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REVIEW

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Effective immunotherapy against cancer

A question of overcoming immune suppression and immune escape?

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Abstract During the last decade, the breakthroughs in understanding of the molecular mechanisms responsible for immune activation and the advent of recombinant DNA technologies have changed the view on immunotherapy from "a dream scenario" to becoming a clinical reality. It is now clear that both cellular immunity comprising T and NK cells, as well as strategies based on antibodies, can provide strong antitumoral effects, and evidence is emerging that these strategies may also cure patients with previously incurable cancers. However, there are still a number of issues that remain unresolved. Progress in immunotherapy against cancer requires a combination of new, improved clinical protocols and strategies for overcoming mechanisms of immune escape and tumor-induced immune suppression. This review discusses some of the salient issues that still need to be resolved, focusing on the role of oxidative stress and the use of antioxidants to alleviate the immune hyporesponsiveness induced by reactive oxygen species (ROS).

Keywords Immune escape · Immune suppression · Immunotherapy · Oxidative stress

Abbreviations

HLA	Human leukocyte antigen
KIR	Killer cell immunoglobulin-like receptor
NKR	Natural killer cell receptor
ROS	Reactive oxygen species
TAA	Tumor-associated antigen

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Introduction

Tumor immunoediting

In the early 20th century, the immunologist Paul Ehrlich [37] first conceived of the idea that tumors arise continuously in our bodies and that the immune system scans for and eradicates these tumors before they become clinically manifested. Experimental evidence that tumors could be repressed by the immune system came from the use of tumor transplantation models in the mid-20th century [51]. The results from these models strongly suggested the existence of tumor-associated antigens (TAAs) and formed the basis of the "immunosurveillance theory" postulated by Sir Macfarlane Burnet and Lewis Thomas [17, 18, 150]. According to this theory, spontaneously developing carcinomas are efficiently eliminated by the immune system recognizing specific determinants expressed by the tumor cells. Burnet claimed that the presence of such a tumor scanning system was an evolutionary necessity and as important for survival as the protection against infections. Soon, a critique against this concept was aroused. Experimental data challenged predictions of the immunosurveillance theory. There was no increased incidence of spontaneous or chemically induced tumors in athymic, nude mice, as compared to wild-type animals even though long observation periods of up to 420 days were employed [129, 145]. If tumors eventually appeared in immunocompromised mice these were often virally induced lymphomas [68]. All these lines of evidence argued against immunological surveillance. However, recent evidence from knockout (KO) mice lacking components of the IFN-γ signaling pathways, perforin, or

recombination activating gene 1/2 (RAG-1/2), has revived the discussion of a potential immunosurveillance against tumors [33]. These mice display enhanced sensitive to methylcholanthrene (MCA)–induced tumor formation when compared with wild-type mice. RAG-2–deficient mice also developed spontaneous epithelial tumors of predominantly intestinal origin. Further support of immunosurveillance against tumors in humans is provided by the positive correlation between the presence of lymphocytes in a tumor as detected by immune histochemistry, and increased survival of patients with colorectal cancer [106]. The experimental revival of the immunosurveillance concept has extended the phenomenon, and it is now referred to as "tumor immunoediting" [33]. Tumor immunoediting includes both immunosurveillance and immune escape. It refers to the protective capacity of the immune system against tumors (i.e., immunosurveillance) and to the consequences of the proposed immune selection pressure (i.e., immune escape). However, the process of tumor immunoediting is somewhat distinct from immune escape, as it includes the observation that tumors that grow in immunocompromised hosts become more immunogenic and are easily rejected when transplanted into immunocompetent mice. In the absence of immune selection, tumors may develop an immunogenic phenotype. Evidence for immune escape upon immune-mediated selection pressure is abundant both in mice and humans. Re-passage of transplantable tumors through immunocompetent hosts leads to the development of tumor variants with reduced immunogenicity [157, 158]. In humans, the immune selection pressure combined with the genetic instability of tumor cells frequently gives rise to deletions and/or altered expression of several molecules important for immune recognition, such as components of the antigen processing and presentation pathway [45, 139]. Several aspects of the original version of the immunosurveillance theory were probably incorrect, and most tumor immunologists would probably agree that it is not a primary property of the immune system to fight cancer. From an evolutionary perspective, there is no advantage to protecting a species from a disease occurring after fertile age [171]. Most tumors arise late in life when the evolutionary pressure cannot exert its influence. Nevertheless, as outlined above, most tumors are antigenic and can be eradicated by the immune system given the right circumstances [162]. Lack of immunogenicity is therefore a result of the tumor's inability to induce an immune response. Understanding the particular conditions under which antitumor immune responses can be raised is the main challenge for cancer immunotherapy.

Immunotherapy against cancer

Although there are anecdotes telling of immunization attempts against cancer performed during the 18th century, it is not until the last decade that the antigens and the T-cell receptors responsible for immune recognition of tumors have been identified. Still, the progress of vaccination against cancer is dependent on new insights in the fields of basic cellular and molecular immunology. Fundamental properties of the immune system such as formation of long-term memory, tolerance, immunodominance, and anergy remain to be fully understood. The use of mature dendritic cells (DCs) both to break tolerance when trying to induce tumor immunity [107] and to induce peripheral tolerance in the case of autoimmune disorders [156] clearly demonstrates that more knowledge is needed to elucidate the basic mechanisms of immune activation.

Targeting specific tumor antigens with cancer vaccines

Based on the discovery and identification of tumor-associated antigens, it is now possible to target tumors specifically by antibodies or by cytotoxic T lymphocytes (CTLs) [123]. This may be achieved either by specific vaccination or adoptive transfer of in vitro generated antitumor CTLs. Several different strategies of vaccination have been tried, including delivery of tumor antigens in the form of peptides, proteins, naked DNA, or whole tumor cells [35, 124, 152]. Vaccines based on DCs pulsed or transduced with tumor antigens have also been tested with varying degrees of success [38]. Altogether these efforts clearly demonstrate that it is possible to induce tumor-specific T-cell responses and in a few patients even objective clinical responses. Herein, the immense literature on the development of cancer vaccines will not be reviewed. The discussion will be limited to some of the obstacles for effective immunization against cancer. Most defined tumor antigens are products of normal genes that are overexpressed or selectively expressed in tumor tissues [47]. Thus, one of the major hurdles for effective vaccination is to overcome the central and peripheral tolerance to these "self" antigens. The existing T-cell repertoire specific for self-antigens is limited to low quality "low avidity" T cells that may recognize a peptide-pulsed target but not endogenously presented antigens on tumor cells [34]. Nevertheless, it has been shown that immunization can be effective even though the response is limited to low avidity CTLs [104]. Attempts have also been made to create more immunogenic antigens by molecular manipulation. By inserting an amino acid change in the peptide epitope, it is possible to produce an antigen that binds more strongly to the relevant HLA molecule and therefore stands a higher chance of breaking tolerance against self-proteins [124]. Vaccination of melanoma patients with a modified peptide, $gp100_{209–217}$ (210 M), from the gp100 melanocyte differentiation antigen was able to induce an immune response that recognized the native peptide antigen and HLA-A2–matched tumors. In combination with IL-2, this peptide induced objective clinical responses in 13 out of 42 treated patients [124].

Bone marrow transplantation and adoptive immunotherapy

Although vaccination against cancer is a promising approach, some of the most encouraging data regarding immunotherapy against cancer stems from studies employing adoptive transfer of tumor reactive T cells and perhaps also of NK cells. Bone marrow transplantation (BMT) has recently been used as immunotherapy of solid tumors with promising results [21]. The intersection of BMT and more specific immunotherapy based on the knowledge of defined antigens is particularly interesting, as illustrated by two recent studies. First, Dummer et al. [32] exploited the homeostatic proliferation phase of T cells occurring after sublethal irradiation (conditioning) to induce strong CD8 T-cell responses against tumor-associated self-antigens. This was based on the concept that homeostatic proliferation occurring in situations of lymphopenia is driven by recognition of self-MHC/peptide ligands similar to those that control positive selection of T cells in the thymus [93]. Adoptively transferred T cells, educated during the homeostatic reconstitution in lymphopenic and tumor-bearing animals, grew to become tumor specific. Similarly, Dudley and colleagues recently reported remarkable clinical responses after adoptive transfer of tumor-specific, tumor-infiltrating lymphocytes (TILs) into melanoma patients that were conditioned with low doses of chemotherapy [31]. Around 50% of the patients experienced objective clinical responses. Other studies provide further evidence that the posttransplant period is optimal for redirecting immune responses toward tumor antigens [6, 149]. The reasons for the beneficial effect of lymphopenia during immunotherapy remain unclear. It may involve the presence of homeostatic proliferation, but other factors such as depletion of immunoregulatory $CD4^+CD25^+$ T cells may also contribute. In another study, T-cell clones specific for the Melan-A/MART-1 or the gp100 melanoma differentiation antigens were repeatedly infused into patients with metastatic melanoma along with low doses of IL-2 [168]. Objective tumor responses were seen along with preferential localization of the infused T cells to the tumor sites. However, adoptively transferred T cells are rapidly eliminated from the circulation and the effect of adoptive immunotherapy is therefore short lived. This may be due to homeostatic forces contracting the lymphocyte population to normal levels. Alternatively, in vitro expanded T cells may have a limited lifespan due to lymphocyte senescence [112]. In other models, EBV-specific T cells were shown to have the potential of long-term survival when adoptively transferred to a recipient [57]. This property was recently exploited by expressing a chimeric tumor-specific TCR in EBV-specific T cells [127]. These T cells survived long-term in vivo and provided a source of T cells capable of killing both EBV-infected cells and tumor cells expressing the antigen recognized by the chimeric receptor. It will be important to define the factors that influence the functional properties and the survival of in vitro–expanded T cells to be used for adoptive immunotherapy against cancer. Yet another breakthrough comes from the potential of NK cells in the setting of allogeneic bone marrow transplantation [128]. NK cells are negatively regulated by inhibitory HLA class I–binding receptors including the killer cell immunoglobulin-like receptors (KIRs) [75]. Briefly, it was demonstrated that the alloreactivity of NK cells, as governed by a donor–recipient KIR-HLA mismatch, correlated with increased survival, more successful grafting, and decreased risk of graft-versus-host disease (GVHD). The mechanism behind the latter observation was dissected in a mouse model revealing that the alloreactive NK cells eliminated GVHD by killing the host antigen-presenting cells, thus preventing these cells from presenting host antigens to donor T cells which initiates the GVH reaction [143]. The striking survival advantage for patients with AML who received KIR mismatched bone marrow transplants clearly demonstrates that the immune system is capable of eradicating tumor cells and suggests that NK cells provide an excellent cellular source for adoptive immunotherapy. Alternative strategies of adoptive immuno–gene therapy that are based on gene transfer into immune effector cells that are infused back to the patients are also very promising. For examples it was possible to eradicate systemic B-cell tumors using human T cells that were genetically targeted to the CD19 antigen [16]. Other approaches involve transfection of human PBMCs with selected T-cell receptors with specificity for tumor-associated antigens (TAAs) [22]. This strategy has advantages compared with conventional adoptive T-cell therapy. For instance, one can select pre-made constructs with specificities for the tumor of each patient and avoid the difficulties associated with T-cell cloning. Although results from different approaches of immunotherapy bring hope for a successful future for this field, it is clear that much improvement is needed before immunotherapy will become a powerful therapeutic tool in the clinic. Several issues that need to be considered will be discussed below.

Timing the immunotherapeutic approach with stage of disease

Perhaps the most important explanation for why immunotherapy has not been more successful in the clinical setting relates to the timing of immunotherapeutic intervention with respect to the disease progression. To date, most vaccinations against cancer have been performed in the late stages of the disease when all other treatment modalities have failed. This is a major difference compared with vaccines against viral or bacterial antigens, as patients with advanced cancer are often immune suppressed and not as responsive to immunization (see further below and references [66, 113]). Furthermore, most vaccines against infectious diseases are prophylactic and allow the immune system

to be prepared already at the onset of infection. This is in sharp contrast to cancer vaccines performed in "late stage" disease when a large number of tumor cells are present. Although there are striking examples of regression of large tumors [125, 126], it is likely that vaccines against cancer will operate more efficiently when administrated early during tumor progression or directly after conventional treatment modalities, such as surgery or chemotherapy. One example of this possibility comes from a vaccination study against lymphoma with an idiotype protein vaccine in combination with GM-CSF [10]. Eight out of 11 patients that were in their first clinical complete remission after standard chemotherapy but still had molecular signs of disease, as determined by the presence of cells with the 14;18 translocation, went into molecular remission upon vaccination. Similar mechanisms may account for the curing potential of the graft-versus-leukemia (GVL) effect seen in BMT against acute leukemias. The GVL effect of T and NK cells follows upon chemotherapy, and patients are often in complete remission before being subjected to the bone marrow transplantation. Thus, the immune system may efficiently eradicate minimal residual disease. In situations of larger tumor burden, adoptive transfer of high numbers of T or NK cells may be needed.

Impediments to immunotherapy

As immunotherapy becomes more efficient, the immunological selection pressure against tumor cells will increase. In combination with the inherited genetic instability of tumor cells, this will lead to the development of immune escape variants [65]. Below, several ways by which tumors may escape the immune system will be discussed with references to the current thinking on how to counteract these mechanisms. This review will give a particular emphasis on tumor-induced immune suppression and the role of oxidative stress for decreased T-cell function, as this may constitute a substantial impediment for effective immunotherapy against cancer. It should be stressed that the development of tumor-induced immune suppression is perhaps more of a reflection of the large tumor burden in "late stage" disease, than a consequence of the rules of the micro-evolution and selection pressure.

Loss of antigen processing and presentation

The presentation of antigens in the context of HLA molecules is crucial both during T-cell priming and during the effector phase of an adaptive immune response. Alterations in antigen processing and presentation are commonly seen both during viral infection and in malignancies. Viruses have adapted to the immune-mediated selection pressure in numerous ingenious ways, affecting almost every part of the biochemistry of the MHC presentation pathway [169]. For instance, Epstein-Barr viruses (EBVs) express a protein (EBNA-1) that carries a repeated sequence (Gly-Ala repeat) that makes it inaccessible for proteasomal degradation into peptides [81]. The herpes simplex virus (HSV)–derived protein ICP47 and the cytomegalovirus (CMV)–derived protein US6 interfere with TAP and prevent peptide translocation into the ER [42]. In tumors, the antigen presentation pathway is disrupted as a consequence of mutations and/or deletions of one or several genes encoding components of the antigen-processing machinery. Complete HLA loss is a common event in several murine and human tumors [44, 140]. Total loss of HLA class I is usually associated with alteration of β_2m expression as the MHC class I molecule is not properly assembled in the absence of this molecule. Mutations of β_2m along with loss of heterozygosity (LOH) of the second allele has been described and may be associated with progression of disease in the course of adoptive immunotherapy [120]. However, also the loss of proteasomal subunits such as LMP-2 and LMP-7, as well as of peptide transporters TAP1 and TAP2, leads to total loss of HLA class I and was reported in several different tumor types including cervical carcinoma, small cell lung carcinoma, non–small cell lung cancer, prostate carcinoma, and renal cell carcinoma [140]. Tumors often display a selective loss of HLA haplotype, locus, or allele. Haplotype losses may be the result of LOH on chromosome 6 [118], whereas several mechanisms may account for the locus down-regulation. Sequential loss of several alleles in successive metastasis from a single patient who underwent immunotherapy suggests that immune-mediated selection pressure plays a pivotal role in selecting the HLA loss variants [78]. A study of several melanoma cell lines demonstrated low or no expression of gene products of the HLA-C locus [92]. An interesting aspect of the loss of certain HLA alleles comes from the study of Ikeda et al. [60] where they demonstrate that tumor cells that had lost HLA-Cw7 upon progression could be sensitized to tumor antigen–specific CTLs. These CTLs were incapable of killing tumors derived from an earlier biopsy, before any loss of HLA had occurred, although they expressed the same tumor antigen. The reason turned out to be that the expression of HLA-Cw7 by early tumors enabled ligation of inhibitory KIR, expressed by the CTLs. Thus, loss of HLA resulted in increased sensitivity to CTLs. Similarly, tumor cells that lose HLA class I should become more sensitive to NK-cell–mediated lysis because they fulfill the criteria of "the missing self hypothesis" [86]. Indeed, this has been reported to be the case. Loss of β_2m leads to increased NK sensitivity and effective tumor elimination [62]. However, NK cells may express inhibitory NK receptors (iNKRs) belonging to different families that only partially overlap, and the failure of one receptor-ligand system may be compensated for by another; i.e., lack of HLA-C/KIR signaling may be compensated for by the HLA-E : CD94/NKG2C

interaction [88]. Furthermore, NK cells are dependent also on activation signals, which is why lack of inhibition is not always enough. The balance of activating and inhibitory signals to NK and T cells will be discussed below.

Antigenic loss variants

An unfortunate consequence of efficient immunotherapy is that the immune system selects for tumor variants that are no longer recognized, i.e., that have lost the targeted antigen. Antigenic variation is the hallmark of many successful pathogens and may be the most difficult obstacle for the induction of long-lasting immunity against many infectious diseases including HIV [46]. This phenomenon was recently demonstrated in the study of Yee et al. [168], where the histology of recurrent metastasis following adoptive transfer of Mart-1–specific T cells was examined. In three out of five patients studied, the recurrent metastasis lacked expression of Mart-1 while expression of gp100 and tyrosinase remained intact. Similar observations have been made in the course of peptide and DC immunizations [151]. These results strongly suggest that one has to target multiple tumor antigens, or perhaps antigens that are crucial for the growth of the tumor cells. In the advent of antigen loss variants, the tumor cells would stop dividing and thereby lose their malignant potential. There are several reported successful attempts of immunotherapy using multiepitope vaccines. For instance it was reported that one could obtain immune responses to several epitopes by using genetic constructs coding for a series of epitopes ordered in a ''string-of-beads'' fashion [153]. However, the use of several epitopes together leads to yet unpredictable patterns of immunodominance. Immune responses to immunodominant epitopes within a multiepitope construct may undermine responses to subdominant epitopes. The factors governing immunodominance are largely unknown but will be important to elucidate, as they influence the efficacy of multiepitope vaccines as a solution for the escape of antigenic loss variants.

The Fas ''counterattack''

The Fas ''counterattack'' refers to the possibility that tumor cells attack and kill T cells that home in to the tumor. This concept was based on the observation that FasL-expressing sertoli cells were not rejected when transplanted into allogeneic mice [163]. It was concluded that FasL expression was a key feature of immunological privilege supported by the finding of FasL expression in the eye [49]. This held great promise for future clinical applications as FasL-expressing transplants would be tolerated by the recipient. The first example of FasL expression on tumors was in colon cancer, rapidly followed by studies of melanoma [52]. FasL expression had functional consequences, as it induced apoptosis of Fas-expressing Jurkat T cells and tumor-infiltrating lymphocytes. FasL expression was also reported on ovarian carcinoma leading to signaling defects and apoptosis of tumor-associated lymphocytes [117]. More recently, the concept of a tumor-mediated counterattack has been revisited [119]. When trying to transplant FasL-expressing islet β-cells, one observed granulocyte infiltration and a rapid rejection of the transplant [2]; hence the opposite to tolerance. Similar observations has been made in tumor models in which FasL-expressing tumors were rapidly rejected [4]. Furthermore, several researchers failed to see expression of FasL on tumors previously reported to express this molecule [39]. The Fas-mediated apoptosis of infiltrating lymphocytes seemed rather to be the consequence of T-cell activation and activation-induced cell death [170]. The Fas death receptor signaling pathway may instead be a very important component of host defense against tumors [24]. Not surprisingly, escape variants that are resistant to death receptor signaling have evolved (discussed below).

Escaping death receptor signaling

Two death receptor signaling systems that contribute to the immunosurveillance of tumors are FasL and TNF-related apoptosis-inducing ligand (TRAIL) [24, 147]. Death receptors have cytoplasmic sequences—death domains—which transmit the apoptotic signal upon ligation. This triggers a cascade of caspases involving caspase 8 (also known as FLICE), caspase 3, and caspase 6 and 7. Tumor cells may become resistant to death receptor signaling by expressing an inhibitor of caspase 8—cellular FLICE inhibitory protein (FLIP) [29]. Overexpression of FLIP was shown to mediate tumor cell escape in vivo [96]. Tumors also display loss of Fas expression which renders them resistant to apoptosis [74]. Alterations may appear further downstream in the death receptor signaling pathway, including functional impairment of FADD and caspase 10 by inactivation mutations [142]. Similarly, tumors escape death receptor signaling by losing the expression of TRAIL receptors or downstream signaling components [56]. Yet another intriguing way for tumors to escape recognition by CTLs and NK cells is by overexpressing PI-9 (in mice known as SPI-6) [95]. PI-9 is a serine protease inhibitor that inactivates granzyme B which is one of the important components of granule-mediated CTL killing [154]. One suggested physiological role of SPI-6 is to protect activated DCs from being killed by the induced CTLs during a Th1 response [97].

Engaging inhibition-blocking activation

The balance between activation and inhibition of T and NK cells is dependent on the quality of a number of

receptor–ligand interactions. Evidence suggests that tumors may evade T- and NK-cell recognition by shifting the balance toward inhibition. Ectopic expression of the nonclassical HLA class I molecule, HLA-G, has been described in several different tumors including melanoma, renal cell carcinoma, breast carcinoma, cutaneous lymphoma, and ovarian carcinoma [59, 77, 91, 111]. HLA-G expression protects tumor cells from lysis by T and NK cells via ligation of inhibitory receptors LIR-1 and/or KIR2DL4. The HLA-G leader sequence–derived peptide (Gsp) binds another nonclassical HLA class I molecule, HLA-E, and thereby promotes a strong interaction with CD94/NKG2 receptors expressed on NK cells and a subset of T cells [91, 160]. It has been demonstrated that the strength of the inhibitory signal depends on properties of the leader sequence peptide that is bound to the HLA-E molecule [160]. While some peptides, including the HLA-G leader sequence–derived peptide, mediate strong interaction with the CD94/NKG2 heterodimer and thereby promote significant signaling, other peptides seem to silence this pathway [100]. HLA-G also shuts down other effector functions such as the production of IFN-γ and Fcγ receptor–mediated activation of LIR-1–expressing macrophages [23]. Soluble HLA-G1 was furthermore demonstrated to induce apoptosis of $CD8^+$ T cells via a Fas-FasL dependent mechanism [41], perhaps constituting a refined version of the counterattack concept. The secreted form of HLA-G1 induced the expression of functional FasL in activated T cells, and this was shown to be the result of the interaction between the conserved α3 domain of the HLA-G1 molecule and the CD8 molecule. Similarly, soluble HLA class I molecules, including the nonclassical HLA-E and HLA-F molecules, as well as alleles of HLA-C, were shown to induce apoptosis of NK cells upon ligation of activating receptors [144]. Again, this was dependent on the up-regulation of FasL in stimulated NK cells resulting in suicide along with killing of neighboring Fas-expressing NK cells (Fratricide). An alternative way for tumors to avoid being killed by NK and T cells is by shedding decoy ligands for activating receptors. Tumors were found to secrete soluble MICA, which is the ligand for the activatory receptor NKG2D [50]. It was demonstrated that soluble MICA is frequently found in sera of cancer patients at levels sufficient to cause down-regulation of NKG2D on tumor-infiltrating and circulating $\alpha\beta$ and $\gamma\delta$ T cells and NK cells. As NKG2D is involved in direct recognition of MICA-expressing tumors, as well as in costimulating T-cell responses, this had functional consequences, down-modulating the reactivity and cytokine production capacity of antitumor CTLs. The expression of ligands that have the capacity to turn off the function of immune effector cells could potentially abolish an otherwise effective T- or NK-cell–mediated tumor recognition. Such inhibitory pathways can be targeted. For instance, it has been shown that mouse NK cells that were preincubated with mAbs against inhibitory receptors had a more potent antitumor effect when adoptively transferred into tumor-bearing mice [69]. Alternatively, one may select or genetically engineer NK- and T-cell clones to express less of inhibitory receptors and perhaps more of activating receptors, prior to adoptive transfer. Another interesting possibility would be to target the nonclassical HLA class I molecules that are ectopically expressed by tumors. This could be done either by humanized specific antibodies or by generating HLA-G or HLA-E allo-specific T cells. With an increasing knowledge of the receptor–ligand interactions that govern T- and NK-cell activation, it may be possible to augment antitumor responses and dampen undesirable reactivity of the immune system.

Suppression of antitumor responses by regulatory T cells

Recently, a subset of T cells, coexpressing CD4 and CD25 (IL-2R α chain), with unique immune regulatory properties was described [130]. These regulatory T cells (T_{reg}) seem to play a pivotal role in immune homeostasis, protecting the host from several T-cell–mediated autoimmune disorders including type 1 diabetes, hypothyroidism due to thyroiditis, and pernicious anemia due to gastritis [122]. $CD4^+CD25^+$ regulatory T cells dramatically suppress the function and proliferation of $CD4^+CD25^-$ and $CD8^+$ T cells [115]. Much of the immune regulatory function of these cells has been attributed to their capacity to secrete immune-suppressive cytokines such as TGF-β and IL-10 which may severely inhibit CTL responses as will be further described below [122]. Although beneficial in protecting the host from autoimmune disorder, regulatory T cells may also dampen antitumor responses. When T_{reg} were depleted in mice, transplantable tumors were efficiently rejected by the host immune system [141]. T_{reg} has been shown to infiltrate several types of human tumors, including non–small cell lung cancer, and pancreas, breast, and ovarian carcinomas [85, 165]. Regulatory T cells are also found at a higher frequency in the peripheral blood of cancer patients and may induce peripheral ignorance of tumor cells, facilitating metastatic spread of the disease [85]. However, in a recent report by Edinger et al. [36], a novel application of regulatory T cells was demonstrated. Cotransplantation of T_{reg} with allogeneic BM could inhibit graft-versus-host reaction of transplanted T cells while preserving their graft-versus-tumor potential, thus curing animals with leukemia and lymphoma. T_{reg} inhibited the massive proliferation of alloreactive T cells associated with the onset of GVHD without affecting their function.

Tumor-induced immune suppression

Mice with experimental tumors and patients with cancer show evidence of decreased immunological potency [66]. This is manifested by decreased proliferation, decreased Th1 cytokine production, and a poor cytolytic capacity

of TILs and PBLs. On a molecular level, this is associated with decreased levels of signaling molecules such as CD3ζ, $p56^{lck}$, $p59^{fyn}$, ZAP70, JAK3, and STAT5, and the blocking of nuclear factor-κB [71, 72, 101, 114, 159]. The underlying mechanisms for immune dysfunction in cancer patients are probably multifactorial. A large number of factors, including old age and cachexia, contribute to the poorly functioning immune system in cancer patients [112]. However, the role of the tumor itself was nicely demonstrated in mice with fibrosarcoma [132]. Upon inoculation with tumors, these mice became immune suppressed, as manifested by impaired antitumor reactivity of T cells associated with decreased expression of signaling molecules. This phenotype was completely reversed following surgical removal of the tumor, unmasking a population of primed T cells that were capable of rejecting a subsequent tumor challenge.

Mechanism of tumor-induced immune suppression

The unresponsiveness of the immune system in patients with cancer has been attributed to the secretion of immune-suppressive cytokines such as IL-10 and TGF-β. Indeed, and as mentioned above, these are the cytokines that may be responsible for the suppressive effect of regulatory T cells. IL-10 is frequently detected in high amounts in cancer patients [116]. This cytokine may skew immune responses away from a protective and antitumor Th1 response [64]. IL-10 also inhibits differentiation, maturation and functional status of DCs [25], thus interfering with the induction of antitumor responses. Furthermore, IL-10 may suppress T-cell–mediated immunity by down-regulating the function of TAP molecules and the expression of MHC class I molecules on target cells [131]. The immune-suppressive properties of IL-10 have been adopted by the Epstein-Barr virus which has a gene product (BCRF1) with striking homology to the mammalian IL-10 sequence [103]. TGF-β is another cytokine that inhibits activation, proliferation, and activity of lymphocytes [40]. This cytokine is often found at high levels in malignancies and is associated with poor prognosis and lack of response to immunotherapy [48].

Suppressor macrophages and free radicals

In the mid-1970s, macrophages from tumor-bearing hosts were found to inhibit proliferative responses of splenocytes in a non–antigen-dependent manner [67]. Splenocytes from tumor-bearing individuals had proliferative responses that were 90% below normal. However, if these "suppressor macrophages" were removed, the proliferation was completely restored. Adoptive transfer of macrophages from tumor-bearing mice facilitated tumor growth in the recipient [167]. Several human tumor types, including carcinomas of the breast, bladder, and cervix, show an association between the levels of infiltration of tumor-associated macrophages and poor prognosis [13]. Such protumor effects by macrophages may depend on secretion of proangiogenic cytokines and enzymes [76], secretion of immune suppressive cytokines [87], and release of free radicals including NO [1] and hydrogen peroxide [138]. Free radicals may act on immune cells and render them hyporesponsive or even apoptotic [66]. It is important to stress that the action of H_2O_2 may be beneficial to the host as a component of innate immunity against bacteria and other pathogens [7]. There is also abundant evidence for a potent proapoptotic and antitumor role for free radicals [102]. Furthermore, radiation therapy is dependent on the generation of free radicals and their capacity to induce DNA damage and apoptosis of tumor cells [11]. Thus, the role of tumor-associated macrophages in tumor progression is controversial.

Consequences for immune cell function and signaling

Early studies suggested that macrophages inhibited T- and NK-cell function by releasing hydrogen peroxide (H_2O_2) [138]. The addition of catalase, an enzyme that specifically catalyzes the reduction of H_2O_2, rescued NK cells from the inhibitory effects of activated macrophages [138]. This was further supported by other investigators showing that histamine, an inhibitor of H_2O_2 production, could rescue NK cells from the detrimental effects of activated macrophages [54]. Later, it was shown that several other features of tumor-induced immune suppression, including loss of Th1 cytokine production, and decreased expression of signaling molecules including the CD3ζ chain, were induced by activated or tumor-infiltrating macrophages [3, 73]. These effects could be mimicked by exogenous H_2O_2 and were restored in the presence of catalase, an enzyme that catalyzes the reduction of H_2O_2 to $H_2O + O_2$. Interestingly, tumor cells have also been found to produce large amounts of hydrogen peroxide [146], which could potentially induce T-cell dysfunction of tumor-infiltrating T cells. The hyporesponsiveness induced by oxidative stress has been investigated in several models. Hydrogen peroxide, added exogenously to freshly isolated and noncultured PBLs, selectively targeted Th1 cytokine production in $CD45RO^+$ memory/activated T cells [89]. The loss of Th1 cytokine production in $CD45RO^+$ T cells was associated with a block of NF-κB activation. This suggested that H_2O_2 inhibited proximal signaling events in $CD45RO^+$ T cells, leading to diminished activation of NF-κB and impaired production of cytokines regulated by this transcription factor [8]. Although not shown, the reason for such selective targeting of $CD45RO^+$ cells may be related to the role of the CD45 molecule in T-cell receptor signaling. CD45 is a protein tyrosine phosphatase (PTP) that preferentially removes phosphates bound to regulatory tyrosine residues (C-terminal tyrosine residues) on src family kinases. This is essential for T-cell activation and

sustained TCR signaling as it allows lck and fyn to be phosphorylated at their active sites [155]. CD45 exists as several isoforms due to alternative splicing of three consecutive exons (4, 5, and 6, designated A, B, and C) in the extra cellular domain [155]. Naïve T cells predominantly express the larger CD45RA isoforms and, over the course of 3 to 5 days after activation, they switch to express the smallest CD45RO isoform [14]. Compatible with the rapid onset of a memory response, $CD45RO^+$ CD4 and CD8 T cells are highly sensitive to antigen and secrete a broad range of cytokines upon stimulation [108]. Interestingly, this responsiveness to antigen correlates with the colocalization of the CD45RO isoform with CD4/CD8 and the TCR at the cell surface [28, 30, 79, 80]. The association of the CD45RO isoform with other signaling molecules may be responsible for the more efficient response to antigen by memory T cells. However, it may also explain the enhanced sensitivity of this subset to H_2O_2. It is well known that H_2O_2 functions as a PTP inhibitor [19]. It can therefore be speculated that H_2O_2 interferes with proper TCR signaling, by blocking the PTP activity of CD45RO. Consequently NF-κB activation and cytokine production is blocked. Such inhibition of CD45 PTP activity would have less functional consequence for $CD45RA^+$ cells, as the CD45RA molecule is less active in promoting TCR signaling due to its distant localization from the T-cell receptor [79]. Another recently described phenomenon that may help in understanding the selective targeting of memory/activated T cells by oxidative stress is the enhanced efficiency by which the CD45RO molecule forms inactive homodimers [166]. This is particularly interesting as H_2O_2-mediated inhibition of PTP activity is known to involve formation and stabilization of homodimers [15]. It should be stressed though that the H_2O_2-mediated inhibition of other PTPs in T cells may instead directly promote kinase activity and induce signaling [61]. Indeed, there is a renewed interest in the role of H_2O_2 as a second messenger [121]. For instance, H_2O_2 may mimic the effects of an antigen, leading to stimulation of resting B cells. As BCR engagement leads to H_2O_2 production stimulating neighboring BCR in an antigen-independent, paracrine fashion, it was suggested that this results in a rapid amplification of the BCR signal [121]. Furthermore, anti-CD3 stimulation of mature T cells leads to production of ROS including hydrogen peroxide and superoxide anion [27]. It has been proposed that the oxidative burst by macrophages serves the sole purpose of stimulating T and B cells [121]. Along these lines, several papers have reported that hydrogen peroxide activates NF-κB in lymphocytes and induces production of IL-2 [134, 136]. However, other studies have shown that IL-2 production upon T-cell activation is independent of ROS production [27]. Recently a sharp analysis of the molecular mechanisms underlying ROS-induced signaling defects in hyporesponsive cells was reported [20]. T cells that were cocultured with activated neutrophils exhibited decreased expression of CD3ζ, $p56^{lck}$, and LAT. Another signaling molecule, PI3 K, was spared, indicating a differential effect of oxidative stress on different components of the T-cell signaling machinery. Two sequential effects of oxidative stress were described: protein modification and protein degradation. While $p56^{lck}$ and LAT were sensitive to protein degradation, CD3ζ was relatively resistant to degradation. In contrast, loss of the CD3ζ molecule was shown to be dependent on protein modification. This was demonstrated using two antibodies with distinct binding motifs on the CD3ζ molecule. When using an antibody recognizing the cytoplasmic domain of the molecule, CD3ζ expression was decreased in a dose-dependent manner by coculture with ROS-producing granulocytes. However, the recognition of CD3ζ by an antibody directed toward the membrane proximal domain was unaffected. Moreover, protein modification seemed to be the rate-limiting step for induction of hyporesponsiveness as function could not be restored by blocking degradation.

The complexity of redox regulation of immune responses and the dual properties of hydrogen peroxide as a second messenger and a negative regulator of immune reactivity are interesting. Perhaps several important properties of hydrogen peroxide can be attributed to its capacity to shut down protein tyrosine phosphatases, leading to activation or inhibition of signaling cascades depending on which tyrosine residues are available for the targeted PTP.

Induction of systemic immune suppression?

Reviewed above is some of the evidence suggesting that oxidative stress may play a role in tumor-induced immune suppression. The chain of evidence includes the role of hydrogen peroxide producing tumor-infiltrating macrophages/granulocytes, and one may argue that such effects could only operate locally in the tumor environment. As several reports also describe signs of immune suppression in peripheral blood of patients with advanced cancer, the mechanisms behind systemic immune suppression have been sought. In a recent study the existence of activated and H_2O_2-secreting granulocytes in PBLs correlated with functional and molecular defects in peripheral T cells of patients with advanced cancer [135]. The release of free radicals by granulocytes may be the principal mechanism for generating a state of persistent oxidative stress in cancer patients. For instance, it has been shown that colorectal cancer patients have lower serum concentrations of free radical scavengers such as vitamin E and C along with increased levels of 8-oxoGua, which serves as a DNA marker for oxidative stress [43]. Excluding the unlikely explanation that universally, patients and normal individuals had different eating habits, it may be concluded that the decreased levels of scavengers resulted from enhanced consumption by the state of oxidative stress present in the patients. One may not cure cancer by altering the

redox status in patients with malignancies. However, it may be very important to relieve patients from oxidative stress in order to make them responsive to immunotherapy with the ultimate goal of curing their disease.

Alleviating immune suppression with antioxidants

Epidemiological studies on the effect of dietary factors in prevention of cancer have largely centered on fat and vitamin intake. Evidence indicates that an increased intake of fat and red meat is associated with a higher risk of colorectal, breast, and prostate cancer [12]. In contrast, high consumption of fruits and vegetables is associated with a reduced risk of several cancers including, lung, oral, pancreas, larynx, esophagus, bladder, and stomach. A molecular explanation for this epidemiological observation is lacking, although it is likely that multiple components of the diet are responsible. A role for vitamin E in decreasing the incidence of prostate cancer was suggested in a large primary prevention study that is being followed up in the recently launched Study of Selenium and Vitamin E Cancer Prevention Trial (SELECT) [26, 53]. Vitamin E is a naturally occurring lipid-soluble antioxidant that acts as a scavenger and terminates the cascade of free radical formation induced by toxic and carcinogenic compounds [83]. It is found at high concentrations in membranes of T cells and is essential for normal immune function. Deficiency of vitamin E is associated with increased sensitivity to infection and incidence of tumors [9]. Supplementation of high doses of dietary vitamin E improves the compromised immune function in elderly and in patients with HIV infection [82, 99]. Similar results have been obtained in animal models of aging mice and rats, with evidence of enhanced proliferative capacity and IL-2 production after intake of vitamin E–enriched diets [148]. We recently investigated the possibility of enhancing the immune function in patients with advanced (Dukes' C and D) colorectal cancer by reconstituting their antioxidant defenses [90]. This was tested in a pilot clinical trial in which patients were given high doses of dietary vitamin E along with vitamin C and selenium at the recommended daily intake (RDA) doses for a period of 2 weeks. Vitamin C and selenium were included in the treatment regimen to recycle vitamin E to its reduced state and thus allow optimal function of vitamin E [9]. Colorectal cancer patients provided a suitable study group as several features of immune suppression were previously reported in these patients. For instance, they exhibit a decrease in signaling transduction molecules of peripheral T cells that is associated with severity of disease [94, 105]; there is evidence for reduced CD4 counts leading to altered CD4/CD8 ratios [5]; and Th1 cytokine production is significantly impaired [55]. Interestingly, surgical removal of the tumor burden leads to a normalization of the cytokine production capacity indicating that the immune suppression is specifically induced by the tumor [55]. In 10 out of 12 treated patients, we observed an increase in the number of T cells capable of producing IL-2 in response to PMA/Ionomycin [90]. The enhanced IL-2 production after vitamin E treatment may be the central mechanism behind the immune-stimulatory effects of vitamin E. IL-2 can reverse T-cell anergy and has a potent capacity to induce T-cell proliferation via both auto and paracrine loops acting at the IL-2 receptor expressed by activated T cells [137]. Decreased CD4/CD8 ratios have been associated with immunological dysfunction in patients with AIDS [109] and different types of cancer, including multiple myeloma [70], colorectal carcinoma [5], and Hodgkin's disease [63]. Increased apoptosis of $CD4^+$ T cells may also contribute to the transient immunodeficiency that follows allogeneic BMT [84]. The altered CD4/CD8 ratios in multiple myeloma and colorectal carcinoma correlated with the stage of disease, being more reduced in advanced stages [5, 70]. Others have demonstrated that TILs display a decreased CD4/CD8 ratio while the balance of lymphocyte subsets remained unaltered in peripheral blood [133]. Evidence from human and mouse studies, suggests that vitamin E increases CD4 counts [109]. In our study, vitamin E significantly increased the overall CD4/CD8 ratios in colorectal cancer patients. This may be of particular importance for the individuals with CD4 counts and CD4/CD8 ratios much below normal. Indeed, a stratified analysis of the five patients with CD4/CD8 ratios below the reference interval for healthy individuals revealed a significant increase after vitamin E treatment. $CD4^+$ T helper cells are essential for induction of $CD8^+$ cytolytic T-lymphocyte immunity due to their ability to drive the maturation of DCs [98]. There is experimental evidence that peptide-based vaccination may become more efficient if tumor antigen–specific $CD4^+$ T-cell–mediated responses are concurrently induced by including HLA class II tumor peptides in the vaccine [110]. The search for such HLA class II–restricted epitopes within previously known and new tumor-associated antigens has been intensified during the last couple of years [164]. Furthermore, CD4 T cells can have a more direct role in eliminating tumors during the effector phase [58]. For any of these mechanisms to be efficient, one is dependent on the presence of functional $CD4^+$ T cells. Antioxidants such as vitamin E may set the stage for efficient immunotherapy against cancer by inducing Th1 production and restoring CD4 counts. Future strategies may also involve genetic transfer of antioxidant genes such as catalase into adoptively transferred tumor-specific T cells to allow optimal function during the effector phase at the tumor site.

Conclusions

Since the description of the first tumor antigens by Pierre Van der Bruggen and Thierry Boon in 1991 [161], we have been facing a new era of tumor immunotherapy.

Along with a tremendous increase in the understanding of basic principles of immunity and novel technologies, we now have the tools to ask fundamental questions of immune-mediated tumor recognition in clinical trials. A better understanding of immune escape mechanisms and the mechanism underlying tumor-induced immune suppression will help in designing novel and more efficient protocols for immunotherapy. However, as for all currently available treatments for cancer, one will have to define the responsive patient groups (types of cancer), the optimal stage of disease, and the ideal combination with other treatment modalities. It is likely that different strategies of immunotherapy will be beneficial under different circumstances. The promising results from several clinical trials of adoptive immunotherapy including transfer of tumor-specific CTLs and the GVL effect of allogeneic BMT [21, 31, 128] indicate that treatment of advanced stages of cancer with large tumor burden may require adoptive transfer of high numbers of tumor-reactive T and NK cells.

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