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Immunotherapeutic modulation of the suppressive liver and tumor microenvironments

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Abstract

The liver is an immunologically unique organ, consisting of resident hematopoietic and parenchymal cells which often contribute to a relatively tolerant microenvironment. It is also becoming increasingly clear that tumor-induced immunosuppression occurs via many of the same cellular mechanisms which contribute to the tolerogenic liver microenvironment. Myeloid cells, consisting of dendritic cells (DC), macrophages and myeloid-derived suppressor cells (MDSC), have been implicated in providing a tolerogenic liver environment and immune dysfunction within the tumor microenvironment which can favor tumor progression. As we increase our understanding of the biological mechanisms involved for each phenotypic and/or functionally distinct leukocyte subset, immunotherapeutic strategies can be developed to overcome the inherent barriers to the development of improved strategies for the treatment of liver disease and tumors. In this review, we discuss the principal myeloid cell-based contributions to immunosuppression that are shared between the liver and tumor microenvironments. We further highlight immune-based strategies shown to modulate immunoregulatory cells within each microenvironment and enhance anti-tumor responses.

The Immunosuppressive Liver Microenvironment

The liver is an immunologically unique microenvironment constantly exposed to various antigens such as microbial products from intestinal bacteria. As such, there are numerous cellular and molecular components that are involved with maintaining a tolerogenic liver microenvironment, yet which still endow this organ with the necessary capabilities for the development of immune responses (1). The capability of inducing tolerance is beneficial in specific situations such as allogeneic transplantation, although opportunistic infections such as hepatitis B and other malignancies may exploit this situation and result in chronic disease. The liver contains a different cellular distribution of lymphocytes, such as the higher proportion of NK and NKT cells compared to other lymphoid organs such as the spleen. DC and macrophages present within the liver are primarily responsible for antigen presentation, although nonlymphoid hepatocytes and liver sinusoidal endothelial cells also have limited antigen presentation capabilities.

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Resident Kupffer Cells and Macrophages Contribute to an Immunosuppressive Liver Microenvironment

Kupffer cells (KC), identified based upon CD68 (microsialin) expression and as a subset of CD11b⁺/F4/80⁺ cells, are the largest group of tissue resident macrophages located in the liver and lie within the periportal area of the hepatic sinusoids. A major function of KC is the phagocytosis of particulates, apoptotic cells and microorganisms present within the portal circulation (1). KC have APC functions with antigen uptake and processing capabilities and express low levels of MHC class II and costimulatory molecules at a steady state. Upon encounter with an antigen, KC can release a variety of reactive oxygen species (superoxide anions, hydrogen peroxide and NO) as well as pro-inflammatory cytokines such as TNF α , IL-1 and IL-6. However, KC have been shown to induce tolerance in models of liver allografts and tolerance to soluble antigens encountered within the circulation (2-4). The implicated tolerogenic mechanisms have included expression of immunoregulatory cytokines/modulators such as IL-10, TGF- β and IDO (indolamine 2,3 dioxygenase), nitric oxide (NO) and Fas (5,6). However, a recent study has also implicated the abundant production of prostaglandins such as PGE₂ and 15-deoxy-delta12, 14-PGJ₂ (15d-PGJ₂), that lead to T cell suppression (3). In addition, the expression of the regulatory costimulatory molecule, B7-H1 (PD-L1) on KC has also been implicated in reducing the inflammation induced in a partial liver warm ischemia/reperfusion model system (7), whereas stimulation via the PD-L1/PD-1 axis can be detrimental in a malignant setting such as human hepatocellular carcinoma (8).

Contribution of dendritic cells towards a tolerogenic liver microenvironment

Multiple subsets of hepatic DC are present within the liver consisting of conventional DC, herein referred to as DC (CD11c⁺ MHC class II⁺ CD11b⁺ or CD8 α ⁺) and pDC (CD11c^{low};B220⁺) (9-12), as well as the controversial NKDC subset that has been noted by some groups (13). The major DC subset is the pDC, which can make up more than 50% of the DC present in this organ. Liver DC are strategically situated around the portal tracts to capture exogenous antigens. Previous studies involving characterization of the entire liver DC populations have shown reduced expression of costimulatory molecules and reduced production of pro-inflammatory cytokines, often in reference to an immature state and resulting in lower allogeneic immunostimulatory properties in mixed lymphocyte reactions compared to their splenic counterparts (11,14). However, detailed analyses of specific subsets have shown there are drastic biological activities within the heterogeneous DC population. Hepatic DC can cross-present antigen to induce activation and proliferation of CD8⁺ T cells in the liver, in a DC-dependent manner, as transient ablation of DC with diphtheria toxin in CD11c-GFP-diphtheria toxin receptor (DTR; (15)) mice dramatically reduced OT-I T cell proliferation (16). One report revealed CD11c⁺CD11b⁺CD8 α ⁻ and CD11c⁺CD11b^{low}CD8 α ⁺ DC had comparable allostimulatory properties and proinflammatory cytokine production similar to their splenic counterparts while the pDC population resulted in minimal T cell proliferation and cytokine production (14). The authors concluded the difference between the liver and spleen is the greater degree of pDC present in the liver and the overall relative paucity of the cDC present, which is reversed in the spleen. Further confirmation was obtained with human liver DC demonstrating lower allo-proliferation and T cell hypo-responsiveness following restimulation and a higher propensity to induce Tregs (17). However, it has also been demonstrated that there are some inherent differences in liver cDC such as the expression of IL-10 and IL-27 compared to splenic DC, which have higher IL-12 production (18). Damage to the liver results in an inflammatory response and chronic inflammation leading to liver fibrosis was dependent on

DC-produced TNF, resulting in increased T cell proliferation and NK cell activation (19). Dependent upon the stimulus, the sterile inflammatory process of liver ischemia/reperfusion injury induced IL-10 production by DC to inhibit the action of CCR2-recruited inflammatory monocytes to the liver, thereby reducing IL-6, TNF and reactive oxygen species (ROS) production and minimizing hepatic injury (20,21). In addition, liver DC displayed decreased expression levels of TLR4 resulting in reduced cytokine expression upon exposure to LPS (22). The reduced expression of TLR4 may be strategically based upon the constant exposure to microbial products that the liver receives. When exposed to high levels of LPS beyond normal physiological levels (≥ 100 ng/ml), the allogeneic C3H/HeJ T cell response was partially restored to the proliferative response of splenic DC and increased the production of Th1 cytokines by T cells (22). However, stimulation of liver DC with anti-CD40 resulted in comparable allogeneic T cell proliferative response as seen with the spleen. Furthermore, the exposure of hepatic DC to the LPS endotoxin induced a “cross-tolerance” effect by attenuating IL-12 production in CpG stimulated DC (23).

The increased frequency of pDC in the liver may also contribute to the tolerogenic microenvironment, as these cells have been shown to play a role in regulating adaptive immunity in the liver (9,11). Although pDC are potent type I IFN producing cells that can initiate T cell responses (24-26), studies analyzing the liver DC subsets in mice and humans have demonstrated that liver pDC are responsible for T cell hypo-responsiveness (14,17). Potential mechanisms for this include the increased production of IL-10 by pDC, an inherent biological preference towards non-Th1 T cell polarizing environment and enhanced proliferation of Tregs (27). In vitro studies of hepatic pDC supplemented with exogenous IL-12 or neutralizing anti-IL-10 antibody improved the ability of Flt3L-expanded hepatic pDC to stimulate T cell proliferation, to levels similar to splenic pDC. Furthermore, the intrinsic biology of hepatic pDCs reveal some functional differences between their splenic and DC counterpart such as a decreased Delta4/Jagged1 Notch ligand ratio further promoting a Th2 type T cell response (27) and a higher expression of the nucleotide-binding oligomerization domain (NOD)2 (28). In mice injected with muramyl dipeptide (MDP), a bacterial peptidoglycan, a selective increase in the expression of the negative TLR-signaling regulator, interferon regulatory factor 4 (IRF-4), and B7-H1 was observed (28). The authors also demonstrated decreased IFN α serum levels upon CpG administration to MDP-treated mice. However, it is also worth noting that hepatic pDC produce less type I IFNs compared to splenic pDCs (28). Further supporting the tolerogenic nature of hepatic pDC, Goubier *et al.* demonstrated liver pDCs mediated oral tolerance to 2,4-dinitrofluorobenzene [DNFB] and ovalbumin (OVA) antigen resulting in CD8⁺ T cell tolerance in a CD4⁺ T cell independent manner, thereby preventing T-cell mediated contact hypersensitivity involved with ear/footpad swelling and rapidly inducing antigen specific T cell anergy or deletion (29). Depletion of pDC using mAbs such as Gr-1 and 120G8 restored the cell-mediated DTH response.

Therapeutic targeting of hepatic DC

As previously indicated, there exists a rather limited number of DC present in the liver. Expansion of hepatic DC has been accomplished with the administration of recombinant protein or vectors expressing granulocyte/macrophage colony stimulating factor (GM-CSF) or Flt3L (30-34). In one report, the systemic administration of an adenovirus expressing GM-CSF into mice resulted in a 400-fold increase in hepatic DC that could reverse the tolerogenic phenotype of hepatic DC as evident by the increased expression of costimulatory molecules, increased antigen processing, increased pro-inflammatory cytokine expression and T cell stimulatory capacity (31). Although GM-CSF can induce the expansion of DC systemically as well as other myeloid cells, administration of Flt3L leads to the expansion of both conventional DC and pDC (35). The combined administration of Flt3L and CpG, a

Toll-like receptor 9 agonist, enhanced the costimulatory expression with higher secretion of IFN α leading to improved activation of NK, NKT and CD8⁺ T cells (31,33). One potential drawback to the administration of Flt3L has been the recently reported dependency for Tregs upon the Flt3-Flt3L signaling axis (36,37). To overcome these drawbacks, combined therapies that target different cellular components will need further examination. One potential option may be regulating glycogen synthase kinase-3 activity in DC through mTOR signaling modulation since this can inhibit DC-mediated Treg conversion (38). On the other hand, rapamycin-conditioned DC have also been found to promote tolerogenicity (39). Thus, a fine balance must be achieved to further enhance the positive effects while minimizing the negative effects of the treatment to gain the desired therapeutic outcome.

Hepatocellular carcinoma (HCC) has been shown to directly interact and alter the function of hepatic DC in vivo as well as bone-marrow derived DC with in vitro co-cultures using tumor culture supernatants. In general, DC remained in an immature state with low levels of co-stimulatory molecule expression, reduced T cell proliferation and generation of Tregs (40,41). Using immunohistochemistry, one study demonstrated the number of DC and the increased presence of CD8⁺ T cells within HCC nodules positively correlated with improved tumor-free survival time following surgical resection (42). Therefore, DC-based therapies may be beneficial in the therapy of HCC, and are currently being examined for use in treatment of a variety of malignancies and diseases (43,44). Methods to improve the antitumor properties of DC include the manipulation of these cells for increased expression of immunostimulatory molecules such as via the adenoviral mediated expression of CD40L on DC (45) or the enhancement of DC-NKT cell interactions by pulsing DC with the glycosphingolipid, alpha-galactosylceramide (46). In both studies, the modified DCs were able to induce protective immunity against the tumor and/or improved survival. Another possibility is to decrease immunoregulatory mediators either secreted by the tumor or the DC themselves to improve response. Tumor derived PGE₂ and TGF- β have been shown to affect the cytokine secretion by TLR7/TLR9-stimulated pDC and migration capabilities; however, cyclooxygenase inhibitors and TGF- β antagonists may improve the stimulatory capacity (47). In addition, the removal of the DC-derived immunosuppressive IL-10 may further improve the immunostimulatory capacity of DC-based therapies (48-50).

Myeloid derived suppressor cells in the liver

MDSC are a heterogeneous population containing myeloid progenitor cells and immature myeloid cells, present in healthy individuals; however, a variety of pathological conditions induces an expansion of this population due to a maturation blockade to a fully differentiated myeloid cell. Although accumulations of MDSC are found within tumors, the increase is also observed in distant peripheral sites such as the spleen, blood and bone marrow. Interestingly, the liver has recently been shown to be a preferred site for the homing and expansion of MDSC (51). The accumulation of MDSC in the liver with tumors originating from the abdominal/gastrointestinal region such as early preinvasive pancreatic neoplasia and advanced colorectal cancers may not be as surprising due to proximity of the tumor to the liver (52); however, the appearance of MDSC in this particular organ accelerated the formation of liver metastasis. This phenomenon is not limited to abdominal/gastrointestinal tumors as the accumulation of MDSC was also observed in subcutaneous tumors of different origins, to levels comparable with the spleen (51). Both migration and increased hematopoiesis within the liver are involved with the expansion, either due, but not limited, to the expression of GM-CSF (53) or the chemokine CXCL1/KC (52), a granulocytic chemoattractant, or stem cell factor (SCF) (54). Trafficking and accumulation of MDSC may also be dependent upon gp130, a common receptor for IL-6 cytokine family members, signaling within hepatocytes through hepatic acute phase proteins such as serum amyloid A, produced in response to infection and inflammation (55). Not only will MDSC

inhibit the function of effector T cells and expand the Treg populations (56), but recent evidence has also shown decreased NK cell cytotoxicity and cytokine production through cell-cell dependent contact mechanisms with the NK receptor, NKp30, in human hepatocellular carcinomas patients (57). Moreover, the expression of membrane bound-TGF β on MDSC, and not Tregs, can also contribute to reduced IFN γ expression, NKG2D and cytotoxicity by NK cells (58). Depletion of MDSC, using Gr-1 depleting ab, was capable of restoring NK cell activity. However, opposing effects were observed in a lymphoma tumor model system (RMA-S), where the MDSC from tumor-bearing mice expressed the NK-cell NKG2D activating receptor, RAE1 (59). Despite the differing effects on NK cells, TGF β knockout mice were still capable of suppressing T cell proliferation *in vitro* in anti-CD3/anti-CD28 and OVA pulsed-D011.10 splenocyte cultures (60). Another mechanism for T cell dysfunction involves crosstalk between MDSC and resident KC for the induced expression of PD-L1 (51).

Therapeutic targeting of MDSC in the liver

Since the liver has recently been demonstrated to be a site for the accumulation of MDSC, therapeutic approaches that directly target/effect MDSC within the liver microenvironment have only recently emerged. The use of antibody-based therapies has proven to be effective for treatment of autoimmune diseases and cancer. As recently demonstrated, the administration of anti-cKit antibody to mice bearing MCA26 colon carcinoma cells in the liver, resulted in a dramatic enhancement in T cell proliferation that was associated with reduced numbers of MDSC and Treg in the bone marrow and spleen and reduced angiogenesis (54). Furthermore, the combination treatment of intra-tumoral injection of a replication defective adenovirus encoding IL-12 combined with agonistic anti-4-1BB and anti-cKit antibodies significantly improved survival to 70% compared to mice that eventually succumb when treated only with Adv.mIL-12 and anti-4-1BB antibody (54). Improved therapeutic responses and survival were achieved by combining the AdV.mIL-12 and anti-4-1BB antibody treatment with administration of sunitinib, a multi-tyrosine kinase inhibitor (30). Another therapeutic option is the modulation of the PD-1/PD-L1 axis. The *in vivo* administration of anti-PD-L1 antibody to mice bearing mammary DA-3 tumors blocked the MDSC-enhanced expression of PD-L1 on KC and slowed tumor growth (51). The modulation of MDSC for the reversal of tolerogenic responses is beneficial not only in a malignancy setting, it can moreover be exploited to reduce liver inflammation and inflammation-related liver damage as well as to achieve the tolerogenic status desired for transplantations (61).

Cancer-related inflammation

Solid tumors of varying etiology and anatomical location are frequently associated with inflammatory cells. Although cell-mediated, cytolytic activities by innate immune cells are critical for the successful eradication of tumors, it has become increasingly evident that certain components of the immune system may actually facilitate tumor initiation and/or progression as well potentially metastatic spread. The tumor-promoting role for cancer-related inflammation has been well reviewed (62-64). Unfortunately, it is becoming increasingly evident that anti-tumor strategies (e.g. vaccination, adoptive T cell transfer, and immunotherapy) will likely fail unless the immunosuppressive tumor microenvironment is overcome. In this section, we focus on the tumor-associated factors which have been shown to increase the frequency and function of key immunoregulatory cells, namely regulatory DC, Tregs, tumor-associated macrophages (TAMs) and MDSC. In this section, we review the contributions of these suppressive cell types to tumor progression and the molecular mechanisms that promote their development, recruitment and/or expansion within the tumor microenvironment. Many of these are similar to those pathways described previously in the

liver. We discuss therapeutic strategies which show promise for the mitigation of the immunosuppressive tumor microenvironment and altering the balance of inflammation in favor of durable anti-tumor responses.

Immunoregulatory dendritic cells within the tumor microenvironment

Tumor infiltrating dendritic cells (DC) have been observed in a variety of human cancers and experimental mouse tumor models (65,66). In general, an increased presence of mature DC, particularly within tertiary lymphoid structures, corresponds to successful therapeutic outcomes (67,68). The infiltration of specific DC subsets may also enhance the overall protective antitumor immune response (69). However, there is increasing evidence that despite the presence of DC within the tumor, stromal elements from the tumor microenvironment, derived either from the tumor or infiltrating cells, can express mediators such as PGE₂ and TGF- β , and convert immunostimulatory DC into regulatory DC. These regulatory cells can express arginase (70,71), have reduced expression of T cell chemoattractants such as CCL19 (72) and induce CD4⁺ T cells to express IL-13 which can contribute to the functional suppression by MDSC (66,73). Ligands expressed on tumor cells, such as bone marrow stromal antigen 2 (CD317) can interact with the immunoglobulin-like transcript 7 receptor on pDC and regulate type I IFN production via a negative feedback mechanism (74). The involvement of regulatory DC in tumor development was confirmed by conditionally ablating DC populations utilizing CD11c-DTR mice which had a significant delay in ovarian tumor growth and enhancement in vascular apoptosis and chemotherapeutic efficacy (75).

Recognizing the powerful capabilities of DC for the induction of more potent anti-tumor responses, a variety of approaches for the expansion and activation of these cells have been evaluated in preclinical and clinical trials. These methods have been extensively reviewed by others (43,44,76). DC can be expanded *ex vivo* with GM-CSF/IL-4 and *in vivo* with either GM-CSF or Flt3L. A concern with GM-CSF/DC-based DC approaches is the potential for undesirable expansion of MDSC, for which a critical role of GM-CSF has been described (77). Thus, a current therapeutic challenge will be the enhancement of DC immunogenicity in such a way that will not deleteriously alter the balance of immunoregulatory mediators within the tumor microenvironment.

Regulatory T cells within the tumor microenvironment

Tregs are a subset of CD4⁺ T cells that directly and indirectly suppress effector T, NK and NK-T cell activation, proliferation and cytokine production (78,79). An increased frequency of Tregs within solid tumors is correlated with poor prognosis (80,81). Tregs have also been shown to be elevated in the peripheral tissues and blood of tumor-bearing hosts (80,81). Tumor-secreted factors, including TGF- β , contribute to Treg accumulation as well as expression of the FoxP3 transcription factor which is important for the survival and function of Tregs (82,83). Tregs subvert host immunity via many mechanisms [Reviewed in (78,79)] and their removal or negation is likely to be a critical component of any successful therapy.

Factors contributing to Treg accumulation within tumors

The accumulation of Tregs within the tumor microenvironment may be the result of proliferation, recruitment or conversion whereby CD4⁺ T cells acquire FoxP3 expression and suppressor phenotype. IL-2 is essential for the development, maintenance, and function of CD4⁺/CD25⁺/FoxP3⁺ Tregs (78,79) and patients receiving systemic IL-2 therapy for the treatment of metastatic renal cell carcinoma had elevated intra-tumoral Tregs (84). In contrast to effector T lymphocytes, Tregs express higher levels of the chemokine receptor, CCR4. The chemokines CCL17/TARC and CCL22/MDC bind to CCR4 and have been

implicated in Treg recruitment in human (80,81,84) and murine (85) tumors. Tumor cells and macrophages within the tumor microenvironment are potential sources of CCL22 (80,81,85). Thus, an anti-inflammatory cascade can be envisioned whereby tumor-associated CCL17 or CCL22 expression recruits Tregs to promote an anti-inflammatory microenvironment. Furthermore, alternatively activated (“M2 phenotype”) macrophages within the tumor microenvironment preferentially produce CCL17 and/or CCL22 (86) to also serve as another important source of Treg-recruiting cytokines. In turn, the Tregs produce cytokines, such as IL-10 and TGF β , which polarize macrophages towards the M2 phenotype and further potentiates CCL17 and CCL22 production. M2 macrophages and MDSC also produce TGF β (87) and TNF α , which have been shown to be critical for the development of highly suppressive populations of FoxP3⁺ Tregs (88). Additionally, MDSC promote the development of functionally suppressive, FoxP3⁺ Tregs through a cell-contact dependent manner (56). It is evident that a progressing tumor profoundly influences its own immune microenvironment such that M2 macrophages predominate, by which the ensuing production of Treg-recruiting factors amplifies the development of an immunosuppressive milieu.

Therapeutic modulation of Tregs in the tumor microenvironment

Various strategies have been used to achieve transient depletion of Tregs and tumor rejection in mice. Unfortunately, the most common approaches involve the use of anti-CD4 or anti-CD25 depleting antibodies (89-95), IL-2 immunotoxins (96,97) and cyclophosphamide (98), which also removes effector T cells. Moreover, these strategies may ultimately fail to achieve durable anti-tumor responses, since tumor-associated Tregs rapidly rebound subsequent to their removal (99), reestablishing an immunosuppressive microenvironment and potentially abrogating any short-term result. An alternate strategy is to target the chemokines that recruit Tregs to the tumor microenvironment. Hoelzinger, et al recently reported that neutralization of the CCL1 chemokine prevented conversion and suppressor function of Tregs (100). The shRNA-mediated blockade of the CCR5 chemokine pathway similarly blocked Treg trafficking to pancreatic tumors and inhibited tumor growth (101). We recently demonstrated that combination therapy consisting of IL-2 and agonistic anti-CD40 antibody removed functionally-suppressive FoxP3⁺ Tregs specifically from the tumor microenvironment through a pathway that coincided with the reduced expression of CCL17 and CCL20 chemokines that recruit Tregs into tumors (102). Interestingly, this same therapy significantly increased Tregs in peripheral tissues, such as the spleen, demonstrating that alterations of Treg populations specifically within the tumor microenvironment best correlated with therapeutic outcome. The mechanism for this selective reduction may be due to reduced Treg recruitment, but it is also known that host Fas expression is a critical component of the successful synergy with IL-2/anti-CD40 combination therapy (103). In this regard, the intriguing observation that induction of Fas expression on Tregs may aid in mediating Treg removal (104) has caused us to investigate whether Fas expression on Tregs is a component of the removal of these cells via Fas ligand expressing T or NK effector cells following IL-2/anti-CD40 therapy. IL-2/anti-CD40 combination therapy also had the added benefit of preventing the recruitment of MDSC into the tumor microenvironment, by also significantly downregulating the chemokines that govern MDSC recruitment to tumors (102). Functional blockade of Tregs has also been achieved through the use of proinflammatory stimuli, such as IL-6 and TLR agonists such as CpG-ODN (100).

Tumor-Associated Macrophages and Myeloid-Derived Suppressor Cells

The degree of macrophage infiltration into tumors has been directly correlated with the extravasation and metastatic potential of tumors (105,106). Although the complex interactions between macrophages and tumor cells are incompletely defined, it is evident

that the macrophage-dependent production of proteases, growth factors and cytokines regulates tumor seeding and the metastatic process. For example, macrophage-derived colony stimulating factor (CSF)-1 was directly implicated in regulating breast cancer metastasis (105). Elevated CSF-1 levels are frequently observed in solid tumor patients and are explicitly linked with the degree of macrophage infiltration into primary tumors and poor prognosis. Although macrophages can also be important effector cells, they are extremely heterogeneous and exquisitely sensitive to discrete alterations in the local cytokine and molecular microenvironment. Tumor-associated macrophages (TAMs) are exposed to a diverse array of tumor-derived signals, such as TGF- β , IL-10, VEGF and macrophage colony stimulating factor (M-CSF) skewing them towards a pro-tumor phenotype. In the presence of these molecules, monocytes differentiate into M2 macrophages which are most closely associated with enhanced TGF- β and IL-10 expression, thereby forming an amplification loop whereby these cells promote the further differentiation of newly-recruited macrophages towards the M2 phenotype. M2 macrophages also produce high levels of IL-1 receptor antagonist (107), which further enables the progressing tumor to subvert host immune responses.

Myeloid-derived suppressor cells (MDSC) represent a further sub-population of heterogeneous macrophages characterized by variable expression of Ly6G, Ly6C and Gr1 antigens but which share immunosuppressive properties (108-111). MDSC promote tumor progression not only by producing many of the same immunosuppressive cytokines as TAMs, but through a number of novel mechanisms as well. MDSC can suppress T cell activation by a diverse array of mechanisms including the production of arginase, nitric oxide and reactive oxygen species (73,108,112-114), nitration of the T cell receptor (115,116), cysteine deprivation (117), interfering with T cell trafficking (118) and the induction of Tregs (119) and T cell tolerance (111,116).

Factors contributing to TAM/MDSC accumulation in tumors

The chemokine-mediated recruitment of macrophage subsets is also subject to the variable expression of certain chemokine receptors on the cell surface. The chemokine monocyte chemoattractant protein (MCP)-1 has been strongly associated with the recruitment of M2 macrophages that facilitate tumor development (120,121). In contrast, chemokines whose expression are regulated by interferon gamma (IFN γ), such as CXCL9/Mig, CXCL10/IP-10 and CCL5/RANTES, are more closely associated with classically activated ("M1") macrophages which play important roles in anti-tumor responses (120,121). Consistently, the expression of these Th1/M1 chemokines among leukocytes from patient tumors is associated with improved prognosis (122-125). Among TAMs, MDSC represent an important component due to their potent immunoregulatory abilities. The chemokines CXCL5/ENA-78 and CXCL12/SDF-1 have been shown to mediate the recruitment of MDSC into solid tumors (87).

MDSC accumulate in most cancer patients and experimental animals with cancer (108,109), where they can limit the efficacy of host and therapy-mediated anti-tumor responses. Indeed, the direct correlation between tumor burden and frequency of MDSC strongly supports the conclusion that tumor-derived factors may promote MDSC accumulation. Corzo, et al recently showed the hypoxic environment established within the tumor microenvironment, acting via the hypoxia-responsive transcription factor, HIF-1 α is critical for the development of functionally-suppressive MDSC (126). The tumor-associated cytokine, GM-CSF, also supports the generation of CD11b⁺Ly6G⁻Ly6C⁺ suppressor subsets capable of inhibiting T cell proliferation and anti-tumor function (77). As previously mentioned, since GM-CSF is commonly used for *ex vivo* expansion of dendritic cells in cell-based immunotherapies, the adverse side-effect of MDSC expansion indicates that GM-CSF based therapies should be

carefully evaluated. Tumor-derived GM-CSF also appears capable of regulating MDSC suppressor function, in addition to the recruitment of these cells. Dolcetti and colleagues recently showed that GM-CSF, but not G-CSF, induced the preferential expansion of CD11b⁺/Gr1^{int} and CD11b⁺/Gr1^{Lo} subsets of MDSC that were potent suppressors of CD8⁺ T cell activation (53). Tumors thus reorient the differentiation of myeloid cells into M2 macrophages or MDSC that express increased levels of VEGF, IL-10 and COX-2. Increased COX-2 and PGE₂ expression are also frequently over-expressed in the tumor microenvironment (127), functionally reducing antigen-presentation and Th1 cytokine production (128,129). PGE₂ further contributes to immune suppression by upregulating Th2 cytokine production, FoxP3 expression in Tregs (130) and arginase expression in myeloid cells (131). PGE₂ has been implicated in MDSC recruitment by acting directly on cell surface receptors of MDSC (132) and Fas-dependent accumulation of MDSC (133). PGE₂ and other factors contained within tumor exosomes can also be secreted by the tumor and taken up by bone marrow myeloid cells, where they may also contribute to MDSC accumulation by switching the development of these cells towards the MDSC pathway (134). Thus tumor-associated accumulation of PGE₂ is an important component of the re-orientation of tumor-associated macrophages towards arginase-expressing M2 and MDSC populations which promote tumor development. MDSC accumulation within tumors can also be caused by pro-inflammatory cytokines such as IL-1 β , IL-6 (135-137) and S100 proteins (138,139), underscoring the complex mechanisms whereby inflammation can promote subversion of the host immune system and tumor progression. An improved understanding of the factors which contribute to MDSC accumulation within tumors will hopefully lead to the development of improved strategies for mitigating this process.

Therapeutic Targeting of TAMs and MDSC

Since macrophages play critical roles in regulating the growth and metastatic potential of tumors, their therapeutic removal holds promise for the treatment of metastatic disease. Qian et al, showed that macrophage ablation, through a number of different genetic and biochemical means, blocks tumor cell seeding of the lungs, inhibits tumor progression and reduces the rate of metastasis (106). Although TAMs are critical components of an immunosuppressive tumor microenvironment, these cells, like all macrophages, retain a considerable degree of functional plasticity that is dependent upon their molecular microenvironment. Cytokines, such as IL-12, for example, have shown great potential for rapidly altering TAM function to a pro-immunogenic profile that is characterized by increased levels of TNF α , IL-6, IL-15 and IL-18 expression accompanied by reduced or abrogated TGF- β and IL-10 expression (140,141). IL-12 also reverses the pro-angiogenic and pro-metastatic properties of TAMs and elicits more potent cell-mediated immune responses against tumors. In our own studies, we have shown in a mouse model of metastatic renal cell carcinoma that the combination therapy of IL-2 and agonistic anti-CD40 antibody potently induces host IL-12 expression and IL-12-dependent anti-tumor responses (103), which is accompanied by the reorientation of TAMs towards an anti-tumor, M1 phenotype associated with reduced arginase expression and increased production of TNF α , IL-6 and anti-angiogenic chemokines (102). Another combination therapy involving the use of the Toll-like receptor 9 ligand, CpG, along with anti-IL-10 receptor antibody therapy could similarly reorient tumor-infiltrating M2 macrophages into M1 cells that could help mediate tumor rejection (49). Recently, the combination of anti-CD40 and CpG ODN immunotherapy with cytotoxic chemotherapy also resulted in synergistic anti-tumor effects in C57BL/6 mice bearing established B16 melanoma or 9464D neuroblastoma accompanied by the repolarization of TAMs towards the M1 effector phenotype (142). These studies highlight the potential for dramatic, synergistic anti-tumor responses achieved by combinations of immunotherapeutic agents but not by either agent alone.

Similarly, MDSC have been depleted using antibodies which recognize the Gr1 antigen (143). It is apparent, however, that such strategies are not selective for MDSC, since neutrophils, eosinophils and pDC also have variable yet constitutive expression of Gr1 and MDSC eventually rebound. Nevertheless, Gr1 depletion studies have demonstrated the potential for improved anti-tumor responses [(143) and our unpublished observations using orthotopically implanted Renca tumors]. We now review the more targeted approaches which involve the inhibition of factors essential for MDSC development, recruitment and/or function.

Targeting MDSC development

It is hopeful that as the list of factors which promote the development of MDSC expands, this will result in the availability of new therapeutic targets for redirecting the differentiation of these cells into more mature myeloid cells which lack immunosuppressive properties. One promising pathway is the blockade of receptor tyrosine kinases, such as SCF/c-kit ligand. SCF plays an important role in the regulation of hematopoiesis in the bone marrow. SCF is expressed by many human and murine tumors and its blockade inhibited MDSC development, Treg development and tumor-specific T cell anergy (54,144). Interestingly this blockade also prevented tumor angiogenesis, underscoring the potential role for MDSC in blood vessel formation within the tumor. More recently, the receptor tyrosine kinase inhibitor Sunitinib (Sutent), similarly prevented MDSC accumulation in tumor-bearing mice (30,145) and renal cell carcinoma patients (146). Underscoring the complex relationship between MDSC and Tregs, both SCF blockade (144) and Sutent (30,147) also reduced Treg development and their associated production of IL-10 and TGF- β . Sutent and other receptor tyrosine kinase inhibitors thus can be used, potentially in combination with additional immunotherapies, for the reversal of immune suppression within the tumor microenvironment and promotion of cell-mediated immune responses. Another approach for promoting the differentiation of MDSC into mature granulocytes is all-*trans*-retinoic acid (ATRA), a derivative of vitamin A which promotes the differentiation of myeloid progenitor cells into mature dendritic cells and macrophages (148). Administration of ATRA into sarcoma-bearing mice induced the differentiation of MDSC into mature myeloid DCs capable of presenting antigen and inducing effector T cell responses (149). The treatment of MDSC isolated from renal cell carcinoma patients with ATRA also promoted the *ex vivo* differentiation of these cells into fully competent antigen-presenting cells (148). These findings demonstrate that MDSC-mediated immune suppression is reversible.

Other promising approaches for the therapeutic targeting of MDSC development are anti-inflammatory therapies, since pro-inflammatory cytokines such as IL-1 β and IL-6 are frequently present in the tumor microenvironment and promote MDSC accumulation (119,136,137). The reduction of inflammation through the use of the naturally occurring IL-1 receptor antagonist, IL-1 receptor blockade (136), or PGE₂ blockade (132,133) can reverse MDSC development and accumulation. The involvement of IL-6 and other cytokines in MDSC development has underscored the role for common signaling by downstream transcription factors (e.g. STAT family). Stat3 is constitutively active in MDSC and a key regulator of MDSC development and function, by mediating the upregulation of anti-apoptotic, proliferative, and pro-angiogenic molecules (150-152). Stat3 inhibition, either through the use of small molecule inhibitors (153), blocking peptides, peptidomimetics or platinum complexes (154) could be of therapeutic benefit, provided the biologic requirement for Stat3 signaling in a diverse array of normal biologic pathways is not adversely affected. The removal of MDSC following Sutent therapy (30,146) may also be related to its ability to abrogate Stat3 signaling. The involvement of S100 inflammatory proteins (138,139), not only in the accumulation of MDSC, but also via autocrine production by MDSC and tumor cells, are also attractive candidates for therapy. Blocking antibodies

against these proteins and their carboxylated glycan ligands reduce MDSC levels in tumors (139) and have been noted for anti-tumor efficacy in murine oncogenesis (155).

Targeting MDSC accumulation

Therapeutic manipulation of MDSC recruitment is another strategy for the mitigation of MDSC-mediated immunosuppression within the tumor microenvironment. Recruitment of MDSC is principally mediated by two chemokine axes: CXCL5/ENA-78 binding to the CXCR2 receptor or CXCL12/SDF-1 binding to the CXCR4 receptor (87). These chemokines are produced by M2 macrophages and tumor cells themselves, thereby achieving a high level within the tumor microenvironment serving to recruit MDSC and further amplify this process. The negation of specific chemokine axes is attractive for several reasons. First, it tends to elicit the more selective targeting of MDSC cells while avoiding substantial impact on T effector cells and other leukocytes (121). Second, the therapeutic modulation of chemokine profiles has potential for the rapid amplification of more desirable M1 macrophage populations. We and others have shown, for example, immunotherapeutic regimens which elicit strong levels of Th1 cytokines such as IL-12 and IFN γ , dramatically restructure the chemokine and myeloid composition of the tumor microenvironment so that the IFN γ -dependent chemokines (RANTES, MIG, IP-10, and MIP-1 γ) and M1 phenotype of macrophages predominate concomitant with the reversal of MDSC frequency and function (102,141). Consistently, the expression of these Th1 chemokines is associated with favorable prognosis in patients with metastatic RCC (122,123). Interestingly, our work showed the combination of IL-2 and agonistic anti-CD40, each shown as separate agents to be important for the promotion of Treg and MDSC development, respectively (156), achieve the surprising ability for selectively removing both suppressor cell types from the tumor microenvironment (102). Since the transient, local depletion of Tregs (104) and MDSC (157) can occur via the Fas pathway, it will be very interesting to evaluate whether the dependence of IL-2/anti-CD40 therapy on host Fas expression (103) is directly related to Fas-mediated loss of these suppressor cell populations following combination therapy. The anti-cancer drug trabectedin was also shown to be capable of inhibiting the expression of tumor-promoting chemokines, macrophage recruitment and tumor-associated vascularization (158). Combination immunotherapy in the form of CCL16 chemokine administration plus the injection of CpG and anti-IL10 receptor antibody similarly polarized tumor-infiltrating myeloid populations from M2 into M1 which paralleled innate and adaptive immune cell-mediated anti-tumor responses (49). An added benefit of these approaches is that reorientation of TAMs towards the M1 phenotype helps remove potential sources of Treg-recruiting chemokines (CCL17 and CCL20), which predominately originate from M2-polarized macrophages (102,159).

Several chemotherapeutic drugs have shown promise for removing MDSC populations. Docetaxel was reported recently to inhibit MDSC accumulation in 4T1-Neu mammary tumor-bearing mice (160). Interestingly, docetaxel treatment preferentially targeted M2/ mannose receptor positive MDSC while sparing M1 macrophages, further supporting investigation of docetaxel in combination with other immunotherapeutic strategies. Gemcitabine also removes MDSC (161,162) through the selective induction of apoptosis in these cells (163). Gemcitabine has been effectively used either as a single agent or in combination with cisplatin, paclitaxel or anti-inflammatory agents in numerous clinical trials (164,165) and is considered among the primary treatment options for the treatment of non-small cell lung cancer.

Targeting MDSC function

Although MDSC induce T cell tolerance and mediate immunosuppression via a multitude of molecular mechanisms, considerable efforts have shown promise for interfering with MDSC suppressor activity. One of the principal targets of these approaches is the removal of arginase or nitric oxide synthase (NOS) 2, which comprise critical components of MDSC immunosuppressive activity (73,110,114,166,167). Nitroaspirin is a classic aspirin molecule covalently linked to a NO donor group currently under evaluation in phase I/II clinical trials. Orally administered nitroaspirin inhibited the enzymatic activities of MDSC, normalized the immune status of tumor-bearing mice and functioned as an effective adjuvant for cancer vaccination (168). The principal mechanism whereby NO-aspirin achieves these effects is through the feedback inhibition of NOS and arginase expression and activity. NO-aspirin also inhibited protein nitration, within the tumor microenvironment, thus inhibiting antigen binding to the TCR (115,116). The inhibition of either COX-2 or PGE₂ can also reverse MDSC-mediated suppression, since these enzymes are important for tumor promotion via a number of different mechanisms, arginase expression and MDSC suppressor function (131-133). Other anti-inflammatory agents, such as IL-1 receptor antagonist or triterpenoid compounds have been shown to reduce MDSC levels and function, in part via the reduction of peroxynitrite and reactive oxygen species generation (136,162). Recently, phosphodiesterase-5 (PDE5) inhibitors were shown to augment antitumor immune responses by interfering with the arginase and NOS-dependent suppressor machinery of MDSC (112). Treatment of tumor-bearing mice with the PDE5 inhibitor sildenafil, in particular, downregulated arginase and NOS2 expression in MDSC isolated from different organs and led to the dramatic restoration of effector CD4⁺ and CD8⁺ T cells. The use of other selective arginase or NOS inhibitors, namely Nor-NOHA and 1-NMMA respectively, similarly enhanced effector T cell responses. PDE5 inhibitors are currently in clinical use for nonmalignant conditions, such as erectile dysfunction, cardiac hypertrophy and pulmonary hypertension. Recent demonstration of their anti-tumor potential (112) further supports investigation for their applicability as cancer therapeutics.

Despite the critical role that NOS2 expression plays in MDSC suppressor function, it is also evident that NO can also be a key component of anti-tumor pathways. The dual nature of NO is related, in part, to the local concentration of NO. Low concentrations of NO promote HIF-1 α and/or MAP kinase-mediated tumor growth (169). In this regard, the NO-mediated upregulation of HIF-1 α may contribute to MDSC expansion (126). In contrast, high steady-state concentrations of NO result in P53 phosphorylation and the associated tumor cell apoptosis, cell cycle delay and DNA repair (169,170). High NO levels also impair the activity of matrix metalloproteinases (MMPs), which regulate matrix remodeling and the metastatic process (171,172). We showed recently that combination immunotherapy consisting of IL-2 and agonistic anti-CD40 antibody induced sufficiently high levels of macrophage-dependent NOS2 expression within the tumor microenvironment such that M1 macrophage responses predominated and tumor metastasis was inhibited (173). IL-2/anti-CD40 potently induced the expression of IL-12, a key regulatory cytokine with the potential for skewing macrophages towards an M1 phenotype (140,141). The tumor-targeted delivery of a nitric oxide donor, JS-K, also significantly inhibited tumor metastases by itself or in combination with IL-2 or anti-CD40 (173). Although NOS inhibition during immunotherapy abrogated the anti-metastatic effects of IL-2/anti-CD40 therapy, divergent effects on primary tumor burden were identified. Whereas NOS2 (iNOS) deficiency had no impact upon primary tumor size, the inhibition of multiple NOS isoforms (via L-NAME in drinking water) resulted in significantly reduced primary tumors. Thus, other NOS isoforms, perhaps derived from tumor-associated vasculature, might be more central to the control of primary tumor growth. These data point to critical roles for various NOS isoforms in the regulation of primary tumor growth and tumor metastasis following combination immunotherapy.

Moreover, these findings demonstrate that macrophages and macrophage-dependent NO production can be appropriately manipulated for treatment of metastatic disease.

Conclusions

Myeloid cells are critical to the establishment of a tolerogenic liver microenvironment as well as the progression and metastatic potential of many solid tumors. It is also clear, however, that heterogeneous macrophage and dendritic cell populations are exquisitely sensitive to alterations in their microenvironment and thus amenable to the immunotherapeutic-mediated alteration in their phenotypes. Among DC populations, pDC also represent highly plastic cell types which comprise one of the major DC subtypes in the liver. Tumor-derived factors such as VEGF, TGF- β , IL-10 and PGE₂, help polarize macrophage and DC responses towards those which favor tumor progression. In contrast, pro-inflammatory cytokines, particularly IL-12, have demonstrated considerable potential for the reorientation of macrophages, in peripheral organs as well as within the tumor microenvironment, towards a more desirable phenotype which can support durable anti-tumor responses.

Many tumor-derived molecules are also critical for the development and function of MDSC, a highly suppressive population of immature myeloid cells. MDSC contribute to immune tolerance and establishment of a suppressive tumor microenvironment. A central reason for limited success in generating potent anti-tumor responses using vaccine and adoptive cell strategies is the failure of these approaches to overcome the suppressive tumor microenvironment. In reviewing the factors which contribute to the development, recruitment and/or function of Tregs, M2 macrophages and MDSC, it emerges that these suppressor cells frequently use overlapping and shared molecular pathways during both the normal immune “shaping” of the tolerogenic liver microenvironment as well as during the progression of tumors. Targeting these points of convergence may thus hold promise for the reorientation of macrophages and the concomitant removal of Tregs. Liver-associated or tumor-derived PGE₂ and TGF- β , for example, each contribute to the development and accumulation of Tregs and MDSC in their respective locations. Chemokine pathways also represent a particularly attractive therapeutic target in that they function to recruit and activate certain leukocyte populations, and also contribute to the rapid amplification of the recruitment of suppressor cell populations. For example, agents which help polarize macrophages towards an M1 phenotype have the added benefit of reducing the production by M2 macrophages of Treg-recruiting chemokines. Several drugs have also been highlighted in this review for their ability to regulate Treg and/or MDSC accumulation or function. These approaches hold considerable promise for the removal of suppressive cell populations in both the liver and tumor microenvironments, for the enhancement of anti-tumor responses in these compartments. Conversely, the use of factors which promote MDSC development, recruitment and/or function should be efficacious for the control of undesirable immune responses, as in the case of liver transplantation or liver inflammatory diseases. The therapeutic efficacy of these molecules may be greatest when they are used in combination with other drugs or immunotherapeutic agents. Indeed, in our studies and those of many others, the combination of two or more immunotherapeutic agents has shown great potential for generating dramatic and synergistic anti-tumor responses, often not recapitulated using the components as mono-agent therapy. Among pro-inflammatory cytokines, IL-12 and IL-12-based therapies demonstrate the marked potential for inducing M1 macrophage polarization, enhanced DC effector functions and an overall shift away from the suppressive features of M2 macrophages and tolerogenic DC populations in both the liver and tumor microenvironments.

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