lack of a tumor-destructing immune response is that tumoral cells do not provide adequate “co-stimulatory signals” such as those provided by professional APCs. Many tumors lack expression of accessory or co-stimulatory molecules, which are required to cooperate with antigens to activate T cells. In this case, unresponsiveness of T cells to many tumor antigens should be considered as ignorance rather than tolerance. Optimal T cell activation requires two distinct signals. The first signal is mediated by MHC-restricted, antigen-specific triggering of the T-cell receptor (TCR)-CD3 complex that activates a number of protein tyrosine kinases and thereby controls various downstream signaling pathways, and the second by an antigen-independent mechanism, is termed co-stimulation. The second signal is provided through receptors on the T cell surface, of which the prototype is CD28. CD28 binds B7-1 (CD80) and B7-2 (CD86), which are constitutively or inducibly expressed by professional APCs, activated B cells, and tumoral cells. In the absence of CD28 co-stimulation, T cells enter a state of anergy. The CD28 T-cell co-stimulatory receptor family includes cytotoxic T-lymphocyte antigen 4 (CTLA-4/CD152), inducible co-stimulator (ICOS), OX40 (CD134), CD40 ligand (CD40L/CD154), and programmed death 1 (PD-1). Each member of the CD28 family has distinct functions, depending on the nature of the stimuli and the antigenic history of the T cells on which it is expressed. CD28-mediated co-stimulation in conjunction with TCR engagement enhances the magnitude and duration of T-cell responses, increases the secretion of IL-2, and prevents the induction of anergy. In addition to CD28, several other molecules provide co-stimulatory signals, including CD2, CD5, CD44, and CD9. Antigen-specific tolerance or anergy and suboptimal activation of NK can occur following MHC-class-I-restricted antigen presentation with lack of expression of co-stimulatory ligands. To overcome tolerance and enhance cellular immunity, different strategies have been proposed as the equipment of tumor cells with co-stimulatory molecules such as B7, blockade of inhibitory signals on T cells (e.g., through cytotoxic T-lymphocyte antigen 4) and grafting of T cells with antigen-triggered, recombinant co-stimulatory receptors. Figure 1 shows the main T cell functions and co-stimulation in tumor immunology.

2. Immunosuppressive Cytokines (VEGF, IL-10, PGE2, TGF-β)

T cells, B lymphocytes, macrophages, monocytes, and tumor cells produce a variety of cytokines and chemokines that can negatively affect maturation and function of immune cells. VEGF is one of these. It is currently assumed that all tissues have the potential to produce this growth factor. Its synthesis is stimulated when cells become deficient in oxygen or glucose and during inflammatory reactions. Furthermore, most tumor cells tend to overexpress VEGF constitutively. In vitro studies show that VEGF inhibits DC differentiation and maturation through suppression of the transcription factor NF-κB in hematopoietic stem cells. In patients with lung, head and neck, and breast cancers, increased plasma concentrations of VEGF were associated with decreased function and...
number of mature DCs. In addition to VEGF, increased concentrations of IL-10 are frequently detected in the serum of patients with cancer. IL-10 is produced by Th2 cells, some activated B lymphocytes or macrophages, and by many cancer cells. IL-10 can be secreted at a higher rate by metastatic cancer cells than by lymphocytes. It down-regulates the inflammatory response of cell-mediated immunity. In particular, IL-10 can inhibit DC differentiation from stem cell precursors and compromise maturation and the functional status of DCs. IL-10 also inhibits antigen presentation, IL-12 production, and induction of T helper type 1 responses in vivo. IL-10 also enhances spontaneous DC apoptosis as well as susceptibility to autologous NK cell lysis. IL-10 may protect tumor cells from CTLs by down-regulation of HLA class I and II and ICAM-1 (intercellular adhesion molecule 1). The loss of HLA class I expression may also be due to IL-10-mediated down regulation of TAP1 and TAP2 proteins in tumor cells. The proinflammatory factor prostaglandin E2 (PGE2) is another cytokine expressed by human tumors as a result of enhanced expression of the enzyme cyclooxygenase 2. PGE2 increases the production of IL-10 by lymphocytes and macrophages and inhibits IL-12 production by macrophages. TGF-β is mainly synthesized by T lymphocytes and monocytes and is usually considered a
signal to switch off the immune responses.\textsuperscript{224} High concentrations of TGF-\(\beta\) are also frequently found in cancer patients and are associated with disease progression and poor responses to immunotherapy.\textsuperscript{235,236} In fact, TGF-\(\beta\) signaling in T cells has been shown to stimulate Stat 3 activation in tumor cells \textit{via} IL-6.\textsuperscript{237} Suppression of TGF-dependent IL-6 trans-signaling prevented colon cancer progression \textit{in vivo}.\textsuperscript{237} In addition to potentially being produced by some tumor cell lines, TGF-\(\beta\) may also be released by cells dying apoptotically.\textsuperscript{238} TGF-\(\beta\) inhibits the activation, proliferation, and activity of lymphocytes \textit{in vivo}.\textsuperscript{239} TGF-\(\beta\) inhibits T cell proliferation with cell cycle arrest typically in G1 phase\textsuperscript{240,241} presumably through the induction of cell cycle inhibitors and suppression of growth factors as IL-2.\textsuperscript{242} Some findings indicate that TGF-\(\beta\) is important for the control of T cells in the periphery. TGF-\(\beta\) has also been shown to block differentiation of both CD4+ and CD8+ naive T cells into effector cells as well as Th1 and Th2 differentiation from naive CD4+ T cells.\textsuperscript{243–245} TGF-\(\beta\) prevents the differentiation of Th1 cells and also completely abrogates Th2 differentiation\textsuperscript{244,245} through suppression of the expression of both the transcription factor T-bet, the master regulator of Th1 commitment\textsuperscript{246} and of the transcription factor GATA-3, and the master regulator of Th2 cell commitment.\textsuperscript{247,248} Furthermore, recent data suggest that TGF-\(\beta\) suppresses immunity \textit{via} regulatory T cells (Treg).\textsuperscript{249}

All of these mechanisms (tumor dependent, host dependent, tumor and host dependent) can be grouped according to whether the final immune evasion is due to defective immune recognition or to immune suppression as summarized in Figure 2.

5. RELATIONSHIP AMONG TUMOR, STROMA AND CELLULAR MEDIATED IMMUNITY: THE CENTRAL ROLE OF CYTOKINES AND GROWTH FACTORS

The experimental studies detailed above clearly show that tumor cells, stromal cells, and T lymphocytes interact with each other in determining tumor progression. In the displayed illustration, all previous data on tumor growth are integrated with the concomitant reported mechanisms (Table I) of impaired immune surveillance. Figure 2 depicts these interactions. For a simplified view, three principal effects should be considered: tumor growth, defective immune recognition, and immune suppression. These three effects concur in determining immune evasion. In particular, tumor cell survival and proliferation are likely to be the result of growth factors and cytokine production mostly by stromal and tumoral cells. In fact, many \textit{in vitro} and \textit{in vivo} observations have been reported on the secretion of IL-1, IL-6, IL-8, TNF\(\alpha\), inflammatory, or pro-tumorigenic cytokines\textsuperscript{224,250–265} and VEGF, EGF, FGF, PDGF, IGFs by tumoral and/or stromal cells.\textsuperscript{266,267} These cytokines\textsuperscript{251–265} and growth factors\textsuperscript{266–276} can exert pro-survival, proliferative, and angiogenic actions by binding to specific receptors on the tumoral cell. Interactions between steroid and peptide growth factor receptors signaling pathways occur and growth factors, such as EGF and IGF-I, may promote activation of steroid receptors even in the absence of the ligand.\textsuperscript{267} Moreover, IL-6, PDGF, and EGFR have been reported to constitutively activate Stat 3\textsuperscript{250} while NF-kB transcription factors have been reported to be constitutively activated by inflammatory cytokines.\textsuperscript{161} Through this pathway, survival, proliferation, and angiogenesis of tumor cells are stimulated, while apoptosis is inhibited. In addition, a defective death receptor signaling may be a further mechanism in inhibiting tumor cell apoptosis. Therefore, an autocrine and paracrine loop mainly fostered by stromal and tumoral cells themselves directly and indirectly contribute to maintaining a continuous, strong stimulus for survival and proliferation of tumoral cells in the tumor environment. Impairment of acquired immunity follows defective immune recognition by the occurrence, alone or combined, of tumor variants from natural selection due to genomic instability, HLA class I antigen abnormalities,
nonclassical HLA class I molecule upregulation, or epitope immunodominance and lack of co-stimulatory molecules in tumor cells. Moreover, some tumor secrete cytokines and substances that suppress DCs in the tumor microenvironment. In addition, this may be due to clonal diversification or CD4+ anergy. CD4+ anergy is explained as full activation of resting T cells that not only requires an antigen-specific signal provided by engagement of the TCR with the appropriate peptide/MHC complex, but in addition, a second co-stimulatory signal delivered by APCs is required. Hence, in the absence of this co-stimulatory signal, T cells are rendered "anergic." Recently, during a search for genes that maintain T cell quiescence, it was found that Tob, a member of an antiproliferative gene family, is highly expressed in anergic T cell clones. Suppression of Tob with an antisense oligonucleotide augmented CD3-mediated responses and abrogated the requirement of co-stimulation for maximal proliferation.

**Figure 2.** Main tumor cell (a), tumor stroma (b), and tumor infiltrating lymphocytes (TILs) (c) mechanisms and interactions. Within the three cell types, different mechanisms are described or included in the numbered ovals; the arrows toward a number or a letter indicate an interaction between different cells; (b) macrophages, fibroblasts, dendritic (DC), and endothelial cells; (c) CD4+ (TH1, TH2) and CD8+ T cells, CD4+CD25+ Treg, NK cells, NKT cells, myeloid suppressor cells, B lymphocytes; (a) steroid and peptide receptors with possible cross talk; (a2) increased proliferation = myc, cyclin D1/D2 increase, p53 decrease; increased angiogenesis = VEGF, IL-8, COX-2, HIF1 increase, p53 decrease; increased survival and decreased apoptosis = bcl-xl, mcl-1 increase, p53 decrease. Interaction among different mechanisms. Tumor cell (a): lack of costimulatory molecules can account for CD4+ anergy; cancer cells expressing functional FASL and/or TRAIL induce apoptosis of FAS+ target cells; microvesicles trigger mechanisms; constitutive Stat3 and NFκB activation inhibit DCs' differentiation and maturation; tumor stroma (b) and tumor infiltrating lymphocytes (c): defective DCs' maturation and inhibition also account for CD4+ T cells dysfunction; cytokines and growth factors, target cytokines, and GF receptors (a) contributing to the autocrine and paracrine loop; (also see text). For an easy lecture, a schema representative of the three principal effects (tumor growth, defective immune recognition, and immune suppression (italics) and their main mechanisms (within brackets)) that concur in determining immune evasion (bold) is shown in the lower part of the illustration. AICD, activation-induced cell death; DR, death receptor; IL, interleukin; pTyr, tyrosine phosphorylation; AG, antigen; TGFβ, transforming growth factor β; PGE2, prostaglandin E2; Stat, signal transducer and activator of transcription; NFκB, nuclear factor-κB; FasL, Fas ligand FADD, Fas-associated death domain.

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**Medicinal Research Reviews**

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and cytokine secretion. Thus, T cell quiescence is an actively maintained phenotype that must be suppressed for T cell activation to occur.\textsuperscript{278} Constitutive activation of Stat 3 contributes to impairment of innate immunity.\textsuperscript{150,155} In particular, suppression of TILs by CD4\textsuperscript{+}CD25\textsuperscript{+} T reg, myeloid suppressor cells and by inhibiting factors mainly produced by tumoral and/or stromal cells (VEGF, IL-10, PGE2, and TGF\textbeta) and a decrease in inflammatory cytokines and chemokines converge to avoid cellular immune attack on tumor cells. These considerations may be summarized by the concept that in clinically detectable tumors, stimulated proliferation, survival, angiogenesis, and inhibited apoptosis increase tumor burden, which in turn further impairs innate and acquired immunity (Fig. 2).

A. A Possible Explanation of Clinical Results From Advanced Breast Cancer Immune Manipulation

Based on the described interactions among tumor cells, TILs, and tumor stroma, it can be inferred that a cell type that has arisen (tumoral cell) or arrives through blood or lymphatic vessels (monocytes) to the tumor site is likely to be strongly conditioned by the pre-existing environment. Therefore, a vicious circle may be activated and maintained. With an increase in tumor burden, all of these actions are likely to become stronger due to the increase in cytokines, as well as growth and immunosuppressive factor production. Accordingly, the tumor burden is higher, while the chances of successful treatment are lower.

1. Immunosuppressive Activity at the Tumor Site

Clinical data in advanced breast cancer patients treated with cytokines or monoclonal antibodies against growth factor receptors may be interpreted by the above-described relationship at the tumor site. IL-2 is a T cell growth factor. Use of rIL-2 after autologous stem cell transplantation may activate the T cell reconstitution known to be impaired in this setting and hopefully induce a GVT effect.\textsuperscript{279} IL-6 has been characterized as a B cell growth and T cell differentiation factor.\textsuperscript{280} IL-12 induces IFN\gamma production by T lymphocytes and NK cells, TH1 differentiation, and increases NK and CD8 cytotoxicity.\textsuperscript{224} Immune stimulation with IL-2, IL-6, or IL-12 did not significantly improve clinical outcome even when immunological effects were observed.\textsuperscript{9} Thus, these exploratory studies were not further followed by phase II and III trials. The failure of these immunoactive cytokines may be due to the above-mentioned prevailing immunosuppressive actions of the tumor environment. This suppression of cell-mediated immunity results from multiple functions of different cells and likely applies to any immunological effector cell that takes part in the tumor microenvironment. Thus, it is not surprising that overall the external administration of one or more immunoactive cytokines cannot significantly change the final outcome. This also may explain the limited success of previous adoptive immunotherapies with lymphokine-activated killer (LAK) cells or TILs with or without systemic IL-2, and/or cells (target or effector) triggered with IL-2 or other immunostimulatory cytokines or cytotoxic molecules as a result of genetic engineering.\textsuperscript{281,282}

2. Targeted Immunotherapy

Clinical studies investigating the usefulness of monoclonal antibodies against c-erbB-2 and VEGF (trastuzumab and bevacizumab, respectively) have produced positive, although limited, results. The erbB family of receptors plays a major role in promoting breast cancer cell proliferation mainly through two important downstream signaling routes. One is via the Ras-Raf-MAP kinase pathway, through activation of MAPKs, ERK1, and ERK2, which regulates transcription of molecules that are linked to cell proliferation, survival, and transformation. The other is via phosphatidylinositol-3-kinase (PI3K) and the downstream protein serine/threonine kinase AKT. AKT transduces signals that trigger a cascade of
responses from cell growth and proliferation to survival and motility. Another route for signaling is via the stress-activated protein kinase pathway, involving protein kinase C and Jak/Stat. VEGF, as already mentioned, is the most commonly studied vascular growth factor, specific mitogen, and survival factor for endothelial cells and a key promoter of angiogenesis. An unimportant increase in the OS of both of these immunotherapies with antibodies may be because any targeted growth factor represents only a small fraction of the many potentially involved and because growth factor receptor stimulation or inhibition is only one of the multiple transduction signals involved along a molecular pathway. At the tumor site, the multiple cellular interactions are likely to use many different molecular pathways with multiple stimulatory and inhibiting mediators that finally inhibit apoptosis, favor survival, and stimulate proliferation and angiogenesis. So far, only a few of these molecular pathways have been fully elucidated. Therefore, as summarized in Figure 2, in this complex system that includes many complementary and perhaps interchangeable signaling pathways, therapeutic interventions that address single or even a few molecular targets are unlikely to significantly hinder the final dominant effect that is tumor burden increase. In addition to the inherent complexity of the system, the high natural mutagenicity of tumoral cells can permit them to easily and rapidly short circuit at any level the interruption of critical points in the signaling pathway by the production of new mediators and/or activation of alternative routes.

3. Cytokines, Antiestrogens, and the Immune System
Interferons have antiproliferative activity as they down-regulate oncogene expression and induce tumor suppressor genes (IFNα and β) or promote tumor cellular apoptosis (IFNγ). As to this last action, all-trans-retinoic acid (AT) acts synergistically with IFNγ. In addition to this antiproliferative activity, IFNα and β increase the expression of MHC-class-I molecules in tumor cells, promote a Th1 type of immune response, stimulate proliferation and prolong survival of cytotoxic lymphocytes, induce maturation and improve function of DCs, and increase NK activity and TILs. IFNγ increases expression of MHC-class-I and class-II molecules, activates monocytes and macrophages to induce a cytokine cascade and a successive complete activation of various subsets of T or B cells; moreover, it induces differentiation of CD4+ to TH1 cells. Due to both of these antiproliferative and immunological actions, IFNs alone or combined in some previously mentioned clinical trials have been used for immune manipulation of advanced breast cancer. Another effect observed in vitro and in patients affected by advanced breast cancer treated with relatively low doses of IFNβ was estrogen and progesterone receptor enhancement. However, no hormone receptor modulation was found for IFNγ and results reported for IFNα were conflicting. Therefore, IFNs, mainly IFNβ and α were given with the antiestrogen tamoxifen in a few studies. In these studies, the principal rationale for adding IFNβ or IFNα was to induce the synthesis of hormone receptors to prolong the response or to overcome resistance to tamoxifen. Many patients were recruited with negative receptors or resistant to previously administered tamoxifen. Therefore, before adding IFNs, no patient had been selected for response or stable disease during tamoxifen. In all of these studies, a tamoxifen–IFN association was neither shown to overcome resistance to tamoxifen nor to significantly improve the OS. In contrast, in an ongoing pilot study, showing 5–10-year OS to be much more prolonged than in historical controls and in other comparable series treated only with antiestrogens, stable disease or response to antiestrogen was the principal selection criterion to recruit patients. Moreover, all patients continuously taking antiestrogens received immune stimulation by cyclic administration of IL-2 following immune effector cell priming with IFNs. Therefore, all patients selected for hormone dependency were under the cytostatic action of
antiestrogen on tumor cells. This action has not yet been completely clarified; however the following points have been reported: antagonization of estrogen and Ca channels, reduction of membrane fluidity, inhibition of protein kinase and P glycoprotein,\textsuperscript{284–287} lowering of circulating IGF levels,\textsuperscript{288,289} and IGF-1 production,\textsuperscript{290} and reduction of key regulatory enzymes.\textsuperscript{290} Moreover, in MCF-7 cells, tamoxifen treatment resulted in decreased total protein synthesis.\textsuperscript{291} The arrest of cell cycle in G0-G1 is one final result,\textsuperscript{284} which is likely to depend on the direct antagonization of estrogen at the promoter regions of DNA with downregulation of the genes favoring proliferation, survival, and angiogenesis. The decrease in total protein synthesis and key regulatory enzymes could reduce the production of other growth factors besides IGF and of pro-tumorigenic cytokines. Therefore, both the direct (by growth factor receptors) and alternative (by constitutive Stat 3 and NF-kB activation) signaling pathways that foster autocrine and paracrine loops may be down-regulated. Reduction of membrane fluidity and the antagonization of Ca channels may increase the amount of the Ca++ in the cell, thus triggering apoptosis through induction of downstream caspases.\textsuperscript{292} Finally, G0-G1 cell cycle arrest is likely to decrease the genomic instability and frequency of HLA class I antigen abnormalities, and hinder the defect of recognition of tumor by immune effector cells. Following these fundamental effects of the antiestrogens, immune evasion could be limited and a decrease in tumor immunosuppressive actions could occur as well. If so, this particularly favorable condition induced by antihormones on tumor cells would permit a more efficacious stimulation of cell-mediated immunity by IL-2 and an inflammatory cytokine-mediated immune attack on tumor cells.\textsuperscript{9,32} Immune attack and decreased tumor proliferation could delay hormone resistance and clinically turn into prolongation of response or stable disease. This condition between tumor and immune system could be maintained or broken at any time consistent with hormone resistance phenomena likely due to the extension of metastatic tumor burden and the level of hormone dependency. In this case, the multiple tumor cell actions re-activate and increase the reported autocrine and paracrine loops of survival, proliferation, and angiogenesis with the concomitant inhibitory effect on cell-mediated immunity. Thus, the initial prevailing negative complex role of tumoral cells at the tumor site environment is again established. This interpretation is compatible with the data from the above-mentioned pilot study.\textsuperscript{32,33} In fact, in this study, after a mean follow-up of 59 ± 37 months, the estimated times for the OS from first-line antiestrogen salvage therapy was 103 months (p<0.0001; 95% CI, 69–136), and the OS from diagnosis of distant metastases it was 106 months (p<0.0001; 95% CI, 90–123).\textsuperscript{33} In addition, a significant prolongation of a CEA-TPA CA15.3 tumor marker panel lead time at the progression of metastatic disease occurred.\textsuperscript{293} Furthermore, 3 out of 32 patients (9.4%) with multiple repetitions have been surviving well for more than 7 and up to 16 years (data not shown). During clinical benefit (i.e., CR or PR or SD), stimulation of cellular immunity occurred after IL-2, while it did not occur during disease progression.\textsuperscript{33} Further recent laboratory immunological assessment confirmed findings on cellular immunity and showed different changes of proinflammatory cytokines, CRP, and inhibiting factors consistent with associated clinical benefit or with disease progression.\textsuperscript{294} In the other reported study, the subset of 11 patients with relatively prolonged survival, similar to the entire population, were unresponsive to previous hormonetherapy and were recruited to maintenance IFNβ, retinyl palmitate, and tamoxifen treatment following complete remission by chemotherapy.\textsuperscript{20} Although no immunological parameters were evaluated in the study, the relatively low production of growth and immunosuppressive factors due to the reduced tumor burden could have favored a limited modulation and stimulation of immune-effector cells by IFNβ and retinyl palmitate combined with concomitant synergistic antiproliferative and antiangiogenic actions that potentially they, and occasionally tamoxifen, could have provided\textsuperscript{20}. 

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6. CONCLUSION

In conclusion, although many experimental studies on the relationships between tumor and immunity are available, a relatively limited number of clinical trials with positive, but limited results regarding immune manipulation in advanced cancer patients have been reported. So far, the principal hypotheses interpreting the relationship between cell-mediated immunity and tumors have not received enough clinical support for an overall validation. Here, an interpretative model has been developed after having considered more recent data on cell-mediated immunity and the genetic mechanisms that regulate it, as well as the action of cytokines and growth factors. This model is based on the relationships among tumors, stroma, and cell-mediated immunity with three principal final effects (tumor growth, defective immune recognition, immunosuppression) that concur in determining immune evasion. This may be used to interpret actual clinical data on immune manipulation of advanced breast cancer and could help to improve clinical results.

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