Chapter 5

THE ROLE OF IMMUNE CELLS IN THE TUMOR MICROENVIRONMENT

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Abstract: Interactions between tumor infiltrating leukocytes and tumor cells have been of great interest because of the possibility that immune cells either interfere with tumor progression or actively promote tumor growth. The tumor microenvironment is shaped by cells entering it, and their functions reflect the local conditions. Successive changes occurring at the tumor site during tumor progression resemble chronic inflammation. This chronic inflammatory reaction seems to be largely orchestrated by the tumor, and it seems to promote tumor survival. Molecular and cellular mechanisms linking the inflammatory reaction and cancer are emerging, and this review summarizes the current understanding of interactions between inflammatory and cancer cells in the tumor microenvironment.

Keywords: TIL; immune evasion; immune surveillance; inflammation; cancer

1. INTRODUCTION

Tumor development involves multiple genetic changes, which occur in the progeny of the transformed cell over many years, accumulate and result in the establishment of a malignant phenotype characterized by uncontrolled growth (Zhang *et al.*, 1997). In parallel, a variety of alterations occur in surrounding normal tissues, leading to establishment of the tumor microenvironment. These changes are necessary to assure survival of the tumor at the expense of surrounding normal tissue cells. To meet tumor requirements and sustain its growth, several successive stages of changes have to occur in the tumor microenvironment. In many respects, the tissue changes arising in response to tumor formation resemble the unfolding process of chronic inflammation. Inflammation is a normal component of wound healing or tissue repair. In fact, tumors have been described as wounds that do not heal (Dworak, 1986). Inflammation, when it occurs, generally consists of the initial ischemia, resulting in the interstitial and cellular edema (an immune reaction associated with appearance in tissue of immune cells) and, finally, the appearance of blood capillaries and lymphatics necessary for feeding of the repaired tissues (Ribatti *et al.*, 2003; Aller *et al.*, 2004). These phases of inflammatory response progress from an anerobic tissue environment (ischemia) to the development of oxidative metabolism, which uses oxygen to produce energy in the form of ATP (Mareel and Leroy, 2003; Balkwill and Mantovani, 2001). Inflammation appears to be a ubiquitous tissue response common to many normal conditions, including embryonic development, as well as disease states. Its involvement in shaping the tumor microenvironment has been recognized, and it has been referred to as the "host reaction" to the tumor.

The tumor appears to be able to induce an inflammatory response in the host early on, because the presence of immune cells has been noted even in pre-cancerous or benign lesions (Kornstein *et al.*, 1983; von Kleist *et al.*, 1987; Vacarello *et al.*, 1993). This has been interpreted as an attempt of the host immune system to interfere with tumor growth, otherwise referred to as "immune surveillance". However, the developing tumor is not a passive participant in this interplay. On the contrary, it takes advantage of the host response in order to: (a) use immune cells for elimination of sensitive tumor cells and their gradual replacement by those resistant to immune intervention ("immune selection") and (b) use the host as a participant in creating the microenvironment favorable to tumor progression ("immune evasion"). To this end, lymphocytes, macrophages and dendritic cells (DC) infiltrating the tumor, together with fibroblasts and extracellular matrix forming a scaffold supporting its expansion, contribute to establishing an inflammatory milieu that nourishes the tumor. Thus, the host becomes a participant in the

establishment of the tumor by providing structural and trophic elements required for cancer progression.

In this chapter, the role of immune cells in the tumor-host interactions will be considered, with the emphasis placed on capabilities of these cells to modify the tumor microenvironment. To date, the role of tumor cells in shaping their microenvironment has been widely discussed (Bogenrieder and Herlyn, 2003). Yet, it is clear that as a component of the inflammatory reaction, immune cells accumulating at the tumor periphery or those infiltrating the tumor stroma or nest of tumor cells are also involved in modulating tumor progression.

1.1 Immune cells in the tumor microenvironment

Immune responses to malignant cells can be categorized as locoregional or systemic. *In situ* or local responses refer mainly to tumor infiltrating lymphocytes (TIL), which accumulate in many human solid tumors and whose role in tumor progression remains controversial. TIL isolated from various human tumors are functionally compromised as compared to normal circulating or tissue-infiltrating lymphocytes. Systemic immunity to tumors, as measured in the peripheral circulation or by delayed-type hypersensitivity (DTH) responses in patients with cancer, is difficult to demonstrate, and tumor-specific responses are particularly elusive. Non-specific proliferative or cytotoxic responses of peripheral lymphocytes in cancer patients are variably impaired (Whiteside, 1993). More recent data indicate that the same functional impairments seen in TIL are found in circulating as well lymph node lymphocytes of patients with cancer (Reichert *et al.*, 2002). In aggregate, the available evidence suggests that in the presence of tumors, both local and systemic antitumor immunity is compromised in patients with cancer. This does not mean that patients with cancer are immunodeficient. Their responses to bacterial and viral antigens remain unimpaired, and only tumor-specific immunity is deficient.

1.2 T cells

Among various cells present at the tumor site, T cells ($CD3^+TCR^+$) have received the most attention. They are by far the largest component of mononuclear tumor infiltrates in all human tumors (Mihm *et al.*, 1996), although some tumors may be also generously infiltrated by macrophages (Lin *et al.*, 2001). The hypothesis that TIL-T represent autotumor-specific cytolytic T cells (CTL) has been promoted; however, early limiting dilution studies performed with TIL-T from various human tumors indicated that the frequency of such CTL was low as compared to peripheral blood T

lymphocytes (PBL-T) (Miescher *et al.*, 1987). Nevertheless, evidence exists that Vβ-restricted clones of T cells are present in some freshly isolated TIL, and that TIL can selectively recognize and kill autologous tumor cells in some cases (Weidmann *et al.*, 1992). Using tetramers and multi-parameter flow cytometry, it has recently been possible to determine the frequency of tumor-peptide-specific (tetramer$^+$) T cells among TIL with greater accuracy in various human tumors. We reported, for example, that TIL obtained from patients with head and neck cancer (HNC) were significantly enriched in wildtype (wt) p53 epitope-specific T cells, as compared to autologous peripheral blood mononuclear cells (PBMC) (Albers *et al.*, 2005a). The frequency of these tetramer$^+$ TIL was highly variable and ranged from 1/800 to 1/5,000 of CD3$^+$CD8$^+$ TIL, which recognized the wt $p53_{264\text{-}272}$ peptide, compared to the mean frequency of 1/6,000 such cells among autologous PBMC (Albers *et al.*, 2005a). Furthermore, our recent analysis of TcR Vβ restrictions in paired TIL and PBMC of patients with HNC indicated the presence of the same Vβ restrictions in both. These oligoclonal expansions of T cells in TIL and PBMC cells were observed in 8/10 patients with HNC and in 0/10 PBMC samples obtained from normal donors. Binding of an HLA-A2.1/p53 tetramer to a high percentage of a Vβ-restricted CD8+ T cell population suggested that the restrictions were tumor related (Albers *et al.*, 2005b). This type of evidence is also available for melanoma (Weidmann *et al.*, 1992 and 1993), and it indicates that TIL may indeed be enriched in tumor-specific T cells.

The phenotypic analysis of T cells in human tumors shows that TIL-T are memory lymphocytes, expressing either CD8 or CD4 markers, although the CD4/CD8 ratio may be highly variable from one tumor to another (Whiteside, 1993). In several studies, the CD4/CD8 ratio was found to be reduced as a result of enrichment in CD8$^+$ T cell, in contrast with nonmalignant inflammatory infiltrates, which consist largely of CD4$^+$ T cells (Whiteside, 1992; Whitford *et al.*, 1990). Some reports have linked a high tumor content of CD8$^+$ T cells with a better prognosis (Baxevanis *et al.*, 1994; Naito *et al.*, 1998), although no consistent data supporting this finding has been obtained. In cervical cancer or in renal cell carcinoma, TIL enrichment in CD8+ T cells seems to be associated with disease progression and a poor prognosis (Sheu *et al.*, 1999; Nakano *et al.*, 2001). When functional attributes of freshly-isolated TIL-T were examined in conventional *ex vivo* assays, their proliferative and anti-tumor functions were found to be significantly depressed compared with equivalent functions of normal T cells. It appears that TIL obtained from advanced or metastatic lesions are more functionally impaired than those from early lesions, suggesting that tumor burden or the potential of more aggressive tumors to suppress immune cells might determine the functional status of TIL-T. The

cytokine profile of these cells was also different from that of normal activated T cells, as either no or little type 1 cytokines (IL-2, IFN-γ) were produced by TIL-T and, instead, these cells preferentially secreted down-regulatory cytokines, IL-10 and TGF-β (Vitolo *et al.*, 1993). These functional characteristics of TIL-T were inconsistent with respect to their phenotype, which identified them as predominantly HLA-DR$^+$CD25$^+$ activated T lymphocytes (Whiteside, 1993). More recently, this inconsistency was addressed by examining the hypothesis that TIL-T were enriched in CD4$^+$CD25$^+$ regulatory T cells. Recent reports confirmed the accumulation of regulatory CD4$^+$CD25$^+$ T cells (T_{reg}) at the tumor site in several human malignancies (Liyanage *et al.*, 2002; Woo *et al.*, 2001). This enrichment in T_{reg}, which are in principle responsible for down-regulation of TIL functions in the tumor microenvironment, could explain the discrepancy between the observed "activation" phenotype (HLA-DR$^+$CD25$^+$) of TIL and their functional impairments. Until recently, however, it was not possible to make a firm distinction between activated CD4$^+$ T cells and T_{reg} due to the lack of specific phenotypic markers for the T_{reg} population. T_{reg} were identified based on the ability to suppress activity of other immune cells in *ex vivo* mixing experiments (Shevach, 2000). We have observed a significant increase in the proportion of CD4$^+$CD25$^+$ T cells in TIL isolated from HNC (a mean of 30 % in TIL vs. 11 % in autologous PBMC; n=17) (Albers *et al.*, 2005a). When the newly available antibodies to FOXp3, a transcription factor expressed in T_{reg} (Ramsdell, 2003), and antibodies to glucocorticoid-induced TNF receptor (GITR) or to CTLA-4 antigen (Stephens *et al.*, 2004; Lee *et al.*, 1998) were used for staining and flow cytometry of permeabilized TIL obtained from patients with HNC, we observed that nearly all CD4$^+$CD25$^+$ TIL were positive for these markers. In contrast, only 1-2% of CD4$^+$CD25$^+$ T cells in PBMC were stained with the antibodies recognizing T_{reg}. These preliminary data indicate that tumors indeed "beckon regulatory T cells" (Shevach, 2004). However, these results have to be confirmed by functional studies demonstrating suppressive capabilities of CD4$^+$CD25$^+$ T cells isolated from TIL and PBMC of patients with HNC, similar to those recently performed by Curiel and colleagues with TIL obtained from tumor tissues and ascites of patients with ovarian carcinoma (Curiel *et al.*, 2004). A tentative conclusion that can be drawn from the data available so far is that TIL are enriched in tumor-specific effector cells as well as T_{reg}, and that functional paralysis of TIL may, in part, be attributed to suppressive effects mediated by T_{reg}, which accumulate in the tumor microenvironment.

Functional impairments observed with TIL isolated from solid tumors can be confirmed by *in situ* studies of signaling molecules in TIL-T. Such studies show that expression of the TcR-associated ζ chain as well as that of NF-κB, the transcription factor regulating expression of a number of

immune and inflammatory genes, is significantly decreased in TIL-T compared to their expression in T cells obtained from the peripheral circulation of normal donors (Reichert *et al.*, 1998a; Li *et al.*, 1994). This is particularly evident for TIL evaluated *in situ* or isolated from advanced or metastatic lesions. In a study comprising 132 cases of human oral cell carcinomas, expression of ζ in TIL-T was found to be an independent and highly statistically significant biomarker of prognosis and survival in patients with stage III and IV disease (Reichert *et al.*, 1998b). The patients with tumors infiltrated by T cells with low or absent ζ expression had significantly shorter 5-year survival compared to the patients with tumors infiltrated by T cells with normal ζ expression (Reichert *et al.*, 1998b). Stimulus-dependent activation of NF-κB was found to be impaired in T cells of patients with renal cell carcinoma (RCC). In some patients, the primary defect was the failure of the transactivating complex RelA/NF-κB1 (p50) to accumulate in the nucleus following T-cell activation due to impaired phosphorylation and degradation of the inhibitor IκBα (Uzzo *et al.*, 1999a; Ling *et al.*, 1998). In other patients, NF-κB activation was defective despite normal stimulus-dependent degradation of IκBα (Uzzo *et al.*, 1999b). In both situations, this defective state could be induced by exposure of normal T cells to supernatants of RCC, and the soluble product responsible was identified as an RCC-derived ganglioside (Uzzo *et al.*, 1999b). Impaired NF-κB activity may contribute to reduced T-cell function seen in TIL-T present in RCC, since this transcription factor controls expression of a number of genes encoding cytokines, their receptors and other membrane regulatory molecules essential for T-cell activation (Baeuerle and Baltimore, 1996; May and Ghosh, 1998). It is important to note that defects in function of the ζ chain and activation of NF-κB are observed in TIL-T as well as circulating T cells of patients with cancer (Kuss *et al.*, 1999; Whiteside, 2004). Thus, these signaling defects in T cells are both local and systemic and seem to be related to the tumor burden.

Taken together, functional studies of TIL-T obtained from a variety of human solid tumors in many different laboratories indicate that these cells are functionally incompetent, possibly because of the inhibitory effects mediated by activated T_{reg} in the tumor microenvironment or by factors produced and released by the tumor itself. In addition, it appears that the loss of functions in TIL-T rather than the number or phenotype of T cells infiltrating the tumor may be the important factor for predicting patient survival.

1.3 Macrophages

$CD45^+CD14^+$ cells are commonly found in human tumors and are referred to as tumor-associated macrophages (TAM). Normal macrophages are phagocytic and antigen-presenting cells, which play an important role in the control of infections. In contrast, TAM are re-programmed to inhibit lymphocyte functions through release of specific cytokines, prostaglandins or reactive oxygen species (ROS). It is hypothesized that re-programming of macrophages occurs in the tumor microenvironment as a result of tumor-driven activation (Al-Sarireh and Eremin, 2000). Possibly, macrophages are the main contributors to removal of dying tumor cells, and the more rapidly proliferating tumors are especially attractive to these scavengers. Evidence has accumulated indicating that invasiveness of tumors, e.g., human primary colon carcinomas, is directly related to the number of TAM detected in the tumor (Al-Sarireh and Eremin, 2000). In invasive breast cancer, an increased TAM count is an independent predictor of reduced relapse-free survival as well as reduced overall survival (Leck *et al.*, 1996). The available data support the active role of TAM in tumor-induced immunosuppression, on the one hand, and in the promotion of tumor growth on the other. The mechanisms that contribute to TAM-mediated inhibition of immune cells are probably numerous, but much attention has been recently devoted to the role of NADPH-dependent ROS, such as superoxide anion or hydrogen peroxide as potential inhibitors of TIL (Kiessling *et al.*, 1996; Hansson *et al.*, 1996). T-cell proliferation and NK-mediated antitumor cytotoxicity are profoundly inhibited by macrophage-derived ROS *in vitro* (Hansson *et al.*, 1996). T and NK cells isolated from human tumors have a decreased expression of CD3ζ and FcγRIII-associated ζ, respectively, and this down-modulation of ζ, a critical signal-transducing molecule for TCR can be induced by ROS produced by TAM (Malmberg *et al.*, 2001). The changes observed in TIL: a loss of normal ζ expression accompanied by a decreased ability to proliferate and subsequent apoptotic cell death, correspond to similar changes induced in T and NK cells co-cultured with activated macrophages (Hansson *et al.*, 1996). Removal of macrophages from these cultures restores T and NK cell functions (Hansson *et al.*, 1996). The overall conclusion from these studies is that immuno-inhibitory activities of TAM, whether due to oxidative stress or to release of inhibitory cytokines, such as IL-10, contribute to making the tumor microenvironment a particularly unfriendly milieu for immune cells. In this, TAM appear to reinforce effects mediated by tumor cells, which also produce ROS, PGE2 and a variety of immuno-inhibitory cytokines (Whiteside *et al.*, 2005). Also, TAM were reported to be associated with angiogenesis and prognosis in breast carcinomas (Leek *et al.*, 1996).

1.4 Dendritic cells

Dendritic cells (DC; *Lin*-CD80+CD86+HLA-DR+ cells) are the most potent antigen presenting cells (APC). They are a heterogenous population of highly motile cells that originate from the precursors in the bone marrow and migrate through the blood stream to peripheral non-lymphoid tissues, capturing antigens (Banchereau *et al.*, 2000). They then travel to the lymphoid tissues, where antigen presentation to T cells takes place. The DC compartment is comprised of subpopulations of morphologically and functionally distinct cells, depending on their hematopoietic origin, maturation stage or tissue localization (Banchereau and Steinman, 1998). The two main subpopulations are myeloid-derived DC (DC1) and lymphoid-derived DC (DC2). While in man, this distinction is somewhat blurred, phenotypic and functional differences exist between monocyte-derived $CD11c^+$ DC (MDC) and $CD11c^-CD123^+$ (IL-$3R\alpha^{high}$) lymphoid-derived DC (Banchereau and Steinman, 1998; Liu, 2001). The proportion of lymphoid DC is substantially lower than that of myeloid DC, and most of these cells belong to a relatively rare subset of DC known as plasmacytoid DC (pDC), which produce IFN-α in response to viruses (Liu, 2001). Human tumors are frequently infiltrated by MDC but rarely by pDC, although the presence of the latter is associated with poor prognosis (Treilleux *et al.*, 2004). The DC maturation stage determines their functionality: immature DC are primarily responsible for antigen uptake, while mature CD83+ DC primarily serve as antigen-presenting cells (Liu, 2001).

In tumor-bearing hosts, DC are responsible for the uptake, processing and cross-presentation of TAA to naïve or memory T cells, thus playing a crucial role in the generation of tumor-specific effector T cells. Antigen presentation by DC leads to T cell proliferation, which results in either immunity or tolerance, depending on the stage of maturation of the presenting DC. In human solid tumors, DC are often present in substantial numbers, and they express attributes of immature DC (Gabrilovich, 2004). Because antitumor immune responses are inefficient in tumor-bearing individuals, it has been suggested that DC, like T cells, are subverted by the tumor (Gabrilovich, 2004). Phenotypic and functional alterations in DC infiltrating human tumors as well as DC recovered from the peripheral circulation of patients with cancer have been reported (Almand *et al.*, 2000; Hoffmann *et al.*, 2002). Tumor-associated DC (TADC) appear to be immature (lack of expression of CD80 and CD86) and have reduced allostimulatory activity (Troy *et al.*, 1998). TADC are at a particular disadvantage, because tumors or tumor-derived factors have been shown to induce DC apoptosis or impair their maturation (Esche *et al.*, 2001; Gabrilovich *et al.*, 1996a). Co-culture of murine or human DC (obtained from isolated $CD34^+$ precursors or plastic-

adherent PBMC, respectively) with a variety of tumor cell lines for 4-48h resulted in apoptotic death of DC, as verified by morphology, TUNEL assays, annexin binding, caspase activation, and DNA laddering (Esche *et al.*, 1999; Shurin *et al.*, 1999). Tumor cells induced DC apoptosis by direct contact or through release of soluble factors. Furthermore, TADC isolated from human tumors contain a high proportion of apoptotic cells (Shurin *et al.*, 1999). Tumor-induced apoptosis of DC was inhibited in the presence of IL-12 and IL-15, an indication that cytokines might regulate DC generation and survival. In fact, these cytokines were shown to stimulate expression of Bcl-2 and Bcl-XL in DC and to protect them from tumor–induced apoptosis (Shurin *et al.*, 1999 and 2001). Experiments performed by Shurin and colleagues further demonstrated that tumor-derived factors, e.g., gangliosides, inhibited DC generation and their function *in vitro* (Shurin *et al.*, 2001). This suppressive effect of gangliosides on DC was found to be mediated by tumor-derived VEGF, a known anti-dendropoietic factor (Gabrilovich *et al.*, 1999). Importantly, cytokines (IL-12, IL-15 and FLT3L) were found to promote DC generation and their functions by exerting a protective anti-apoptotic effect, while tumor-derived factors caused apoptosis in mature DC and inhibited differentiation of hematopoietic precursors into DC. Very recent studies indicate that tumors and tumor supernatants can down-regulate expression of antigen presenting machinery (APM) components in DC, thus interfering with the capacity of these cells to process antigens and present the processed epitopes to T cells (Whiteside *et al.*, 2004; Tourkova *et al.*, 2004). Again, tumor-derived gangliosides were identified as a factor responsible for down-modulation of APM components in DC co-incubated with the tumor (Tourkova *et al.*, 2004). These studies underscore the role of the microenvironment in shaping the functional potential of DC and perhaps other tumor-infiltrating immune cells.

Numerous reports in the literature suggest that despite functional impairments of TADC, their presence in tumors is associated with improved prognosis (Becker, 1993; Reichert *et al.*, 2001). DC infiltrations into tumors have been correlated to significantly prolonged patient survival and reduced incidence of recurrent or metastatic disease in patients with bladder, lung, laryngeal, oral, gastric and nasopharyngeal carcinomas (Reichert *et al.*, 2001; Tsujitani *et al.*, 1987; Lespagnard *et al.*, 1999; Furukawa *et al.*, 1985; Goldman *et al.*, 1998; Giannini *et al.*, 1991). In contrast, patients with lesions reported to be scarcely infiltrated with DC had a relatively poor prognosis (Tsujitani *et al.*, 1990). Fewer DC were observed in metastatic than primary lesions (Murphy *et al.*, 1993). In a recent study, we demonstrated that the number of S-100$^+$ DC present in the tumor was by far the strongest independent predictor of overall survival as well as disease-free survival and time to recurrence in 132 patients with oral carcinoma,

compared with such well established prognostic factors as disease stage or lymph node involvement (Reichert *et al.*, 2001). Another striking observation we made concerns the relationship between the number of DC in the tumor and expression of the TcR-associated ζ chain in TIL. The paucity of DC in the tumor was significantly related to the loss of ζ expression in TIL, and these two factors had a highly significant effect on patient overall survival. The poorest survival and the greatest risk was observed in patients with tumors that had small number of DC and little or no ζ expression in TIL ($p=2.4x10^{-8}$). Our data suggest that both the number of DC and the presence of functionally unimpaired T cells in the tumor microenvironment are important for overall survival of patients with cancer. Interactions of DC and T cells in the tumor appear to sustain TcR-mediated, and presumably tumor-directed, functions of the T cells infiltrating the same area. It is possible that DC protect T cells from tumor-induced immune suppression, although the mechanism responsible for such protection remains unknown and is being actively investigated.

The correlation between TADC presence in the tumor and patient overall survival or relapse free survival has not been confirmed in other more recent studies, in which immunostaining for MDC and pDC subsets was performed. Thus, in primary breast cancer, a strong association of mature DC with $CD3^+$ T cells was observed but did not correlate with prognosis (Treilleux *et al.*, 2004). Rather, it was the infiltration by pDC that predicted a poor survival in the same series. While this and all other reports based on immunostaining of tumor sections may suffer from a bias related to selection of patients, tissues, antibodies used for staining, and methods for cell enumeration, it appears that similar to T-cell infiltrates, the biologic significance of DC presence in the tumor remains undefined.

1.5 B cells

B lymphocytes ($CD19^+$, $CD20^+$) are uncommon components of human solid tumors. While anti-tumor antibodies (Abs) are frequently detected in the circulation of cancer patients, it has been assumed that these Abs are made and secreted by plasma cells situated in the tumor draining lymph nodes, spleen or other lymphoid tissues. However, in some carcinomas, plasma cells may be present and, occasionally, represent a substantial infiltrating element (Kornstein *et al.*, 1983). More recent reports indicate that lymphoplasmacytic infiltrates are relatively common in pre-malignant cervical lesions as well as cervical carcinomas (O'Brien *et al.*, 2001) and in medullary ductal breast carcinomas (Coronella *et al.*, 2001). In cervical carcinomas, using antibody phage display, it was possible to show that infiltrating B cells and plasma cells represent an antigen-induced response to

human papillomavirus (HPV) infection or transformation (O'Brien *et al.*, 2001). In medullary ductal breast carcinoma, the presence of B and plasma cells is associated with improved prognosis (Fisher *et al.*, 1990), and this has generated considerable interest in the role of these B cells and their products in tumor progression. Several independent studies examined the hypothesis that lymphoplasmacytic infiltrates represented a host humoral response driven by tumor-derived neo-antigens (Coronella *et al.*, 2002; Hansen *et al.*, 2001; Nzula *et al.*, 2003). The data based on patterns and levels of TIL-B IgG H chain hypermutation suggested that tumor-infiltrating B cells are undergoing antigen-driven proliferation and affinity maturation *in situ*. Abs produced by TIL-B may be tumor-specific or may specifically bind an intracellular protein, such as β-actin, translocated and presented to the cell surface upon tumor cell apoptosis (Hansen *et al.*, 2001). It has been suggested that dissection of the intratumoral Ab response using Ig variable region analysis might identify those that bind with high affinity to TAA for the purpose of their isolation (Kotlan *et al.*, 2003). The implication of the presence of ectopic germinal centers in breast cancer and perhaps other solid tumors is that Ab production can occur in the tumor microenvironment under certain circumstances. The biologic significance or the prognostic importance of this is unknown, although it might be that the ability to make Abs *in situ* may be an important aspect of host defense.

1.6 Other leukocytes

Human tumors are sometimes infiltrated by granulocytes, and nests of eosinophils may be seen in association with tumor cells in various squamous cell carcinomas, for example. By far the most frequent cell in tumors has characteristics of the immature myeloid cell (iMC). It expresses CD33, a common myeloid marker, but lacks markers of mature myeloid or lymphoid cells and HLA-DR (Almand *et al.*, 2001). The iMC are equivalent to murine Gr-1$^+$CD11b$^+$ cells, which have been shown to inhibit IFN-γ production by CD8$^+$ T cells in response to epitopes presented by MHC class I molecules on the surface of these cells (Almand *et al.*, 2001). This inhibition is apparently mediated by ROS produced by iMC, such as H_2O_2, which suppress CD3ζ expression by T cells (Otsuji *et al.*, 1996). Indeed, granulocyte-derived H_2O_2 has been shown to be involved in the inhibition of IFN-γ production and the suppression of CD3ζ expression in circulating T cells of patients with advanced malignancy (Schmielau and Finn, 2001). In tumors, where the hypoxic environment prevails, H_2O_2-generating iMC might contribute to creating conditions favoring T-cell suppression.

2. INTERACTIONS BETWEEN THE TUMOR AND INFILTRATING LEUKOCYTES

In aggregate, recent phenotypic and functional assessments of tumor-associated leukocytes have provided a broad cross-sectional view of the types of cellular infiltrates present in human solid tumors. While the nature and cellular composition of these infiltrates vary from one tumor type to another or even among individual tumors of the same histologic type, their presence in human solid tumors is a consistent feature. Furthermore, the phenotype, numbers and location in the tumor (i.e., stroma vs. intraepithelial) of infiltrating leukocytes have been in many instances correlated to prognosis and patient survival (see, e.g., Phillips *et al.*, 2004). However, no unified conclusions have emerged to date in this respect, because for every report linking ample leukocyte infiltration to a better prognosis, a report can be found claiming the opposite. It is clear that the presence of leukocytic infiltrates in the tumor is either good or bad but not neutral. There are several aspects of this dilemma that are particularly intriguing, as discussed below.

It is possible to consider leukocytic infiltrates as a component of the inflammatory process representing a "host reaction" to the tumor. In fact, establishing parallels or links between inflammation and cancer has become a popular quest (Balkwill and Coussens, 2004). The initial goal of inflammation is the destruction of the invader, which in this case is the tumor. Hence, the "immune phase" of tumor-driven inflammation involves an influx of anti-tumor effector cells to the affected tissue. In comparison to vigorous cellular and antibody responses that are generated in tissues during the infection by an exogenous pathogen, however, inflammatory responses to tumors are weak. At least one reason for the lack of robust immune responses to the tumor is the absence of a "danger signal" in the tumor microenvironment (Gallucci and Matzinger, 2001; Matzinger, 1998). The immune system perceives infections with bacteria or viruses as "danger" and the tumor as "self." It responds vigorously to contain the external danger introduced by pathogens and only weakly, if at all, to tumor-associated antigens (TAA), which are largely self antigens or altered self antigens. It may be that the attraction of T_{reg} to the tumor is related to an attempt by the host to ill advisedly (in this case) regulate host response to self. Interestingly, inflammatory infiltrates in tumors generally contain few, if any, NK cells which mediate innate immunity and are rich in perforin- or granzyme-containing granules (Whiteside *et al.*, 1998). NK cells are exquisitely attuned to distinguish self from non-self by virtue of a complex system of inhibitory and activating receptors expressed on their surface (Lanier, 2003). They represent "the first line" of defense against pathogens, and their conspicuous

absence from tumor infiltrates or even pre-cancerous lesions (Lanier, 2003) suggests that the host's response to the tumor is indeed different in strength and quality from that initiated by exogenous pathogens.

Tolerance to self that needs to be overcome before a full-scale immune response to the tumor can develop is one reason for ineffective anti-tumor host defense (Janeway 1992). But there are others as well. The nature of the tumor microenvironment appears to be quite unique. On the one hand, the tumor creates stress signals, which mobilize the host to initiate an inflammatory cascade. On the other, the tumor microenvironment is characterized by the presence of multiple immuno-suppressive factors and by the excess of antigens produced and released from proliferating or dying tumor cells (Whiteside and Rabinowich, 1998). Thus, inflammatory cells arrive into this environment to be faced by conflicting signals, which orchestrate the local response. Consequently, a somewhat precarious balance is established between the host and the tumor, which clearly favors the tumor, and which has at least two aims: a) to cripple the host immune system so that the tumor can survive, and b) to utilize infiltrating cells and their products for supporting tumor survival. Ample evidence exists to support the existence of both these mechanisms (Whiteside *et al.*, 2005). While tumor escape from the host-mediated surveillance in its various forms has been recently in the limelight (reviewed in Whiteside *et al.*, 2005), those elements of the local inflammatory response that mediate trophic functions and thus support tumor growth have to be recognized as well. Thus, once recruited to the tumor microenvironment, various leukocytes are subjected to non-specific or tumor-specific signals and in response may produce a variety of soluble products, including cytokines and antibodies. In theory, antitumor effects of these products combined with direct cytolytic activity of infiltrating effector cells against tumor targets should result in tumor demise. In most cases, however, the tumor also releases soluble factors, including cytokines, lipids, polyamines and TAA that suppress immune cells and at the same time stimulate tumor growth and survival.

3. CHARACTERISTICS OF THE TUMOR MICROENVIRONMENT

Among the factors that may determine the nature of inflammatory infiltrates found in the tumor is the hypoxic environment. It is created early in tumor development through activation of hypoxia responsive genes in tumor cells (Denko *et al.*, 2003), and it obviously favors influx of those inflammatory cells that depend on the glycolytic pathway for survival, namely, phagocytic macrophages and granulocytes (Sitkovsky *et al.*, 2004).

These cells can not only survive in the hypoxic environment but contribute to it by hyperproduction of ROS upon their activation. The tumor milieu, in which apoptosis of rapidly expanding tumor cells is a common feature, provides ample activating signals for phagocytic cells and leads to the generation of ROS. In most inflammatory responses, activities of ROS are mediated by the NF-κB pathway, which in turn is regulated by hypoxia and/or re-oxygenation (Hanada and Yoshimura, 2002). It has been recently proposed that NF-κB activates signaling pathways in both cancer cells and tumor-associated inflammatory cells, thus promoting malignancy (Pikarsky *et al.*, 2004). If progression to malignancy is indeed regulated at the level of NF-κB and a pro-inflammatory mediator TNF-α or other pro-inflammatory cytokines, as some of the animal models of cancer seem to indicate, the missing link between inflammation and cancer may have been identified (Pikarsky *et al.*, 2004; Greten *et al.*, 2004). These cancer models also underscore the importance of the tumor microenvironment, and its interactions with infiltrating inflammatory cells, in cancer progression. The data suggest that the NF-κB pathway is regulated differently in normal vs. malignant tissues. NF-κB is present as an inactive complex in the cytoplasm of many cells, including inflammatory and tissue cells. During inflammation, activation of NF-κB initiated by, e.g., binding of TNF-α to its receptor (TNFR1) expressed on inflammatory cells in the microenvironment initiates regulated expression of cytokine genes which control cell proliferation and cell death. Tumor cells depend on these cytokines for proliferation, and leukocytes activated in the tumor microenvironment are re-programmed to continually release these cytokines. Responding to this cytokine cascade, tumor and stromal cells produce a panoply of soluble factors with biologic effects ranging from enhancement of cell proliferation, matrix remodeling, vessel growth, inhibition of cellular differentiation to sustained release of pro-inflammatory mediators. Inhibition of NF-κB activation in tumor cells favors cell death and arrests tumor progression. This model is consistent with observed correlations between the numbers and maturation stages of inflammatory cells in the tumor, levels of cytokines produced and tumor prognosis (Balkwill, 2004). The role of TNF-α in driving tumor progression has been emphasized by Balkwill and colleagues (Malik *et al.*, 1998), and it offers an interesting example of the efficiency of tumors in their ability to usurp the normal biologic process of inflammation to promote tumor progression.

As tumor cells successively modify their microenvironment, they often adopt the phenotypic characteristics of immune cells. They co-opt signaling molecules, chemokines, selectins and their receptors normally expressed by leukocytes to serve for tumor migration, invasion and metastasis. It is likely that soluble factors produced during the immune phase, such as colony-

stimulating factor (CSF-1), could contribute to this adoption by tumor cells of a myeloid-like phenotype. The plasticity of tumor cells allows them to express chemokines and chemokine receptors, which usually function as chemoattractants and activating factors in leukocytes. Functions associated with neutrophils, such as the production of extracellular proteases, including matrix metalloproteinases (MMPs) that modify the extracellular matrix and fit it into the tumor scaffolding, are also adopted by tumor cells (Engbring and Kleinman, 2003). The use of a leukocyte-like metabolism by tumor cells, i.e., their ability to metabolize glucose via the glycolytic pathway and to synthesize ROS, is another example of how the properties of leukocytes are co-opted to maintain the hypoxic state in the tumor microenvironment (Borregaard and Herlin, 1982). Masquerading as inflammatory cells, tumor cells acquire the ability to further alter the microenvironment, migrate by responding to signals and pathways normally reserved for the cells of the immune system and establish metastases to organs rich in resident macrophages, where conditions are favorable for proliferation (i.e., lung, liver and bone). Thus, the leukocytes infiltrating the tumor contribute to the maintenance of the cytokine-rich microenvironment, which facilitates adoption of the leukocyte-like phenotype by tumor cells.

In inflammation, the immune phase is followed by the appearance of blood vessels and lymphatics in the repaired tissue. The process of angiogenesis is a prominent component of the tumor microenvironment (Bamias and Dimipoulos, 2003). Vascular endothelial growth factor (VEGF) is produced by most tumors and plays a crucial role in the development of tumor vasculature (Gabrilovich *et al.*, 1996b). Increased levels of VEGF in the plasma of patients with cancer were shown to correlate with a poor prognosis (Ellis and Fidler, 1996). Evidence points to VEGF as one of the factors responsible for inducing defects in DC differentiation in the tumor. As TAM play an important role in angiogenesis, it is not surprising that the tumor re-programs the myeloid precursors to express the secretory phenotype, which serves to promote vessel development, rather than their maturation to DC capable of priming T cells for antitumor responses. The appearance of blood vessels in the tumors signals another major change in the tumor microenvironment, namely a switch from hypoxic to oxidative metabolism. Oxidative phosphorylation with an increase in ATP synthesis is necessary to drive tumor cell proliferation, and it is enabled by angiogenesis promoted by combined activities of tumor-infiltrating leukocytes and tumor cells.

Once established, the tumor microenvironment is not a friendly place for infiltrating leukocytes. While they are clearly conscripted by the host to interfere with abnormal tissue growth, once in the tumor, they come in contact with a variety of soluble factors that impede their maturation, inhibit

their functions or simply induce their apoptosis (reviewed in Whiteside *et al.,* 2005). Cytokines are known to be present in tumors and are known to affect maturation, differentiation or functions and survival of immune cells (Toi, 2002). They include M-CSF, GM-CSF, IL-6, IL-10, TGFβ and other tumor-derived soluble factors (Whiteside *et al.*, 2005). The degree of impairment of immune cells in the tumor microenvironment differs widely in individual tumors. While the inhibitory effects are the strongest in the tumor, they are not confined locally but are systemic, especially in patients with advanced disease (Whiteside, 2002). Functional abnormalities and apoptosis of T lymphocytes are seen not only at the tumor site but are common in the circulation of patients with cancer. However, it is important to note that these patients generally have normal immune responses to recall antigens and can mount normal primary responses, except for those with terminal cancers. The mechanisms involved in selective and persistent inhibition of antitumor immune responses in patients with cancer are numerous and have been reviewed (Whiteside *et al.*, 2005). Tumors grow progressively and metastasize despite prominent leukocyte infiltrations, largely because they evolve strategies for escape from immune intervention (Whiteside *et al.*, 2005). Consequently, the fate of immune cells infiltrating the tumor is to be corrupted by the tumor into helping its progression, to lose their functional attributes or to die. Tumor aggressiveness depends on the efficiency with which this can be accomplished.

Successive changes that take place during chronic inflammation in the tumor imply that the nature and composition of the inflammatory infiltrates change as well. The characteristic feature of the tumor microenvironment is that it undergoes alterations in concert with tumor progression. It is for this reason that snapshots of the tumor microenvironment obtained by immunohistology of tumor sections or studies of TIL isolated from tumors at one stage of their progression provide an incomplete picture of cellular interactions *in situ*. Therefore, correlations of the numbers or phenotype of inflammatory cells in the tumor with clinical data or with patient prognosis may not be informative. Indeed, conflicting reports available in the literature regarding the significance of immune cells in the tumor microenvironment reflect the difficulties in interpretation of events that unfold and change in the context of host-tumor interactions.

4. CONCLUSIONS

New developments in molecular immunology in conjunction with newly developed animal models, as discussed above, have altered our perception of *in situ* interactions between infiltrating leukocytes and tumor cells. More

recent evidence favors the view of the developing tumor as a site of chronic inflammatory reaction that is orchestrated not by the host but by the tumor. Its success in co-opting functions of leukocytes toward promoting tumor survival depends on a variety of molecular mechanisms, and these are beginning to be elucidated. At least one link between inflammation and cancer in the form of the NF-κB pathway, which can either promote survival of cells with the malignant phenotype or sustain the production of pro-inflammatory cytokines in inflammatory cells within the tumor, has been identified. This discovery dramatically emphasizes the fact that the same molecular pathway can be harnessed to mediate opposite effects, depending on the context in which they operate. It also reminds us that dysregulated production of pro-inflammatory cytokines and chemokines can and does lead to tissue pathology. An understanding of the complex role of inflammatory infiltrates in the tumor progression is essential for devising novel and more effective anticancer therapies of the future.

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