Immune Signatures of Murine and Human Cancers Reveal Unique Mechanisms of Tumor Escape and New Targets for Cancer Immunotherapy

Rebecca E. Sadun, Suzanne M. Sachsman, Xiaoying Chen, Kamilee W. Christenson, William Z. Morris, Peisheng Hu, and Alan L. Epstein

Abstract

Purpose: Despite lymphocyte infiltration of tumors and the activation of tumor-draining lymph nodes, malignant tumors are able to "escape" from both innate and adaptive immune responses. For immunotherapy to be successful, it must reverse these escape mechanisms, which necessitates explicit and tumor-specific elucidation of tumor escape strategies.

Research Design: To identify relevant escape mechanisms in murine tumors and in two corresponding human cancers, real-time reverse transcription-PCR was used to measure a panel of genes associated with T-cell activation and inhibition pathways.

Results: Comparative analysis of the expression levels of these immunomodulatory genes showed astonishing similarities in expression patterns between murine and human breast cancers but profound variability in the expression of immunomodulatory genes in colorectal cancers. For human ductal adenocarcinoma of the breast, down-regulation of dendritic cell maturation marker CD83 and T-cell activation gene CD28 was observed as well as a notable increase in the expression of the immunoinhibitory gene B7-H4. By contrast, colorectal adenocarcinoma cases showed high variability in tumor escape mechanisms, indicating a need to produce immune signatures for individual patients to identify appropriate immunotherapeutic targets.

Conclusions: These results show that certain tumors, such as ductal carcinoma of the breast, show consistent immunologic abnormalities that can be used as targets for immunotherapy. These findings also show the importance and feasibility of determining the immune signatures of patients’ tumors to select appropriate immunotherapeutic strategies. Ultimately, these results advocate for the determination of immune signatures as part of the customary repertoire of clinical diagnostics for cancer.

Immunotherapy defines the process by which immune mediators are able to recognize and attack tumors (1, 2). The ability of the immune system to recognize and destroy tumor cells is supported by observations of spontaneous T-cell responses in human malignancies (3), spontaneous CTL-induced tumor regression (4), and the involvement of innate immune effectors in the identification and elimination of tumor cells (5). Nevertheless, although the immune system may on occasion succeed in eliminating nascent tumor, immunoediting places a strong selection pressure on tumor cells. Because tumors are often genetically unstable (6), immunoediting confers a survival advantage on tumor cells, enabling them to develop strategies for evading the immune system. Consistent with the multihit model of oncogenesis (7), the acquisition of tumor escape mechanisms may be a necessary “hit” for a malignancy and is hence considered by many to be “the seventh hallmark of cancer” (8). Tumor escape strategies include mechanisms as varied as clonal deletion (9), induction of peripheral anergy (10), and epigenetic modification (11). In each instance, the tumor exploits immunologic signaling pathways to create a microenvironment favorable to tumor growth (12). All such mechanisms of tumor escape pose a great obstacle to the widespread use of immunotherapy for the treatment of cancer (13). The reliable identification of the mechanisms being used by a given tumor therefore becomes important if immunotherapeutic treatments are to reverse tumor escape pathways.

One of the first identified mechanisms of tumor escape came from the observation that certain tumors secrete immunosuppressive cytokines, such as interleukin (IL)-10 and transforming growth factor-β (TGF-β; ref. 14). In more recent years, an increasing number of tumors have been observed to attract...
suppressor T cells, known as T regulatory cells, which may help establish tolerance at the tumor site (15). Most recently, the field has focused on the various mechanisms by which tumors prevent effector T-cell activation by impeding activating “second signals” and critical “expansion signals” that are usually delivered through members of the B7/CD28 (16) and tumor necrosis factor/tumor necrosis factor receptor superfamilies (17, 18). Each of these superfamilies pathways has immunostimulatory and immunoinhibitory members, and it has been hypothesized that the overall ratio of immunostimulatory to immunoinhibitory molecules determines whether T cells become activated or anergic in response to an antigenic exposure (19).

Although many potentially relevant immunoinhibitory mechanisms have been identified in recent years, most studies on tumor escape have explored individual tolerogenic genes or small sets of genes, commenting on which cancers show involvement of that mechanism. Alternatively, some studies have investigated one specific tumor, elucidating the array of immunologic genes with altered expression. These two disparate approaches paint a patchy picture of immune escape mechanisms. Using immunotherapy to treat patients with cancer, however, necessitates a more complete understanding of all of the major mechanisms of tumor escape at play for a given tumor as well as an understanding of the differences in tumor escape between different cancer types.

Although DNA array-based “immunogenomics” has been proffered as a theoretically promising approach to this question (20), microarray experiments thus far have failed to provide a clear story of tumor escape, in part because of the great challenge involved in integrating the vast number of modulated genes into coherent immunomodulatory pathways (21). Exceptions include the occasional cancer-specific gene network, such as the HMGB1/amphoterin pathway in gastric cancer (22), but such information does not approach providing a systematic delineation of the critical genes in each of the common cancers. Consequently, the field still lacks the fundamental knowledge that would enable the determination of the most appropriate immunotherapies for a given tumor. This becomes especially important as new single-molecule immunotherapy reagents, including anti-CD25 antibody (23, 24), anti-CTLA-4 antibody (25), PD-L1 blockade (26), and PD1 blockade (27), are processed with a homogenizer and reverse transcribed with a bovine serum, L-glutamine, and penicillin/streptomycin antibiotics as described previously (29).

**Materials and Methods**

**Cell culture and reagents.** 4T1, A20, CT26, MAD109, and RENCA murine tumor cell lines were purchased from the American Type Culture Collection and maintained in RPMI 1640 with 10% fetal bovine serum, i-glutamine, and penicillin/streptomycin antibiotics as described previously (29).

**Mice.** Six-week-old female BALB/c female mice were purchased from Harlan Sprague Dawley. Institutional Animal Care and Use Committee–approved protocols and institutional guidelines for the proper humane care and use of animals in research were followed in all experiments. Mice were injected s.c. in the flank with a 0.2 mL inoculum of 5 × 10⁶ viable cells of the murine tumor cell lines. Tumors were allowed to develop, and mice bearing each tumor type were sacrificed on days 2, 4, 7, 14, and 21 (n = 2 of each tumor per day). Control mice were sacrificed on day 10. Tumor and tumor-draining lymph nodes were harvested from each mouse and preserved in Qiagen RNAlater.

**RNA isolation and reverse transcription.** RNAlater-preserved tissues were processed with a homogenizer and reverse transcribed with a Qiagen total RNA isolation kit. RNA was subjected to DNase using Ambion’s RNAqueous-4PCR kit and reverse transcribed using Invitrogen SuperScript III with 4 mL RNA/reaction. First-strand synthesis was verified by nonquantitative RT-PCR with murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer, and reverse transcriptase–negative controls were used to verify RNA purity.

**Human specimens.** Human samples, both cancerous biopsies and normal tissue controls, were purchased as PCR-ready samples from Oncomatrix. When possible, matched lymph node specimens were also purchased. All human samples were reverse transcribed and analyzed as described above using 4 mL RNA/SuperScript III first-strand reaction. To control for age-related changes in the intensity of immune response, all breast cancer samples and breast tissue control samples were obtained from patients between the ages of 42 and 47. Colon tumor specimens were obtained from patients between the ages of 63 and 67, and colon control specimens were all patient matched. All tumor samples were graded by a surgical pathologist as moderately differentiated.

**Real-time primers.** Real-time RT-PCR primers were designed using Primer3 software or purchased from SuperArray:

1 http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi
### Gene Expression Profiles of Immunomodulatory Genes in Murine Tumor Models

#### Murine

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<td>5'–GGCAGACTTCCACCATGCAAGA-3'</td>
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#### Real-time RT-PCR

Real-time reactions were done with 1 µl cDNA, 0.5 µl of each forward and reverse primers (25–40 pmol/µl), 12.5 µl Stratagene Brilliant SYBR Green Master Mix, and 10.5 µl Millipore water. Reactions were run for 40 cycles on a Stratagene Mx3000P cycler. Each sample was amplified in triplicate runs and GAPDH served as the housekeeping gene control for normalization.

#### Quantitative analysis

For each sample, the triplicate Ct values from each primer were averaged. cDNA concentration differences were normalized by the equation 2^(-A gene mean Ct - GAPDH mean Ct). Fold increases and fold decreases in gene expression were calculated as the ratio of normalized-case to normalized-control. For analysis by hierarchical clustering, fold values were log transformed using Cluster 2.11 software (30) and exported for display in TreeView 1.60 software (both ex files available online).

### Results

#### Expression profiles of immunomodulatory genes in murine tumor models

Using real-time RT-PCR, the gene expression of 11 immunomodulatory genes was analyzed for each of the five murine tumor models. SD bars were often too small to be visible in bar graphs, confirming minimal genetic variability in the BALB/c mouse strain and verifying high precision of the methodology. Of the eight immunoinhibitory genes, IDO, IL-10, TGβ-β, PD-L1, and PD-L2 were consistently and robustly up-regulated across all tested cancers (Fig. 1). CTLA-4 and PD1 showed marked up-regulation in three of the five tumor models (CT26, A20, and 4TI), the same three models that respond to immunotherapy with a combination immunotherapy protocol consisting of anti-CD25 and a targeted liver expression chemokine (LEC) fusion protein (23). In addition, the three tested immunostimulatory molecules were all moderately decreased in the tumors of CT26-, MAD109-, A20-, and RENCA-bearing mice but were increased in 4TI-bearing mice.

Tumor-draining lymph node expression patterns were markedly different from the intratumoral expression described above. With a few noteworthy exceptions, the panel of immunomodulatory genes showed generalized down-regulation in the tumor-draining lymph nodes in comparison with control lymph nodes. This is despite the fact that tumor-draining lymph nodes were visibly congested and enlarged at the time of surgical excision. For a subset of molecules, those for which antibodies for immunohistochemistry were commercially available (CD25, CD80, CD83, CD86, PD1, and PD-L2), real-time RT-PCR data were substantiated with immunohistochemical analysis, confirming close correlation

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between mRNA quantitation and protein expression (data not shown).

**Expression profiles of immunomodulatory genes in human tumor models.** To investigate the ability of murine intratumoral gene expression data to predict immunotherapy targets for human tumors, two human tumor types were analyzed using the same methods. In mice, colorectal CT26 and breast 4T1 tumor models bore similar signatures, prompting us to choose human colorectal and ductal breast adenocarcinomas to study alongside their murine counterparts. Tumor-draining lymph nodes were not analyzed because of reduced tissue availability.

For these studies, an expanded panel of 25 immunomodulatory genes was investigated. These genes were selected based on their role in T-cell activation and tolerance induction. In the eight examined ductal adenocarcinomas of the breast (Fig. 2), three immunoinhibitory genes were consistently and significantly increased: TGF-βR (P = 0.003), IL-10R (P = 0.006), and B7-H4 (P = 0.008). Additionally, two immunostimulatory genes were profoundly down-regulated in all breast cancer specimens: CD28 (P < 0.0001) and CD83 (P < 0.0001). Interestingly, several purported immunoinhibitory genes, such as FoxP3, IDO, PD-1, PD-L1, PD-L2, and CTLA-4, showed significantly decreased expression, suggesting that they are not responsible for producing tumor escape in human ductal adenocarcinoma of the breast. Remarkably, all targets showed consistent expression patterns throughout the progression of breast cancer tumors, indicating that gene expression was not stage dependent.

Immunomodulatory gene expression patterns were less consistent across the 11 colorectal cases examined (Fig. 2). Whereas breast cancer profiles showed three universally increased immunoinhibitory genes and two universally decreased genes, no change in gene expression was 100% consistent across all colorectal cases. The immunostimulatory genes Lag3 and IL-6R showed generalized down-regulation in most cases, and PD-L2 expression was significantly increased in half of the tested cases. Nevertheless, these gene expression patterns were not sufficiently generalized to make blanket predictions about appropriate and inappropriate immunotherapeutic targets for the treatment of colorectal cancer. Indeed, the only widespread result was decreased expression of immunoinhibitory B7-H4, which notably was one of the most profoundly increased genes in the tested breast cancer cases.

**Temporal patterns of gene expression.** In addition to assessing overall trends in expression profiles, changes in the expression of specific genes over time were also assessed. To direct a therapy at a specific molecular target, it is important to know not just which molecules to target but also at what stage(s) of tumor growth it is appropriate to target them. In theory, some targets have stage-independent expression patterns, whereas others show stage-dependent dynamic changes in expression. To prove this point, Fig. 3 shows two important categories of up-regulation: namely, consistently increased expression (Fig. 3A), which is characterized by murine PD-L2, and peaking and waning expression (Fig. 3B), which is characteristic of molecules such as murine PD1. These expression patterns help establish the temporal window during which the proposed therapy is most likely to produce significant clinical results.

In human ductal adenocarcinoma of the breast, as with the murine cancers, many genes did indeed fall into one of the two aforementioned categories. B7-H4 (Fig. 3C) and TGF-βR, two immunoinhibitory genes, both showed patterns of consistent increase throughout the stage progression of ductal tumors. IDO expression, however, peaked and then fell (Fig. 3D). Colorectal cancers, in contrast, were heterogeneous both within and between stages and consequently failed to show stage-dependent expression patterns.

**Grouped cell analysis of immunomodulatory gene expression.** Enhanced gene expression could indicate either increased proliferation/infiltration of the expressing cell type or specific up-regulation of the gene by expressing cells. Similarly, decreased expression could be due to decreased proliferation/infiltration of the gene by expressing cells.

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**Fig. 1.** Murine tumor immunomodulatory gene expression profiles. TreeView display of log-transformed fold change values in murine tumors (A) and tumor-draining lymph nodes (B). Increased expression is indicated by the intensity of red, whereas decreased expression is indicated by the intensity of green. Black, no change; gray, missing data point. Vertical lines, separate tumor models. For each model, left to right represents increasing days after tumor implantation. Horizontal line, separates immunoinhibitory genes (top) from immunostimulatory genes (bottom). Note that different tumor models display different immune signatures, but certain key genes show consistent involvement across all tumors (e.g., PD-L2 and GITRL).


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Infiltration or to specific down-regulation of the gene by one or more of the expressing cell populations. To differentiate between changes in cell number and changes in gene expression per cell, we grouped gene expression fold changes by expressing cell type (Fig. 4). Uniformly increased or uniformly decreased gene expression across a cell type indicated a change in proliferation/infiltration of that cell type, whereas gene-specific increases or decreases, above or below the baseline of the cell, indicated specific up-regulation or down-regulation of that gene.

Grouped cell analysis revealed several immunoinhibitory molecules to be increased as the result of specific up-regulation and several immunostimulatory molecules to be decreased as the result of specific down-regulation (Fig. 4). In human breast cancer, the down-regulation of immunostimulatory CD28 and CD83 was especially notable. Additionally, all stages of ductal adenocarcinoma showed a specific up-regulation of immunoinhibitory genes B7-H4, IL-10R, and TGF-βR. By contrast, in colorectal cancer grouped cell analysis, no immunoinhibitory gene showed completely consistent specific up-regulation, although several cases did show specific up-regulation of the immunosuppressive cytokines IL-10 or TGF-β.

Figure 2. Human breast and colorectal cancer immunomodulatory gene expression profiles. TreeView display of log-transformed fold change values from human adenocarcinoma of the breast (A) and colorectal adenocarcinoma (B). Increased expression is indicated by the intensity of red, whereas decreased expression is indicated by the intensity of green, and black indicates no change. For each, tumor samples appear in order from left to right in terms of progressive stage by pathologic diagnosis. Horizontal line, separates immunoinhibitory genes (top) from immunostimulatory genes (bottom). Note that the breast ductal adenocarcinoma cases show remarkable homogeneity in their immune signatures, whereas the colorectal adenocarcinoma cases were fundamentally heterogeneous. Clinically significant target are those that appear in red above the horizontal line (e.g., TGF-βR, IL-10R, and B7-H4 in breast cancer) or in green below the line (e.g., CD28 and CD83 in breast cancer).

To complement this deductive analysis, flow cytometry was used to examine treatment-induced changes in murine cell populations (data not shown). Specifically, treatment with the LEC fusion protein was found to decrease CD49b+ cells by 70%, increase CD11b+ cells by 20%, and nearly double the population of CD4+CD25+ cells. LEC treatment also shifted the CD8+ population to being increasingly CD62L+, suggestive of a central memory T-cell population.

In addition to the key targets identified via grouped cell analysis, Fig. 4 also shows the specific up-regulation of certain immunostimulatory molecules (such as GITR in human breast cancer) and the specific down-regulation of certain immunoinhibitory molecules (such as B7-H4 in human colorectal cancer), changes unlikely to contribute to the state of tolerance. Increased immunostimulatory genes and decreased immunoinhibitory genes, although identifiable via this expression analysis, are not designated rational targets for immunotherapy, as their altered gene expression is more likely the effect of a failing immune response than the cause of the immune evasion.

Discussion

Immunotherapy has emerged as a promising approach to the treatment of cancer but still suffers under the weight of two substantial challenges: the need to overcome established immunologic tolerance and the need to avoid induction of autoimmune disease. Both challenges can be satisfactorily overcome only through knowledge of the mechanisms of tolerance used by different tumors. Such knowledge would enable the identification of therapies that can directly reverse tumor escape, which would enable immunotherapy to combat tolerance without creating the autoimmune complications characteristic of indirect, systemic immunotherapeutic approaches.

Nevertheless, systematic analysis of the different escape mechanisms used by different tumors had been precluded to-date by several difficulties inherent to such a study. These challenges include the questionable connection between murine immune findings and human immune responses, the recent identification of dozens of new immunomodulatory molecules, uncertainties about the location of tolerance induction or potential locations for tolerance reversal, and background differences in individual patients’ basal immune states. Although these are all indisputable challenges, the present study has attempted to address each and is therein capable of offering novel insights into the tumor escape mechanisms of different tumor systems. This study also takes the first step toward demonstrating the feasibility of fingerprinting tumor escape by determining immune gene expression levels to guide the selection of immunotherapeutic strategies.

The murine data presented in Fig. 1 show that different tumors do indeed use different mechanisms for tumor escape. The range of immunomodulatory gene expression within any given murine tumor type was <10% because of the genetically identical background of the inbred strain, showing the precision of the methodology. Nevertheless, the range of immunomodulatory gene expression between murine tumor types did indeed differ, often more than 100-fold, showing the discriminating capacity of the methodology. Interestingly, some immunomodulatory genes were consistently up-regulated, such
as PD-L2, whereas the expression of others varied considerably across tumor models, such as with CTLA-4. Additionally, the results displayed in Fig. 1 prove that tumors spanning a range of immunogenicity, from very low to very high, all induce changes in the expression of immunologic molecules, and in all cases such changes can be discriminated by real-time RT-PCR.

Although quantitative RT-PCR data provide only a proxy of protein expression, the technique has distinct advantages over immunohistochemistry, immunofluorescence, or flow cytometry. First, the real-time RT-PCR approach enables the simultaneous screening of a large number of genes in the same sample. Importantly, quantitative RT-PCR also has the sensitivity to quantify small changes in the expression of candidate genes, even those with high baseline expression levels. Quantitative RT-PCR can therefore be used much like microarrays to create a “gene signature” that has enhanced statistical power while being an inexpensive and relatively simple technology that can be translated to the clinical laboratory more easily than microarrays or flow cytometry.

The success of this methodology with murine tumor analysis was complemented by analysis of human tumor biopsies of breast and colorectal cancers. This enabled examination of the correlation between the tumor escape profile of a given murine tumor model and the corresponding human tumor. For the human studies, an even larger panel of immunomodulatory genes was used than for the mouse studies, and Fig. 2 highlights those of the 25 genes that reveal key tumor escape targets for human breast and colorectal adenocarcinomas. Focusing first on breast cancer, several genes present themselves as potential causes of tolerance and thus good candidates for target by immunotherapy. Significant and specific down-regulation of CD28 and CD83 suggest routes for costimulatory or cytokine therapy. CD28 is the receptor through which the activating second signal is delivered to the T cell (31), whereas CD83 is a marker of dendritic cell maturation (32). The decrease of both these molecules suggests that tumor escape in human breast adenocarcinoma may be attributed, at least in part, to T-cell down-regulation of CD28 and dendritic cell maturation block (with CD83 down-regulation being either the cause or the effect of this block). In considering the potential of immunotherapy to reverse these escape mechanisms, IL-12 is known to stimulate CD28 expression (33), whereas IL-4, granulocyte macrophage colony-stimulating factor, and tumor necrosis factor-α are known to promote dendritic cell maturation and expression of CD83 (34). Such cytokines can be delivered to the tumor site via common immunobiology techniques, such as Fc binding to immune effector cells or antibody targeting to the tumor, enabling tumor-specific stimulation and reversal of tumor escape.

In addition to the down-regulation of these two immunostimulatory molecules, all tested human breast cancer specimens showed significant and specific up-regulation of the immunoinhibitory molecule B7-H4, a member of the B7 family. This finding is corroborated by previously published investigations.

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**Fig. 3.** Temporal patterns of gene expression. Gene expression was examined over the course of the development of murine 4T1 breast tumors (days 4-21) and over the progressive stages of human ductal adenocarcinomas (stages I-III). Patterns were categorized as either consistently progressive (A and C) or peaking and waning (B and D) for purposes of determining appropriate windows for therapeutic intervention.
Fig. 4. Specific up-regulation and down-regulation of immunomodulatory genes in human breast and colorectal cancers. Immunomodulatory genes were grouped by expressing cell type to determine whether increased gene expression was attributable to increases in cell number (consistent increase or decrease across all cell markers) or specific up-regulation or down-regulation of gene expression (singular increase or decrease in specific cell markers above or below baseline expression of other markers of the cell population). Solid black arrows, specifically up-regulated immunoinhibitory molecules; open arrows, specifically down-regulated immunostimulatory molecules.
which have shown, using immunohistochemistry, that B7-H4 is up-regulated and expressed on the surface of many breast and ovarian tumor cells (35, 36). Although the receptor for B7-H4 remains unknown, experiments using a human B7-H4-Fc fusion protein (hB7-H4lg) showed the ability of B7-H4 to suppress secretion of cytokines and T-cell proliferation (37). These features make B7-H4 a good target for an antibody blockade that would reverse the immunosuppressive function of this molecule, enhancing ongoing immunotherapeutic strategies in breast cancer.

Remarkably, the breast cancer results from Fig. 2 show consistent expression patterns throughout the progression of breast cancer tumors rather than showing stage-dependent gene expression. This finding is critical in that it suggests that patients with either early or late diagnoses can be treated with the same immunotherapeutic regimen.

Unlike human breast cancer specimens, however, the sampled colorectal tumors did not display stage-consistent gene expression, nor did they display patient-to-patient consistency. Potential explanations for colorectal gene expression variability include heterogeneity of patients’ gender (whereas all studied breast cancer patients were female), age-dependent changes in patients’ immune responses (breast cancer patients were ages 42-47, whereas colorectal cancer patients were ages 63-67, a time when patient immune responses are known for showing blunting; ref. 38), or differences between tumors that arose in the rectum from those that arose in the colon. None of these factors alone correlates with the observed gene expression pattern variations, although a complex combination of these factors could perhaps be explanatory.

Alternatively, colorectal adenocarcinoma may represent a diverse family of tumors, histopathologically similar but genetically distinguishable, each tumor subtype inducing immunologic tolerance through a distinctive mechanism. These studies thus present a possible means for teasing apart this immunologically heterogeneous group of colorectal tumors, enabling subclassification that could enhance prognostic precision and promote treatment response prediction. Additionally, the finding of colorectal immune gene heterogeneity argues in favor of analyzing individual patient’s tumors, rather than relying on a classic signature, to achieve a basis for predictive judgment about treatment of colorectal adenocarcinoma patients.

Figure 2 also shows the ability of the described real-time RT-PCR panel to go beyond identifying a list of the differentially expressed immunomodulatory genes, helping to guide the selection of which genes are the most appropriate targets for immunotherapy. As an example, in human breast cancer, several immunoinhibitory molecules showed decreased expression and several immunomodulatory genes showed increased expression, perhaps a reflection of the unsuccessful immune response that was mounted against the tumor. Invariably, the decreased expression of immunoinhibitory CTLA-4 would argue against the use of anti-CTLA-4 for these breast cancer patients, much as the increase of immunomodulatory GITR in breast cancer would argue against the use of GITR agonist treatment, a currently proposed immunotherapy strategy (39). The potential therapies of anti-CTLA-4 and GITR-Fc fusion protein thus stand in contrast to antagonistic antibodies to TGF-β and IL-10 receptors, to B7-H4 blockade, or to IDO chemical inhibition, all of which do derive support from Figs. 2 and 4 as potentially beneficial treatments for breast cancer patients.

Figure 3 provides important insights into the clinically relevant time window for each of these highlighted molecular targets. The examination of the temporal patterns of immunomodulatory gene expression helps identify, for example, that antibodies directed at B7-H4 would be appropriate therapy at any stage of ductal adenocarcinoma of the breast and would perhaps become an increasingly useful treatment strategy during later stages of cancer development. Treatment inhibiting IDO, however, would be best if delivered during an intermediary phase of the disease, when the expression of immunoinhibitory IDO is greatest; this immunotherapy, therefore, would have a constraining window, and this window should guide the decision to treat with the IDO chemical inhibitor 1-MT (40). These results emphasize that, for certain markers, determination of the immune signature is a critical prerequisite to identifying those patients for whom a given immunotherapy will be of value.

Ultimately, the dramatic intratumoral immunomodulatory gene expression changes seen in human breast and colorectal tumors are encouraging in that they suggest that immune gene markers may be useful for both monitoring immunotherapy treatment and directing new treatment strategies. Tumor-draining lymph node immunomodulatory gene expression analysis may reveal additional targets that are especially prominent in the lymph node, but there seems to be sufficient tumor-induced immune dysregulation at the site of the tumor to justify targeted therapy, which has the profound advantage of minimizing the autoimmune sequelae that often result from systemic administration of immunostimulating therapies. Tumor-specific immunotherapies can be achieved by creating targeted antibody fusion proteins, such as B7.1/NHS76 (41), which have been developed in our laboratory.

Given the existence of many already developed immunotherapies, another potential future application of this study entails probing currently used cancer immunotherapy reagents for their tolerance reversal mechanism(s) of action. Using the knowledge of a gene expression signature that is characteristic of a given murine tumor, it should be possible to treat that tumor model with successful immunotherapies and ask the question of which genes are modulated. One would look specifically for which previously up-regulated immunoinhibitory genes are down-regulated in a dose-dependent fashion and which previously down-regulated immunomodulatory genes are up-regulated. Strong correlation between reversal of tolerogenic gene expression and tumor regression would suggest a tolerance reversal mechanism of action for the immunotherapy reagent.

Continuation of these studies could provide a 3-fold effect on research into cancer immunotherapy. First, as the Broad Institute’s “Connectivity Map” becomes available, it will be possible to query the database for Food and Drug Administration–approved drugs and small molecules that are known to induce a gene expression profile opposite of that seen to be induced by developing tumors (42). These treatments are key in that they will be rapidly available for clinical trials. Second, the extension of this line of investigation to examine the effects of known immunotherapy reagents on immunomodulatory gene expression profiles will help determine the manner in which already developed immunotherapies work to reverse tolerance
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