[Frontiers in Bioscience 15, 291-308, January 1, 2010]

Immunoregulatory cells of the tumor microenvironment

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1. ABSTRACT

Current immune therapies for cancer have been disappointing. The various approaches to immunotherapy for cancer so far tried clinically include adoptive immunotherapy, vaccination strategies, and administration of anti-tolerogenic antibodies. Each of these approaches basically involves the inhibition or circumvention of immune tolerance, activating immune effectors that have the capability to recognize and lyse tumor. Unfortunately, only a relatively small population of patients respond to these therapies, and most of the responses are not durable. There is mounting evidence that immune interventions employing anti-tolerogenic strategies are insufficient to control tumor, as the tumor microenvironment is generally immunosuppressive. The present review summarizes the current knowledge on the cellular constituents of tumor (excluding tumor cells themselves) that contribute to this immunosuppressive microenvironment.

2. INTRODUCTION

Tumor represents an abnormal growth directly derived from normal host tissues. Therefore, the majority of antigens expressed by tumor are immunologically indistinct from normal tissues, hidden from the immune system by the same tolerogenic mechanisms that protect against autoimmunity. It therefore seems intuitive that inhibition of tolerance would successfully control the growth of a malignancy.

As a result of that teleologic reasoning, past attempts at harnessing the immune response for the purpose of controlling cancer have involved inhibition or avoidance of immune tolerance, activating immune effectors that have the capability to recognize and lyse tumor. Such strategies include adoptive immunotherapy and vaccination strategies (1,2), as well as administration of antibodies that induce cell-mediated immunity (3-6). Unfortunately, only a

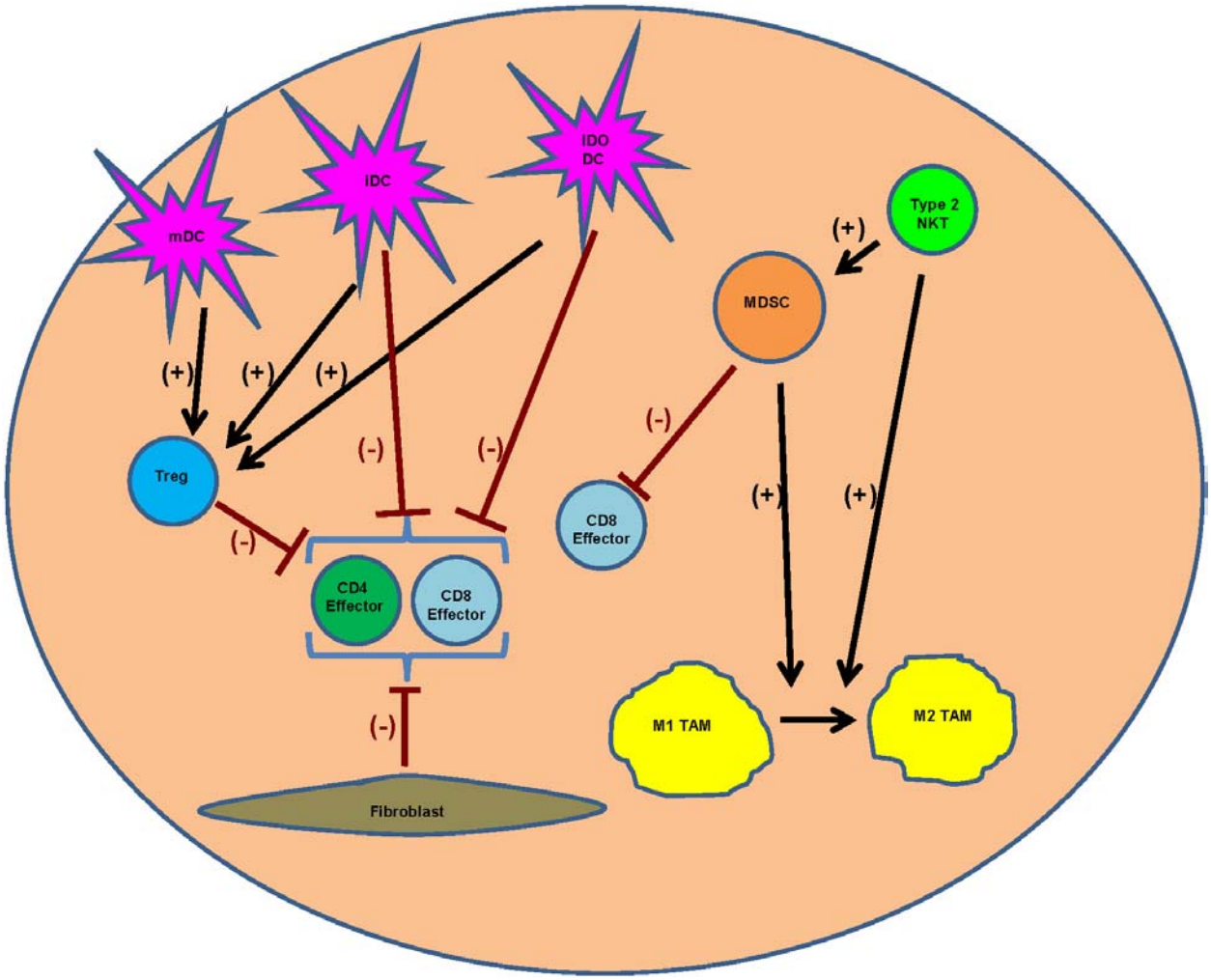

Figure 1. Immune interactions between non-tumor stromal cells within the tumor microenvironment. Many immunoregulatory cells challenge CD8+ and CD4+ T cell effector function within tumor. Immature DC (iDC) may recognize T cell receptors, but do not provide proper co-stimulation, resulting in CD4+ and CD8+ T cell inactivation. IDO secreting DC metabolize tryptophan required for CD4+ and CD8+ T cell proliferation. iDC and IDO-DC can induce Treg formation. Mature myeloid DC expand the peripheral Treg population through CD80/CD86-CD28 interactions and IL-2. Treg mediate direct suppression of CD4+ and CD8+ effector cells. Fibroblasts induce anergy and apoptosis in T cells. MDSC inhibit T cell function by depleting L-arginine and by producing peroxynitrites, resulting in the downregulation of the CD3ζ chain. Type 2 NKT cells secrete IL-13, which activates immunosuppressive activity in MDSC and promote conversion of M1 macrophages to the M2 phenotype.

relatively small proportion of patients respond to these therapies, and most of the responses are not sustained.

There are numerous reasons for the lack of success in inducing a reliable and durable response with immunotherapy. Tumor may escape recognition by downregulation of MHC class I molecules or by loss of expression of the targeted antigen (7-9). There may be insufficient co-stimulation, inducing T cell anergy (10). The active destruction of T cells or inhibition of T cell function has also been reported, as tumor elaborates a wide range of immunosuppressive factors (11). Finally, tumor may contain various populations of immunoregulatory cells which further contribute to the immunosuppressive microenvironment (Figure 1).

Our own fundamental studies have demonstrated that, even if a large number of tolerogenic mechanisms have been inhibited and despite priming of large numbers of T cells which recognize endogenous antigens on tumor, tumor emerges despite an immune response that is sufficiently strong to induce autoimmunity (12). We believe that this experimental situation simulates an optimal multimechanistic vaccination strategy and that this demonstrates the limitations of the current immunotherapeutic strategies utilized clinically. Evidence so far obtained through this model suggests that the immunosuppressive tumor microenvironment is responsible for failure of protection against the tumor.

Tumor is more than just a collection of transformed host cells. Tumor is also comprised of a

melange of stromal cells, including endothelial cells, various inflammatory cells, and fibroblasts. Some of these stromal cells contribute to the immunosuppressive microenvironment. The roles of these immunomodulatory inflammatory cells on tumor biology and immune escape comprise the focus of this review.

3. REGULATORY T CELLS

A number of regulatory T cell subtypes have been described in the literature. In general, they can be classified as natural regulatory T cells (nTreg) or inducible (adaptive) regulatory T cells (iTreg). Inducible Treg develop from conventional T cells, arising from repetitive antigen stimulation, under the influence of functionally polarizing cytokines or due to interference with costimulatory signals. These include CD4+Foxp3- regulatory cells (Tr1), which mediate suppression via IL-10 (13,14); CD4+CD25- (Th3) cells, which immunosuppress through a transforming growth factor (TGF)-beta dependent mechanism and which also secrete IL-4 and IL-10 (15); as well as CD8+ regulatory cells that can be Foxp3+ or Foxp3- (16-18). Each of these regulatory T cell subtypes inhibit the antigen specific immune response in a cytokine or cell contact-dependent manner, preventing autoimmunity. CD4+CD25+Foxp3+ suppressor cells have perhaps been best described in the literature. These occur naturally, generated in the thymus, although they may also be inducible in the periphery (19-21).

CD4+CD25+Foxp3+ T cells represent the population that is best known to infiltrate tumor. Other regulatory T cell populations have not been well studied in the context of tumor. Therefore, CD4+CD25+Foxp3+ T cells will represent the primary focus of this section.

The most specific marker for this population is the nuclear transcription factor Foxp3. Foxp3 is a critical regulator of CD4+CD25+ regulatory T cell development and function; its expression correlates with the capability to immunosuppress (19). The IL-2R-alpha chain (CD25) is highly expressed in Tregs, which increases the affinity of IL-2 to its receptor complex (22). CD127 (IL-7 receptor) is expressed at low levels (23,24). Glucocorticoid-induced TNF receptor (GITR), a member of the TNF receptor family, is present on CD4+CD25+Foxp3+ T cells (25,26), although it is also present in a small proportion of CD4+CD25- T cells capable of immunosuppression (27). Cytotoxic T lymphocyte associated antigen (CTLA)-4 appears at the cell surface when these cells are activated (28,29). Neither GITR nor CTLA-4 are considered specific markers for regulatory T cells.

Antigen stimulation results in suppressive function, but the proliferative response of CD4+CD25+Foxp3+ cells to antigen stimulation depends on the experimental conditions. *In vitro* studies have found CD4+CD25+Foxp3+ T cells do not proliferate following agonistic T cell receptor (TCR) ligation (30,31). *In vivo*, these regulatory cells behave differently. They are seen to proliferate at a high rate, possibly in response to self antigen recognition or commensal bacteria (32,33). Suppressive activity of CD4+CD25+Foxp3+ cells is enhanced with repeated stimulation by antigens displayed by antigen presenting cells (APCs), including dendritic cells and B cells (34,35,35). Despite some evidence indicating that CD4+CD25+Foxp3+ cells are naturally anergic to activation via the TCR (31), they do require TCR-mediated stimulation to exert their suppressive function and to inhibit the expansion of conventionally responsive CD4+ and CD8+ T cells (36,37).

Activated CD4+CD25+Foxp3+ cells suppress T cell function (inhibiting responder IL-2 production) in an antigen non-specific fashion, and this requires cell-to-cell contact (38,39). High levels of CTLA-4 on regulatory T cells appear to be important in conferring the suppressive phenotype (29). Stimulated regulatory cells also produce IL-10 and/or TGF-beta, depending on experimental conditions (34,40), although neither of these cytokines appear to be essential for their suppressive function (29,34,40). Thus, regulatory cells are capable of inducing their suppressive effects through direct cell-to-cell contact, as well as through soluble mediators.

There is much evidence to support an important role of CD4+CD25+ cells in suppressing tumor immunity. In animal models, CD4+CD25+ regulatory T cells have been observed to be present in tumor. Their depletion enhances tumor immunogenicity (41,42). There is also mounting clinical evidence of the impact of Treg. CD4+CD25+ T cells with immunosuppressive characteristics comprise a significant proportion of tumor infiltrating lymphocytes in breast and gastrointestinal adenocarcinomas (43-45), head and neck cancers (46), lung cancer (47,48), and ovarian cancer (49). Treg exist in markedly high proportions in the peripheral blood of patients with cancers as compared to controls, reportedly increasing with tumor progression (43,50,51). The presence of higher numbers of CD4+CD25+ T cells is associated with a worse prognosis (49,51). Experimental and clinical evidence therefore strongly suggest that CD4+CD25+ Treg alter tumor biology, which has a direct impact on patient outcomes.

4. DENDRITIC CELLS

Dendritic cells (DC) represent a central point of intersection between the innate and adaptive elements of the immune system. They have long been recognized as the most potent and efficient professional APC, capable of stimulating naïve T cells to become fully competent. They have also been found in some circumstances to have immunoregulatory, or tolerogenic, properties.

Different DC subsets vary in their functional properties. Immature DC function mainly to capture antigen, then gain the capability to stimulate T cells as they mature in the presence of appropriate “danger” signals. Immature DC express low levels of co-stimulatory molecules (CD80, CD86, ICOS ligand), and are unable to form a stable immunological synapse with T cells. Rather than stimulating conventional effector T cells, they induce effector T cell anergy and they activate regulatory T cells

(34,52,53). Recently, semi-mature DC were described, which originated from exposure of immature DC to TNF-alpha (54). These cells have upregulated co-stimulatory molecules (like mature DC), but they produce only low levels of the pro-inflammatory cytokines IL1-beta, IL-6, TNF-alpha, and IL-12p70. Semi-mature DC predominately encourage the development of inducible regulatory T cells. Finally, in addition to the myeloid DC subsets described above, plasmacytoid DC have been described, which express lymphoid markers and are thought to be lymphoid in origin (55,56). Plasmacytoid DC freshly isolated from blood can induce CD4+ T cell anergy (57,58). Thus, while DC are widely considered to be potent activators of T cell mediated immunity, there is a variety of DC subsets which have the capability to tolerize.

The effects of tolerizing DC on regulatory T cells is not unilateral, however. In addition to DC being capable of activating regulatory T cells, there is growing evidence that regulatory T cells are reciprocally capable of modifying the maturation status of DC and other APC (18,59-61). In general, CD4+CD25+ T regulatory cells induce a tolerogenic DC phenotype: maturation is inhibited, they are less capable of presenting antigens, and there is a diminished production of proinflammatory cytokines (62,63). The mechanism for tolerizing DC is unclear, but cell-to-cell contact appears to be required (62). Another population of regulatory T cells present in humans, characterized by their CD8+CD28- phenotype, have also been found to render APC tolerogenic (18). This effect is accompanied by upregulation of immunoglobulin-like transcript 3 and immunoglobulin-like transcript 4, inhibitory receptors essential to their tolerogenic phenotype. It is not clear why this reciprocal tolerization occurs between Treg and DC except that this may serve as a mechanism for accelerating immunoregulatory effects in the event of an exuberant inflammatory response.

Normally, immature DC reside in the periphery as well as in lymphoid organs, where they encounter antigens. Upon activation and maturation, they migrate via the lymphatic vessels into draining lymph nodes, where they present processed antigen to naïve T cells. Tumors are also known to contain significant numbers of DC (64,65). Tumor infiltrating DC have been reported in breast cancer (65), ovarian cancer (66), renal cell carcinoma (67), lung cancer (68,69), and melanoma (70). Several investigators have demonstrated that they are mainly comprised of the immature phenotype, expressing relatively low levels of costimulatory molecules (64,65,71). Plasmacytoid DC have been described as the prevailing population in ovarian cancer, head and neck squamous cell cancer (HNSCC) and melanoma lesions (72-75). Tumor infiltrating DC have also been reported to have a reduced capacity to take up antigen (64) as well as a reduced ability to stimulate T cells (76). DC isolated from ovarian carcinoma constitutively express B7-H1 (PD-L1), which is known to inhibit T cell function (66). Moreover, DC in tumor-draining lymph nodes overexpress indoleamine 2,3-dioxygenase (IDO). IDO-expressing DC induce Treg and have a number of other immunomodulatory activities (77).

Clinically, the effect of these tumor-infiltrating DC on tumor biology is less clear, as several studies have reported the presence of tumor-infiltrating DC to be associated with a relatively good prognosis (69,78,79). Thus, while tumor-infiltrating dendritic cells appear to be functionally immunosuppressive, their actual influence on tumor biology and the tumor-bearing host is unclear.

5. TUMOR ASSOCIATED MACROPHAGES (TAMs)

Macrophages have numerous functions and considerable phenotypical heterogeneity, depending on the tissue in which they reside and also on the local microenvironment. Prototypical tissue-specific functions include bone resorption (osteoclasts), phagocytosis of inhaled particulate matter (alveolar macrophages) and hepatic clearance of bacteria, toxins and debris (Kupffer cells). Non-residential macrophage phenotype and function appears to be more plastic, shaped by the microenvironment. This differentiation is reflected by their general classification into classically activated (M1) or alternatively activated (M2) macrophages.

M1 macrophage activation occurs in response to microbial products (eg: LPS) or proinflammatory cytokines such as IFN-gamma or TNF-alpha(80,81). M1 macrophages express opsonic receptors such as Fc-gamma-RIII (CD16) (82). They typically express high amounts of IL-12 and low levels of IL-10, and they elicit proinflammatory cytokines such as IL-1, IL-6 and TNF-alpha (80,83). M1 macrophages express high levels of inducible nitric oxide synthase (iNOS/NOS2), and produce reactive oxygen species (ROS) and reactive nitrogen species (RNS) (80,81,84). M1 macrophages are considered to be potent effector cells that kill microorganisms and tumor cells.

M2 macrophages are derived from the presence of anti-inflammatory molecules such as glucocorticoid, hormones, IL-4, IL-12, IL-13 and IL-10 (80,81,85-87). In contrast to M1 macrophages, they typically produce high amounts of IL-10 and very little IL-12 (80,88). M2 macrophages function poorly as APC; curtail Th1 adaptive immunity by producing IL-10, TGF-beta and prostaglandin E2 (89); scavenge debris; promote tissue remodelling and repair; and encourage angiogenesis. M2 macrophages preferentially express non-opsonic receptors such as mannose receptor and scavenger receptors (80). Thus, M2 macrophages could be considered immunoregulatory and proneoplastic.

Sica and co-workers (80) recently suggested that M2 macrophages can be classified to subtypes which are derived from different stimulatory milieus. M2a macrophages are generated as a result of exposure to IL-4 and IL-13. They promote a Th2-biased adaptive immune response and also support the killing of encapsulated parasites. M2b macrophages appear following stimulation with immune complexes and toll-like receptor agonists or IL-1R ligands. This subpopulation activates the Th2 response and also appears to regulate inflammation and immunity. M2c macrophages emerge from stimulation

with IL-10, TGF-beta or glucocorticoids and these macrophages have the capability to regulate the immune response as well as to promote matrix deposition and tissue remodelling (90).

One particularly important feature of M2 macrophages is that they elaborate arginase 1 (ARG1). ARG1 expression is stimulated in mouse macrophages by Th2 cytokines (91); TGF-beta (92); macrophage stimulating protein (93); and GM-CSF (94). Increased uptake of the amino acid L-arginine results in reduced extracellular levels of L-arginine, which culminates in the impairment of the ability of activated T cells to proliferate. Macrophages that produce ARG1 (but not macrophages that produce NOS2) cause loss of the CD3-zeta chain and inhibit T cell proliferation (95). L-arginine depletion similarly impairs the expression of the CD3-zeta chain of the T cell receptor (96). Expression of ARG1 by M2 macrophages is therefore an important mechanism for their immunosuppressive effects.

Tumors have frequently been reported to contain large numbers of tumor infiltrating macrophages (TAM). The phenotype of TAM may change as tumor progresses, depending on the type of tumor. In lungs containing pre-malignant lesions, macrophages express ARG1 but not NOS2. As invasive carcinoma develops, the pulmonary macrophages express NOS2 but not ARG1 (97). On the other hand, in early melanoma lesions, TAM expressing NOS2 prevail over ARG1 (98). These data demonstrate a degree of plasticity of TAM which may be due to a changing tumor microenvironment or to differential recruitment of various macrophage subsets as tumor progresses.

In the majority of reports on established tumors, TAM have the phenotype and function attributed to M2 macrophages. TAM express high levels of macrophage scavenger receptor 1 and the mannose receptor and they also express ARG1 (80,99,100). Low amounts of proinflammatory cytokines such as IL-12, TNF-alpha, and IL-1 are elicited, but high amounts of the immunosuppressive cytokine IL-10 are produced (80,100,101). TAM produce low amounts of nitric oxide (102); only a minority express iNOS, and they are poor producers of ROS (103). TAM have poor antigen presenting capacity and can suppress T cell activation and proliferation (80,81). Mediators involved in immunosuppression include prostaglandins, IL-10, TGF-alpha, and IDO metabolites. The cytokine and metabolite milieu produced by TAM is typical of M2 macrophages and would be expected to support a response by regulatory T cells.

In addition to promoting an immunosuppressive microenvironment, TAM may promote tumor progression through their support of angiogenesis. TAM can produce proangiogenic factors such as TGF-beta, VEGF, PDGF, bFGF, and angiogenic chemokines such as CXCL1, CXCL5, CXCL8, and CXCL12 (80,104). Indeed, the density of microvessels in tumor correlates with the level of TAM infiltration in cancer (105,106). TAM have been found to accumulate preferentially in poorly vascularised, hypoxic regions of tumor where they secrete proangiogenic factors, taking on the M2 macrophage program (107-109). This appears to be dependent on HIF-1-alpha, as suppression of HIF-1-alpha inhibits migration of TAM to these hypoxic regions (110), although hypoxic macrophages upregulate both HIF-1-alpha and HIF-2-alpha (108,109).

TAM may also affect tumor invasiveness and its capability to metastasize because of their involvement in tissue remodelling. Enzymes such as matrix metalloproteinases, plasma urokinase-type plasminogen activator (uPA), and its receptors (uPAR) are produced by macrophages. These enzymes are involved in the breakdown of the extracellular matrix, and their expression correlates with a much more aggressive tumor phenotype (111). It is thought that these enzymes are involved in the dissolution of the basement membrane, which acts as a point of anchorage within the primary tumor. Thus, because of the actions of TAM on the extracellular matrix, tumor cells are endowed with the capability of escaping from the primary tumor, metastasizing to distant organs through lymphatics and the vasculature. The matrix remodelling activities of TAM are therefore important contributory factors in tumor growth as well.

Experimental and clinical observations support the proneoplastic role of macrophages, although it is difficult to discern how much of that phenomenon is related to immune suppression as opposed to other mechanisms. In APC(Delta716) mice, macrophage depletion results in suppression of gastric tumorigenesis by mechanisms that are not directly immune mediated (112). In a murine model of mesothelioma, depletion of macrophages using liposomal clodronate reduces the size and number of tumors (113). There are also clinical series in which TAM are associated with a worse prognosis (114-117). The observation that the presence of TAM is associated with an impaired response to cancer immunotherapy suggests that at least some of the effect seen in clinical series is immune-mediated (118).

6. MYELOID DERIVED SUPPRESSOR CELLS

Tumor development is frequently accompanied by an accumulation of myeloid cells in the periphery, as well as in the tumor. These myeloid derived suppressor cells (MDSC) have only recently been the focus of investigation. A number of MDSC subsets have been described, and it has been speculated that they may be related to TAM.

In mice, MDSC are commonly defined as CD11b+Gr-1+ cells, which are predominately present in bone marrow, but also exist in spleen and blood. The marker Gr-1 identifies at least two populations comprising polymorphonuclear cells (CD11b+Gr-1^{High}) and mononuclear cells (CD11b+Gr-1$^{Int/Low}$) (119). It is the monocytic fraction which expresses the common alpha chain of the receptor for IL-4 and IL-13 (IL-4R-alpha) and which has immunosuppressive functions (120). Other

markers on murine MDSC include CD31 and ER-MP58 (121). In humans, the MDSC phenotype is not well defined and probably consists of a number of related subpopulations. MDSC have been variably described as: CD34+ (122,123); Lin-CD33+CD15- (positive or negative for HLA-DR) (124); CD11b+ CD14- HLA-DR$^{Neg/Low}$ CD15+ (125); and CD11b+CD14+ HLA-DR$^{Neg/Low}$ cells (126).

MDSC have a number of immunoregulatory actions. Given the heterogeneity of this population, it may be that not all of these actions can be attributed to the same subset. One way that MDSC incite immunosuppression is by controlling the metabolites contained within their microenvironment. MDSC regulate T cell responses mainly by controlling the availability of L-arginine. They typically express high levels of ARG1 and, like M2 macrophages, the low levels of L-arginine in their microenvironment influence T cell function by impairing the expression of the CD3-zeta chain of the T cell receptor (96). MDSC also produce substantial amounts of ROS and RNS, which impair DC maturation (119). Reactive nitrogen species such as peroxynitrites impair the response of CD8+ T cells to antigen (127). In addition to the immunosuppressive effects of MDSC on the microenvironment, they can inhibit T cell function through cell-to-cell contact. This primarily affects CD8+ T cell function by inhibiting their secretion of IFN-gamma (128). The effect on CD8+ T cells requires the co-expression of ARG1 and NOS2 (96,129) in MDSC and it also appears to be dependant on IL-13 and IFN-gamma (120,130). Other immunoregulatory effects attributed to MDSC include the expansion of regulatory T cells (131,132); inhibition of NK cell activity in a contact-dependant fashion (133); impairment of NKT cells (134); and polarization of macrophages to the M2 phenotype (135).

The presence of tumor is associated with increased numbers of circulating MDSC. This has been described in head and neck cancer, lung cancer, and breast cancer (124), as well as melanoma (126), and renal cell carcinoma (125). Tumor infiltrating MDSC have been well described in animal models (120,136). Unfortunately, in human studies, it is much more difficult to completely characterize the tumor infiltrating inflammatory response in sufficient detail to fully distinguish TAM from MDSC. Therefore, in studies on human tissue in which TAM are described, it is possible that at least some of the macrophage-like population may actually represent subsets of MDSC.

While it is difficult to determine the effect of circulating and tumor-infiltrating MDSC on prognosis, there is evidence that MDSC are at least partly responsible for the failure of some vaccine trials. For example, in a recent trial of a GM-CSF-based vaccine, increased numbers of MDSC were seen following vaccination. This increased MDSC activity was not found in patients receiving non-GM-CSF-based vaccines. In that trial, the presence of MDSC was associated with a lack of immunologic response to the vaccine (126).

7. NKT CELLS

CD1d restricted natural killer T (NKT) cells consist of at least two subsets. Type 1 semi-invariant TCR-alpha chain-expressing NKT cells express V-alpha14J-alpha18 in mice and V-alpha24J-alpha18 in humans. They have NK-like cytolytic activity, produce large amounts of both Th1 and Th2 cytokines, and they are known to be involved in anti-tumor immunity (137,138). Type 2 variable TCR-alpha chain-expressing NKT cells appear to have an immunosuppressive role.

In a fibrosarcoma model, type 2 NKT cells expressing CD4 were found to produce IL-13, which induced production of TGF-beta by CD11b+Gr-1+ MDSC, which subsequently resulted in suppression of CD8+ T cell mediated immunity against tumor (139,140). The immunosuppressive role of NKT cells has also been demonstrated in a number of other tumor models, including CT26 colon carcinoma (141,142), a 4T1 mammary carcinoma model (142,143), and an orthotopic K7M2 osteosarcoma model (144). In this latter model, the effects of NKT cells on tumor immunity did not appear to be dependent on IL-4R-alpha signalling, IL-13, or TGF-beta, suggesting that other mechanisms of CD1d restricted NKT cell mediated immunosuppression exist. Selective stimulation of type-2 NKT cells by sulphatide significantly enhances growth of CT26 colon carcinoma lung metastases (145). It has been postulated that sulphatide, which can be found in tumors as well as many other tissues, stimulates type 2 NKT cells to induce immunosuppression in tumor bearing individuals (146). NKT cells have been shown to regulate Treg function in an autoimmune myasthenia model (147), but interactions between NKT cells and Treg have not been demonstrated in tumor models. Moreover, in clinical cases of cancer, there is little information on the influence of type 2 NKT cells on the tumor microenvironment.

8. FIBROBLASTS

The tumor framework is maintained by fibroblasts, cells which deposit the extracellular matrix, maintain the basement membrane and produce matrix metalloproteinases and other enzymes responsible for tissue remodelling. Generally, fibroblasts are not considered immunoregulatory cells. However, in the context of the tumor microenvironment, fibroblasts have been shown to have some immunosuppressive properties.

Mesenchymal stem cells from bone marrow, which have the capability to differentiate into bone, cartilage and fat, are known to have immunomodulatory effects. They cause direct suppression of T cell proliferation (148-150); induce T cell anergy (151); promote T cell apoptosis (152); inhibit dendritic cell maturation (153-155); and inhibit function of B cells (156) and NK cells (157). Recently, it was shown that this capability to immunosuppress was a fundamental property shared by all stromal cells (158,159). Chondrocytes, as well as fibroblasts from synovial joints, lung and skin can each inhibit T cell proliferation. Stromal cells require

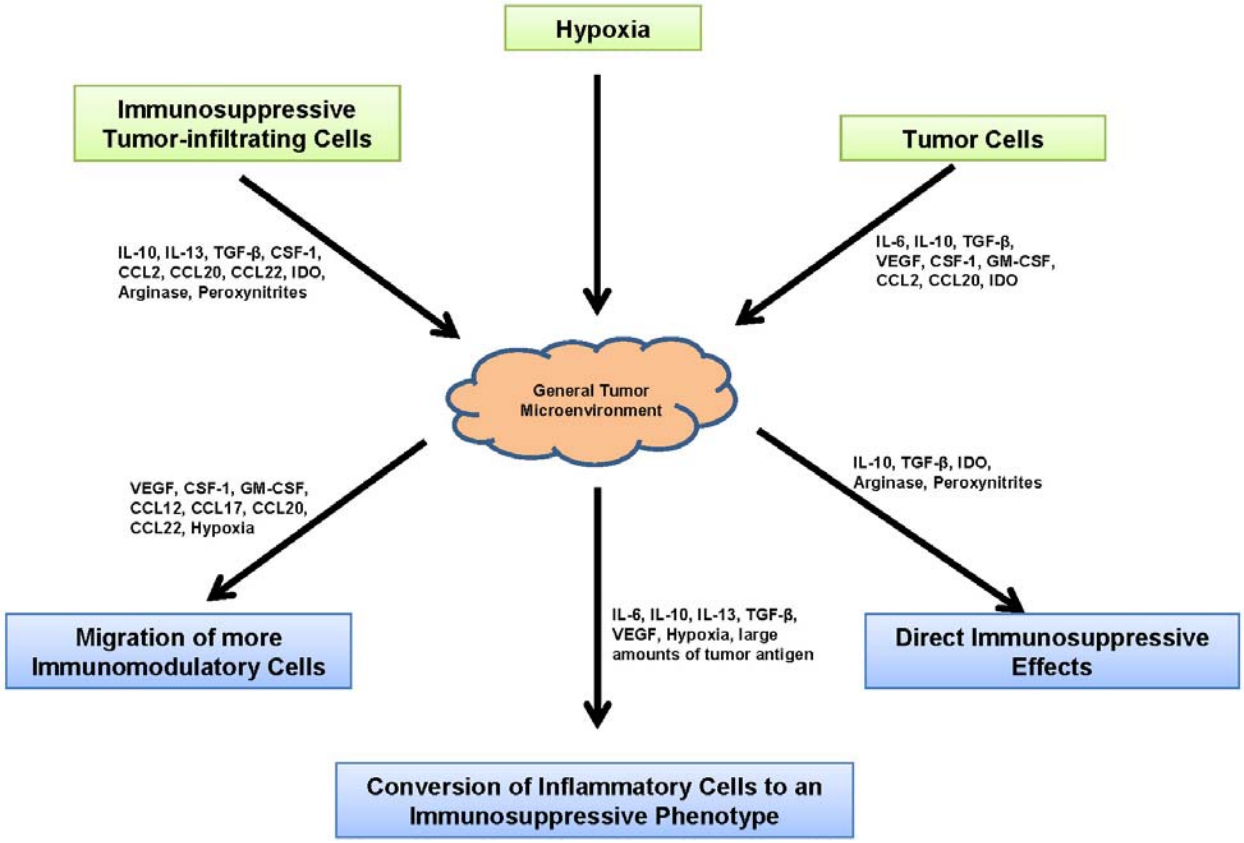

Figure 2. Immunosuppressive mediators of the tumor microenvironment. Tumor-infiltrating immunosuppressive stromal cells, tumor cells, and hypoxia contribute to a generally immunosuppressive microenvironment. Factors within this environment encourage the migration of more immunosuppressive cells, convert inflammatory cells to an immunomodulatory phenotype, and directly inhibit tumor immunity.

licensing by activated T cells to acquire antiproliferative functions (158,159). However, the role of IFN-gamma in this fibroblast-mediated suppression is not completely clear as only one of the reports demonstrated that IFN-gamma may be important in mediating these effects (159). In that report, IFN-gamma induced IDO in fibroblasts, an enzyme known to have a number of immunosuppressive effects (160).

9. RECRUITMENT OF IMMUNOREGULATORY CELLS TO TUMOR

Tumor elaborates its own signals to attract immunosuppressive cells to its lair. Factors derived from proinflammatory cells infiltrating tumor further modify the tumor microenvironment, encouraging recruitment of suppressive immune effectors meant by nature to prevent uncontrolled immunity (Figure 2). What is not completely clear is whether the cells that are recruited to tumor are intrinsically immunosuppressive or whether the tumor microenvironment induces an immunosuppressive phenotype. There are data that support each of these possibilities.

Tumor appears to have interesting effects on CD4+CD25+Foxp3+ Treg. In patients with ovarian cancer, Curiel and co-workers demonstrated the preferential migration of CD4+CD25+ cells to tumors and ascites. Because macrophages contained in malignant ascites produce large amounts of CCL22 (which was shown to induce migration of regulatory T cells into tumor in an animal model), this group speculated that the recruitment of Treg was secondary to the presence of macrophages within the tumor microenvironment (49). This supports the hypothesis that recruitment of tumor infiltrating Treg is secondary to the primary inflammatory response to tumor. Tumor may itself also elaborate substances capable of attracting Treg. In head and neck cancer patients, the number of circulating CD4+CD25+ T cells is significantly reduced following curative resection (161). Once in the tumor, the presence of TGF-beta may promote expansion of infiltrating Treg (162). Tumors have been described to produce IL-10 (163,164). High concentrations of TGF-beta and IL-10 are known to induce CD4+CD25- T cells to develop suppressive function (165). Moreover, once in tumor, the presence of large amounts of secreted tumor antigens may stimulate Treg (166). Thus, Treg appear to traffic to tumor due to chemokines elaborated by other inflammatory cells. Once there, their immunosuppressive phenotype is promoted by various tumor-derived factors.

The presence of DC in tumor seems to be secondary to signals elaborated by tumor. Tumor cells produce MIP-3-alpha/CCL20, which is selectively chemotactic to immature DC expressing CCR6 (65). Once in the tumor, factors in the microenvironment are present which may inhibit maturation. Cytokines secreted by tumor which are known to inhibit DC maturation include IL-10, TGF-beta, VEGF, and IL-6 (167-171).

Macrophage recruitment to tumors is mediated by a number of chemokines and cytokines, including CCL2/MCP-1, VEGF and macrophage colony stimulating factor (M-CSF, or CSF-1). Most human carcinomas produce CCL2 and its levels of expression correlate with increased infiltration of macrophages (80). CCL2 produced by TAM may accelerate trafficking of monocytes to tumor (172). In breast cancer and esophageal cancer, CCL2 levels correlate with the extent of macrophage infiltration, which appears to be related to a propensity for lymph node metastases and clinical aggressiveness (173,174). VEGF-mediated recruitment of TAM is dependent on VEGFR2 expression on macrophages (175,176). Finally, high expression of macrophage colony stimulating factor (CSF-1) in peritumoral liver tissue and TAM infiltration is associated with poor survival after resection of hepatocellular carcinoma (117). Thus, a number of factors that promote macrophage trafficking to tumor have been identified.

Mobilization of MDSC also appears to be a result of factors elicited by tumor. MDSC are seen in higher numbers in animals and humans with tumor; tumor burden is correlated with the number of circulating MDSC; and resection of tumor is associated with diminished numbers of MDSC (177-179). One example of a tumor-derived factor that could incite MDSC mobilization is GM-CSF. Some tumors have been shown to elaborate GM-CSF (180-182). High serum levels of GM-CSF are accompanied by higher numbers of circulating MDSC (89,120,126). Thus, tumor appears to stimulate MDSC mobilization and migration to its locale.

The nature of the monocytic infiltrate in tumor is intriguing. TAM and tumor-infiltrating MDSC originate from common progenitors, but it is not clear how plastic their differentiation is. It has been postulated that circulating MDSC could differentiate into TAM (183), particularly M2 polarized TAM (135). It is therefore possible that TAM and MDSC co-exist in the tumor because they are actually of common origin and really represent different stages of differentiation incited by factors in the tumor microenvironment. Another possibility is that MDSC and macrophages are already terminally differentiated prior to migration to tumor and that the two populations represent distinct populations with different functions. It was recently observed in a 4T1 murine mammary carcinoma model that direct cell-to-cell contact between MDSC results in IL-10 production by MDSC, which inhibits IL-12 production by macrophages (135). This cross-talk between MDSC and macrophages inhibits tumor immunity. More investigations are required to delineate the relationship between MDSC and macrophages in tumor.

It has recently been suggested that macrophage phenotype changes during the natural history of tumor (184). This was mostly suggested because of the observation that tumors derived in the inflammatory premalignant state initially contain M1 macrophages. Then, as tumor grows, the TAM consist mainly of M2 macrophages. This is thought to be mainly due to a defective NF□B function in TAM as tumors grow (100,185). Hypoxia, which occurs in some regions of established tumors, may also affect the monocytic infiltrate. The transcription factors HIF-1-alpha and HIF-2-alpha are both upregulated in macrophages exposed to hypoxia and this results in increased secretion of VEGF and MMP7. Hypoxia also encourages the expression of IL-10, arginase, and PGE-2; typical of M2 macrophages (109). These observations suggest that the phenotype of TAM may be mostly a result of changes in tumor microenvironment as tumor evolves, rather than differential recruitment of a particular subtype of macrophages.

Another well documented mechanism for the emergence of an immunosuppressive tumor microenvironment involves IDO. The tumor cells themselves are a source of IDO (186,187). Tumor infiltrating dendritic cells may also express IDO. IDO-expressing DC potently suppress T cell responses and induce tolerance to tumor derived antigens (188,189). IDO causes local depletion of tryptophan. The local depletion of tryptophan and the production of toxic tryptophan catabolytes inhibits activation of NK cells (190). Thus, IDO elaborated by the tumor as well as by tumor infiltrating DC, is a contributing factor to the immunosuppressive microenvironment in some tumors.

Until recently, the origin of tumor fibroblasts was not well defined. In particular, it was not clear whether they were derived from the local milieu in which the tumor emerged or whether they migrated to tumor from afar. Studies in an insulinoma model revealed that about 25% of tumor fibroblasts are bone marrow-derived (191). The origin of the remainder of these cells was not delineated. It is possible that the rest of the stromal fibroblasts represent locally derived cells. Whether there are phenotypical differences (eg: elaboration of immunosuppressive factors) between these two subpopulations of stromal cells is not yet known.

10. SUMMARY

Present immunotherapeutic strategies for cancer typically involve the inhibition of immune tolerance. While some successes have been seen in individuals, cancer immunotherapeutics have not had dramatic successes. This may partly be because of the immunosuppressive tumor microenvironment. While it is well known that the tumor cells themselves produce immunomodulatory factors, tumor stroma also contains a number of immunoregulatory cell populations. Some of these may be recruited to the tumor secondary to chemokines produced by a primary immune response or by the tumor itself. However, very little is understood about

the factors responsible for recruitment of these immunoregulatory cells to tumor.

It is becoming obvious that, in order for immunotherapy to be successful in the context of malignancy, not only do we require the capability of priming tumor-specific immunity, we also need to sustain that immune response. This may be possible by inhibiting the immunosuppressive actions of tumor and its stromal constituents, either by blocking inhibitory factors or by (functionally) depleting immunoregulatory effectors. Blocking factors that encourage the migration of any immunosuppressive cell populations to tumor may be useful on a therapeutic basis. Finally, it is possible that some of the inhibitors of angiogenesis, especially VEGF antagonists, may be useful in encouraging the immune response against tumor. Our recent recognition of the immunosuppressive qualities of the tumor microenvironment has therefore provided new opportunities for therapeutic innovation for cancer.

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Abbreviations: APC: antigen presenting cell, ARG1: arginase 1, CTLA: cytotoxic T lymphocyte associated antigen, DC: dendritic cell, GITR: glucocorticoid-induced TNF receptor, HNSCC: head and neck squamous cell cancer, LPS: lipopolysaccharide, MDSC: myeloid derived suppressor cell, NK: natural killer cell, NKT: natural killer T cell, RNS: reactive nitrogen species, ROS: reactive oxygen species, TAM: tumor associated macrophage, TCR: T cell receptor, TGF-beta: transforming growth factor beta, Treg: regulatory T cell, uPA: urokinase-type plasminogen activator, uPAR: urokinase-type plasminogen activator receptors

Key Words: Tumor Microenvironment, Tolerance, Suppression, Anergy, Cytotoxic T cells, regulatory T cells, Tumor Associated Macrophages, Review

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