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EDITORIAL

# Pancreas Cancer and the Role of Soluble Immunoglobulin-Like Transcript 3 (ILT3)

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## Summary

Attempts to ameliorate the poor prognosis of pancreatic cancer have been largely unsuccessful. Interventions to enhance patients' immune responses to malignancies have been also largely unsuccessful. We now describe an immune-escape mechanism mediated by the inhibitory receptor immunoglobulin-like transcript 3 (ILT3) which may be responsible for such failures. Using a humanized severe combined immunodeficiency (SCID) mouse model, we demonstrate that soluble and membrane ILT3 induce $CD8^+$ T suppressor cells and prevent rejection of allogeneic tumor transplants. Furthermore, we found that patients with carcinoma of the pancreas produce the soluble ILT3 protein, which induces the differentiation of $CD8^+$ T suppressor cells and impairs T cell responses in mixed lymphocyte culture. These responses are restored by anti-ILT3 mAb or by depletion of sILT3 from the serum. Immunohistochemical staining of biopsies from the tumors and metastatic lymph nodes suggest that $CD68^+$ tumor associated macrophages represent the major source of soluble ILT3. Alternative splicing, resulting in the loss of the ILT3 transmembrane domain may contribute to the release of ILT3 in the circulation. These data suggest that ILT3 depletion or blockade is crucial to the success of immunobiotherapy, particularly in pancreatic cancer.

## Introduction

The treatment of patients with advanced pancreatic cancers remains a serious challenge. The outcome for this conditions is extremely poor, with the median survival of patients treated with the best supportive care within approximately 3 to 6 months [1].

Single-agent gemcitabine is still the standard treatment for advanced pancreatic cancer, which has demonstrated some improvement in disease-related symptoms and a little benefit in survival. Gemcitabine in combination with other chemotherapeutic agents as gemcitabine/oxaliplatin and gemcitabine/capecitabine has in recent phase III trials shown some encouraging results.

The same agent (gemcitabine) in combination with bevacizumab and cetuximab in recent Phase II trials showed no benefit. "How can we change this bleak landscape?" wrote Dr. Saif in a recent article [2].

The answer is probably in studying therapeutic interventions which involve immunologic approaches. Maybe the solution is in immunobiotherapy in pancreas cancer. Probably by precisely targeting our therapy with the epidermal growth factor receptor agents as well as other biological agents by identifying those patients who are most likely to derive benefits and achieve meaningful response. Development of novel concepts and new agents are urgently needed.

This manuscript will examine a potential target for immunobiotherapy of pancreas

cancer and the mechanisms through which tumors evade destruction by immune effector cells, and possible strategies for overcoming immune escape by tumor cells so that immune self defense against cancer cells will effectively function.

## Immune Response to Pancreas Cancer

A complete understanding of the general molecular events that define the evolution of pancreas cancer is vital to the development of specific targeted therapies. Development of tumor targeted vaccine will serve someday as a major preventive measures or as adjuvant in chemotherapy. Vaccine may be most effective during early stages in tumor development, before tumors in their microenvironment evolve protective mechanisms to evade immune cells that could kill them. Mechanisms of tumor evasion from immune attack must be better understood, and blocked in order for immune cells to effectively reach the tumor cells and kill them [3].

There is an emerging recognition that tumor growth elicit specific immune responses mediated by $CD8^+$ and $CD4^+$ T reg cells, which participate in innate and adaptive immunity, that may delay progression and be potentially harnessed to eradicate malignant disease [4].

Many innovative approaches have focused on the activation of $CD4^+$ Th capable to provide the help required for the growth and differentiation of tumor specific cytotoxic T cells. Because activated dendritic cells are highly efficient in stimulating immune response numerous clinical studies have used dendritic cells loaded in various ways with tumor associated antigens, dendritic cell vaccines, to induce CD8 and CD4 responses against complexes formed by tumor specific peptides with MHC class I and class II molecules [5]. However, most immuno-therapy trials have met with limited success, failing to demonstrate significant clinical responses [6]. It is believed that combination of immunotherapy such as adoptive transfer of in vivo primed T cells and post-transplant vaccination may foster enhanced memory T cell responses [7, 8].

## Mechanisms of Escape from Immune Surveillance

Unfortunately, the theory of “natural selection or survival of the fittest” applies to tumor cells as to the intact organism. Survival of the cancer cells is affected by the entire host and particularly by the micro-environment surrounding the tumor in which act the natural and adaptive immune defenses. As the tumor cells continue to evolve, the “strongest” ones survive because they have developed means to render anergic the immune system. To metastasize, cancer cells have to acquire several dozen genetic alterations, in contrast with the handful typically necessary to initiate a primary tumor [9]. Pancreas cancer arise in the inside lining of the organ: to metastasize the tumor cells must break cellular bonds to dislodge itself, break down the tenacity of the connective tissue, change shape and sprout “legs” that can pull it trough the densely packed tissue. After accomplishing this escape, the metastatic cell passes through a capillary into the blood stream, where rotated and can be ripped apart by the force of circulation or can passes into the lymphatic network. In the lymph nodes the tumor cells encounter the immune cells and can be destroyed or can survive. Finally the surviving cells develop a micrometastasis and face an hostile environment before to develop into a macrometastasis.

Several are the mechanisms induced by tumor cells to escape from immune surveillance: decreased MHC expression, decreased antigen presentation, produced by soluble Muc1 mucin (antigen CA-15-3), induction of T cell anergy, deletion or tolerance, produced by TGF-beta or TGF-alpha, induction of $CD4^+$ Tregs or $CD8^+$ suppressor T cells, inhibition of NK-cell activity, increased ligand expression for phosphatase domain 1 (PD1) (B-7) family, defective signaling through death receptor ligands as Fas ligand (FASL) and TNF-related apoptosis-inducing

ligand (TRAIL), lack of appropriate costimulation and production of immunosuppressive cytokines [10, 11, 12].
In recent years the role of tumor associated macrophages has been extensively investigated. Tumor associated macrophages have been proposed as a particular phagocyte population that is committed to produce high levels of IL-10, exhibits little toxicity for tumor cells and promotes tumor-cell proliferation. Furthermore, IL-10 may inhibit the tumoricidal capacity of macrophages by suppressing the production of many pro-inflammatory molecules responsible for killing of the cancer cells. In this paper we will describe another important function of these cells.

## Role of Soluble Immunoglobulin-Like Transcript 3 (ILT3) in Cancer

In our laboratory we have discovered the inhibitory effect of serum and membrane ILT3 which represent an additional mechanism that contributes to impaired T cell responses in patients with pancreas and colon cancer.
In previous studies, we demonstrated that chronic *in vitro* stimulation of human T cells with peptide-pulsed autologous antigen presenting cells or with allogeneic antigen presenting cells resulted in the generation of MHC class I restricted $CD8^+$ T suppressors that inhibit the activation and effector function of Th and cytotoxic T cells with cognate specificity [13, 14]. Alloantigen specific $CD8^+$ T suppressors induce the up-regulation of ILT3 and ILT4 on monocytes and dendritic cells, rendering them tolerogenic. ILT3 and ILT4 are inhibitory receptors Ig-like transcript 3 and 4.
We recently demonstrated that the extracellular region of ILT3 is endowed with immunomodulatory properties. Both membrane-bound ILT3 (mILT3) and soluble ILT3(sILT3) inhibited T cell proliferation in mixed lymphocyte culture (MLC), anergizing $CD4^+$ Th cells, suppressing the differentiation of IFN-gamma producing $CD8^+$ cytotoxic T cells, and inducing the differentiation of alloantigen-specific $CD8^+$ T suppressors in primary 7-day MLC [15].
In view of recent studies unraveling the role of Tregs/T suppressors and tolerogenic dendritic cells in tumor growth and metastasis, we explored the possibility that mILT3 and sILT3 participate in the induction of T cell anergy and differentiation of Tregs cells in patients with cancer [16, 17, 18, 19].
Using a hu-severe combined immunodeficiency (SCID) mouse model we found that recombinant ILT3-Fc (rILT3-Fc) protein and mILT3 inhibit T cell mediated rejection of human tumor allografts and induce the differentiation of allospecific $CD8^+$ T suppressor cells.
However, $CD4^+$ T cells from the same tumor-bearing hu-SCID mouse had no regulatory activity. This finding is consistent with our previous in vitro studies in which we found that $CD4^+$ T cells allostimulated in the presence of soluble or membrane ILT3 had no Treg activity, yet because they became anergic, they were unable to provide the help required for functional differentiation of IFN-gamma producing $CD8^+$ cytotoxic T cells. Instead, alloactivated $CD8^+$ T cells from these cultures differentiated into T suppressors, which acted in an allorestricted manner on priming antigen presenting cells inducing them to upregulate the inhibitory ILT3 receptor [20].
Since soluble ILT3 inhibits the rejection of allogeneic human tumors, it is apparent that, if present in sera from patients with cancer, it may have a similar effect on autologous tumors even if they express tumor associated antigens.

## Experience in Cancer Patients

The inhibitory activity of sera from cancer patients on the reactivity of autologous T cells or of T cells from healthy individuals has long been known [21, 22]. Elevated IL-10 serum levels were shown to correlate with poor clinical outcome [23]. More recently there has been increasing evidence that sera from patients with malignant disease contain soluble forms of NKG2D ligand which can

potentially impair NKG2D-mediated immune function by blocking NKG2D receptors on natural killers and T cells [24, 25, 26].

Our study showed that soluble ILT3, present in a relatively high percentage of patients with various malignancies including a large cohort of patients with pancreatic cancer, inhibited strongly T cell responses in MLC. This inhibitory effect was partially abrogated by anti-ILT3 mAb indicating that it was caused by soluble ILT3. Furthermore, T cell allostimulation in cultures containing $sILT3^+$ sera from cancer patients resulted in the differentiation of allospecific $CD8^+$ T suppressor cells, consistent with the results obtained in the hu-SCID mouse model.

It is now believed that the main obstacle tempering successful immunotherapy and active vaccination resides in the immunosuppressive effect displayed by regulatory $CD4^+CD25^+$ regulatory T cells which provide a crucial tumor evasion mechanism [16, 27].

Stage related increases in the frequencies of $CD4^+CD25^+$ regulatory T cells were reported in numerous malignancies [27].

$CD8^+$ T suppressors act in an antigen specific manner inhibiting the T cell priming capacity of the antigen presenting cells with which they interact or display their regulatory activity by producing IL-10, (similar to $CD4^+CD25^+$ Tr1 cells) have been described both in human and in rodents [28, 29, 30, 31, 32, 33, 34, 35]. Their contribution to tumor escape from immunosurveillance, however, has received less attention although the high frequency of non-cytolitic tumor associated antigen specific $CD8^+$ T cells found in patients with metastatic melanoma may reflect the presence of T suppressors rather than Tc effector cells [36, 37].

Our present results indicate that membrane and soluble ILT3 may promote the differentiation of $CD8^+$ T suppressors within the tumor microenvironment or in sentinel lymph nodes. We found intensive membrane ILT3 staining of tumor associated $CD68^+$ macrophages in colorectal and pancreatic carcinoma as well as in melanoma. The frequency of ILT3 expressing macrophage in tumor infiltrated lymph nodes was also much higher than that seen in normal lymph nodes (Figure 1).

There is an increasing evidence that tumor associated macrophages activated by the immunosuppressors and IL-10 (known to induce ILT3) play an important role in tumor progression and metastasis [38]. Tumor associated macrophages were shown to possess poor antigen-presenting capacity, suppress T cell activation, and secrete a wide range of growth and proangiogenic factors as well as metalloproteinases. It has been

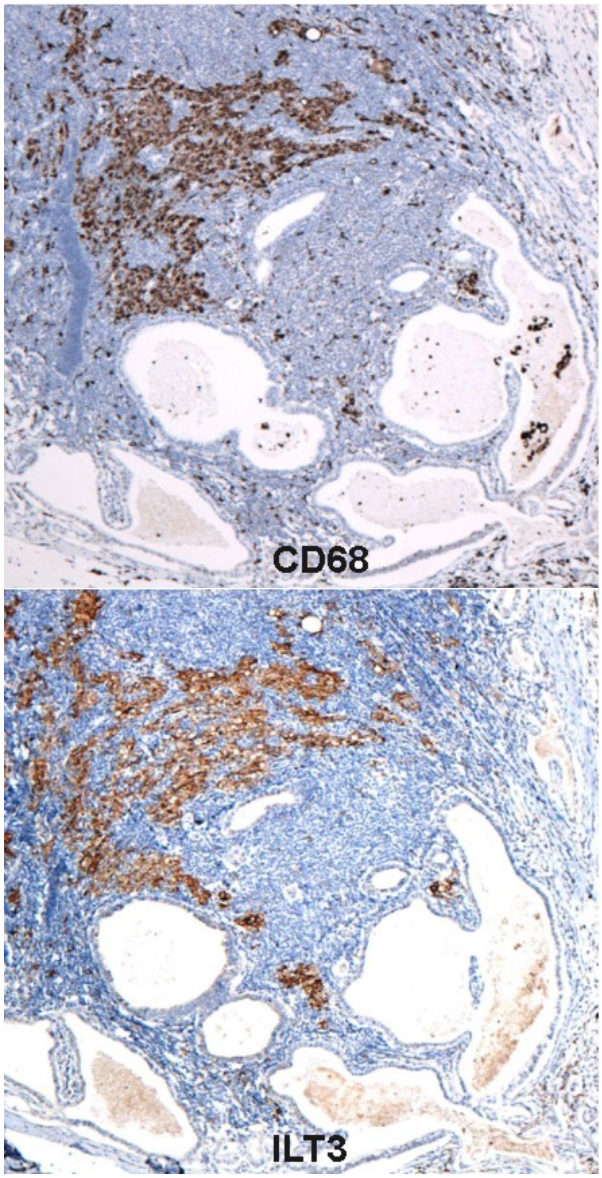


**Figure 1.** Immunohistochemical staining of macrophages with anti-CD68 and anti-ILT3 antibodies in a lymph node from a patient with metastatic pancreas carcinoma.

suggested that cytokines present in the tumor microenvironment have the potential to induce the differentiation of recruited macrophages into tumor associated macrophages which, in turn, produce growth factors and extracellular matrix enzymes facilitating tumor proliferation and invasion of surrounding tissue [39, 40]. Recently, a population of tumor associated macrophages, characterized by B7-H4 expression and capacity to suppress tumor associated antigen-specific T cell immunity, was identified in human ovarian carcinoma [40]. Also in non small cell lung cancer macrophage expression of IL-10 was found to correlate with stages. IL-10$^{+}$ tumor associated macrophages were higher in patients with stages II, III and IV and in those with lymph nodes metastases as compared to patients with stage I non-small cell lung cancer [41]. These are M2 macrophages as described by Mantovani *et al*. [38].

The mechanism(s) leading to the generation of soluble ILT3 is currently unknown. By analogy to NKG2D ligand is possible that soluble ILT3 production is associated with post-translational proteolytic cleavage. An alternative, not mutually exclusive mechanism, is that sILT3 is encoded by alternative splice variants lacking exon 7 which corresponds to the transmembrane domain. This latter possibility is supported by our finding of ILT3 gene transcript lacking exon 7 in malignancies in which large amounts of sILT3 was detected by ELISA. Other immunoregulatory receptors such as cytotoxic T cells A-4 and phosphatase domain 1 have also been shown to have alternative variants which encode soluble forms of the protein, lacking the transmembrane domain [42, 43].

Whatever the mechanism of soluble ILT3 production might be, our data suggest that neither tumor vaccines nor adoptive therapy with tumor associated antigen-specific T cells is likely to be successful in patient with high levels of ILT3.

We conclude that plasmapheresis with immunoabsorption of serum ILT3 may be necessary as a preliminary step before active or passive (adoptive) immunotherapy is initiated in patients with cancer or as immune-enhancing treatment before chemotherapy.

Furthermore, identifying and blocking the ligand of ILT3 on activated T cells may offer new strategies to enhance T cell immunity in cancer and this new approach could be very important for pancreas cancer's treatment.

**Keywords** LILRB4 protein, human; Macrophages; Pancreatic Neoplasms; Receptors, Cell Surface; T-Lymphocytes, Regulatory; Tumor Escape

**Abbreviations** ILT3: immunoglobulin-like transcript 3; mILT3: membrane-bound ILT3; MLC: mixed lymphocyte culture; rILT3-Fc: recombinant ILT3-Fc; SCID: severe combined immunodeficiency; sILT3: soluble ILT3

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