Tumor-derived immune tolerance: A mechanism of tumor evasion

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Abstract
Although great progress has been made in developing immunotherapeutic interventions, little clinical success has been achieved. A reason for this may be related to the profound immune defects in patients with advanced cancer. Immune suppression mediated by tumors has been recently recognized.

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Tumor-derived immune suppression is mediated by multiple mechanisms including: i) alternative immune recognition; ii) secretion of soluble factors such as TGF-β, IL-10, and VEGF; iii) expression of immune-suppressive molecules PD-1L and FasL; and iv) release of exosomes that inhibit overall anti-cancer immunity. In particular, exosomes (tumor-secreted microvesicles) can act as systemic antigen-presenting agents capable of delivering death signals that play a critical role in specifically inhibiting anti-tumor immune responses.

Diversity of anti-cancer immunity

The era of molecular biology has allowed for gene-specific deletion in animals. This means that genes associated with immune responses can be “knocked-out” of animals as a strategy to study the importance of a specific gene in an in vivo setting. Speaking in very general terms, there are two pathways that the immune system can take when it is activated. The first type is called “Th1”, which is involved in destroying cells of the body that are infected from the inside, such as virally infected cells. The second type of immune response is called “Th2”, which is responsible for killing targets that reside outside of the cells of the body [1, 2]. Since cancer consists of cells of the body that have distinguishing properties from the other cells (i.e., high proliferation, ability to metastasize, etc.), it is intuitively plausible that the Th1 path of the immune response would be the one responsible for the control of cancer, if the immune response is involved at all. Indeed, Coley’s toxin (and its constituents) was identified early in the 20th century to be a potent inducer of the Th1 cytokine TNF-alpha, as well as an activator of this general immune response pathway [3, 4]. The discovery of sine qua non transcription factors mediating Th1 and Th2 immunity allowed the hypothesis that Th1 pathways are critical in cancer progression to be tested in animal models. Transcription factors for Th1 immunity are T-bet [5] and STAT4 [6], and for Th2 are GATA-3 [7] and STAT6 [8, 9]. When tumors of various kinds are grown as xenografts in STAT6 knockout animals (which are predisposed to Th1 responses due to the ablation of the STAT6-dependent Th2 pathway), these tumors are either spontaneously rejected [10], or immunity to them is achieved with much higher potency than in wild-type animals [11]. Furthermore, in STAT6 knockout animals, immunologic resistance to metastasis formation is observed [12]. On the other hand, STAT4 knockout mice (which lack STAT4-dependent Th1 capability and, therefore, have only Th2 immunity) have accelerated cancer formation after treatment with chemical carcinogens [13].
While the above suggests the importance of the Th1 immune response in controlling tumors, animal data does not always translate efficiently to the clinic. Accordingly, to better understand the implications of animal studies for human patients we turn our attention to situations where immune suppression is induced either by a spontaneous human genetic abnormality or in response to a medical condition. Generally speaking, natural killer (NK) cell activity is associated with Th1 immune responses and tumor immunity [14]. Patients with the congenital abnormality Chediak-Higashi Syndrome are characterized by absent or severely diminished NK function. In this population, the overall incidence of malignant tumors is 200-300 times greater than that of the general population [15]. Another example of an in-born trait associated with immune deviation is patients born with a specific polymorphism of the IL-4 receptor gene known to be associated with augmented Th2 responses. Multivariate regression analysis has shown that this polymorphism is an independent prognostic factor for shorter cancer survival and more advanced histo-pathological grade [16]. In addition to inborn genetic abnormalities, the immune suppressive regimens used to prevent transplant rejection are associated with selective inhibition of Th1 responses [17-19]. In support of the concept that suppression of Th1 immunity is associated with cancer onset, the incidence of cancer in the post-transplant population is markedly increased in comparison to controls living under similar environmental conditions [20-25]. In terms of disease associated immune suppression, HIV infected patients also have a marked predisposition to a variety of tumors, particularly (but not limited to) lymphomas, as a result of immunodeficiency [26].

Although the above examples support a relationship between immune suppression (or Th2 deviation and cancer proliferation) the opposite situation (immune stimulation resulting in anticancer response) is also documented. Numerous clinical trials using antigen specific approaches such as vaccination with either tumor antigens alone [27, 28], tumor antigens bound to immunogens [29, 30], tumor antigens delivered alone [31] or in combination with costimulatory molecules by viral methods [32], tumor antigens loaded on dendritic cells ex vivo [33-35], or administration of in vitro generated tumor-reactive T cells [36] have all demonstrated some, albeit modest, clinical effects. Furthermore, the influence of the immune system on cancer does not necessarily lead to regression. It is documented that inappropriate immune responses (broadly speaking, Th2 responses) can actually stimulate tumor growth [37, 38]. Accordingly, these data all support the presumed recognition of cancer by the immune system and the notion that the immune system, if stimulated properly, may induce cancer regression.
Immune recognition of cancer: “self” vs “non-self”

The concept that cancer is recognized by the immune system has been a topic of intense discussion for more than a century. Philosophically speaking, the argument revolves around one central aspect: since cancer originates from “self” tissue normally protected from immune attack, what is the basis for immune-targeting of tumor cells? The traditional concept of immunology, which teaches that the main purpose of the immune system is to distinguish between “self” and “non-self”, suggests that since cancer is “self” there should be no immune response against it. Current-day immunological advances, however, have struck down this notion.

The philosophical question posed at the beginning of this discussion (how can the immune system recognize cancer when cancer is part of “self”), is answered in the following manner. The immune system is not responsible for seeing only “self” versus “non-self” but actually seeing and responding to different variations of “self”. The tumor, in its quest for proliferative advantage, ability to metastasize, and need for formation of new blood supply, actually expresses new molecules at levels that are recognized by the immune system. Immunological recognition of molecules needed for the tumor to have the "cancer phenotype" has been well-documented. We will not provide a detailed overview of the data supporting this concept here but we will provide some examples. Specifically, the proliferative advantage of tumors is associated with growth factor receptor upregulation. Accordingly, immune responses to such receptors are known to exist naturally or to be inducible [39, 40]. The same is true for matrix metalloproteases involved in tumor extravasation and metastasis [41, 42], as well as for angiogenic factors involved in formation of new blood vessels [43, 44]. The question remains: if the immune system can recognize cancer cells, why does it not eradicate it? Further, why has the clinical implementation of cancer immunotherapy yielded such poor results at the bedside?

Cancer-derived immune suppression

The development of a successful immune response to cancer is hindered by numerous factors, including primarily the ability of the tumor to cause suppression of a productive host immune response to cancer. The interaction between the tumor and the immune system has been likened to pregnancy, in which an allogeneic graft (the fetus) rapidly develops without rejection by an immunologically competent host. The ability of the fetus to evade the maternal immune response of the mother is not due to anatomical barriers, since maternal immune cells have been shown to cross the placenta and enter the fetus [45]. What seems to occur in pregnancy is similar to the cancer patient in
that a selective depletion of immune components occurs, while other immunological parameters are left intact. Both in pregnancy and cancer a specific depletion of certain T cells occurs via numerous common mechanisms such as increased FasL activity [46-48]. Before elaborating on specific mechanisms by which FasL kills immune system cells, we will first discuss some of the historical work that led us to the notion that cancer suppresses the immune system.

Although great progress has been made in developing immunotherapeutic interventions, little clinical success has been achieved. A reason for this may be related to and illustrated by the example of the profound immune deficiencies associated with advanced prostate cancer patients. Immune suppression mediated by tumors has been extensively described. In the 1970s "blocking factors" were reported in the sera of cancer patients, which cause inhibition of lymphocyte function [49]. Factors secreted by tumors, including TGF-β, IL-10, and VEGF, have been shown to be crucial in tumor evasion of the host immune response [50]. Additionally, T cell apoptosis-inducing molecules such as FasL are found circulating in the plasma of tumor patients, and their appearance is correlated with poor prognosis [51]. Although this type of immune suppression is antigen-nonspecific, specific T cell deletion is also seen in patients (reviewed in [52, 53]). Antigen specific deletion implies: a) that the T cell receptor (TCR) interacts with tumor MHC and tumor antigen, and b) the tumor provides a death signal to the T cell. Such a signal could be an active death signal, such as tumor-expressed FasL, or absence of a survival signal, such as lack of co-stimulation [54, 55]. The question then arises of how the tumor can modulate systemic T cell function, especially in experiments in which the serum from cancer patients induces apoptosis in T cells bearing a cancer-specific TCR [56].

**Exosomes: Cancer secreted microvesicles that induce distant and systemic immune suppression**

Microvesicles, termed exosomes, have been shown to possess powerful immune stimulatory functions both *in vitro* and *in vivo* [57]. Dendritic cell (DC) and B cell exosomes contain high quantities of MHC I, MHC II, and CD86, the key components necessary to activate T cells [58, 59]. Administration of exosomes from tumor antigen-pulsed DCs into tumor-bearing mice results in potent priming of anti-tumor responses and tumor regression [57]. In contrast, exosomes secreted by intestinal cells, termed tolerosomes, are important in the antigen-specific tolerization associated with oral tolerance [60]. Tumor cells themselves have been shown to secrete
exosomes that possess MHC I and tumor antigen. These tumor-derived exosomes can be taken up by DCs in vitro and used as a potent source of tumor antigen for vaccination [61]. Exosomes derived in vitro from leukemia [62] and melanoma cells [63] express bioactive FasL, however, no studies have described FasL-expressing exosomes in clinical patient samples. The importance of FasL in antigen-specific elimination of T cells was functionally demonstrated in our previous work in which DCs transfected with FasL could induce antigen-specific tolerance in a murine model of transplantation [64].

Research independent of that described above identified other microvesicular-like structures, which were termed “exosomes”. Originally defined as small 80-200 nanometers in diameter, these exosomes were observed initially in maturing reticulocytes [65, 66]. Subsequently it was discovered that exosomes are potent mediators of communication between DCs and other antigen-presenting cells. Exosomes secreted by DCs were observed to contain extremely high levels of MHC I, MHC II, costimulatory molecules, and various adhesion molecules [67]. In addition, DC exosomes contain antigens that DCs had previously engulfed [68]. The ability of exosomes to act as “mini-antigen presenting cells” has stimulated cancer researchers to generate DCs containing tumor antigens (by “pulsing” DCs with those antigens), collect exosomes secreted by the tumor antigen-pulsed DCs, and use these exosomes for immunotherapy. Such exosomes were shown to be capable of eradicating established tumors when administered in various murine models [69, 70]. The ability of dendritic exosomes to potent ly prime the immune system raised the possibility that exosomes may also possess a tolerance-inducing or immune-suppressive role. Since it has been established that exosomes have a high concentration of tumor antigens, the further possibility arose that exosomes may induce tolerance through an abortive T cell activation process leading to anergy [71]. Specifically, it is known that numerous tumor cells, and exosomes derived from them, express the T cell apoptosis-inducing molecule Fas ligand [71-73].

The recent recognition that tumor-secreted exosomes are identical to the tumor secreted microvesicles described in the 1980s [74], has stimulated a wide variety of research into the immune suppressive ability of microvesicles. Specifically, immune suppressive microvesicles were identified not only in cancer patients [75, 76], but also in pregnancy [77-79], transplant tolerance [80, 81], and oral tolerance [82, 83] situations. Accordingly, one ideal method of stimulating the immune response of a cancer patient would be the removal of these immunosuppressive microvesicles from circulation through the use of an extracorporeal filter.
Isolation and detection of the exosomes secreted by cancer cells

For over 20 years, soluble factors secreted by tumor cells called “blocking factors” have been described in the serum of cancer patients. Hellstrom et al. showed that lymphocytes from cancer patients could inhibit the growth of autologous tumors only in absence of autologous serum. Serum from healthy controls, or allogeneic cancer patients, would not block tumor inhibition [84, 85]. The authors postulated that serum blocking factors, responsible for abolishing the ability of lymphocytes to inhibit tumor growth, were most likely MHC and tumor antigen. More recently, Maccalli et al. demonstrated that specific inhibition of healthy lymphocytes could be achieved by addition of serum from patients with melanoma. Interestingly, when antibody to MHC I was added to the serum, the suppressive effects were abrogated [56]. These observations led us to postulate that tumor-derived exosomes, which are known to possess both MHC I and tumor antigen, may cause the killing of T cells. Our previous study revealed that FasL is expressed on cancer exosomes. This strongly supports this hypothesis and implies a potential causative role [71].

![Figure 1](image_url)

**Figure 1. Time dependent production of exosomes.** The human prostate cancer cell lines DU-145, PC-3 and LNCaP were allowed to grow to 50% confluence in the media supplemented with 10% fetal calf serum. 10ml samples of media were extracted at the indicated timepoints, Exosomes were purified using a modification of the procedure described by zitvogel et al (10). Separation of the cellular debris was performed by centrifugation at 7,000g for half-hour followed by pelleting of the exosome through centrifugation at 100,000g for 3 hours, at which point it was then followed by one wash in PBS. Exosomal proteins were assessed using the Bradford Assay.
While it has been established that immunological cells secrete exosomes [86], it is uncertain whether prostate cancer cells also behave in this matter. Normal prostatic epithelial cells secrete microvesicular bodies, termed prostatosomes. These prostatosomes function to promote the mobility of spermatozoa [87]. In comparison to recent reports describing tumor-derived exosomes [57, 62, 63], we asked whether prostate cancer cells also secrete exosomes. The human prostate adenocarcinoma cell lines LNCaP, DU-145 and PC-3 were used for validating detection of exosomes produced by prostate cancer. LNCaP is derived from lymph node metastasis and is androgen-dependent, whereas DU-145 and PC-3 are androgen independent and are derived from brain and bone metastasis, respectively [88-90]. Purification of exosomes from tissue culture media of all three cell lines was performed by ultracentrifugation at 24, 48 and 72 hours of culture. A time-dependent increase in exosomal concentration was observed (Fig. 1), which indicated exosomes were actively secreted from the cell lines and were not the result of tissue culture artifact or fetal calf serum-derived exosomes.

**Tumor-derived exosomes inhibit T cell responses**

Microvesicles have been known to be secreted by tumor cells since the early 1980s, originally described by Dr Doug Taylor [91]. They were estimated to be between 50-200 nanometers in diameter and associated with a variety of immune inhibitory effects. Specifically, it was shown that such microvesicles could not only induce T cell apoptosis, but also block various aspects of T cell signaling, proliferation, cytokine production, and cytotoxicity [76, 92, 93]. Although much interest arose in the biology of microvesicles, little in the way of therapeutic applications have developed as a consequence since the microvesicles were relatively poorly characterized at the molecular level.

In order to demonstrate immunological activities of exosomes, we assessed the ability of exosomes derived from DU-145, PC-3 and LNCaP cell lines to modulate proliferation of PHA-stimulated T cells. The exosomes from in vitro activated lymphocytes was used as a non-cancerous control [94]. When purified exosomes from the cell lines were added to the activated T cells, a dose-dependent inhibition of proliferation was observed (Figure 2A). In contrast, addition of control lymphocyte exosomes had no effect on T cell proliferation (Fig. 2A). To exclude the possibility that fetal calf serum in the culture media may contain exosomes or exosome-like factors, we ultracentrifuged the culture media alone using the same exosome purification procedure and assessed the ability to alter T cell proliferation. There was no alteration of proliferation observed (data not shown).
In order to rule out the possibility that immune-inhibitory soluble factors were not responsible for the suppression of T cell proliferation, exosome-free ultracentrifuge supernatants from each of the above-mentioned cell lines were added to PHA-stimulated T cells. The exosome-free supernatants possessed no suppressive effects, however, with the exception of DU-145 supernatant which was found to stimulate T cell proliferation. These findings are in accord with our previous report [95] stating that the DU-145 cells secrete a T cell proliferation-inducing factor (Fig. 2B). Further confirmation of exosome-induced suppression of proliferation was performed by demonstrating that the inhibitory activity was not retained after passage of exosomes through 0.1 mm filters (data not shown).

**Figure 2. Inhibition of T cell proliferation by prostate cancer derived exosomes.**

**A.** Exosomes from prostate cancer cell lines inhibit proliferation of human peripheral blood T cells. Exosomes were purified as described in Figure 1. Protein concentration of exosomes was detected by the Bradford assay (Biorad). Exosomes were added at the indicated concentrations to PHA (10 µg/ml) activated T cells for 72 hours. Tritiated thymidine was added for the last 18 hours of culture at 1µC/well. Cells were subsequently harvested and thymidine incorporation was assessed by scintillation counting.

**B.** Exosome free supernatants from prostate cancer cell lines do not inhibit T cell proliferation. Supernatants of 48 hour incubated confluent PC 3, DU 145 and LNCaP cell lines, or control human T cells were collected and ultracentrifuged as described in materials and methods. The exosome free supernatants were added to cultures of PHA activated T cells for 48 hours. Tritiated thymidine was added for the last 18 hours of culture at 1 µC/well. Cells were subsequently harvested and thymidine incorporation was assessed by scintillation counting.
Tumor-derived exosomes induce apoptosis of activated T cells

Fas ligand is a type II membrane protein belonging to the TNF family whose expression is observed in a variety of tissues and cells, such as activated lymphocytes and the anterior chamber in the eye. Fas ligand, which induces apoptotic cell death in various types of cells, targets cells via its corresponding receptor, CD95/APO1. Fas ligand not only plays important roles in the homeostasis of activated lymphocytes but it has also been implicated in establishment of immune-privileged status in the testis and eye, and as a mediator of mechanism by which tumors escape immune mediated killing. Accordingly, and given the expression of Fas ligand on a variety of tumors, we and others have sought and successfully demonstrated that Fas ligand is expressed on exosomes secreted by tumor cells [71]. Due to the ability of exosomes to mediate a variety of immunological signals it was proposed that, at the beginning of the neoplastic process, tumor-secreted exosomes selectively induce antigen-specific T cell apoptosis by activating the T cell receptor. In turn, activated T cell receptor upregulates expression of Fas on the T cell. Subsequently, the Fas ligand molecule on the exosome induces apoptosis. This process may be occurring through a direct interaction between the tumor exosome and the T cell, or it may be occurring indirectly. A possible indirect interaction would be through tumor exosomes binding to DCs; then, as T cells subsequently associate with DCs in lymphatic areas, exosomes bound by DCs would form stable interactions with T cells through DC adhesion/costimulatory molecules. The stable interaction would lead to T cell-mediated apoptosis of tumor cells. In the context of more advanced cancer patients (where exosomes reach higher systemic concentrations than in patients with early stage tumors) the induction of T cell apoptosis occurs in an antigen-nonspecific but Fas ligand and MHC I-dependent manner.

Exosomes derived from Jurkat T cell lines and melanoma cells have been reported to induce T cell apoptosis [62, 63]. On the other hand, exosomes have been previously described to act as antigen-presenting vesicles and a potent source of tumor antigens [96]. However, to our knowledge, no assessment of immune-suppressive activities of exosomes has been performed from prostate cancer-derived cell lines. Accordingly, we assessed the capacity of prostate cancer-derived exosomes to promote apoptosis in primary T cells. A dose-dependent induction of apoptosis in PHA-activated T cells was observed with exosomes derived from cell lines representing both androgen dependent (LNCaP) and independent (PC-3, DU-145) prostate cancer (Fig. 3).
Figure 3. Exosomes from prostate cancer cell lines induce apoptosis in human peripheral blood T cells. Exosomes were purified as described in Figure 1. Protein concentration of exosomes was detected by the Bradford assay (Biorad). Exosomes were added at the indicated concentrations to PHA (10µg/ml) activated T cells for 72 hours. Apoptosis was assessed using annexin-V staining and analyzed by flow cytometry.

Summary

Overall immune suppression is seen in cancer patients [98]. Attempts to enhance anti-tumor immunity have been pursued through the use of drugs, cytokines, and vaccines. However, little therapeutic efficacy has been successfully achieved in clinical trials. Amongst the mechanisms underlying tumor progression, immune suppression appears to be a critical factor in preventing cancer destruction by the host immune system. Thus, tumor induced immune suppression remains a formidable obstacle to the success of immunotherapy. Cancers may inhibit immune responses in antigen-specific and non-specific manors.

Exosomes are small vesicles containing high concentrations of MHC I and tumor antigens. They have been successfully used to induce anti-tumor immunity in animal models. On the other hand, tumor-derived exosomes can also mediate T cell suppression. In this chapter we describe a novel mechanism of cancer-mediated immune suppression by which FasL present on the surface of the tumor exosome induces T cell apoptosis. These findings could open the door to the concept that modulation of tumor-derived exosomes in cancer patients (possibly by removal of exosomes from the plasma of cancer patients by ultrapheresis or other methods) may be a strategy to reverse or ameliorate cancer-mediated immune suppression.
References


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