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Prognostic significance of regulatory T cells in tumor

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Since entering the immunological stage several decades ago, regulatory T cell biology has been realized as fundamentally important in the prevention of autoimmune conditions, induction of transplant tolerance and the immune response to cancer. The role of regulatory T cells in tumor immunobiology is still being elucidated. Currently, regulatory T cells are implicated in the dampening of antitumor T-cell responses both through direct and indirect means. A number of investigators have demonstrated that regulatory T cell density and location may serve as independent prognostic factors in several types of cancer and are alternately detrimental or beneficial to patient survival. In this article, we will review the characteristics and functional phenotype of classical regulatory T cells, describe their distribution and quantification in tumor-bearing hosts and summarize recent studies investigating the prognostic significance of regulatory T cell number and locality in various cancers.

Regulatory T (T_{Reg}) cells are a subpopulation of $CD4^+$ T cells with suppressive functionality. In healthy individuals, perhaps the most important role of regulatory T cells is to maintain immune tolerance to self-antigens, which prevents development of autoimmune disease. T_{Reg} cells are also responsible for limiting tissue damage during ongoing and resolving immune responses, maintaining oral and fetomaternal tolerance and restraining asthma and allergy. In settings of organ transplant and cancer, the suppressive function of T_{Reg} cells is currently being manipulated to improve patient health and survival. Investigators of transplantation biology are exploring ways to increase the number of alloantigen-reactive regulatory T cells in transplant recipients to minimize grafted tissue damage and prevent organ rejection.[1] In cancer patients, where regulatory T cells contribute to the dampening of the antitumor immune response, combination therapies that include the inhibition of regulatory T cell function have been explored. Although few Stage III trials of T_{Reg} inhibition have reached their clinical endpoints, analysis of T_{Reg} cells in tumor environments can still yield useful information about patient prognosis and tumor growth, and may eventually lead to new, more successful treatment regimes.

Definition

Regulatory T cells, originally termed suppressive T cells, were first described by Gershon *et al.*[2,3] in the early 1970s as thymus-derived lymphocytes that tolerized bone marrow–derived lymphocytes to antigenic challenge. Research in the laboratory of R.J. North subsequently demonstrated that T cells expressing CD4 and CD25 from tumor-bearing mice abrogated tumor rejection; this suggested the existence of a tumor-suppressor T cell population[4–6]. Many years later, after more than a decade of intense skepticism regarding the suppressive cells' existence, Sakaguchi *et al.* ascertained that the interleukin-2 (IL-2) receptor α-chain (also called CD25) could be used to identify them.[7] Later studies in the same laboratory, as well as studies from Rudensky *et al.*, established the transcription factor forkhead box P3 (FoxP3) as both a key intracellular marker of CD4+ CD25+ regulatory T cells and necessary factor for development and proper function of these cells,[8–10] which was described early on as prevention of autoimmune conditions (e.g., colitis[11]) and suppression of CD8+ T cell homeostatic proliferation.[12] Beginning with these reports, the field of regulatory T cells has expanded and progressed rapidly. In fact, several distinct regulatory T cell populations have been proposed, including CD8+ subsets. These include thymically derived CD8+ CD25+ T cells that utilize cytotoxic T-lymphocyte–associated antigen-4 (CTLA4) and transforming growth factor β (TGFβ) to suppress cell proliferation and activation,[13] as well as a CD8+ CD28− T cell population from the periphery that targets immunoglobulin-like transcripts 3 (ILT3) and 4 (ILT4) on dendritic cells (DCs).[14] Our group has identified CD8+ T cells[15,16] in human ovarian cancer that secrete the suppressive cytokine interleukin-10 (IL-10). Interestingly, a CD8+ regulatory T cell population specific for heme oxygenase-1 has recently been identified.[17] This population, isolated from the peripheral blood of cancer patients, inhibited proliferation, cytotoxicity and cytokine production of other cell immune cells. Groux *et al.* identified a FoxP3- CD4+ population (termed T_R1 cells), which may also suppress through IL-10 *in vitro*.[18] Weiner characterized a CD4+TGFβ+

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population (T_H3) that exerts suppressive action *in vivo* through TGFβ.[19] Both aforementioned populations are likely derived from the periphery. Classic regulatory T cells (T_{Reg}), CD4+CD25+FoxP3+ T cells, differentiate in the thymus and migrate to the periphery.[20,21] They constitutively express leukocyte common antigen isoform RO (CD45RO), glucocorticoid-induced tumor-necrosis factor receptor–related protein (GITR) and CTLA-4.[22–26] Fascinatingly, research from Arne Akbar's group has demonstrated that functional T_{Reg} may be induced from memory CD4+ T cell populations found in inflamed skin.[27] These antigen-specific CD4+ T cells were isolated, rendered anergic and then tested for suppressive capacity. Interestingly, in parallel with their newly acquired suppressive ability, FoxP3+ mRNA and protein expression in these cells increased profoundly, while CD25, GITR and CTLA-4 expression were all up-regulated. Finally, a recent paper from the laboratory of Shimon Sakaguchi presents the possibility of further categorizing naturally occurring T_{Regs} into three subgroups: CD45RA+ $FoxP3^{lo}$ resting T_{Reg}, termed "rTreg" by the authors, CD45RA− $FoxP3^{hi}$ activated Treg (aTreg) cells and cytokine-secreting CD45RA-$FoxP3^{lo}$ nonsuppressive T cells.[28] Ongoing investigations into phenotype, function and associations with disease states will likely contribute to knowledge of an even wider range of regulatory T cell populations in the future. Regardless, it is important to emphasize that regulatory T cells must be defined not only by phenotypic markers but also by their suppressive activity *in vivo*.

Distribution in Tumor-Bearing Hosts

In healthy mice and humans, T_{Reg} cells are found primarily in the thymus, peripheral blood, lymph nodes and spleen. They constitute 5–10% of the resident CD4+ T cells in each of these organs.[29–31] In bone marrow, however, T_{Reg} cells account for a remarkable 25% of CD4+ T cells.[32] Bone marrow is the preferential site of metastasis for some cancers (such as breast, lung and prostate), suggesting that the suppressive environment here is conducive to tumor growth. In tumors themselves, however, there are a number of ways that T_{Reg} cells might accumulate: trafficking to the tumor under the influence of chemokine ligand 22 (CCL22),[33] differentiation[15,16,34–37] or expansion[38–40] within the tumor stroma and conversion from normal T cells.[41–44] Many tumors express tumor-associated antigens (TAAs), molecules found not only on tumor cells but also on certain populations of normal cells. The work of several groups has identified multiple mechanisms of suppression by TAA-specific T_{Reg} cells. These include induction of IL-10, which can drastically suppress antigen-presenting cell (APC) and T cell function,[45] induction of TGFβ, which may suppress natural killer (NK) cell function,[46] competitive consumption of interleukin-2 (IL-2), which is a survival factor for conventional T cells,[29,47,48] perforin- and granzyme-dependent killing of T cells and APCs,[49,50] CTLA-4 induction of indolamine 2,3-dioxygenase (IDO)-expressing APCs, which suppress T cell activation and promote tolerance,[51,52] and finally, induction of B7-H4 expression on APCs, which renders them immunosuppressive.[53,54] Thus, T_{Reg} cells target both T cells and APCs to create a generally tolerant tumor microenvironment.

Mouse tumors

Tumor-associated T_{Reg} cells have been studied largely with reagents that target T_{Reg} cells in tumor-bearing mice. Treatment with CD25-specific antibody (PC61) *in vivo* suppressed growth of several tumor types.[55,56] These early studies demonstrated a correlation between reduced T_{Reg} numbers and reduced tumor volume. Interestingly, depletion of total CD4+ T cells corroborated these data and lead to improved tumor immunity and rejection of tumors.[57–59] Several groups confirmed these data with CD25-depletion alone or in concert with other treatments, such as anti-CTLA4 antibody,[58] anti-B7H1 antibodies (Zou *et al.*, unpublished observations), exogenous interferon-α (IFNα)[60] or interleukin-12 (IL-12),[61] adoptive transfer of DCs[60,62] and irradiated tumor cells.[63] Adoptive transfer of human[33] or mouse[64,65] T_{reg} cells into mice have also provided a direct functional connection between T_{Reg} cell presence and reduced tumor immunity. One study examined B16 melanoma-bearing mice that received tumor-specific CD8+ T cells with either classic T_{Reg} cells or with CD25− CD4+ T cells.[65] CD8+ T-cell–mediated tumor immunity was abrogated in mice receiving classic T_{Reg} cells, but not CD25− CD4+ T cells. These studies demonstrate that T_{Reg} cells inhibit murine TAA-specific immunity.

Human tumors

June *et al.* observed increased numbers of T_{Reg} cells in patients with nonsmall cell lung cancer and ovarian carcinoma when compared to healthy patients.[66] Since this study in 2001, several other groups have made similar observations in the peripheral blood of patients with various types of cancer, including pancreatic and breast cancer,[67] colorectal cancer (CRC),[68,69] gastric and esophageal cancer,[70,71] leukemia and lymphoma,[72,73] melanoma,[74,75] lung and ovarian cancer[66,69] and hepatocellular carcinoma.[76]

Quantification of Regulatory T Cells in Tumor

Regulatory T cell numbers may be evaluated in the tumor and tumor microenvironment in multiple ways. It is common for these cells to be identified on the basis of CD25 and/or FoxP3 expression. Quantification may be presented as a percentage of CD4+ T cells or total (CD3+) T cells, or as absolute number per area (such as mm^3) in a given tissue, as in the recent study by Haas *et al.*[77] Additionally, data may take the form of the ratio of regulatory T cells: effector T cells. Typically, tissue samples are investigated either by creating tissue microarrays and using immunohistochemistry or processing the tissue and examining cell populations via flow cytometry. Importantly, it is known that cell types other than T_{Reg} cells express what were formerly thought of as unique T_{Reg} markers: both CD25 and FoxP3 can be expressed on activated T cells.[78–80] A recent publication has suggested that

analysis of DNA methylation of the FoxP3+ gene may be a more conclusive way to identify T_{Reg} cells[81]: apparently, a certain region within the FoxP3 gene (TSDR, T_{Reg}-specific demethylated region) is demethylated. Because this demethylation is not found in other cell types—including those known to express FoxP3, like activated T cells—it allows for enumeration of T_{Regs} via DNA quantification methods such as PCR. When presenting T_{Reg} quantification data, it is important to consider it in the context of the numbers and locations of other lymphocytes associated with cancer prognoses, such as T-helper-17 (Th17) cells, tumor-associated macrophages (TAMs), and other APCs, especially DCs.

Association with Pathological and Clinical Outcome

Thus far, we have reviewed the current data on regulatory T cell phenotype, quantification and function in the tumor microenvironment. Much of the data suggests that a higher T_{Reg} cell number within the tumor microenvironment would imply a worse prognosis. In many cases, this is true. However, regulatory T cell infiltration in and around the tumor can be beneficial, depending on the type of tumor in question.

Ovarian cancer

A study in 2003 by Zhang *et al.* found significant differences in the distributions of progression-free survival and 5-year overall survival (OS) of 186 epithelial ovarian cancer patients according to the presence or absence of intratumoral CD3+ T cells.[82] Intratumoral T cells correlated with delayed recurrence or death of patients. T cell presence was additionally associated with microenvironmental expression of lymphocyte-attracting chemokines and increased intratumoral levels of IL-2 and IFNγ. While this study did not specifically address the presence or prognostic significance of T_{Reg} cells, it supports the notion that T cell presence within the tumor is beneficial—the logical hypothesis, then, would be that suppression of intratumoral T cell activation might decrease survival. A subsequent study from the laboratory of Kunle Odunsi demonstrated that epithelial ovarian cancer patients with higher numbers of intraepithelial CD8+ T cells had improved survival (55 *versus* 26 months) compared with patients with lower numbers.[83] Interestingly, no survival association was found for CD3+ tumor infiltrating lymphocytes (TILs). However, the patients with high versus low intraepithelial CD8+/CD4+ TIL ratios had median survival of 74 and 25 months, indicating that CD4+ TIL (or subpopulations thereof) might negatively influence the actions of CD8+ TIL. With this in mind, the investigators examined the survival of patients based upon intratumoral CD8+/T_{Reg} ratios. They found that patients with high ratios lived more than two times longer than those with lower ratios (58 *vs.* 23 months), and the study concluded that T_{Reg} presence in tumor tissue can negatively impact patient prognosis. Our group published a study in 2004 in which we examined 104 patients with ovarian carcinoma.[33] In these patients, we found that human tumor T_{Reg} cells suppressed tumor-specific T cell immunity and contributed to tumor growth *in vivo*. T_{Reg} cells were not only more populous in ascites and tumors of patients with Stage III and Stage IV disease, but were also associated with a strikingly high death hazard and reduced survival-patients with 341 or more T_{Reg} cells per ten high-power fields (HPF) had a 25-fold higher risk of death than patients with 131 or fewer T_{Reg} cells in the same number of fields. It is important to note that both tumor cells and TAMs produced the chemokine CCL22, which mediated T_{Reg} recruitment to the tumor. In 2007, we published a report describing the relationship between B7-H4, T_{Reg} cells, and the survival of 103 patients with ovarian cancer.[84] We had previously showed that ovarian tumor cells and TAMs expressed B7-H4.[53] Our data demonstrated that B7-H4 expression in tumor microenvironmental macrophages was significantly correlated with intratumoral T_{Reg} numbers, and that both of these variables were associated with poor patient outcome. Tumor T_{Reg} cells enabled local macrophages to spontaneously produce IL-10 and IL-6, through which the macrophages stimulated their own B7-H4 expression. Along with the 2004 study[33] and our more recent work demonstrating that T_{Reg} cells induce B7-H4 on APCs (including macrophages),[54] our data support the notion that T_{Reg} cells may convey suppressive activity to APCs through B7-H4 induction in human ovarian cancer. These four studies present a rather convincing picture that increased T_{Reg} cell presence and function in ovarian cancer correlates with reduced survival. The data detailing involvement of other cell types in T_{Reg} cell recruitment and function in the tumor emphasizes the necessity to comprehensively evaluate the cellular network in the tumor microenvironment.

Gastric cancer

Kawaida et al. published a study in 2005 documenting an increased number of CD4+ $CD25^{hi}$ T cells in regional lymph nodes in gastric cancer patients when compared to control mesenteric lymph nodes from the same patients.[85] Functional tests of these cells confirmed inhibitory activity corresponding to T_{Reg} cells. A subsequent report by Kono *et al.* investigated the proportion of CD4+ CD25hi FoxP3 mRNA-expressing T cells in total PBMC CD4+ T cells in 72 patients with gastric cancer and 42 patients with esophageal cancer.[86] Although they did not provide concrete numbers in the text of the study, the authors state that there were significant differences in the prevalence of CD4+CD25hi T cells between the early and advanced disease stages, both in gastric cancer (∼2% in stage I *vs.* ∼7% in stage IV) and esophageal cancer (about 2.5% in stage I *vs.* ∼8% in stage IV). They also state that in both cancers, the patients with a high proportion of CD4+ CD25hi T cells showed poorer survival rates (about 40 *vs.* 93% after 6 years for gastric cancer, and 25 *vs.* 55% in esophageal cancer) in comparison to those with a low proportion. This study utilized small groups and was not specific about grouping parameters. A 2008 study by Mizukami *et al.*

demonstrated a relationship between the localization of T_{Reg} cells and clinical outcome in 80 patients with gastric cancer.[87] Although the populations of Foxp3+ cells in patients with Stage IV cancer (107.4 cells per five randomly selected high-magnification fields *vs.* 47.2) were significantly larger than those with stage I cancer, this study did not find a significant difference between survival of patients with low levels of T_{Reg} cells (fewer than 34.5 cells per five randomly selected fields) and those with high T_{Reg} levels (more than 34.5) in the tumor. Localization patterns of infiltrating $Foxp3^+$ cells in the tumor were divided into three groups: a peritumour group (more frequent in Stage I), a diffuse group and a follicular group (defined as patients in whom the population of $Foxp3^+$ cells mainly occupied the lymphoid follicles of the submucosal layer compared with any other region of the tumor; more prevalent in Stages II–IV than Stage I). Interestingly, patients with a diffuse pattern of FoxP3+ cells had significantly poorer 10-year survival (60%) than patients with a peritumoral pattern (90%). This suggests that T_{Reg} location, rather than number, might be more important when forecasting the survival of patients with gastric cancer. Shortly afterward, Mizukami *et al.* published another report in which they investigated the frequency of FoxP3+ T_{Regs} within total CD4+ cells in TILs, regional lymph nodes and peripheral blood lymphocytes (PBLs) of gastric cancer patients ($n = 45$).[88] As might be expected, the frequency of T_{Reg} cells in TILs was significantly higher than in normal gastric mucosa (12.4 *vs.* 4.1%) both in early and late disease. Interestingly, the frequency of CCL17+ or CCL22+ cells among intratumoral CD14+ cells (monocytes and macrophages) was significantly higher than that of normal gastric mucosa, and this frequency correlated significantly with tumor-infiltrating T_{Reg} numbers. The investigators confirmed in an *in vitro* migration assay that T_{Reg} cells could be induced to migrate by CCL17 or CCL22. This study supports the notion suggested previously by our group[33] that chemokines secreted by monocytes and macrophages within the tumor environment are important for T_{Reg} trafficking into the tumor. More recently, Haas *et al.* published an investigation of T_{Reg} prognostic significance in 52 patients with intestinal-type gastric cardiac cancer.[77] Although the group found no relationship between the numbers of T_{Reg} cells (or macrophages) infiltrating the tumor and patient survival, they did observe that patients with larger T_{Reg} populations in the tumor stroma (>125.9 FoxP3+TILs/mm^2) had a median survival time of 58 months while those with smaller populations (<125.9 FoxP3+TILs per mm^2 of tissue) had a median survival time of 32 months. Interestingly, they also discovered that patients with higher (above 2.9) stromal CD68+ (a glycoprotein expressed on monocytes and macrophages)/FoxP3+ cell ratios in primary tumor had shorter median survival time than those with lower ratios. This data again supports the concept of an immunosuppressive and/or tumor-promoting role for APC in the tumor microenvironment. Haas *et al.* propose that their findings suggest that inflammatory processes within the tumor stroma of gastric cardiac adenocarcinomas may have direct effects on patient outcome. In opposition to the probable protumor role for macrophages, it is feasible that large numbers of stromal T_{Reg} may inhibit local cancer-promoting inflammatory processes. Therefore, it is possible in patients with chronic inflammation-associated cancers such as gastritis-associated gastric adenocarcinoma and ulcerative colitis–associated colon cancer (see discussion below), T_{Reg} may be protective.

Pancreatic cancer

In 2006, Hiraoka *et al.* performed a study of the clinical significance of T_{Reg} in the progression of pancreatic ductal adenocarcinoma.[89] On investigation of tumor tissue and draining lymph nodes of 198 pancreatic ductal adenocarcinomas, their associated premalignant lesions and 15 non-neoplastic pancreatic lesions, the investigators found an increased T_{Reg} prevalence in the ductal adenocarcinomas compared with that in the stroma of non-neoplastic lesions. This increase significantly correlated with certain clinicopathologic factors, including distant metastasis, advanced tumor stage and higher tumor grade. Interestingly, the investigators documented that infiltration of intraepithelial CD8+ cytotoxic T cells into pancreatic ducts was prominent in low-grade premalignant lesions but diminished during the progression of both pancreatic intraepithelial neoplasias and intraductal papillary-mucinous neoplasms. Conversely, numbers of stromal T_{Reg} cells increased during this progression. Patients with a low frequency (less than the average 34.6% of total intratumoral CD4+ T cells) of tumor-infiltrating T_{Reg} cells had significantly longer survival than those who had a high frequency (more than the average 34.6%) of intratumoral T_{Reg} cells.

Anal cancer

A study by Grabenbauer *et al.* explored the prognostic significance of T_{Reg} cells and TIL subsets in 38 anal squamous cell carcinoma patients treated with radiochemotherapy.[90] Although they found no prognostic effects for T_{Reg} or macrophages, the investigators did determine that higher numbers of cytotoxic TIL numbers (>0.6 granzyme B+ TILs per 100 tumor cells) served as indicators of poor prognosis (3-year survival rate of 47 *vs.* 89% in patients with fewer than 0.6 granzyme B+ TIL per 100 tumor cells). Additionally, 3-year survival rates for patients with low numbers of TILs (defined as $\leq$3.8 CD3+ per 100 tumor cells or $\leq$1.5 CD4+ per 100 tumor cells) were 89 and 95%, respectively, and 54 and 48%, respectively, in cases with high numbers (>3.8 CD3+ per 100 tumor cells or >1.5 CD4+ per 100 tumor cells). It appears here that lower numbers of TILs indicate better patient outcome. However, the prognostic significance of these cell populations must be considered in light of the fact that the patients examined had already been treated with radiochemotherapy, and it is therefore possible that the

remaining tumor cells may have arisen from radiation-resistant precursors.

Colorectal cancer

A recent study by Salama *et al.* assessed the survival correlations of CD8+, CD45RO+ and FoxP3+ T cell frequencies in tumor and normal colonic tissue from 967 patients with Stage II or Stage III CRC.[91] The investigators found that CD8+ and CD45RO+ cell densities (cells per mm^2) were lower in tumor than in normal tissue, but FoxP3+ cell density was higher. FoxP3+ cells were not associated with any histopathologic features other than tumor stage: interestingly, lower numbers of FoxP3+ cells correlated with more advance tumor stages. Further examination demonstrated that tumor stage, vascular invasion, and FoxP3+ density in normal and tumor tissue all served as independent prognostic factors. High FoxP3+ frequency (more than the median value of 44 FoxP3+ cells/mm^2) in healthy tissue was associated with worse prognosis, while higher frequencies of FoxP3+ cells in tumors (more than the median value of 116 FoxP3+ cells/mm^2) were associated with better survival. In this study, CD8+ and CD45RO+ T cells did not correlate with patient outcome. Two other studies have contributed to our knowledge of T_{Reg} cells in CRC in the past year. Sinicrope *et al.* investigated the prognostic impact of T_{Reg} and CD3+ T cell numbers within 160 Stage II or III colon cancer patients.[92] On comparison with normal colon tissue from the same patients, the investigators determined that densities of both T_{Reg} and CD3+ T cell populations were increased in tumor tissue. Although intraepithelial FoxP3+ cell numbers were not prognostic, higher levels of expression were found to correlate with poor tumor differentiation, advanced patient age and, interestingly, female gender. As for CD3+ T cells, patients with smaller intraepithelial populations experienced reduced disease-free survival (DFS). A low intraepithelial CD3+/FoxP3+ ratio (lower than the first quartile value of all patients) also served as a prognostic indicator for reduced DFS. Both of these variables were found to be prognostically stronger for patients with colon carcinoma than either numbers of lymph node metastases or tumor stage. More recently, Frey *et al.* investigated the prognostic significance of T_{Reg} cells in CRC patients after their tumors had been stratified by mismatch repair (MMR) status.[93] They examined 1,197 MMR-proficient and 223 MMR-deficient CRCs. Fascinatingly, high FoxP3+ numbers (classified as more than 17 FoxP3+ cells per microarray tissue punch, approximately the same area as one 40× field) in the MMR-proficient patients correlated with early T stage, tumor location (rectal) and better 5-year survival rate. In MMR-deficient CRCs, however, larger FoxP3+ populations were associated with an absence of lymph node involvement and absence of vascularization, along with a better 5-year survival rate. Finally, the investigators determined that an elevated FoxP3+ cell frequency served as an independent prognostic factor in MMR-proficient CRC and could predict enhanced survival in these patients.

Liver cancer

In 2007, Kobayashi *et al.* examined the infiltration of FoxP3+ T_{Regs} and CD8+ T cells in the tumor stroma and nontumorous liver parenchyma of patients with liver cancer.[94] Their samples included 323 hepatic nodules (including precursor lesions), early hepatocellular carcinoma (HCC), and advanced HCC, in addition to 39 intrahepatic cholangiocarcinomas and 59 metastatic liver adenocarcinomas. The investigators found that T_{Reg} numbers were significantly higher in HCC than in non-tumorous liver, and higher in primary HCC than in metastatic HCC. In both cases, HCC-infiltrating T_{Reg} cell density was an independent prognostic factor. T_{Reg} frequency was also increased in nontumorous liver (both with and without hepatitis) bearing primary tumors. Higher T_{Reg} numbers within tumor tissue correlated with higher tumor grade and tended to correlate ($p = 0.064$) with fewer infiltrating CD8+ T cells. The patient group with a high prevalence of T_{Regs} (greater than 29% of CD4+ T cells) infiltrating HCC showed a significantly lower DFS and overall survival (OS) rate (27.3 and 45.1 months, respectively) than patients with fewer than 29% of CD4+ T cells identified as T_{Reg} cells (36.2 and 60.3 months, respectively). Contrastingly, there was no significant difference in the OS or DFS between patients with low numbers of tumor-infiltrating CD8+ T and those with high numbers. Kobayashi *et al.* observed that during hepatocarcinogenesis, the prevalence of T_{Regs} increased, while CD8+ T cell numbers decreased. This work supports the notion that primary hepatic cancers develop in liver that is immunosuppressed by large populations of T_{Reg} cells. In the same year, Gao *et al.* published a manuscript detailing their investigation into the prognostic value of TILs in 302 HCC patients after tumor resection.[95] Interestingly, numbers of CD3+, CD4+ and CD8+ TILs were not associated with patient survival. However, fewer intratumoral T_{Regs} (<2.24 per 400× field) in combination with more (>17.74 per 400× field) activated CD8+ cytotoxic cells (CTLs, activation as defined by positive Granzyme B staining), served as an independent prognostic factor for both improved DFS and OS. Patients with high numbers of tumor-infiltrating T_{Regs} and low numbers of CD8+ CTLs (fewer than 17.74 per 400× field) had 5-year OS and DFS rates of 24.1 and 19.8%, respectively, whereas the group with low T_{Regs} and high CD8+ CTLs had rates of 64.0 and 59.4%. Both T_{Regs} alone and activated CTLs alone within the tumor served as independent predictors for OS. Patients with low numbers of intratumoral T_{Regs} had longer OS (70 months) and DFS (69 months) than did those with high numbers of T_{Regs} (higher than 2.24 per 400× field; 51 and 34 months, respectively). Interestingly, the investigators found a correlation between high T_{Reg} cell density and both absence of tumor encapsulation and presence of tumor vascular invasion, which suggests an association of T_{Reg} cells with tumor invasiveness. If a

CD8+ CTL-heavy balance of CTL and T_{Reg} in the tumor microenvironment is indicative of better patient outcome, then therapy which increases CD8+ number and efficacy while simultaneously depleting T_{Reg} cells would be ideal. In 2006, Cai *et al.* explored the potential of intratumoral DCs and T cells to serve as prognostic indicators in 123 patients who underwent surgical resection of hepatocellular carcinoma.[96] Although the investigators did not find a significant correlation between the number grade of infiltrating immune cells in HCC nodules or pericancerous tissues and DFS, they observed that an absolute number of DCs in HCC nodules of 25 or more per ten HPF did correlate with DFS. However, 28 or more DCs per ten HPF in pericancerous tissues had no correlation with survival. As might be expected, more DCs alone or with T lymphocytes (CD3+, CD45RO+ or CD8+), or more CD8+ T lymphocytes alone in HCC nodules strongly correlated to longer tumor-free survival time. It is important to consider this study because it explores numbers and locations of immune cells other than T_{Reg} cells that likely interact with T_{Regs} in the tumor microenvironment. In 2009, we published a study of B7-H1 and PD-1 expression in HCC patients in which we determined not only that B7-H1 expression on Kupffer cells (KC) was increased in tumor tissues compared with surrounding nontumor liver tissues, but also that this expression correlated with poor survival.[97] Additionally, numbers of PD-1+ CD8+ T cells were higher in tumor tissues than in non-tumor tissues, and B7-H1+ KCs and PD-1+ T cells colocalized in the HCC stroma. PD-1+ CD8+ T cells had decreased proliferative ability and effector function, but these attributes were rescued on PD-1/B7-H1 blockade. In summary, it is clear from the aforementioned studies that T_{Regs} are not the only prognostic marker of survival in HCC patients. Zhang *et al.* recently published a study investigating the prognostic potential of Th17 cells in 178 patients with hepatocellular carcinoma.[98] The investigators found that Th17 cell numbers were higher in tumors of HCC patients than in non-tumor tissue, and that these Th17 displayed an effector memory phenotype. It was also determined that intratumoral frequency of IL-17–producing cells, which correlated proportionally with tumor microvessel density, could serve as an independent prognostic factor for OS and DFS. Other studies have documented a proangiogenic role for IL-17.[99,100] In HCC, intratumoral density of Th17 cells is negatively associated with patient outcome.

Head and neck cancer

In 2006, a study from the laboratory of Eric Tartour investigated the prognostic value of various tumor-infiltrating CD4+ T-cell populations in 84 untreated patients with head and neck squamous cell carcinoma.[101] The investigators found that larger populations of tumor-infiltrating CD4+ CD69+ (activated) T cells (greater than 2.6 cells per field using a 40× objective) correlated with both better local control of the tumor and longer patient survival. Interestingly, higher numbers of intratumoral regulatory Foxp3+ CD4+ T cells (more than 1.5 cells per 40× field) were also positively associated with and served as an independent prognostic factor for better regional control of the tumor. CD4+ CD69+ T cells made up the only population within the tumor that significantly influenced OS: more infiltration correlated with better patient outcome. In head and neck cancer, T_{Reg} cells may enact better local tumor control through suppression of inflammatory intermediates.

Breast cancer

Also in 2006, Bates *et al.* performed experiments to assess the clinical significance of T_{Reg} cells in breast cancer patients with pure ductal carcinoma *in situ* (DCIS; $n = 62$), invasive breast cancer ($n = 237$) or from samples of normal breast tissue ($n = 10$). The investigators found increased T_{Reg} numbers in *in situ* and invasive breast carcinomas when compared with normal breast tissue, and larger T_{Reg} populations in invasive tumors than in DCIS. Increased levels (greater than or equal to 15 positively-stained cells per 1 mm diameter invasive tumor cores) of T_{Reg} cells distinguished both patients with DCIS at increased risk of relapse and patients with invasive tumors who would go on to have shorter DFS and OS. High-grade tumors, patients with lymph node involvement and estrogen receptor (ER)-negative tumors all displayed significantly larger numbers of T_{Reg} cells. Patients with larger T_{Reg} populations in ER+ tumors were categorized as high risk. In this study, high numbers of T_{Reg} cells identified patients at risk of relapse after 5 years. Bates *et al.* recommend T_{Reg} as a novel marker for identifying late-relapse patients who might be good candidates for aromatase therapy (which suppresses estrogen production) after tamoxifen treatment.

Lymphoma

Research from the lab of Miguel Piris in 2005 explored the relevance of T_{Reg} and CTL (defined by TIA-1 and Granzyme B) populations in the reactive background of Hodgkin's lymphoma (HL) samples in the prognosis of 257 patients with classic HL (cHL).[102] Previous research reported by Oudejans *et al.* that increased numbers of CTLs were associated with poor patient outcome[103] was met with skepticism.[104] The 2005 report showed that a smaller population of FoxP3+ cells (lowest quartile of total patient numbers) combined with higher numbers of CTL (highest quartile) in the infiltrate served as an independent prognostic factor that negatively influenced event-free survival (EFS) and DFS in cHL patients. Alvaro *et al.* tested four cases in which patients relapsed and discovered that these samples tended to have more TIA-1+ cells and a lower proportion of FoxP3+ cells than at the time of diagnosis. The results of this investigation suggest that a combination of more CTLs with small numbers of FoxP3+ cells in the reactive background may predict a poor outcome in cHL patients. Three years later, Karube *et al.* analyzed the expression of FoxP3 in adult T-cell leukemia and lymphoma.[105] Interestingly, 60 (36%) of the 169 cases examined had FoxP3

expression in lymphoma cells. On closer examination, the investigators found that FoxP3+ and FoxP3− leukemia/lymphoma cases did not differ in clinical stage, age distribution, lactate dehydrogenase and calcium in serum or in overall survival. However, a larger proportion of FoxP3+ cases (8/34) suffered from severe infection; while in FoxP3− cases, only two of 62 patients did so. Karube *et al.* also demonstrated that FoxP3 expression in adult T-cell leukemia/lymphoma indicated certain morphological features (including chromosome abnormalities) and concluded that this T_{Reg} marker is associated with patient immunosuppression.

Melanoma

In 2007, Miracco *et al.* analyzed 66 vertical growth phase primary cutaneous melanomas for correlation of T_{Reg} cell presence with recurrence potential.[106] The investigators discovered that the percentage of T_{Regs} within tumor parenchyma, at its periphery, and among TILs at the tumor–stroma boundary, was significantly higher in patients that experienced recurrence than in those that did not. Interestingly, many of the T_{Regs} identified in these samples were found in close proximity to TAMs, the presence of which has been correlated to poor prognosis in patients with advanced melanoma.[107] Although the Miracco study did not analyze such parameters as distant metastases and patient survival, the preliminary data point to the possibility of using T_{Reg} quantification as a prognostic indicator in melanoma.

Conclusions

Although regulatory T cells share functionality and mechanisms of suppression across cancers, it is important to recognize the individual (tumor-specific) nature of the T_{Reg} component of any given tumor microenvironment. As we have reviewed above, T_{Regs} play an important role in the development and maintenance of tumors and in the abrogation of the immune responses against them. In many cancers, elevated T_{Reg} cell presence and number imply a worse prognosis for the patient in question. However, this is not always the case. As the studies of gastric, colorectal and anal cancer suggest, it seems that T_{Regs} also have a different role. In these reports, increased T_{Reg} populations within the tumor tissue seemed to be beneficial. It is interesting to note that all of these cancers are localized to the gastrointestinal tract, portions of which are sites of the most rapid cell turnover in the human body. It is possible that T_{Reg} cells in this environment are more crucial for restraining inflammation (and thus preventing angiogenesis and other developments beneficial to tumor growth and survival) than for shutting off the host's response to the tumor.[108,109] Chronic inflammation is often linked to cancer development in the gastrointestinal system. Further investigation into this phenomenon is warranted. In addition to tumor location, then, it is important to consider T_{Reg} populations in the context of their upstream and downstream mediators, as well as alongside their cohorts in the tumor microenvironment. It is prudent to acknowledge the recently identified population of myeloid-derived suppressor cells (MDSC), the known functions of which thus far include inhibition of T cell activation, proliferation, migration and cytokine production through a variety of mechanisms.[110] As many studies have described, other T cell subsets and APCs (notably macrophages and dendritic cells) are significant in their relationships to intratumoral T_{Reg} cells in the recruitment of T_{Regs} into the tumor, influence of T_{Reg} function within the tumor environment and in the molecules they (as target cells) upregulate in response to T_{Reg} signaling. For example, IDO, a molecule induced in APC via CTLA-4 expression on T_{Reg}, has been shown to diminish local CD8+ T cell infiltration and responses to allogeneic target cells *in vitro* and *in vivo*.[111] Interestingly, Sørensen *et al.* identified IDO-specific cytotoxic effector CD8+ T cells in the peripheral blood and tumor microenvironment of some cancer patients,[112] demonstrating that the immune system can mount responses against at least some of the mechanisms designed to suppress it. This affirms the importance of evaluating intratumoral T_{Reg} presence in the context of other T cells, especially CD8+ T cells. Indeed, no single molecule or cell examined thus far within the tumor environment can serve as an absolutely independent indicator of patient survival. Therefore, it will be necessary to examine multiple tumor-associated cell populations in tandem with well-defined pathological, clinical and genetic parameters to more accurately predict patient outcome.

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