

Focus on TILs: Prognostic significance of tumor infiltrating lymphocytes in human bladder cancer

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The idea of generating cytotoxic T-lymphocytes that have anti-tumor activity has been the focus of many clinical trials aimed at delivering effective immunotherapy to cancer patients. We have gained insight into the human immune system in cancer patients as a result of these numerous clinical investigations. It is now clear that although various vaccination methods are capable of inducing tumor antigen-specific T-cells in the circulating blood, these immunological responses are infrequently correlated with clinical responses. Therefore, it appears that priming of a T-cell response is not sufficient for tumor regression and other avenues, downstream of the priming phase, need to be investigated. Mechanisms of immune evasion at the effector phase of the anti-tumor phase are currently under investigation, with an increasing focus on the tumor microenvironment. There is evidence indicating that multiple variables may contribute to immune escape, including: regulatory cells; inhibitory ligands on tumor cells, such as PD-L1 and B7x; soluble factors such as TGF- β and IL-10; and nutrient-catabolizing enzymes, such as indoleamine-2,3-dioxygenase (IDO). In addition, there are ongoing efforts to assess the presence and function of effector cells within the tumor microenvironment. Tumor infiltrating lymphocytes (TILs) have been observed in patients with melanoma, colon cancer, and ovarian cancer. TILs in these patients have been associated with favorable clinical outcomes. In the clinical setting of bladder cancer, as compared to melanoma, there is limited data regarding TILs. This review will focus on immunological responses to bladder cancer and ongoing studies to identify factors that are amenable to therapeutic manipulation.

Keywords: human, bladder cancer, tumor infiltrating lymphocytes, prognosis

Bladder cancer

Bladder cancer, the majority of which are diagnosed pathologically as transitional cell carcinoma (TCC) or urothelial carcinoma (UC), is the only solid tumor other than melanoma for which immunotherapy confers a survival benefit. The tumors of the bladder can be classified depending on their depth of invasion. The bladder lining consists of a mucous layer of surface cells, called transitional epithelial cells or the urothelium that expand and deflate depending on the volume of urine present inside the bladder. Disease that affects only the urothelial lining can be papillary and designated as pTa disease. Underneath the urothelium is a thin layer of connective tissue, also called the *lamina propria*, and tumor in this area is designated as pT1 disease. The next layer is a wider zone of muscle tissue called the *muscularis propria*, with disease involvement designated as pT2. Beyond this muscular zone, another layer of fatty connective tissue separates the bladder from other nearby organs and disease extending to this

perivesical fat is designated as pT3. Disease beyond the bladder and extending to nearby organs or structures is designated as pT4 (Figure 1A). Disease that has spread to distant sites from the bladder is labeled as metastatic.

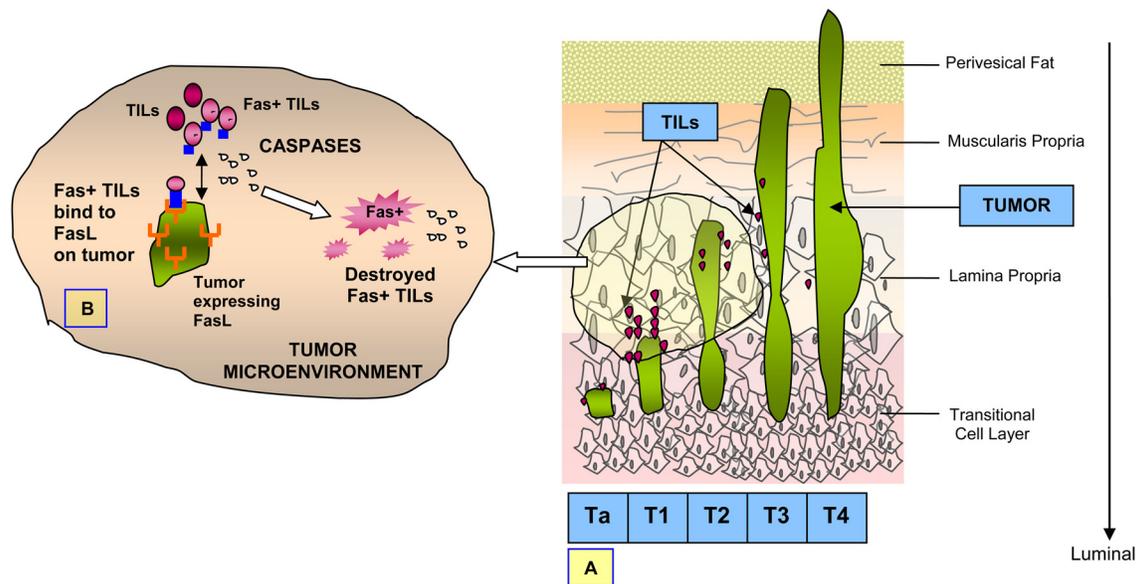
In the setting of non-muscle invasive disease, intravesical bacillus Calmette-Guérin (BCG) is pivotal as an immunological agent in the treatment of early TCC and leads to a cure rate of approximately 90% in select patients, such as those with unifocal and pathological T1 lesions. Intravesical BCG has been used to treat superficial TCC for more than 20 years; randomized trials have shown that BCG results in lower recurrence rates and significantly superior survival rates (1). Although the mechanism of action of BCG is poorly understood, scientific evidence continues to implicate an immunological mechanism. BCG plays a significant role in the maturation of dendritic cells (DCs) by signaling through different Toll-like receptors, as indicated by its upregulation of the DC maturation marker CD83 (2) and secretion of inflammatory cytokines such as IL-12, IFN- γ , and TNF- α . A variety of cytokines has been detected in the urine of patients treated with intravesical BCG, and BCG causes an influx of granulocytes and mononuclear cells into the bladder wall (3-10). Although the significance of these infiltrating cells remains controversial, there are ongoing studies to characterize these cells and assess their functional status.

Immunological alterations that occur with the development of bladder cancer

Apart from the physiological changes in and around the bladder as a result of tumor development, there are several notable immunological changes that occur simultaneously. Changes may be phenotypic in nature, such as the loss or addition of certain molecules on the cell surface of the urothelial cells, or changes may be related to the functionality of certain molecules.

Blood group antigens, major histocompatibility complex (MHC) antigens, and other cellular adhesion molecules which are expressed on the cell surface of normal cells tend to have variable expression on bladder cancer cells. For example, decreased expression and incomplete biosynthesis of certain proteins in the family of blood group antigens have been noted (11-12). It has also been observed that the loss of blood group antigens increases irreversibly with tumor progression. The class I human leukocyte antigen (HLA) in humans, otherwise known as MHC class I antigen, is normally expressed on the urothelial cell surface; however, in urothelial malignancies, it has been observed that there is a loss of HLA class A, B and C expression (13-16). Such a loss of MHC class I antigen expression also directly correlates with tumor progression. Some other molecules that play a role in immuno-oncology and cellular adhesion events also appear to be diminished in their expression

Figure 1



Bladder cancer and TILs. (A) Urothelial carcinomas can be classified as pTa (papillary), pT1 (lamina propria invasion), pT2 (muscle invasive), pT3 (invasion to perivesical fat), and pT4 (locally advanced). (B) Interaction of FasL on tumor cells and Fas-positive T-lymphocytes leads to elimination of Fas-positive TILs, which appears to be a caspase-mediated event.

in the initial stages of bladder malignancies. Molecules such as CD44 and ICAM-1, which normally promote the adhesion and activation of T-lymphocytes, seem to have decreased expression on bladder cancer cells (17-18).

Furthermore, certain subpopulations of circulating immune cells appear to be associated with clinical outcome in UC patients. Innate immune cells, known as natural killer (NK) cells, obtained from circulating peripheral blood mononuclear cells of bladder cancer patients were studied in 67 patients and 29 healthy donors. A decreased NK cell activity in TCC patients was observed as compared to healthy controls and furthermore, it was noted that disease recurrence was associated with decreased NK cell activity (19).

Another factor which appears to impact the immunological response to bladder cancer is cellular architecture. In one study (20), the impact of physical changes in cellular architecture on autologous T-lymphocyte activation was examined. Two autologous bladder carcinoma cell lines were used as targets for T-lymphocytes. In an effort to compare the effect of cell architecture of these targets on the overall cytokine production levels, the cell lines were grown with varying cellular geometry including a two-dimensional monolayer and a three-dimensional spheroid. Interestingly, it was observed that there was a reduction in cytokine production by T-cells after interaction with the three-dimensional structure of tumor cells as compared to the two-dimensional structure of tumor cells. The data implied that information obtained from a two-dimensional structure of the cells in an *in vitro* study may not be comparable to *in vivo* occurrences as mimicked by the three-dimensional tumor microenvironment. Cellular architecture also appears to play a role in the immune response generated in the tumor microenvironment.

Tumor infiltrating lymphocytes in the tumor microenvironment

Tumor infiltrating lymphocytes (TILs) are lymphocytes of the host immune system that have been observed within tumor sites; presumably they migrate to the tumor site in order to combat the growing malignant cells. They are normally activated T cells, natural killer cells and non-T or non-B lymphocytes. These lymphocytes can be physically characterized by cluster differentiation and certain surface-antigen groupings. However, the phenotypic characteristics and the T-cell populations in cultured TILs can be affected by the components of culturing media as demonstrated by Haas *et al.* (21) and Housseau *et al.* (22). In the study by Haas *et al.* (21), the isolation and expansion of human TILs from urological malignancies, including testicular, bladder, and prostate cancer, were examined. The initial TIL population was mainly comprised of CD3+ T-cells, with subpopulations of CD4+ and CD8+ T-cells. The authors observed that over time, the ratio of CD4+ and CD8+ subpopulations varied as a consequence of culture conditions. In the study by Housseau *et al.* (22), human TILs from primary urothelial carcinomas were characterized. The cytolytic properties of these TILs were examined by the establishment of short-term autologous tumor cell lines for obtaining neoplastic targets with minimal phenotype modifications in comparison with fresh tumor cells; five pairs of autologous TIL/tumor cell pairs were examined. Four of the five TIL cultures manifested lysis against their autologous counterparts and three of these four cytotoxic TIL lines demonstrated MHC class I-dependent cytotoxicity, as established by blocking experiments with anti-HLA class I mAb W6.32. One of these three TIL lines, which demonstrated high levels of cytotoxicity, was further examined and was found to have numerous CD8+ T-cells. The depletion of CD4+ cells from this culture indicated that CD8+ MHC class I CTLs were the predominant effectors.

Even though these studies demonstrated that there are effector cells in urothelial carcinomas that are capable of specific cytotoxic activity against tumor cells, the fact remains that these effector cells are incapable of suppressing tumor growth *in vivo*. Some studies have proposed activation-induced cell death (AICD) to be a major cause of death of TILs, thus explaining their inability to control tumor growth (23-25). Other studies have proposed that cancers can become "immune privileged" sites by expressing Fas-ligand (26-28). Fas-ligand (FasL) is a cell surface protein of the tumor necrosis factor family that induces apoptosis on Fas-bearing cells when FasL binds to Fas. Fas-mediated apoptosis is dependent on the activation of different members of a family of cysteinylaspartate proteases called caspases, including caspase-8, -9, and -3, which are responsible for both the initiation of the apoptotic cascade and the execution of the cell damage. Interaction of FasL with Fas+ TILs has been demonstrated to lead to cell death (29). Therefore, expression of FasL by tumor cells may provide a mechanism by which cancer cells escape eradication by TILs (Figure 1B).

In a recent study by Chopin *et al.* (30), FasL expression was observed in 45% ($n = 45$) of TCC samples, while expression of FasL was not observed in normal urothelium ($n = 20$). A correlation existed between FasL expression and high tumor grade and stage (13% in superficial Ta-T1 versus 81% in invasive T2-T4; $P < 0.0001$). Two primary bladder TCC cell lines, established from two FasL-positive invasive bladder TCC tissues, were shown to specifically mediate FasL cell death in two conventional Fas-sensitive T lymphocyte targets. Further analysis of one of these cultures demonstrated its ability to induce FAS-mediated killing of autologous T-lymphocytes *in vitro*. Fas-mediated apoptosis of IFN- γ -producing CD8+ TILs was evident by the detection of activated caspases -8, -9, and -3 expression on these cells, which were found in close association with FasL-expressing TCC cells *in situ*. In short, these results suggest that TCC-expressed FasL may induce apoptosis of anti-tumor T-lymphocytes *in vivo* and may provide new insights into the mechanisms that allow for the suppression of TIL immunological function.

In addition, signaling defects in TILs have also been implicated in the dysfunction of lymphocytes at the tumor site. These include loss of signal-transducing zeta (ζ) chain (31) or reduction in the epsilon chain of p56, Zap70 and p59 expression (32-33). In some experiments, the lost ζ chain was seen to be reversible after treatment with IL-2 (34). Another signaling mechanism that emerged quite recently and which could potentiate the down regulation of TIL proliferation and activation is an alteration in the signaling pathways of IL-2/IL2R (35). It has also been observed that cancer-derived matrix metalloproteinases (MMP) play an important role in cleaving IL2R α chain, thus resulting in host immunosuppression, tumor metastases, and lymphovascular invasion by interfering with the signaling pathways of IL-2 (36).

Other studies indicate that the enzyme indoleamine-2,3-dioxygenase (IDO) is responsible for creating an immunosuppressive environment. IDO, an enzyme classically known for its role in the tryptophan degradation pathway, has recently emerged as an important immunomodulator of T-cell function and inducer of tolerance. The induced expression of IDO by dendritic cells may suppress T-cell responses and promote tolerance either through direct effects on T cells (mediated by tryptophan depletion or tryptophan metabolites) or through effects of IDO on the dendritic cell. Friberg *et al.* (37) observed that the enzyme IDO is responsible for metabolizing tryptophan found on antigen presenting cells, thus

compromising their efforts in anti-tumor cytotoxicity. Furthermore, it has been found that the enzyme IDO is produced by tumor cells in order to resist attacks by the host immune system (38) and thus weaken or even nullify the effects of potential immunosurveillance mechanisms.

Furthermore, it appears that a mechanism of "cancer immunoediting" may be responsible for the development of tumors that are resistant to immune-mediated events. Shankaran *et al.* (39) demonstrated that the immune system may promote the emergence of primary tumors with reduced immunogenicity that are capable of escaping immune recognition and destruction. The hypothesis of cancer immunosurveillance, which states that sentinel thymus-dependent cells of the body constantly survey host tissues for nascently transformed cells, has been demonstrated in several studies (40-42). However, since immunosurveillance represents only one dimension of the complex relationship between the immune system and cancer, the hypothesis of cancer immunoediting was proposed to encompass the various interactions, including elimination, equilibrium, and escape, that occur between the host immune system and developing cancer cells. In the elimination phase, the immune system is able to eradicate developing tumor cells; however, as the immune system continues to exert pressure on the tumor cells, escape variants occur and are able to persist in a state of equilibrium, whereby the immune system keeps further development of these cells at a minimum until finally, the escape phase occurs when the cancer cells have accrued sufficient mutations that enable evasion of the immune system (42).

Therefore, there are many mechanisms which appear to contribute to the inefficiency of TILs in their ability to eradicate tumor cells. Deeths *et al.* (43) proposed that certain tumor cell mechanisms, such as activation-induced non-responsiveness (AINR), may possibly induce CD8+ TILs death, thus jeopardizing the cytotoxic abilities and functions of the host immune system. Other mechanisms, such as down regulation of HLA class I molecules, loss of co-stimulatory molecules, and removal or blocking of tumor antigens by tumor cells also result in immune escape by tumor cells. Contrastingly, some investigators have also explored the possible inherent reasons for the inhibition or the inability of CD8+ TILs to counter the antagonistic tumor cells. Two mechanisms have been proposed: a defect in the cytolytic pathways or an overexpression of inhibitory molecules. Recent studies by Sheu *et al.* (44) illustrate that the down-regulation of perforin completely blocks the cytotoxic mechanisms of TILs. Additionally, they also found that inhibitory NK receptors such as CD94/NKG2A can be up-regulated on TILs by cytokines such as IL-15 and TGF- β which are derived from cancer cells. This can be a further contributing factor for the inertness of cytotoxic TILs in their response towards tumor cells. All of these mechanisms can be seen as potential targets for novel immunotherapy agents that are aimed at overcoming the suppressive and regulatory factors within the tumor microenvironment.

TILs as a prognostic factor in bladder cancer

An early study by Mostofi *et al.* (45) reported a favorable prognosis with higher number of TILs present in TCC. Later, a prolonged recurrence-free survival rate was correlated with an increased number of T-zone histiocytes in TCC indicating a competent host immune system as a favorable factor (46). According to another study (47, 48), denser TILs were associated with invasive (pT3-T4) TCC tumors as opposed to

superficial papillary tumors. It appears that a higher prevalence of TILs is associated with a favorable response, even in the setting of a more invasive disease.

In another study by Tsujihashi *et al.* (49), a comparison of the functional activities of TILs and peripheral blood lymphocytes (PBLs) from 22 bladder cancer patients led to the conclusion that a higher number of TILs was associated with better clinical outcome and fewer occurrences of tumor metastases. TILs and PBLs were isolated from the patients and used against a natural killer (NK) myeloid leukemic line, myeloid K562, a fresh bladder tumor, and a cultured bladder tumor, HT1197, as their targets *in vitro*. The amount of lymphocytes in TILs and PBLs was measured by flow cytometry and showed a predominantly higher cell population of T-cells present in both PBLs and TILs. A ⁵¹Cr release cytotoxicity assay revealed that there was a spontaneous response to the NK target cells by the PBLs which was of 23.4%, compared to the response by the TILs which was of 3.5%. But when the TILs and PBLs were cultured with an immunomodulating cytokine, IL-2, it was noted that immune responses by both the PBLs and TILs were enhanced. However, interestingly, there was a tumor-specific response by TILs which was not observed for the PBLs. The cytotoxic response of IL-2-treated TILs was higher against autologous bladder cancer tumors than against the artificially cultured tumor tissue, HT1197. Therefore the presence of IL-2, a lymphokine known to be released from helper T-cells for the generation of cytotoxic T-cells, boosted the TIL cytotoxic response to the tumor tissues *in vitro* (49-50). These data continue to support the concept that TILs are capable of effective immunity against tumor cells when the appropriate immunomodulatory agents are present.

In another study by Lipponen *et al.* (51), tumor tissues from 514 patients with TCC were analyzed for a correlation between TILs, recurrence-free survival and metastases. Biopsy specimens from tumor tissues were analyzed by histological staining in order to categorize the tumors as papillary or nodular. The quantification of TILs was categorized as: weak or absent; moderately populated; or densely populated. In pTa-pT1 staged, papillary grade tumors, a higher density of TILs indicated an unfavorable prognosis, and these patients were noted to have a lower survival rate as compared to similarly staged patients with a lower density of TILs. In pT3-pT4 staged nodular tumors, a higher rate of survival was observed with a higher density of TILs around the tumor site, predicting a favorable prognosis. Recently, we analyzed the presence of intratumoral CD8 T cells, the expression of MHC class I antigen and the expression of the NY-ESO-1 tumor antigen in UC samples and correlated our findings with clinical outcome. Immunohistochemical staining for intratumoral CD8 T cells in tissue samples from 69 patients with UC showed that patients with advanced UC (pT2, pT3, or pT4) and higher numbers of CD8 TILs within the tumor (8 or more) had better disease-free survival ($P < 0.001$) and overall survival ($P = 0.018$) than did patients with similar-staged UC and fewer intratumoral CD8 TILs (52).

Conclusions and future directions

The presence of tumor infiltrating lymphocytes in bladder cancer is associated with a favorable prognosis but is not sufficient to overcome inhibitory changes within the tumor microenvironment over a prolonged period of time. Migration of lymphocytes from the circulating immune system to the tumor site implies that the host immune system is capable of initiating an anti-tumor response. Unfortunately, changes that

occur as a result of mutations within tumor cells eventually create an immunosuppressive tumor microenvironment that prevents tumor eradication by TILs. Nevertheless, new findings and research on TILs and the tumor microenvironment are ongoing in efforts to reverse the multitude of immunosuppressive mechanisms.

The rational development of immunotherapy applicable for patients with solid tumors, analogous to that used for molecularly targeted therapy, is possible. Improvements in the understanding of immune regulation in the context of cancer, as well as novel agents that are able to target specific immune regulatory pathways, and evolving methodology to characterize human cancer at the cellular and molecular level will result in more informative clinical studies. The knowledge gained through the proposed integration of tumor biology with immunology will be the foundation for the effective application of immunotherapy to the treatment of solid tumors. Our ongoing studies in bladder cancer include utilization of a novel anti-CTLA4 monoclonal antibody aimed at releasing the inhibitory mechanisms on T-cells so as to enhance T-cell activity against tumor cells. We are currently enrolling bladder cancer patients onto a phase I neoadjuvant clinical trial with anti-CTLA4 and will conduct in-depth immunological studies on the obtained tumor tissues to characterize the various TIL populations and their function as compared to TILs obtained from untreated tissues. These types of studies will continue to provide valuable information regarding the significance of various subpopulations of TILs and therapeutic agents that will enhance anti-tumor responses by TILs in bladder cancer patients.

Abbreviations

BCG, bacillus Calmette-Guérin; IDO, indoleamine-2,3-dioxygenase; TCC, transitional cell carcinoma; UC, urothelial carcinoma

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