Chapter 2
T Cell and Antigen-Presenting Cell Subsets in the Tumor Microenvironment

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Abstract The development of successful antitumor immunity depends upon cross talk and collaboration between multiple T cell and antigen-presenting cell subsets. In this chapter, we review and summarize current knowledge regarding the function, interactions, and prognostic significance of each of these populations, as well as their dependence upon one another within the tumor microenvironment.

2.1 Cytotoxic T Lymphocytes

Cytotoxic T lymphocytes (CTLs) have long been the focus of antitumor immune study. The first evidence that T cells could in fact kill tumor cells came from L.R. Freedman and colleagues in 1972[1]. Nearly two decades later, investigators

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observed that high numbers of tumor-infiltrating $CD8^+$ T cells correlated with increased cancer patient survival[2]. Since that time, studies of colorectal cancer[3], hepatocellular carcinoma[4, 5], ovarian cancer[6], esophageal carcinoma[7], leukemia[8], and various other cancers have all indicated similar prognostic value of $CD8^+$ T cell infiltration[9]. Interestingly, this seems to be specific to tumor tissue as circulating tumor-antigen-specific $CD8^+$ T cells have no prognostic significance in melanoma patients[10].

$CD8^+$ T cells use multiple mechanisms to kill tumor cells. They express granzymes, perforin, and ligands of the tumor necrosis factor (TNF) superfamily, including Fas ligand. CTLs use their surface-expressed TNF family members to bind corresponding receptors on the surfaces of tumor cells, engaging an intrinsic death program[11]. Granzymes are formed in the $CD8^+$ T cell only after antigen-specific activation of the cell[12–14]. Once the enzymes have been delivered to the target tumor cell, killing can occur in as few as 20 minutes[15]. The signature cytokines expressed by $CD8^+$ T cells are also important—secretion of interferon-gamma (IFN-γ) and TNF alpha (TNF-α) mediate many antitumor effects. It is not yet clear, however, if these effects occur directly within the tumor cells, or whether they influence other mechanisms that aid in antitumor immunity. IFN-γ is well known for its antiangiogenic properties[16–18] and its stimulatory effects upon macrophages[19, 20]. It is also possible that this cytokine could prompt tumor cells to upregulate antigen-presentation machinery, increase their antigenic properties, and/or induce the expression of Fas[21–26]. Whereas CTL secretion of IFN-γ is directional (toward the immunological synapse and thus the target cell), TNF-α release is not[27]. TNF-α can therefore nonspecifically affect other nearby immune cells or vasculature[28]. There is also evidence for IFN-γ- and TNF-α-mediated destruction of tumor stroma[29]. To be sure, directed studies are required further to elucidate the antitumor effects of TNF-α. However, there are obstacles to efficient CTL and other T cell subset trafficking into tumor tissues[30, 31]. One of the major problems is the lack of a mature, properly developed vascular system within the malignancy. Recent imaging studies have contributed to our knowledge of CTL trafficking and the kinetics of killing in the tumor microenvironment[32, 33].

A discussion of tumor-infiltrating $CD8^+$ T cells cannot exclude the seminal work of Dr. Stephen Rosenberg, who was the first to harvest patients' own tumor-infiltrating lymphocytes (TILs), expand them in culture with IL-2, and reinfuse them to take advantage of their capacity for specific lysis[34, 35]. Although some patients experienced clinically measurable improvement, many did not. To evaluate why induced antitumor responses do not necessarily correlate with clinical responses, we must keep in mind both the immune-manipulating properties of the tumor microenvironment (see the antigen-presentation cell section below) and the simple property that tumor cells less susceptible to specific lysis will live and divide longer than those easily killed by TILs[36]. Any surviving tumor will likely be more resistant to such CTL mechanisms of elimination.

2.2 T-Helper-1 Th1

The Th1/Th2 paradigm was first demonstrated in 1986 by Mosmann and Coffman[37]. In their experimental conditions, T-helper-1 (Th1) and T-helper-2 (Th2) cells could be polarized with IFN-γ and IL-4, respectively. The key transcription factors to control Th1 and Th2 polarization are T-bet and GATA3, respectively. The involvement of helper T cells in the development of anticancer immunity was initially thought to include only the priming and support, through CD40/CD154 interactions with antigen-presenting cells (APCs)[38–40] and secretion of IFN-γ and interleukin (IL)-2, of a fully activated $CD8^+$ T cell response[41, 42]. However, subsequent experiments have shown that the importance of both Th1 and Th2 subsets does not end with $CD8^+$ CTL activation. An elegant mouse study from 1998 demonstrated that both Th1 and Th2 cytokines play essential roles in antitumor immunity. Cytokines secreted by Th1 cells are capable of recruiting and activating macrophages[41]. Macrophage-derived nitric oxide has multiple antitumor properties, including control of macrophage killing of tumor cells[43–45]. A key function of Th1-polarized T cells in tumor-bearing hosts is the secretion of IFN-γ, which can substantially increase the level of IL-12 production by stimulated dendritic cells (DCs)[46]. DC-derived IL-12 serves to polarize naïve T cells to the Th1 phenotype. In this way, Th1 cells can contribute to their own population growth and maintenance. Additionally, an interesting recent paper from the Corthay laboratory has suggested that Th1-derived IFN-γ in the tumor microenvironment elicits both in vivo macrophage killing of cancer cells and macrophage elaboration of the angiostatic chemokines CXCL9/MIG and CXCL10/IP-10[47]. Whether this holds true in human patients remains to be determined.

Patients with Kaposi sarcoma have what appears to be a Th1-like predominance in their TIL and blood, characterized by a high secretion of IFN-γ. These patients also had higher $CD8^+$ T cell numbers. Kaposi sarcoma is often accompanied by a concomitant infection with herpesvirus, so it is possible that this Th1-like phenotype is elicited in reaction to the virus[48]. Kusuda et al. found that a higher proportion of IFN-γ to Th2-type cytokines was strongly associated with better prognosis in patients with ovarian cancer[49]. Intriguingly, a study from the same year found that a high Th1:Th2 ratio in the peripheral blood mononuclear cells of patients with non-small cell lung cancer was actually predictive of shorter survival[50]. IFN-γ and chemokines associated with a Th1 response, including monokine induced by IFN-γ (MIG) and IFN-γ-inducible T cell α chemoattractant, identified renal cell carcinomas that did not recur after surgical resection. In addition, higher expression of MIG was correlated with a favorable prognosis[51], suggesting that the induction of a Th1-type response in kidney cancer patients is beneficial. A very recent report examining gastric cancer showed that higher initial Th1:Th2 ratio (as defined by expression of IFN-γ and IL-4) and higher Th1:Th2 ratio 14 days after surgery indicated better patient prognosis[52]. Tosolini and colleagues recently demonstrated that colorectal patients with high levels of Th1-associated gene expression (T-bet, IRF1, IL12Rb2, and STAT4) in their tumor tissue had longer disease-free survival. When the investigators paired some Th1 information with the expression of genes involved in cytotoxicity

(GNLY, GZMB, and PRF1) and Th17-related genes (RORC and IL17A), they could classify patients into four groups. Those with high Th1/cytotoxicity gene expression and low Th17-associated gene expression had the best 5-year disease-free survival.

2.3 Th2

Th2 cells are well known for their involvement in allergy and the response to helminths and other extracellular pathogens. The development of Th2 cells is controlled by the transcription factor GATA-3 and by exposure to IL-4[53–56]. Many laboratories have investigated the function of Th2 cells in the context of tumor immunity and explored how these cells impact disease development and patient survival. Th2 cells are crucial in recruiting eosinophils to the tumor site[41]. Although a definitive effect of this population on the tumor is still controversial, it has been observed that eosinophils are capable of killing tumor cells via secretion of their cytotoxic protein products[57, 58].

Myriad early reports documented an "unbalanced" or "decreased" Th1:Th2 ratio in malignancy[42, 59]. Some studies have found a predominantly Th2 phenotype in TIL populations of certain cancers[60], where they are skewed by tumor cell expression of IL-10 and serve to counteract the IFN-γ-driven Th1 and CTL antitumor response. Huang et al. demonstrated the Th2 cytokine-expressing capacity of non-small cell lung cancers in 1995[61]. Maeurer and colleagues found a similar cytokine signature in renal cell cancer[62]. A very recent report showed that Th2-type cytokines in the microenvironment of colorectal cancer had no prognostic significance for patient survival[63], which correlates well with a previous study[64]. Although early reports suggested that Th2 cells might contribute to antitumor immunity[65], it now seems that these cells fail to protect the host[66]. There is some evidence in mice, however, that the Th2-associated cytokine IL-4 serves to prime Th1-associated, tumor-specific CTL[67]. Melanoma patients who develop Th2 responses usually experience disease progression[68, 69]. Interestingly, some cancer patients do have tumor-antigen-specific Th2 cells in their blood. Melanoma[70–72] and renal cell carcinoma[73] patients have both been examined in this regard. The Rocken laboratory found that in mice, the human tumor-associated antigen EpCAM could induce Th2 skewing even under heavily Th1-polarizing conditions. Although human patient studies are required, it is possible that tumor cell EpCAM could drive a Th2 response while downregulating Th1 development. This combination of Th1/Th2 skewing could help tumors avoid the host immune response.

Pancreatic cancer, one of the most aggressive malignancies, has an intriguing relationship with Th2. Tumor stroma is typically characterized by a heavy Th2 infiltrate[74]. A recent, elegant study demonstrated that the ratio of Th2:Th1 cells in pancreatic tumors could serve as an independent prognostic marker of patient survival[75]. This study also identified cancer-associated fibroblast-derived thymic stromal lymphopoietin as capable of conditioning myeloid DC. These conditioned myeloid DCs could then produce Th2-attracting chemokines and polarize T cells to

a Th2 phenotype. It seems that Th2 cells in the tumor microenvironment can be induced by multiple tumor-derived factors and that they serve to impede or co-opt the development of antitumor responses.

2.4 Th17

Since their identification within the last half decade, Th17 cells have risen to prominence in studies of nearly every human pathology. While their role in many conditions is rather well understood, their function(s) in the context of tumor immunology remains contentious. Th17 cell effects in the tumor microenvironment are often grouped or confused with those of IL-17, IL-23, and other Th17 "signature cytokines." Data from mouse studies and chemically induced tumorigenesis have further complicated the issue. However, here we will focus exclusively on Th17 studies in the human tumor environment.

Th17 cells are defined as $CD4^+$ T-helper cells whose developmental program is controlled by the transcription factor RAR-related orphan receptor gamma T and multiple cytokines[76]. Human tumor-associated Th17 cells express minimal levels of HLA-DR, CD25, granzyme B, programmed cell death 1 (PD-1), or forkhead box P3 (FoxP3), suggesting that they are not a conventional effector or immune-suppressive cell population. Th17 cells in cancer patients produce high levels of granulocyte-macrophage colony-stimulating factor (GM-CSF), TNF-α, IL-2, and IFN-γ, but no IL-10[77, 78]. Tumor-associated Th17 cytokine products mimic those found in some instances of viral infection[79, 80]. These cytokines may be the primary mediators by which Th17 from cancer patients influence local immune responses. Interestingly, Th17 cells expanded in vitro from TIL populations in melanoma, breast, and colon cancers secrete IL-8 and TNF-α, but no IL-2[81]. Because Th17 cells isolated from both healthy donors[82] and patients with autoimmune diseases[83] produce the same cytokines, it is possible the phenotypes of freshly isolated Th17 cells and those induced in vitro from tumor-associated populations differ.

Tumor-associated Th17 express large amounts of the homing molecules CXCR4 and CCR6, c-type lectin receptor CD161, and the CD49 integrin isoforms c, d, and e, but no CCR2, CCR5, or CCR7[77]. As CCR6 and CD161 have been observed on both Th17 cells from healthy donors and on various cells in inflammatory environments[84–86], they may not serve as Th17-specific molecules.

Many laboratories have studied Th17 populations in the blood and (occasionally) tissues of patients with various cancers (**Box 1**). Throughout our work with ovarian cancer patients, we have made several key observations in regard to Th17 distribution and function. Th17 cell numbers in the tumor-draining lymph nodes and blood of these patients is comparable to that of healthy donors. Th17 cells constitute a numerically small but proportionally high population within the tumor microenvironment in comparison to other immune cell subsets. Within the tumor environment, Th17 levels correlate positively with Th1 cells, cytotoxic $CD8^+$ T cells, and NK cells. Perhaps not surprisingly, their numbers are inversely related to those of

regulatory T (Treg) cells[77, 87]. In vitro expansion data from Su et al. corroborates our findings of higher numbers of Th17 cells in TIL populations than in lymphocyte populations from non-tumor tissue[81]. IL-17 derived solely from Th17 cells in ovarian cancer ascites fluid correlated positively with patient survival and served as a negative predictor of death hazard. The average survival of patients with greater than 220 pg/ml IL-17 in ascites was 78 months, while patients with less IL-17 survived for only 27 months. IL-17 in the tumor microenvironment synergized with IFN-γ to induce the Th1-type chemokines CXCL9 and CXCL10. Ascites levels of CXCL9 and CXCL10 correlated directly with tumor-infiltrating NK and $CD8^+$ T cells, suggesting that these chemokines recruited effector cell populations to the tumor[77]. In agreement with our finding that Th17 cells are protective, Sfanos et al. found an inverse correlation between Th17 cell differentiation stage in the tumor mass in prostate cancer patients and their tumor progression[88]. Malignant pleural effusion from patients with lung adenocarcinoma or squamous cell carcinoma was chemotactic for Th17 cells, and this activity was partially abrogated by chemokine ligand 20 (CCL20) and/or CCL22 blockade. Interestingly, higher accumulation of Th17 cells in malignant pleural effusions predicted improved patient survival[89].

Intriguingly, Derhovanessian et al. demonstrated an inverse correlation between pretreatment circulating levels of Th17 cells in patients with hormone-resistant prostate cancer and time to disease progression[90]. The levels of Th17 cells are usually limited in cancer patients[77, 87]. Increased Th17 in the blood could indicate an underlying infection or other inflammatory state. IL-17 would certainly have an impact on the efficacy of immunotherapy and tumor development speed. IL-17-producing cells are enriched predominantly in the peritumoral stroma of hepatocellular carcinoma tissues, where their levels correlated with monocyte/macrophage density. Consistent with our observations[77], Kuang et al. found that tumor-activated monocytes were better than tumor-associated macrophages (TAMs) in inducing in vitro expansion and proliferation of Th17 from circulating memory T cells[91]. However, not all studies of Th17 in malignancy demonstrate a clear relation to disease progression: a recent study showed no correlation of Th17 numbers with nasopharyngeal patient clinicopathological characteristics or survival[92].

Patients with chronic inflammation have a greatly increased risk of cancer in the affected organs[93, 94]. Because inflammation resulting from infections can often contribute to the development of malignancy, it is necessary to understand the kinetics and targets of inflammation in a discussion of cancer. Our laboratory found that Th1-derived IFN-γ could rapidly induce B7-H1 expression on APCs and stimulate their production of IL-1 and IL-23. B7-H1 signaling abrogated the Th1-polarizing capacity of the APC, while IL-1 and IL-23 directed them toward a memory Th17-expanding phenotype[95]. In the course of inflammation, the acute Th1-mediated response is attenuated by IFN-γ-induced B7-H1 on APCs and is subsequently evolved toward chronic inflammation mediated by Th17 cells. Not only does this data challenge the dogma of Th17 suppression by IFN-γ, it also reinforces the notion that Th17 population kinetics depend strongly on the ongoing immune response and constituents of the cytokine milieu. Disease progression influences both of these factors.

2.5 Treg

T regulatory cells, originally termed suppressive T cells, were first described in the early 1970s as thymus-derived lymphocytes that tolerized bone marrow-derived lymphocytes to antigenic challenge[96, 97]. Subsequent research demonstrated that T cells expressing CD4 and CD25 from tumor-bearing mice abrogated tumor rejection[98–100]. After more than a decade of intense skepticism, Sakaguchi and colleagues ascertained that the IL-2 receptor α-chain (CD25) could be used to identify these suppressive cells[101]. Later studies in the same laboratory and others established the transcription factor FoxP3 as both a key intracellular marker of $CD4^+CD25^+$ Tregs and was a necessary factor for development and proper function of these cells[102–104]. Beginning with these reports, the field of Tregs has expanded and progressed rapidly. In fact, several distinct regulatory T cell populations have been proposed, including $CD8^+$ subsets. These include $CD8^+CD25^+$ T cells from the thymus that utilize TGF-β and cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) to suppress cell activation and proliferation[105], as well as a peripheral $CD8^+CD28^-$ T cell population that targets DC immunoglobulin-like transcripts 3 and 4[106]. We have identified IL-10-secreting $CD8^+$ T cells[107, 108] in human ovarian cancer. A $FoxP3^-CD4^+$ population (termed T_R1 cells) identified by Groux et al. can also suppress through IL-10 in vitro[109]. Weiner et al. characterized a peripherally derived $CD4^+TGF\text{-}\beta^+$ population (T_H3) that exerts suppressive action in vivo through TGF-β[110]. $CD4^+CD25^+FoxP3^+$ T cells, termed "classical T regulatory cells" or T_{Regs}, differentiate in the thymus and then migrate to the periphery[111, 112]. T_{Regs} constitutively express glucocorticoid-induced tumor necrosis factor receptor-related protein, leukocyte common antigen isoform RO (CD45RO), and CTLA-4[113–117]. Recent data presents the possibility of further categorizing naturally occurring T_{Regs} into three subgroups: $CD45RA^+FoxP3^{lo}$ resting T_{Reg}, termed "rTreg," $CD45RA^-FoxP3^{hi}$ activated Treg (aTreg) cells, and cytokine-secreting $CD45RA^-FoxP3^{lo}$ non-suppressive T cells[118]. Ongoing investigations into phenotype and function will likely contribute to the appreciation of an even wider range of regulatory T cell populations in the future.

In humans, T_{Reg} cells are found primarily in the thymus, peripheral blood, lymph nodes, and spleen, where they constitute 5–10% of the resident $CD4^+$ T cells[119–121]. In bone marrow, however, they make up a remarkable 25% of $CD4^+$ T cells[122]. 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 malignancy. In tumors themselves, however, there are a number of ways that T_{Reg} cells accumulate: trafficking under the influence of CCL22[123], differentiation[89, 96, 107, 108, 124, 125] or expansion[126–128] within the stroma, and conversion from other T cell populations[129–132]. Many types of tumors express tumor-associated antigens—molecules found on tumor cells and certain populations of normal cells. Multiple mechanisms of suppression enacted by tumor-associated antigen-specific T_{Reg} cells have been identified. These include the induction of IL-10 and TGF-β, which can drastically suppress APC, natural

killer (NK), and T cell function[133, 134]; competitive consumption of the T cell survival factor IL-2[119, 135, 136]; perforin and granzyme-dependent killing of APCs and T cells[137, 138]; CTLA-4 induction of indolamine 2,3-dioxygenase (IDO)-expression, which promotes tolerance[139–141]; and finally induction of B7-H4 expression on APCs, which renders them immunosuppressive[142]. In these ways, T_{Reg} cells target both T cells and APCs to create a generally tolerant tumor microenvironment.

Increased numbers of T_{Reg} cells have been observed in patients with many types of cancer, including pancreatic and breast cancer[143], colorectal cancer[144, 145], gastric and esophageal cancer[146, 147], leukemia and lymphoma[148, 149], melanoma[150, 151], lung and ovarian cancer[145, 152], and hepatocellular carcinoma[153].

Many studies have examined the prognostic significance of T_{Reg} cells in the tumor microenvironment, and these are reviewed in detail[154]. Briefly, higher T_{Reg} numbers in and around ovarian cancer negatively impact disease progression and patient survival[6, 87, 155]. Work from our laboratory has demonstrated that B7-H4 expression on TAMs and tumor cells correlated with intratumoral T_{Reg} presence[142]. Higher numbers of T_{Regs} in pancreatic cancer also predict more advanced disease and shorter survival[156]. Melanoma is similar: T_{Reg} populations were larger in patients who experienced recurrence than in those who did not. Interestingly, T_{Regs} were often found in proximity with TAMs, the presence of which is associated with poor prognosis[157, 158]. Breast cancer patients with higher T_{Reg} numbers have increased chance of relapse and shorter overall survival[159]. Finally, more liver cancer-associated T_{Regs} correlate with poorer disease-free and overall patient survival[4, 160].

T_{Regs} in other cancers are not so easy to define. Increased T_{Regs} in head or neck squamous cell carcinoma indicate better regional tumor control[161]. Studies in gastric cancer point to T_{Reg} location, rather than number, as an important prognostic factor in that patients with peritumoral T_{Regs} had better overall survival than those with a diffuse T_{Reg} pattern[162]. Another study found that larger Treg populations in the stroma of gastric cancer patients correlated positively with longer survival[163]. Colorectal cancer studies parallel gastric cancer: various studies have found associations of higher T_{Reg} numbers with poorly differentiated tumors or earlier stage and better patient overall survival[164–166]. In lymphoma, fewer T_{Regs} and more CTLs in the reactive background serve as an independent prognostic factor suggesting shorter patient disease-free survival[167]. It is possible that in these cancers, T_{Reg} cells predominantly function to minimize inflammation rather than curb the antitumor response. More careful mechanistic studies will shed light on this hypothesis.

2.6 Myeloid Dendritic Cells

Myeloid DCs are the most frequently studied of the APC subsets. They stimulate the adaptive arm of the immune system by activating naïve T cells[168]. Pulsing of DCs with killed ovarian tumor cells can stimulate tumor-specific blood-derived

T cells, which can produce IFN-γ upon autologous tumor cell encounter[169]. Various other studies demonstrate the potential of tumor-antigen-pulsed DCs to stimulate CTL responses in vitro[170, 171]. The antitumor protection observed upon adoptive transfer of appropriately primed myeloid DCs to tumor patients[172, 173] is rarely seen in the natural development of human tumors[174]. The tumor and its environment produces factors that suppress the development and normal function of DCs[107, 175], which compromises antitumor immunity. In 2003, our laboratory demonstrated low expression levels of the inhibitory molecule B7-H1 on blood- and lymph node-derived myeloid DCs in healthy individuals but observed much higher expression of the molecule on myeloid DCs from tumor-draining lymph nodes and tumors[176] from patients with ovarian cancer. B7-H1 expression on these cells was controlled by IL-10, previously shown to decrease co-stimulatory molecules on DCs[177], and vascular endothelial growth factor, known to inhibit DC differentiation from hematopoietic precursors[175]. Abrogation of B7-H1 signaling enhanced myeloid DC-mediated T cell activation, which correlated with a decrease in T cell-derived IL-10 and an increase in T cell-derived IL-2 and IFN-γ. Interestingly, this treatment also downregulated IL-10 expression and stimulated increased IL-12 expression on myeloid DCs. T cells conditioned with myeloid DCs in which B7-H1 had been blocked could inhibit autologous human ovarian carcinoma growth better than unconditioned T cells when xenotransplanted into nonobese diabetic−severe combined immunodeficient mice. A recent report from the Knustson laboratory showed that in addition to expressing B7-H1, murine ovarian tumor-associated myeloid DCs acquire higher levels of programmed death receptor-1 (PD-1) over time. PD-1 ligation on these cells impeded NF-κB activation, elaboration of numerous cytokines (IL-10, IL-6, IL-12, TNF-α, and GM-CSF) and co-stimulatory molecule upregulation[178].

Hepatocyte growth factor could stimulate papillary thyroid carcinoma cells to secrete MIP-3α (CCL20) and other chemokines to recruit immature myeloid $CD1a^{++}$ DCs to the tumor periphery[179, 180]. By contrast, mature DCs have been documented in colon cancer, albeit at a lower density than in normal colon tissue[181]. Tumor expression of VEGF and TIL expression of TNF-α were associated with higher intratumoral DC infiltration. Interestingly, DC infiltration in metastases was approximately sixfold lower than in the primary colorectal tumors. Studies in breast cancer have revealed that immature DCs infiltrate tumor beds, while mature DCs remain in peritumoral areas[182, 183]. It seems that breast cancer tumor cells prompt intratumoral myeloid DCs to polarize local naïve T cells to an IL-13 (Th2-type cytokine)-secreting phenotype, which facilitated the progression of human tumor growth in a mouse xenograft model[184]. Culture of human multiple myeloma cell lines and primary multiple myeloma cells with myeloid DCs leads to improved survival, proliferation, and enhanced clonogenicity of the tumor cells. These effects can be abrogated by blockade of RANK ligand and APRIL[185]. In primary multiple myeloma samples, myeloid DCs are found to co-localize with tumor cells, suggesting that these interactions may occur in vivo[186].

A few years ago, Huarte et al. demonstrated that $CD11c^{+}DEC205^{+}$ DCs co-expressing α-smooth muscle actin and VE-cadherin played an essential role in

tumor vasculature maintenance[187]. Decelerated tumor growth after depletion of myeloid DCs was associated with vascular apoptosis. Our laboratory's more recent studies demonstrated that both myeloid DCs and macrophages (but not plasmacytoid DCs) from normal donors were capable of inducing Th17 cells from memory but not naïve $CD4^+$ T cells, and myeloid DCs and macrophages in the ovarian tumor microenvironment were similarly capable[77]. The relevance of Th17 induction is discussed in the next section. Altogether, myeloid DCs are thought to be the major functional DC subsets in the malignant microenvironment. Myeloid DC vaccination has been utilized in clinical trials to treat cancer patients, albeit with generally modest results at best. Functional mature myeloid DCs exist in limited numbers within the tumor, and many if not all are phenotypically and functionally altered. Myeloid DCs that are dysfunctional or mediate immune suppression are likely a reason for these thus far unsatisfying clinical observations.

2.7 Macrophages

TAMs form the major APC subset (by number) in solid human epithelial cancers. Several years ago, our group discovered that both tumor cells and microenvironmental macrophages in ovarian cancer expressed CCL22, a chemokine instrumental in attracting Tregs to the tumor environment[87]. Interestingly, because the presence of Tregs predicts poorer survival and is associated with a high death hazard in ovarian cancer patients, TAMs may contribute to their prognoses. Indeed, we subsequently demonstrated that although they are highly B7-H4 positive, ovarian cancer cells do not directly mediate antitumor T cell suppression. However, B7-$H4^+$ macrophages from the human ovarian tumor microenvironment are powerful suppressors of tumor-associated antigen-specific T cell immunity[142]. B7-H4 blockade restored the stimulatory capacity of macrophages and mediated ovarian tumor regression in vivo in NOD/SCID mice. Both IL-10 and IL-6, often found in high concentrations in the tumor environment, can induce B7-H4 expression on macrophages. Contrastingly, two cytokines minimally expressed in the same environment—GM-CSF and IL-4—inhibit B7-H4 expression. Interestingly, forced expression of B7-H4 in macrophages from healthy donors conferred a suppressive phenotype on the cells. As for the prognostic significance of B7-$H4^+$ macrophages in ovarian cancer, we documented an inverse relationship between the intensity of B7-H4 expression on macrophages and patient survival. Importantly, Tregs, typically predictors of poor prognoses in cancer patients[154], could induce B7-H4 expression on myeloid APCs (including macrophages) and were positively associated with B7-$H4^+$ macrophage presence in ovarian tumors[188]. A later observation of Wan and colleagues showed that the mean density of TAMs is significantly higher in ovarian cancer than in benign ovarian lesions and that the average 5-year survival rate in patients with low densities of TAM was significantly higher than in patients with larger TAM populations, agreeing well with our observations. Multivariate analysis demonstrated that TAM infiltration status serves as an independent negative

predictor for overall survival of patients with ovarian cancer[189]. The presence of $CCL17^+$ or $CCL22^+$ cells in $CD14^+$ monocytes and macrophages within gastric tumors correlated directly with Treg cell presence. Tregs were also shown to migrate toward CCL17 and CCL22[162]. A study by Haas et al. demonstrated that a higher ratio of stromal $CD68^+$ (a monocyte/macrophage glycoprotein) cells to $FoxP3^+$ cells in intestinal-type gastric cancer patients correlated with shorter median survival time[163]. Another study from our laboratory examined B7-H1 expression on Kupffer cells in hepatocellular carcinoma and found that it was increased in comparison to normal tissue. This expression correlated with poor survival. Not surprisingly, $B7\text{-}H1^+$ Kupffer cells impaired the proliferation and effector function of $CD8^+PD\text{-}1^+$ T cells from the tumor tissue that was reversed upon B7-H1/PD-1 blockade[157]. Finally, a report from Miracco in 2007 showed that Tregs and TAMs were co-localized in melanoma tumors in human patients[157]. TAM presence in advanced melanoma has also been correlated with poor patient prognosis[158].

As for function within the tumor microenvironment, macrophages display a number of pro-tumor activities. They can modify the extracellular matrix; secrete proangiogenic chemokines such as fibroblast growth factor, monocyte/macrophage chemoattractant protein-1 (MCP-1), and VEGF; and produce the immunosuppressive cytokine IL-10[190–193]. MCP-1 expression in breast tumors and TAMs correlated significantly with the presence of other angiogenic factors and with macrophage infiltration of the tumor. Higher levels of TAMs indicated patients with a higher risk of early relapse[194]. Higher MCP-1 levels in urine correlated with more advanced bladder cancer stage[195]. MCP-1 positive invasive ductal breast carcinomas were poorly differentiated, suggesting a correlation of MCP-1 expression and tumor grade[196]. However, a subsequent study showed that MCP-1 levels did not correlate with TAM infiltration in breast carcinoma[197]. It is therefore likely that MCP-1 is not the only chemokine responsible for attracting macrophages into the tumor microenvironment.

The function(s) and prognostic significance of Th17 cells in human cancer are still under discussion[198, 199]. Although few human studies on the subject are published, it seems that Th17 in established epithelial cancers (like ovarian) act to recruit other effector T cell subsets and in doing so, support antitumor immunity[77]. As discussed, both ovarian cancer-derived myeloid DCs and macrophages are capable of Th17 induction. TAMs are more potent Th17 cell inducers than either tumor-derived myeloid DCs or blood macrophages from healthy volunteers. Th17 cell induction is additionally dependent upon TAM expression of IL-1β and IL-23. Blockade of either cytokine significantly decreases the resultant Th17 population, while concomitant blockade of both further diminishes final numbers. In the tumor microenvironment, Th17 induction is also suppressed by Treg cells[77]. In summary, macrophages are the largest APC subset in ovarian and quite possibly other types of cancer, where they may suppress antitumor immunity through multiple modes of action, including the expression of inhibitory B7 family members, the elaboration of proangiogenic chemokines, and the recruitment of Tregs.

2.8 Plasmacytoid Dendritic Cells

Our laboratory was responsible for some of the first studies of plasmacytoid DCs in the tumor environment. A decade ago, we found that human ovarian cancer cells express extremely high levels of stromal-derived factor-1 (SDF-1), which induced plasmacytoid DC trafficking to the tumor via signaling through CXC chemokine receptor-4 (CXCR4)[107, 200]. Additionally, SDF-1 induced plasmacytoid DC expression of very late antigen-5, which interacted with VCAM-1 to mediate cell adhesion and migration through vessel walls. SDF-1 also protected plasmacytoid DCs from apoptosis induced by IL-10 from TAM. Tumor-associated plasmacytoid DCs could induce interleukin-10 production from nearby T cells, which impeded T cell activation by local myeloid DC. This is evidence that plasmacytoid DCs can undermine antitumor immunity and contribute to a suppressive tumor environment. We have also demonstrated a role for plasmacytoid DCs in promoting angiogenesis in ovarian tumors[201]. SDF-1 attracted plasmacytoid DCs into the tumor, where they induced angiogenesis through the production of proangiogenic mediators including TNF-α and IL-8. Conversely, functional myeloid DCs, although numerically restricted in the tumor microenvironment, could suppress angiogenesis in vivo via elaboration of IL-12. These data suggest that malignant cells attract plasmacytoid DCs through expression of SDF-1 to augment vessel formation while excluding the presence of angiogenesis-inhibiting myeloid DCs. We subsequently observed that plasmacytoid DCs from malignant ascites could induce $CD8^+$ regulatory T cell populations[202], in contrast to macrophage-derived DCs[203] which induced tumor-associated antigen-specific $CD8^+$ T cells with effector functions. $CD8^+$ suppressor cells induced by plasmacytoid DCs were IL-$10^+CCR7^+CD45RO^+$, and could suppress myeloid DC-mediated tumor-associated antigen-specific T cell effector functions via IL-10. Plasmacytoid DC CCR7 was functional, as they migrated efficiently under the influence of the lymphoid homing chemokine MIP-3β. Suppressive populations of $CCR7^+CD45RO^+CD8^+$ T cells are found in the tumor environment of ovarian cancer patients, suggesting the in vivo functionality of tumor-associated plasmacytoid DC. Ovarian cancer-associated plasmacytoid DCs can thus induce $CD8^+$ Treg cells and promote tumor angiogenesis, inhibiting antitumor immunity.

Plasmacytoid DC detection (which occurs in approximately one-tenth of breast carcinoma samples) is correlated with poor prognosis[204]. This phenomenon may be attributed to the fact that cells of at least one type of human cancer (head and neck squamous cell carcinoma) negatively impact the ability of plasmacytoid DCs to elaborate IFN-α upon toll-like receptor stimulation[110]. Fascinatingly, investigators found that treatment of basal cell carcinoma with Imiquimod (a toll-like receptor 7 agonist) could induce myeloid DCs to express perforin and granzyme and plasmacytoid DCs to express TRAIL. Imiquimod-treated myeloid DCs and plasmacytoid DCs could kill human tumor cell lines and MHC I-expressing Jurkat cells, respectively, suggesting a new functionality of DCs in immune (and possibly antitumor) responses[203]. Plasmacytoid DCs have also been seen to accumulate in the peritumoral area of primary cutaneous melanomas, likely as a result of

melanoma cell production of SDF-1. Peritumoral plasmacytoid DCs could produce type I IFNs, but their expression of MxA (myxovirus resistance protein A, an IFN-α-inducible protein) was extremely varied and typically minimal. Intratumoral plasmacytoid DCs have an immature phenotype, suggesting incomplete development, possibly influenced by the tumor itself[205]. Salio and colleagues observed that plasmacytoid DCs from human blood could efficiently prime naïve melanoma tumor-antigen (melan-A)-specific $CD8^+$ lymphocytes to become IFN-γ-producing cells in vitro[206]. Plasmacytoid DCs stimulated with CD40L induced cutaneous lymphocyte antigen and L-selectin (CD62L) expression on primed tumor-associated antigen-specific T cells. These homing receptors could allow effector cell migration to diseased skin. This study also confirmed the presence of plasmacytoid DCs in the peritumoral area of most primary cutaneous melanomas in vivo. Plasmacytoid DC type I IFN-containing supernatant induced upregulation of CD95 and MHC class I and class II molecules on melanoma cells in vitro. Thus, tissue-infiltrating plasmacytoid DCs could have a previously unknown immune-modulating capacity.

2.9 B Cells

As noted above, tumor-infiltrating $CD8^+$ T lymphocytes typically correlate positively with improved survival of cancer patients. B cells have been observed to co-localize with T cells and are known to provide various support functions. However, the association of B cell presence or function with patient prognoses in cancer has not been well studied[207]. Milne and colleagues recently demonstrated that $CD20^+$ tumor-infiltrating B cells could be found in more than two-fifths of high-grade serous ovarian cancer samples[208]. B cell presence here was strongly associated with $CD4^+$ and $CD8^+$ T cells, the activation markers CD25 and CD45RO, and markers of T cell effector function including expression of tumor infiltrating B cells (TIA-1) and granzyme B. Intriguingly, B cells were also associated with T cell expression of FoxP3, a marker that could indicate either activated or regulatory T cells[209, 210]. Intraepithelial B cell numbers correlated positively with improved patient disease-specific survival, while fascinatingly, the combination of $CD8^+$ and $CD20^+$ TILs in the same tumor indicated significantly increased disease-specific survival over tumors that contained one or the other type of TIL. $CD20^+$ cells could support the actions of tumor-associated effector T cells through various mechanisms. In mice, B cells can produce autoantibodies directed against tumor targets[211]. It is possible that tumor-infiltrating B cells can raise the concentration of antitumor autoantibodies in the tumor microenvironment to physiologically relevant levels. Tumor-infiltrating B cells can also secrete granzyme B[212] and TRAIL[213] and induce tumor cell death through both of these mechanisms. New evidence of B cell killer potential is coming to light and will no doubt inform future studies of these cells in the context of malignancy[214].

B cell infiltration of tumors has been examined in multiple tumor settings. They are detected in approximately one-quarter of breast cancers, where they can make up nearly 40% of the TIL populations[215–217]. B cells are early infiltrators of breast

cancer[218]. Tumor-infiltrating B cell phenotypes appear driven by affinity maturation[219–222] and can also be found in tertiary lymphoid structures, where they co-localize with $CD4^+$ T cells, $CD8^+$ T cells, and/or DCs[223–225]. The expression of B cell signature genes in node-negative breast cancer was shown to have positive prognostic significance[226]. In medullary breast cancer, the presence of B cells and T cell subsets appears to be beneficial for patient survival[217, 227, 228]. A very recent study utilizing the 4T1 mouse model of breast cancer identified a subset of activated B2 cells ($CD19^+CD25^{hi}CD69^{hi}$) that proliferated poorly. Interestingly, these cells expressed B7-H1 and their principal function within the tumor environment appeared to be mediating the conversion of $CD4^+$ T cells to Treg cells via production of TGF-β[229]. Whether this conversion occurs in human cancer remains to be seen. B cells have also been examined in non-small cell lung cancer, where their presence in epithelium, tumor stroma, and tumor lysis syndrome (TLS) correlate strongly with better survival[230–232]. B cells in lung cancer are specific for antigens that include the tumor suppressor gene p53 and other molecules typically overexpressed in tumor tissue[233]. Finally, in cervical cancer, peritumoral B cell presence is associated with decreased patient relapse[234].

Interestingly, an earlier study examined $CD19^+$ cell presence in post-chemotherapy effusions from advanced ovarian cancer and found that it was predictive of poorer survival[235]. How then, can B cells be prognostically good in one investigation of ovarian carcinoma while detrimental in another? First, patients in the latter study were subjected to chemotherapy (which can profoundly affect the numbers and functionality of immune cell subsets), while those in the former study were not. Second, the populations delineated by CD19 and CD20 are not precisely the same: while CD20 is present on the surface of all mature B cells[236–238] and CD19 is predominantly expressed on B cells[239], these surface markers have slightly different expression profiles. Finally, the activation state of B cells contributes to their effector or suppressor functions in various pathologies: resting B cells inhibit the antitumor response[240], while activated B cells can aid T cell responses[241].

The roles for B cells in the malignant microenvironment are many. B cells can effect regulatory functions. They can be polarized by Th subsets into subpopulations that produce IFN-γ, IL-12, and TNF-α and promote Th1 skewing, or they can be producers of IL-2, IL-4, TNF-α, and IL-6 that support Th2 development. B cell production of these groups of cytokines feeds back into the maintenance and expansion of the Th populations that initially stimulated their cytokine expression, thereby maintaining and propagating a Th1 or Th2-type cytokine milieu[242, 243]. B cells can also influence T cell memory, survival, and proliferation[244, 245], as well as present antigen to both T cells[241, 246]. In advanced tumors, where DCs may have become suppressive or rare, B cells could serve a greater antigen-presentation role[207]. This, however, could act as a double-edged sword: presentation to helper or cytotoxic T cells might support antitumor immunity, while antigen presentation to Tregs could undermine the antitumor response. B cells can additionally mediate immunosuppressive functions via their cytokine products. The immunoregulators IL-10 and TGF-β can both be produced by B cells[246] and foster downregulation of antigen presentation, suppression of T cell activation, and maintenance of Treg

suppressor function[247–249]. However, we have recently demonstrated several new roles for IL-10 in support of antitumor immunity, including the moderation of tumor-associated suppressive cellular networks including regulatory T cells and myeloid-derived suppressor cells[250]. Further research is warranted to determine whether B cell-derived IL-10 acts solely to suppress or support antitumor immunity, or whether these cells' functions are context-dependent, like so many other immune factors. B cells themselves may serve beneficial or detrimental roles to antitumor immunity depending on their intratumoral phenotype.

2.10 Conclusions

It is evident that different subsets of immune cells infiltrate tumors in different degrees. The detailed molecular and cellular mechanisms controlling the quantity and quality of immune infiltration remain to be fully dissected. It is clear that immune infiltration is different from tumor to tumor and from different clinical stages. Therefore, the pathological relevance of each immune subset tumor infiltration may be generalized and need to be analyzed in a specific situation. It is expected that manipulation of tumor immune cell infiltration should be therapeutically important in treating patients with cancer.

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