Influence of organ site and tumor cell type on MUC1-specific tumor immunity


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Abstract

We investigated the influence of organ-specific parameters on tolerance and immunity to human MUC1. C57Bl/6 mice (wild-type) and C57Bl/6 transgenic for MUC1 (MUC1.Tg) were challenged in the pancreas with Panc02-MUC1, a C57Bl/6-syngeneic pancreatic cancer cell line expressing human MUC1. Wild-type mice produced immune responses to MUC1 when presented on tumor cells growing in the pancreas; however, the responses to tumors in the pancreas were less effective than responses produced by tumor challenge at the s.c. site. Tumor immunity specific for MUC1 was produced in wild-type mice by two different procedures: (i) s.c. immunization of wild-type mice with a low dose of Panc02-MUC1 or (ii) adoptive transfer of spleen and lymph node cells harvested from wild-type mice previously immunized s.c. with Panc02-MUC1. This demonstrates that immune responses to MUC1 presented at the s.c. site can be detected and adoptively transferred. MUC1.Tg mice were immunologically tolerant to MUC1; however, some immunological protection against orthotopic challenge with Panc02-MUC1 was conferred by adoptive transfer of CD4+ and CD8+ T cells from wild-type mice. These results show that it is more difficult to produce immune responses to tumors growing at the pancreatic site than the s.c. site. Panc02-MUC1 cells growing in the pancreas were accessible to the immune system, and immune responses evoked by s.c. presentation of this molecule in wild-type mice were effective in rejecting tumor cells in the pancreas of both wild-type and MUC1.Tg mice. No effective anti-tumor immune responses against MUC1 were produced in MUC1.Tg mice.

Introduction

The prognosis for pancreatic cancer remains unfavorable (1). Surgical procedures are effective against early lesions that have not metastasized; however, virtually all patients with pancreatic cancer have non-operable disease at diagnosis. Chemo-therapy is ineffective and radiotherapy is of limited curative value. Immunotherapeutic strategies including the use of defined tumor vaccines are under investigation as adjunctive therapies for treating pancreatic cancer (2–5). One candidate target antigen for use in pancreatic cancer vaccines is MUC1, a human epithelial mucin expressed on secretory epithelia of the pancreas and other organs. Most pancreatic tumors overexpress MUC1 (6). In addition, MUC1 expressed by pancreatic tumor cells is aberrantly glycosylated compared to normal epithelia of the pancreatic duct, as evidenced by the appearance of tumor-associated oligosaccharide structures such as sialyl Tn (7). This has led us and others to undertake a series of experiments to determine the feasibility of developing tumor vaccines that produce immune responses against forms of MUC1 expressed on pancreatic cancer cells that may not be present on normal ductal epithelia.

One factor often not considered during evaluation of tumor vaccines is organ site of the primary tumor or metastatic lesions. The pancreas has a microenvironment that differs from other organ sites, and includes unique features of cellularity, vascularity and lymphatic accessibility. Moreover, the pancreas is both an endocrine and exocrine organ that produces locally high concentrations of a number of hormones, cytokines and secretory products related to digestion. The influence of these locally produced factors on tumor immunity responses, including the ability of immune effector cells and molecules to penetrate and kill tumors at different sites, is poorly understood. Most previously described models...
to evaluate tumor immunotherapy utilize s.c. sites of tumor challenge or lung colonization following i.v. injection of tumor cells. These organ sites (s.c. and lung) are unusual from the immunological perspective in that they contain relatively high numbers of antigen-presenting cells, they are frequented by circulating lymphocytes, and they are well provisioned with lymphatics and blood vessels. It is not known if mechanisms of immunity that protect against tumors at s.c. sites are effective at organ-specific locations such as the pancreas.

Another factor not widely investigated is the influence of immunological tolerance on immune responses to tumor antigens that are also expressed on adjoining normal cells in the pancreas. The inter-relationships between tolerance and immunity, normal anatomical and cellular expression patterns of antigens, and expression of antigens on tumors are not well understood. For example, MUC1 is expressed on the apical side of normal ductal epithelia of pancreas and other secretory epithelia, an anatomical location that can be considered to be external to the body and possibly sequestered from antibodies and circulating immune effector cells. The accessibility to circulating lymphocytes of MHC molecules expressed on ductal epithelia is not well described. Disruption of the differentiated structure of the pancreas by a developing adenocarcinoma (which is believed to be derived from the ductal epithelia) results in the cell surface exposure of antigens and MHC molecules to the interorgan space in which immune cells may traffic. Released or secreted antigens such as MUC1 may enter circulation, bind to other cells in the region of the tumor, and affect immune responses and other local biological processes.

In humans, it has been impossible to investigate the interplay between parameters of tumor growth, immunological tolerance and the development of anti-tumor responses. It has also been impossible to evaluate the development of autoimmune responses that may result from effective vaccination with antigens that are common to tumors and normal cells. Thus, we developed animal model systems to investigate organ-specific effects on tolerance and tumor immunity. One model of pancreatic tumor growth includes orthotopic injection of a syngeneic pancreas cancer cell line into the pancreas of C57Bl/6 mice (8), which can be used to evaluate tumor immunity to MUC1 in the context of tumor growth in the microenvironment of the pancreas. A second (complementary) model system utilizes a C57Bl/6 strain of mice that is transgenic for human MUC1 and congenic to wild-type C57Bl/6 mice (9), which allows us to investigate immunity and tolerance to MUC1 in the context of an animal strain that shows normal temporal and spatial expression of MUC1.

In the study reported here, we investigated by in vivo methods the nature of the immune response that rejects MUC1-expressing pancreatic tumor cells at both s.c. and orthotopic sites. We established that MUC1-specific CD8+ cells are required to reject pancreatic tumors at s.c. sites and that these CD8+ cells can reject pancreatic tumors growing in the pancreas. Furthermore, MUC1 transgenic mice are immunologically tolerant to MUC1 and demonstrated that immunity against pancreatic tumors expressing MUC1 could be adoptively transferred to the MUC1 transgenic mice from wild-type mice immunized at the s.c. site.

### Methods

**Mice**

C57BL/6 female mice (8–10 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME). MUC1.Tg C57BL/6 (MUC1.Tg) mice were previously described (9). Female MUC1.Tg mice were obtained from a breeding colony at the University of Nebraska Medical Center.

**Tumor cells and injection procedures**

The pancreatic tumor cell line Panc02, which is syngeneic to C57BL/6 mice, was obtained from Dr. J. Nelson (University of Texas M. D. Anderson Cancer Center) (10). This cell line was maintained in McCoy’s 5A media with 10% FBS and no antibiotics. Panc02 was transfected with an expression vector for the human MUC1 cDNA (7). Cloned lines constitutively expressing MUC1 were selected as described previously (8). A cell line expressing high levels of MUC1 was used in the studies reported here and is designated Panc02-MUC1. A cloned control cell line transfected with vector alone was named Panc02-neo.

Subcutaneous tumor challenge was performed as previously described (9). Briefly, 1×10⁶ tumor cells (unless otherwise indicated) were injected s.c. and tumor growth was quantified by calculating tumor volume over time. The experimental endpoint (death) was defined as the time point at which tumor volume reached 1 cm³, whereupon the animals were euthanized.

Orthotopic injection of the tumor suspension into the pancreas was performed as previously described (8). Briefly, suspensions of 1×10⁵ tumor cells were prepared by harvesting the cultured cells in vitro and resuspending the cells in McCoy’s 5A media with no additives. Mice were anesthetized and the pancreas was exposed by traction through a median incision in the upper abdomen. Then 30 μl of the tumor suspension was injected into the gastric lobe of the pancreas. The abdomen was then closed in two layers. The experimental endpoint (death) was defined as the time point at which mice developed a distended abdomen due to ascites or exhibited moribund behavior, at which time the animals were euthanized and examined. Tumor growth of 1 cm³ or greater in these animals was confirmed by gross and microscopic examination of euthanized animals.

Statistical differences between survival for all groups of animals were calculated using the log-rank test.

**Cellular adoptive transfer**

Spleens and axillary lymph nodes were harvested from donor mice that were previously challenged s.c. with 1×10⁶ cells of Panc02-MUC1. Spleens and lymph nodes were manually and mechanically processed to yield a single-cell suspension. Aliquots equivalent to one-half of the total cell suspension from one donor mouse were injected i.p. into each recipient wild-type or MUC1.Tg mouse using established procedures (11). One day later, an orthotopic injection of tumor cell suspension containing 1×10⁵ cells of Panc02-MUC1 or Panc02-neo was performed.

**Depletion of CD4+/CD8+ T lymphocytes**

Rat anti-mouse hybridoma clones GK1.5 (anti-CD4), 53-6.72 (anti-CD8) and SFR3-DR5 (control antibodies) were
Results

Immunity and tolerance to MUC1 expressed on Panc02-MUC1 cells at different organ sites

Data presented in Fig. 1(a) demonstrate that Panc02 tumors expressing MUC1 are rejected by a substantial proportion of wild-type C57Bl/6 mice (70%) challenged at the s.c. site, whereas control Panc02 cells expressing only the product of the neomycin-resistance gene grow progressively in a majority of wild-type mice. MUC1.Tg mice allowed progressive tumor growth for both Panc02-MUC1 and Panc02-neo, resulting in survival curves that were indistinguishable from each other and from the survival curve of wild-type mice challenged with Panc02-neo. These data demonstrate that MUC1 is an immunodominant xenoantigen when expressed on Panc02 cells. MUC1-specific immune responses in wild-type C57Bl/6 reject Panc02-MUC1 tumors at the s.c. site. MUC1.Tg mice are immunologically unresponsive (tolerant) to MUC1. The data showing equivalent growth rates for Panc02-neo and Panc02-MUC1 in MUC1.Tg mice suggest that MUC1 expression has not affected the growth rates or properties of these tumors in vivo.

Data presented in Fig. 1(b) demonstrate that both Panc02-neo and Panc02-MUC1 tumor cells show growth properties in the pancreas that are more aggressive than those observed at the s.c. site. Similar to the s.c. site, Panc02 tumors growing in the pancreas of wild-type mice elicited MUC1-specific immune responses that were not observed in the MUC1.Tg mice.

Subcutaneous immunization of wild-type mice with MUC1-expressing tumor cells protects against subsequent intrapancreatic tumor challenge with Panc02-MUC1

Wild-type mice were immunized s.c. with $1 \times 10^5$ Panc02-MUC1. Three weeks later, these mice and control mice (no immunization) were challenged intrapancreatically with $1 \times 10^5$ Panc02-MUC1 cells (Fig. 2a) or Panc02-neo cells (Fig. 2b). Data presented in Fig. 2(a) shows that mice immunized with Panc02-MUC1 developed no tumors at the pancreatic site, whereas non-immunized mice developed tumors and showed poor survival after orthotopic challenge with Panc02-MUC1 ($P < 0.0001$). Thus, s.c. immunization with Panc02-MUC1 provided protection against subsequent challenge with Panc02-MUC1 in the pancreas.

The data presented in Fig. 2(b) demonstrate that immunity to Panc02-MUC1 was specific for MUC1, since s.c. immunization with Panc02-MUC1 did not provide significant protection against intrapancreatic challenge with Panc02-neo: there was no statistically significant ($P = 0.09$) difference in the survival curves shown in Fig. 2(b). Thus, Panc02 expressing the neomycin-resistance gene remains poorly immunogenic and the tumor immunity observed in the data presented in Fig. 2(a) is primarily due to MUC1-specific responses.

Adoptive transfer of immune cells protect wild-type mice against orthotopic tumor challenge

Wild-type donor mice were immunized s.c. with $1 \times 10^5$ Panc02-MUC1 cells. Three weeks later, spleen and lymph node cells from these mice were adoptively transferred to wild-type recipient mice. Adoptive transfer of non-immune cells from an unmanipulated group of mice to negative control (recipient) wild-type mice was also performed. One day following adoptive transfer, mice in both groups were challenged in the pancreas with $1 \times 10^5$ cells of Panc02-MUC1 (Fig. 3a) or Panc02-neo (Fig. 3b). As shown in Fig. 3(a), a degree of tumor immunity was conferred on the recipient
mice as evidenced by significantly improved survival in the
group of mice that received the adoptive transfer of cells from
immune animals, as compared with the group of mice that
received adoptive transfer of naive cells (P = 0.0044). This
shows that adoptive transfer of immune cells from wild-type
donors is effective in protecting wild-type recipients against
intrapancreatic challenge of Panc02-MUC1.

Data presented in Fig. 3(b) demonstrate that adoptive
transfer of cells from Panc02-MUC1-immunized mice con-
ferred immunity specific for MUC1, since no immune
protection was afforded mice challenged with Panc02-neo.
In addition, the data in Fig. 3(b) demonstrate that the immune
protection resulted from immunization with Panc02-MUC1
cells, since adoptive transfer of naive cells had no effect on
survival. Taken together, these results show that adoptive
transfer of immune cells into naive wild-type mice can be
used to detect MUC1-specific tumor immunity in vivo.

Adoptive transfer of immune cells from wild-type mice prolong
survival of MUC1.Tg recipient mice challenged orthotopically
with Panc02-MUC1

The data shown in Fig. 1 suggested that MUC1.Tg animals
were immunologically tolerant to Panc02-MUC1 tumor cells.
It was of interest to determine if tumor immunity could be
adoptively transferred from wild-type animals into MUC1.Tg
mice. Wild-type donor mice were immunized s.c. with 1×10^6
Panc02-MUC1 cells. Three weeks later, immune cells from
spleens and lymph nodes of these wild-type donors were
adoptively transferred to MUC1.Tg recipient mice. A negative
control for this experiment was adoptive transfer of cells from
non-immunized wild-type mice into a group of MUC1.Tg
recipient mice. On the following day, recipient mice were
challenged in the pancreas with 1×10^5 Panc02-MUC1 (Fig. 4a)
or Panc02-neo cells (Fig. 4b). As shown in Fig. 4(a),
the group of MUC1.Tg mice that received an adoptive
transfer of immune cells from wild-type donors had significantly better
survival than a control group of mice that received an adoptive
transfer of naive cells (P = 0.045). This demonstrates that
adoptive transfer of immune cells from wild-type donors
provides some immunological protection to MUC1.Tg recipi-
ents against intrapancreatic challenge of Panc02-MUC1. As
shown in Fig. 4(b), for animals challenged with Panc02-neo
there was no significant difference between the survival of
recipients of adoptive transfer of cells from wild-type donors
and survival of mice that received an adoptive transfer of
naive cells. Thus, adoptive transfer of immune cells into
MUC1.Tg recipients conferred MUC1-specific tumor immunity
and not immunity to other tumor-associated antigens
expressed on Panc02 cells.

Adoptive transfer of immune cells from MUC1.Tg mice was
not effective in prolonging survival of wild-type mice

The data in Fig. 1 and previously published data (9,11)
support the hypothesis that MUC1.Tg mice are immunolo-
gically tolerant to MUC1; however, the mechanism of tolerance
Influence of organ and cell type on immunity to MUC1

Fig. 4. Adoptive transfer of immune cells from wild-type donors improved survival of MUC1.Tg recipients challenged orthotopically with Panc02-MUC1. Wild-type donor mice were immunized s.c. with 1×10^6 Panc02-MUC1 cells. Three weeks later, immune cells from spleens and lymph nodes of these wild-type donors were adoptively transferred to ‘MUC1.Tg recipient’ mice. A negative control for this experiment was adoptive transfer of the cells from non-immunized wild-type mice into a group of ‘MUC1.Tg’ mice. On the following day, all mice were challenged in the pancreas with 1×10^5 Panc02-MUC1 (a) or Panc02-neo (b) cells.

Both CD4^+ and CD8^+ T cells play a role in cell-mediated immunity against Panc02-MUC1 cells in the pancreas

Antibody-mediated immune responses are ineffective for rejection of Panc-02-MUC1 tumors (8). Thus, we hypothesized that a population of T cells was responsible for MUC1-specific tumor rejection. Depletion of specific T cell populations was accomplished by i.p. injection of wild-type mice with antibodies against CD4 and/or CD8 using established procedures, as described in Methods. Cellular depletion of specific T cell populations for each group of mice was confirmed by flow cytometric analysis of one mouse per group, 1 day before intrapancreatic tumor challenge. The results indicated that <1% of CD4^+ and/or CD8^+ cells remained in depleted mice, demonstrating that depletion was effective (data not shown). As shown in Fig. 6(a), mortality from tumor growth was greater but did not achieve statistical significance for CD4-depleted (P = 0.113) mice and it was statistically greater for CD8-depleted (P = 0.0439) mice, as compared to control mice. The group depleted of both CD4^+ and CD8^+ cells showed the poorest survival (P = 0.0097) suggesting that tumor growth and mortality in mice depleted of both CD4^+ and CD8^+ cells was similar to that of MUC1.Tg mice. This
investigated immune responses to the tumor-associated anti-peptides associated with class II MHC molecules. The CD8^+ cells were required for rejection of Panc02-MUC1 tumors in the pancreas, whereas CD4^+ cells are not required for rejection of Panc02-MUC1 at the s.c. site. Antibodies against CD4^+ and/or CD8^+ (see Methods) depleted T cell populations in wild-type mice. Mice were challenged with Panc02-MUC1 in the pancreas (a) or at the s.c. site (b). The results of adoptive transfer of cells from animals previously challenged with Panc02-MUC1, which improved survival of recipient wild-type mice challenged with Panc02-MUC1 (Fig. 3). No increase was observed in survival of recipient wild-type mice challenged with Panc02-neo, demonstrating that immune responses to non-MUC1 tumor antigens are a minor factor in the anti-tumor responses. In addition, adoptive transfer of immune cells from donor wild-type mice previously immunized with Panc02-MUC1 prolonged survival of MUC1.Tg mice. Control MUC1.Tg mice that received cells from unprimed wild-type animals were unresponsive to MUC1.

The molecular phenotypes of cellular responses to Panc02-MUC1 in wild-type mice were investigated by performing antibody-mediated T cell depletion studies. The results showed that CD8^+ cells were required for rejection of Panc02-MUC1 tumors at the s.c. site. Elimination of CD4^+ cells did not significantly reduce immunity to MUC1 expressed on Panc02-MUC1 in the pancreas, whereas CD4^+ cells were required for rejection of Panc02-MUC1 tumors growing in the pancreatic environment.

Discussion

One goal of our studies was to understand parameters that contribute to effective vaccination against pancreatic cancer. There are no previously reported studies that investigated the influence of the pancreatic organ site on immunity to a specific tumor antigen in the context of immunological tolerance, which is one subject of this report. Orthotopic implantation of tumor cells in mice provides an improved model for growth of tumor cells that includes rapid growth of local tumor and the development of spontaneous metastases (12). We investigated immune responses to the tumor-associated antigen MUC1, which has been a component of several human tumor vaccine trials. MUC1 is a useful target of immunotherapy because it is overexpressed in most pancreatic tumors (6) and it is aberrantly glycosylated by tumors as compared to corresponding normal tissues (7,13).

We asked whether immunization at the s.c. site would protect mice against tumors growing in the pancreas. In wild-type mice, injection of Panc02-MUC1 cells into the pancreas resulted in tumors with aggressive growth properties (Fig. 1) that were less immunogenic than corresponding tumors grown at the s.c. site. Panc02-MUC1 tumor growth was more aggressive in MUC1.Tg mice (Fig. 1), because of immunological tolerance to MUC1 (10). Subcutaneous challenge with Panc02-MUC1 cells produced an immune response to MUC1 that protected against subsequent intrapancreatic challenge (Fig. 2) in wild-type mice, suggesting that immune responses to MUC1 were capable of rejecting tumors growing in the pancreatic environment.

We sought evidence of a cellular immune response to MUC1 expressed on Panc02, in light of previous findings suggesting that antibody responses do not protect against pancreatic tumor growth (8). Similar to previous findings using the B16 melanoma cell line (11), in vitro assays of cytotoxic T cell activity were not informative regarding the tumor immune status of mice challenged with Panc02-MUC1 (data not shown). Evidence of MUC1-specific cellular immunity is seen in the results of adoptive transfer of cells from animals previously challenged with Panc02-MUC1, which improved the survival of recipient wild-type mice challenged with Panc02-MUC1 (Fig. 3). No increase was observed in survival of recipient wild-type mice challenged with Panc02-neo, demonstrating that immune responses to non-MUC1 tumor antigens are a minor factor in the anti-tumor responses. In addition, adoptive transfer of immune cells from donor wild-type mice previously immunized with Panc02-MUC1 prolonged survival of MUC1.Tg mice. Control MUC1.Tg mice that received cells from unprimed wild-type animals were unresponsive to MUC1.

The results in contrast to previous results obtained when the B16.MUC1 melanoma cell line was used for tumor challenge at the s.c. site (11). CD4^+ but not CD8^+ cells were required for rejection of this melanoma cell line (11). This response was not detected by in vitro assays, but instead was detected by in vivo assays of tumor rejection following adoptive transfer of immune cells and/or in vivo antibody depletion of selected cellular populations. Although the mechanism of CD4^+-mediated rejection of B16.MUC1 cells is not known, it is likely that CD4^+ effector cells recognize MUC1 peptides associated with class II MHC molecules. The CD8^+ effector cells that mediate rejection of Panc02-MUC1 cells are predicted to recognize antigen in the context of MHC class I molecules. Taken together, these results suggest that
distinct mechanisms of cellular immunity to the specific tumor antigen MUC1 are produced at the s.c. site and that the type of response produced to MUC1 is in part determined by the tumor on which the antigen is expressed.

It should be noted that our findings regarding CD8-mediated tumor immune responses to MUC1 cells are consistent with those of other investigators (14), who observed that CD8+ T cells were responsible for MUC1-specific tumor immunity against a different tumor cell line challenged at the s.c. site (15,16). Interestingly, CD4+ cells (17) reactive with MUC1 have also been detected by in vitro assays of CTL activity from human patients with different adenocarcinomas, although the in vivo activity of these cells has not been established.

The cells that mediate rejection of Panc02-MUC1 tumors at the pancreatic site were also evaluated by antibody-mediated T cell-depletion studies (Fig. 6). Both CD4+ and CD8+ T cells contributed to MUC1-specific tumor immunity at the pancreatic site of wild-type mice (Fig. 6). The findings that CD4+ and CD8+ cells are required for rejection at the pancreatic site but that CD8+ cells are sufficient for rejection of Panc02-MUC1 at the s.c. site support the hypothesis that immune rejection of the same tumor at different organ sites requires different populations of effector T cells. The mechanisms of cytotoxicity utilized by these T cells and the parameters that contribute to their development will be determined in future studies.

In contrast to findings with wild-type mice, we were unable to induce immune responses against MUC1 in MUC1.Tg mice by directly challenging the animals with tumors. Subcutaneous immunization of MUC1.Tg mice with irradiated Panc02-MUC1 cells did not protect against subsequent intrapancreatic challenge with Panc02-MUC1 cells (data not shown). Adoptive transfer of immune cells from MUC1.Tg donors into wild-type recipients had no protective effect against subsequent orthotopic injection with Panc02-MUC1, in spite of the fact that MUC1.Tg mice produce cytotoxic cells that are detectable by in vitro assay (11). This suggests that transferring the cells into a non-transgenic environment cannot abrogate mechanisms of tolerance which are operative in the MUC1.Tg mice. A recent report has suggested that it is possible to break tolerance to MUC1 in MUC1.Tg mice by immunizing with vaccines comprised of fusions of tumor cells and dendritic cells (18). The evaluation of these and other vaccination strategies in the MUC1.Tg mice is in progress, including genetic modification of tumor cells to express cytokines to enhance the immunogenicity of tumor antigens (19–21).

In summary, we have shown that immune responses elicited to MUC1 expressed on a pancreatic carcinoma in C57BI/6 mice are more effective when presented at the s.c. site as compared to the pancreatic site. Immunization with Panc02-MUC1 at the s.c. site protected against tumor challenge at the pancreatic site; however, there were differences in the nature of the cells required for tumor rejection at these sites. CD8+ cells are required for rejection of Panc02-MUC1 pancreatic tumors at the s.c. site, in contrast to previous findings that CD4+ cells are required for rejection of B16.MUC1 melanoma cells at the same site. Both CD4+ and CD8+ cells are required for rejection of tumors at the pancreatic site. Differences in the effectiveness of distinct T cell effector populations may be caused by several different factors, none of which are well understood in the context of different organ sites. The local environment at different organ sites may affect the properties of tumors that grow at those sites. For example, factors that may be present at locally high concentrations in the pancreas (cytokines, hormones, cellular interactions) may up-regulate or down-regulate expression of molecules on the tumor cells that are related to antigen recognition or immune destruction of the tumor cells (MHC class I and II, Fas ligand, and receptors for tumor necrosis factor and TRAIL). Alternatively, the activity of some T cells or accessory cells required for immune responses may be altered by such a microenvironment (22). These parameters of tumor immunity should be investigated further in future studies and principles gleaned from these studies should be applied to clinical trials in humans.

**References**

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