Prevalence of Regulatory T Cells Is Increased in Peripheral Blood and Tumor Microenvironment of Patients with Pancreas or Breast Adenocarcinoma

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Prevalence of Regulatory T Cells Is Increased in Peripheral Blood and Tumor Microenvironment of Patients with Pancreas or Breast Adenocarcinoma

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Regulatory T cells (T\textsubscript{reg}) that prevent autoimmune disease by suppression of self-reactive T cells may also suppress the immune response against cancer. In mice, depletion of T\textsubscript{reg} by Ab therapy leads to more efficient tumor rejection. T\textsubscript{reg}-mediated suppression of antitumor immune responses may partly explain the poor clinical response to vaccine-based immunotherapy for human cancer. In this study, we measured the prevalence of T\textsubscript{reg} that coexpress CD4 and CD25 in the PBLs, tumor-infiltrating lymphocytes, and regional lymph node lymphocytes from 65 patients with either pancreas or breast cancer. In breast cancer patients (n = 35), pancreas cancer patients (n = 30), and normal donors (n = 35), the prevalence of T\textsubscript{reg} were 16.6% (SE 1.22), 13.2% (SE 1.13), and 8.6% (SE 0.71) of the total CD4\textsuperscript{+} cells, respectively. The prevalence of T\textsubscript{reg} were significantly higher in breast cancer patients (p < 0.01) and pancreas cancer patients (p < 0.01) when compared with normal donors. In tumor-infiltrating lymphocytes and lymph node lymphocytes, the T\textsubscript{reg} prevalence were 20.2% (SE 3.93) and 20.1% (SE 4.3), respectively. T\textsubscript{reg} constitutively coexpressed CTLA-4 and CD45RO markers, and secreted TGF-\beta and IL-10 but did not secrete IFN-\gamma. When cocultured with activated CD8\textsuperscript{+} cells or CD4\textsuperscript{+}CD25\textsuperscript{+} cells, T\textsubscript{reg} potently suppressed their proliferation and secretion of IFN-\gamma. We conclude that the prevalence of T\textsubscript{reg} is increased in the peripheral blood as well as in the tumor microenvironment of patients with invasive breast or pancreas cancers. These T\textsubscript{reg} may mitigate the immune response against cancer, and may partly explain the poor immune response against tumor Ags. The Journal of Immunology, 2002, 169: 2756–2761.

The recent description of a unique lineage of CD4\textsuperscript{+} T cells that suppress T cell effector function has shed some light on the basic mechanisms of immune homeostasis (1). These cells, called regulatory T cells (T\textsubscript{reg}),\textsuperscript{2} are characterized by coexpression of CD4 and the IL-2R \alpha-chain (CD25). T\textsubscript{reg} have been isolated from human PBMCs (2) and mouse spleen (3). These cells are thought to be a functionally unique subset of T lymphocytes that play an important function in maintaining immune homeostasis and protecting the host against autoimmune diseases (4, 5). Mice that are deficient in this subset of T cells develop T cell-mediated autoimmune diseases such as type I diabetes due to insulin (6), hypothyroidism due to thyroiditis (7), infertility due to oophoritis/ovaritis (8), and pernicious anemia due to gastritis (9). In addition to CD4 and CD25 markers, T\textsubscript{reg} also constitutively express CD45RO and CD152 (CTLA-4). Ex vivo studies on these cells reveal a poorly proliferative cell population that secretes inhibitory cytokines such as TGF-\beta and IL-10 (2). They also inhibit the proliferation of CD4\textsuperscript{+}CD25\textsuperscript{−} and CD8\textsuperscript{+} lymphocytes (10). Their mechanism of action remains an area of active investigation.

When T\textsubscript{reg} are depleted using mAb, transplanted tumors in mice are efficiently rejected by the host immune system (11, 12). This finding suggests that T\textsubscript{reg}, which function as a protective mechanism against autoimmunity, may also mitigate the immune response against cancers. A previous study demonstrated that large numbers of CD4\textsuperscript{+}CD25\textsuperscript{−} lymphocytes infiltrate the tumor microenvironment of non-small cell lung cancers and ovarian cancers (13). This may partly explain the poor clinical response to cancer vaccines even after measurable postvaccination increases in tumor-specific CTLs (14). Prevalence of T\textsubscript{reg} in the circulation and tumor microenvironment of cancer patients has not been extensively studied previously, and interactions between human tumors and T\textsubscript{reg} are unknown.

Animal studies indicate that suppressor cells similar to T\textsubscript{reg} that protect against autoimmunity may also impair immune response against tumor Ags. In mice that carry chemically induced fibrosarcoma, CD4\textsuperscript{+} T\textsubscript{reg} down-regulated the activity of effector cells against the tumor (15). Passive transfer of these suppressor T cells from fibrosarcoma-bearing hosts impaired generation of tumor-specific immunity in recipients (16). Similar observations have been made with other murine tumors (17–20). Administration of a monoclonal anti-CD25 Ab that depletes the immune system of CD4\textsuperscript{+}CD25\textsuperscript{+} T\textsubscript{reg} cells has been shown to promote the rejection of tumors derived from leukemia, myeloma, and sarcoma (21). In the recent report by Sutmuller et al. (12), a synergism between treatment with anti-CTLA-4 Ab and depletion of CD25\textsuperscript{+} cells with anti-CD25 Ab was observed. This synergism led to more efficient rejection of transplanted tumors than when treated with either Ab alone.

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3 Abbreviations used in this paper: T\textsubscript{reg}, regulatory T cell; TIL, tumor-infiltrating lymphocyte; LNL, lymph node lymphocyte.

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Immune tolerance to tumor may contribute to progression of the tumor by local invasion as well as metastatic spread. In mice, development of a suppressor T cell population that inhibits the antitumor immune response preceded tumor progression (22). In a different experimental system, advanced stage metastatic lymphoma was rejected by the host if suppressor CD4+ T cells were eliminated by a single dose of vinblastine, a chemotherapeutic agent (23).

These studies combined with other indirect evidence lead us to formulate the hypothesis that invasive cancer in humans is associated with an expansion of Treg that suppress a tumor-specific immune response. To test this hypothesis, we collected samples of peripheral blood, tumor, and regional tumor-infiltrated lymph nodes from 65 patients undergoing surgery for either breast or pancreatic ductal adenocarcinoma. We compared the prevalence of CD4+25 Treg in these samples to that of 35 normal individuals and patients who underwent surgical procedures for benign diseases and carcinoma in situ. In addition, we performed functional analysis on patient-derived Treg confirming their suppressor cytokine profiles and their antiproliferative effect on activated CD4+CD25+ and CD8+ autologous lymphocytes. We show that the prevalence of Treg in the peripheral blood of breast and pancreas cancer patients is increased when compared with normal individuals. Similarly, Treg are present in tumor-infiltrating lymphocytes (TIL) and regional lymph nodes infiltrated by tumor. These cells secrete IL-10 and TGF-β, and prevent activated CD4+CD25+ and CD8+ from proliferating.

Materials and Methods

Patients and normal donors

Following a research protocol approved by the Washington University School of Medicine Human Studies Committee (St. Louis, MO), peripheral blood, tumor, and tumor-infiltrated lymph nodes were obtained from patients undergoing resections for breast or pancreas cancers. Blood samples were also obtained from normal healthy volunteers and patients undergoing surgery for benign diseases as controls. All the breast cancer patients and 13 of 25 pancreatic cancer patients were female. Of 35 patients with breast cancer, 21 carried stage II or higher disease. All except one pancreatic cancer patient carried stage II or higher disease with invasive tumors (Table I). Any patient who had received chemotherapy before obtaining specimens was excluded from the study.

Isolation of Treg from peripheral blood

CD4+ cells were isolated from heparinized blood using Rosette Sep CD4+ cell enrichment mixture according to the manufacturer’s guidelines. The resulting 90% pure CD4+ cells (data not shown) were washed, and CD25+ cells were positively selected by labeling with anti-CD25 tetrameric Ab complex and magnetic colloid, followed by separation using magnetic columns. CD25+ and CD25- fractions were separated and saved. The CD4+ cell enrichment mixture, CD25 tetrameric Ab complex, magnetic colloid, and separation columns were obtained from StemCell Technologies (Vancouver, Canada).

Isolation of Treg from tumor-draining lymph nodes

Fresh lymph nodes obtained from patients were minced with a scalpel, pipetted repeatedly in PBS containing 2% FBS, and strained through a 40-μm mesh to obtain a single-cell suspension. RBC were lysed by incubating in ACK lysis buffer (0.15 M NH4Cl, 10 mM KHCO3, 0.1 mM EDTA, pH 7.4), and washed twice in cold PBS.

Isolation of CD8+ cells

CD8+ cells were isolated from heparinized blood using Rosette Sep CD8+ cell enrichment mixture (StemCell Technologies) according to the manufacturer’s guidelines. The enriched cell population contained >85% CD8+ cells as determined by cell surface staining and flow cytometry analysis.

Isolation of TILs and lymph node lymphocytes (LNLs)

Fresh pieces of tumor and draining regional lymph nodes were minced into 1-mm-size pieces and digested in a buffer containing 1 mg/ml collagenase (Sigma-Aldrich, St. Louis, MO), 2.5 U/ml hyaluronidase (Sigma-Aldrich), and 0.1 mg/ml DNase (Sigma-Aldrich) for 2 h to obtain a single-cell suspension. Resulting cells were washed twice in PBS and used for additional experiments.

Cell culture

T cells were cultured in X-VIVO 15 medium (BioWhittaker, Walkersville, MD) containing 2% autologous serum, 10 U/ml rIL-2 (Endogen, Woburn, MA), and 10 ng/ml of anti-CD28 Ab (clone CD28.2; BD PharMingen, San Diego, CA). For cytokine secretion assays, 1.25 × 105 cells were grown in anti-CD3 Ab (OKT3)-coated round bottom 96-well plates, and culture supernatant was harvested after 72 h. In coculture experiments, 1.25 × 105 of CD4+25+ cells or CD8+ cells were incubated with the specified ratio of the CD4+25+ cells. Again, the culture supernatant was harvested after 72 h for cytokine assays.

Cell proliferation assay

Cell proliferation in coculture experiments was determined by incorporation of radiolabeled thymidine after incubation with medium containing 1 μCi/well (10 μCi/ml) of [3H]thymidine for 16–18 h. Incorporated radioactivity was counted using a scintillation counter.

ELISA

Culture supernatant concentrations of IFN-γ, TGF-β, and IL-10 were measured by using commercially available ELISA kits (BioSource International, Camarillo, CA) according to the protocols provided by the manufacturer.

Immunofluorescence labeling and flow cytometry

PBMC, TIL, and LNL isolated respectively from peripheral blood, fresh tumor samples, and regional lymph nodes were used for two- and three-color cell surface labeling using Abs against CD4, CD25, CTLA4 (CD152), and CD45RO. All the Abs were obtained from BD PharMingen. Labeled cells were analyzed by using FACSCalibur flow cytometer and CellQuest software (BD Biosciences, Mountain View, CA). With each sample, a negative control with isotype-matched control Abs was used to determine the positive and negative cell populations. The p values were determined using the Student t test.

Results

The prevalence of CD4+25+ Treg lymphocytes is higher in patients with invasive breast ductal carcinoma, and with pancreas adenocarcinoma than in normal individuals

We analyzed the PBL from 35 patients with breast ductal carcinoma and 30 patients with pancreas adenocarcinoma by flow cytometry after cell surface labeling for coexpression of CD4 and

Table I. Patient characteristics

<table>
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<th>Study Subjects</th>
<th>Normal</th>
<th>Breast cancer patients</th>
<th>Pancreas cancer patients</th>
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<tr>
<td>Male</td>
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<tr>
<td>Female</td>
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<tr>
<td>Average age (years)</td>
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<td>58.5</td>
<td>66.4</td>
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<tr>
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</tr>
<tr>
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<td>4</td>
</tr>
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CD25 molecules. The prevalence of CD4+25+ cells as a percentage of total CD4+ population was determined by standard determination of quadrant statistics. These percentages were compared with that of 35 normal donors and patients who underwent surgical resections of benign breast and pancreatic lesions. There was no significant difference between the prevalence of CD4+25+ cells in normal donors and patients with benign disease ($n = 6$, data not shown). Representative dot plots of breast (Fig. 1A) and pancreas (Fig. 1B) cancer patients as well as normal donors (Fig. 1C) are shown. Cumulative data for all the patients and normal donors are presented in a bar chart and a scatter chart (Fig. 1, D and E). The prevalence of CD4+25+ cells in breast cancer patients was 16.6% (SE 1.22) and in pancreas cancer patients was 13.2% (SE 1.13), as compared with that of normal donors, which was 8.6% (SE 0.71). The prevalence of CD4+25+ cells in both breast ($p < 0.001$) and pancreas ($p < 0.015$) cancer patients was significantly higher than in normal individuals.

The CD4+25+ T lymphocytes from cancer patients are phenotypically similar to T

reg from normal donors by coexpression of CD45RO and CTLA-4 molecules as well as cytokine profile

To verify that the more prevalent CD4+25+ lymphocytes in cancer patients are indeed similar to well-documented T

reg isolated from normal donors, we compared the cell surface expression of CTLA-4 and CD45RO molecules by three-color staining and flow cytometry. PBL freshly isolated from cancer patients and normal donors were labeled with FITC-conjugated anti-CD4, PE-conjugated anti-CD25, and CyChrome-conjugated anti-CTLA-4 or anti-CD45RO. As shown in Fig. 2A, CD4+25+ cell population was first identified and the expression of either CTLA-4 (Fig. 2B) or CD45RO (Fig. 2C) was analyzed in this gated population. The majority of CD4+25+ cells from cancer patients coexpress CTLA-4 and CD45RO molecules similar to T

reg of normal donors. We further characterized these CD4+25+ cells by analyzing the cytokine expression pattern. CD4+ cells were purified to >90% purity from peripheral blood of normal donors as well as cancer patients by negative selection as described in Materials and Methods. CD4+ cells were separated into CD4+CD25+ and CD4+CD25− fractions by positive selection of CD25+ cells using magnetic labeling. Average purity of CD4+CD25− and CD4+CD25+ were 88 and 82%, respectively (data not shown). Cells from both fractions were cultured and stimulated with plate-bound anti-CD3 and soluble anti-CD28 Abs for 72 h. The culture supernatants were removed and assayed for IFN-γ, TGF-β, and IL-10. CD4+25+ lymphocytes secrete IL-10 and TGF-β, but no IFN-γ, as shown in Fig. 2D. This is in contrast to CD4+25− lymphocytes, which secrete large amounts of IFN-γ, but little TGF-β and IL-10.

**FIGURE 1.** Prevalence of CD4+25+ lymphocytes is elevated in peripheral blood of patients with breast or pancreas cancers. Representative flow cytometry results of PBL from a breast cancer patient (A), pancreas cancer patient (B), and a normal donor (C) are depicted. Isolated PBL labeled with FITC-conjugated anti-human CD4 and PE-labeled anti-human CD25 Abs are shown as described in Materials and Methods. D, Cumulative results from 35 breast cancer patients, 30 pancreas cancer patients, and 35 normal donors. E, The same data are presented in scatter graph format. The prevalence of CD4+25+ cells is presented as a percentage of total CD4+ cells. The prevalence of CD4+25+ cells in breast cancer patients ($p < 0.01$) and pancreas cancer patients ($p < 0.01$) is significantly higher than in normal donors.

**FIGURE 2.** CD4+25+ cells from peripheral blood of patients with breast or pancreas cancer demonstrate T

reg phenotype by coexpression of CD152 (CTLA-4) and CD45RO markers and secreted cytokine profile. PBL is triple labeled with anti-CD4, anti-CD25, and anti-CD152 or anti-CD45RO conjugated with FITC, PE, and CyChrome, respectively. A, Representative flow cytometry data from a breast cancer patient. Using this plot, the T

reg population that coexpress CD4 and CD25 molecules was gated to obtain B and C, which depict the expression pattern of CD152 and CD45RO, respectively. Isolated CD4+ cells were fractionated into CD4+25+ and CD4+25− populations and cultured separately as described in Materials and Methods, and the culture supernatants were analyzed for secreted amounts of TGF-β, IL-10, and IFN-γ. D, Representative results for a normal donor, a breast cancer patient, and a pancreas cancer patient.
CD4\(^{+}\)25\(^+\) T lymphocytes from peripheral blood of cancer patients suppress proliferation and IFN-\(\gamma\) secretion by activated CD8\(^{+}\) T lymphocytes and help CD4\(^{+}\)25\(^+\) lymphocytes

We assessed the suppressor function of CD4\(^{+}\)25\(^+\) T\(_{\text{reg}}\) from cancer patients and normal donors by coculturing CD8\(^{+}\) or CD4\(^{+}\)25\(^+\) cells with CD4\(^{+}\)25\(^+\) cells. Each type of lymphocyte was isolated from the peripheral blood of cancer patients and normal donors as described in Materials and Methods, and resuspended in culture medium containing rIL-2, anti-CD28 Ab, and 2% autologous serum. Either CD8\(^{+}\) or CD4\(^{+}\)25\(^+\) lymphocytes were cocultured with the indicated ratio of CD4\(^{+}\)25\(^+\) cells in anti-CD3-coated 96-well plates. After 72 h of coculture, concentration of IFN-\(\gamma\) in the medium was measured by ELISA. Cell proliferation was determined by incorporation of \([^{3}\text{H}]\)thymidine. Representative results from 14 cancer patients and 5 normal donors are depicted in Fig. 3. The data for all other patients and normal donors not represented in the Fig. 3 are presented in Table II.

CD4\(^{+}\)25\(^+\) lymphocytes infiltrate tumors and regional lymph nodes

To test the hypothesis that T\(_{\text{reg}}\) infiltrate the tumor microenvironment, we isolated lymphocytes from 20 fresh breast and pancreas cancer specimens and tumor-infiltrated lymph nodes, and measured the prevalence of CD4\(^{+}\)25\(^+\) cells. In Fig. 4, A and B are representative results of flow cytometry analyses from TIL and LNL. There was a selection bias toward patients bearing larger and more advanced tumors in the 20 patients from whom we were able to collect tumor samples and tumor-infiltrated lymph nodes. This may partly explain the very high prevalence of T\(_{\text{reg}}\) in the TIL and LNL.

CD4\(^{+}\)25\(^+\) lymphocytes from solid tumors and tumor-draining lymph nodes secrete cytokines that are similar to peripheral blood-derived T\(_{\text{reg}}\)

To confirm that CD4\(^{+}\)25\(^+\) lymphocytes derived from tumor microenvironment and draining lymph nodes exhibit the T\(_{\text{reg}}\) phenotype, we isolated CD4\(^{+}\) lymphocytes from two breast cancer specimens and tumor-draining lymph nodes from two pancreas cancer patients. The CD\(_{\text{4}}\) cells were fractionated into CD25\(^{+}\) and CD25\(^{-}\) fractions by using CD25 microbeads and grown in cell culture as described in Materials and Methods. After 72 h of culture, culture supernatant was analyzed by ELISA for IFN-\(\gamma\), IL-10, and TGF-\(\beta\). The results are shown in Fig. 4D.

CD4\(^{+}\)25\(^+\) lymphocytes from tumor-draining lymph nodes suppress activation of CD4\(^{+}\)25\(^+\) cells

Lymph nodes that are present within the immediate drainage basin of the tumor are likely sites of tumor-specific CTL proliferation. Presence of T\(_{\text{reg}}\) in these lymph nodes may inhibit such CTL proliferation. To test the hypothesis that T\(_{\text{reg}}\) inhibit Th cell function in the draining lymph nodes, we isolated T\(_{\text{reg}}\) from draining lymph nodes of two pancreas cancer patients, and analyzed the secreted cytokine profile and in vitro suppression activity on CD4\(^{+}\) cells. The cytokine profile reveals similar characteristics to peripheral blood-derived T\(_{\text{reg}}\) (Fig. 4D) and suppressor activity on CD4\(^{+}\) cell proliferation and IFN-\(\gamma\) secretion (Fig. 5).

Discussion

This study provides the first evidence for increased prevalence of CD4\(^{+}\)CD25\(^{+}\) T\(_{\text{reg}}\) lymphocytes in the tumor microenvironment as well as the peripheral blood of patients with invasive cancer. In 65 patients with either breast or pancreas cancer, the prevalence of T\(_{\text{reg}}\) in PBL was significantly higher than in 35 normal donors. In the tumor microenvironment, T\(_{\text{reg}}\) were present in large numbers as well. In ex vivo functional assays, we confirmed the regulatory function of these T\(_{\text{reg}}\) by analyzing the cytokine profile and the suppressor activity on CD8\(^{+}\) and CD4\(^{+}\)CD25\(^{+}\) lymphocytes. These findings are the strongest evidence yet that suggests a role for T\(_{\text{reg}}\) in immune tolerance to invasive tumors in humans. Further study using animal models will be necessary to assess whether increased T\(_{\text{reg}}\) in the tumor microenvironment facilitate local tumor growth, whereas increased prevalence of T\(_{\text{reg}}\) in peripheral blood contributes to metastatic spread.

Current research on immunotherapy for cancer mainly focuses on generation of T cell-mediated tumor lysis by using vaccination strategies. Such a strategy remains largely unsuccessful in eliciting
clinically significant regression of established tumors even in patients with detectable vaccine-induced tumor-specific CTL. A few documented cases of tumor regression remain the exception rather than the rule. Explanations offered for this marginal success of immunotherapy include "tolerance" development due to lack of costimulatory molecules on tumors, down-regulation of signal transduction molecules in T cells, apoptosis of T cells upon contact with tumor, tumor-induced dysfunction of APCs, secreted immunosuppressive proteins such as TGF-β by tumor, and emergence of Ag loss variants. However, virtually no experimental evidence on a role for Treg in inhibiting immune responses against human cancer exists.

Tumor-induced tolerance mediated by T cells has been demonstrated in a variety of tumor types in mice. This has been well-documented in a large body of literature which has been reviewed by R. J. North (24). Until recently, the suppressor population was not defined by the coexpression of CD4 and CD25 markers. Identification of CD4^+CD25^+ Treg will now allow us to determine whether the previously described suppressor T cells are indeed the CD4^+CD25^+ Treg.

Experimental evidence suggests that down-regulation of immune response to cancer by concomitant development of suppressor cells may allow progressive local growth of tumors (25). When tumor cells are implanted in mice, an initial slow period of growth is followed by a rapid growth period with local invasion and metastatic spread. The phase of rapid growth and metastatic spread has been shown to be synchronous with the development of CD4^+ suppressor T cells. These suppressor cells, when adaptively transferred, can suppress immune response to the same tumor in a previously immune mouse (26). Mice rendered immunodeficient by thymectomy or gamma irradiation are better able to reject transplanted tumors when infused with tumor-specific T cells than their immunocompetent counterparts (27). These immunocompromised mice were unable to develop suppressor T cells, allowing adoptively transferred cytotoxic T cells to lyse tumor efficiently. Depletion of suppressor cells with cytotoxic agents such as cyclophosphamide (28) and vinblastine (23) appear to have

FIGURE 5. Two peripancreatic tumor-draining lymph nodes were used for isolation of CD4^+ cells, which were further fractionated into CD25^+ and CD25^- fractions. The CD4^+CD25^+ cells were stimulated with plate-bound anti-CD3 Ab and soluble anti-CD28 Abs for 48 h, and cocultured with stimulated CD4^+CD25^- lymphocytes in the indicated ratios. A, With increasing ratio of CD4^+CD25^+ cells present in the culture, the proliferation of CD4^+CD25^- lymphocytes was inhibited. B, The IFN-γ secretion by the same cells were also inhibited, indicating regulatory activity of CD4^+CD25^- cells derived from tumor-draining lymph nodes.
cytolytic effect on suppressor T cells resulting in T cell-mediated tumor rejection.

Our findings provide no mechanistic explanation for the increased prevalence of $T_{reg}$ in patients with invasive cancer. Future studies will explore two possible mechanisms: 1) tumor-mediated induction of $T_{reg}$ expansion, or 2) physiologic expansion of the $T_{reg}$ repertoire as a response to mostly self Ags born by cancer cells. No experimental evidence is currently available to favor either mechanism. However, by using an animal model, one can attempt to answer this question experimentally. If the emergence of $T_{reg}$ is physiological due to the immune response generated by cancer patients with Abs targeting Treg-bearing CD25 and CD152 molecules offer promise. Furthermore, the efficacy of any vaccine-based immunotherapy may be greatly enhanced by combining such vaccines with Abs that deplete Treg.

Acknowledgments

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References


