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Role Of Immature Myeloid Cells in Mechanisms of Immune Evasion In Cancer¹

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Abstract

Tumor affects myelopoiesis by inhibiting the process of differentiation/maturation of antigen-presenting cells from their myeloid precursors and by stimulating an accumulation of immature myeloid cells in cancer patients and tumor-bearing mice. These immature myeloid cells can contribute greatly to tumor progression and promote tumor evasion from immune attack: i) by inhibiting development of adaptive immune responses against tumor in lymphoid organs; ii) by migrating into tumor site and differentiating there into highly immune suppressive tumor-associated macrophages. Immature myeloid cells and tumor-associated macrophages utilize different JAK/STAT signaling pathways and different mechanisms to control T cell responses, which include increased production of TGF-beta, reactive oxygen species, peroxynitrites, as well as enhanced L-arginine metabolism. Understanding of precise mechanisms, which tumors use to affect differentiation of APC from myeloid cell precursors and inhibit T cell responses, could help to develop new approaches for cancer therapy and substantially improve efficiency of existing cancer vaccination strategies.

Phenomenon of expansion of immature myeloid cells in tumor hosts

Recent data from a number of groups have demonstrated that myeloid cells accumulating in tumor-bearing hosts play an important role in tumor non-responsiveness by suppressing antigen-specific T cell responses [1;11;17;26;33;39]. These cells contribute to the failure of immune therapy in patients with advanced cancer and in tumor-bearing mice. In mice, these myeloid cells are characterized as Gr-1⁺CD11b⁺ cells. Myeloid lineage differentiation antigen Gr-1 (Ly6G) is expressed on myeloid precursor cells, granulocytes, and transiently on monocytes [22]. CD11b receptor (Mac-1) is α_M integrin that is expressed on the surface of monocytes/macrophages, dendritic cells (DC), granulocytes, and activated B- and T-lymphocytes. Gr-1⁺CD11b⁺ cells represent about 20–30% of normal bone marrow cells and only 2–4% of all nucleated normal splenocytes. Inoculation of transplantable tumor cells [10;20;26;53] or spontaneous development of tumors in transgenic mice with tissue-restricted expression of oncogenes [33] results in marked systemic expansion of these cells. The proportion of this myeloid cell population in spleen of tumor-bearing mice may reach up to 50% of all splenocytes [25]. Less impressive but significant transient increase of the Gr-1⁺CD11b⁺ cells was also demonstrated in normal mice after immunization with different antigens [13;14;25] or in mice with bacterial and parasitic infections [3;34].

Only a minor proportion of Gr-1⁺CD11b⁺ splenocytes (less than 4%) in tumor-bearing mice express markers of mature antigen-presenting cells such as MHC class II, macrophage marker

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F4/80, or DC marker CD11c [25]. Morphological analysis demonstrated that these cells are comprised of a mixture of myeloid cells such as granulocytes and monocyte-macrophages as well as myeloid cell precursors at various stages of differentiation (unpublished data, SK and DG). In the presence of appropriate growth factors and/or cytokines Gr-1⁺ cells from tumor-bearing host could be differentiated *in vitro* into DC or macrophages [8;25;30]. Since Gr-1⁺CD11b⁺ cell population displays features of undifferentiated myeloid cells and contains precursors of different myeloid cell subsets, these cells have been termed as **immature myeloid cells (iMC)**.

In cancer patients, iMC are defined as cells that express the common myeloid marker CD33 but lack expression of markers of mature myeloid and lymphoid cells and the MHC class II molecule HLA-DR [1]. An accumulation of iMC was associated with the decreased number of DCs in the peripheral blood of patients with head and neck, lung or breast cancer [2]. Advanced-stage cancer was found to promote the accumulation of these cells in the peripheral blood, whereas surgical resection of the tumor decreased the number of iMC. A similar effect of tumor resection was observed in mouse tumor models [46].

iMC as precursors of dendritic cells, macrophages and endothelial cells. Two pathways of iMC differentiation in cancer

In the presence of appropriate growth factors or cytokines Gr-1⁺ myeloid cells derived from tumor-bearing mice can differentiate *in vitro* into CD11c⁺MHC class II⁺ and CD11c⁺CD86⁺ DCs. To evaluate *in vivo* differentiation of Gr-1⁺ cells, an adoptive transfer model to congenic recipients has been used [25]. After adoptive transfer of iMC into naïve congenic mice almost all iMC obtained from control tumor-free mice differentiated into mature CD11c⁺MHC class II⁺ DC and Gr-1⁺F4/80⁺ macrophages within 5 days. In contrast, a substantial proportion of iMC derived from tumor-bearing mice retained the phenotype of immature cells (Gr-1⁺CD11b⁺) and differentiation of macrophages was significantly inhibited. Thus, iMC from tumor-free and tumor-bearing mice significantly differ in their ability to differentiate in a tumor-free environment. When Gr-1⁺ cells from tumor-bearing mice were transferred into tumor-bearing recipients, very few DCs of the donor's origin were found in spleens of recipients [25]. These data were consistent with previously published observations that tumor-derived factors inhibit differentiation of DC from hematopoietic progenitor cells [18;19;35].

Tumor associated macrophages (TAM) are a major inflammatory cell component of tumors [4], and known as promoters of tumor progression and metastases [32;41]. TAM also show potent immunosuppressive features [24;45]. Since iMC derived from tumor-bearing mice could differentiate *in vitro* toward macrophages [25;30] it is plausible that they may represent a source of tumor-associated macrophages (TAM). To investigate this possibility we conducted experiments with adoptive transfer of Gr-1⁺ iMCs into tumor-bearing congenic mice [24]. Recipient's tumors were analyzed 3 days after iMC cell transfer for the presence of donor's cells using multicolor flow cytometry. More than 70 % of donor's cells isolated from the tumor site were positive for F4/80 marker. At the same time, a significant portion of those cells also retained an immature phenotype (Gr-1⁺CD11b⁺) [24]. These results suggest that splenic Gr-1⁺ iMC could be precursors of F4/80 TAM.

Several recent publications pointed to iMCs as a source of endothelial cells and their direct role in tumor vasculogenesis. Yang and co-workers [59] have demonstrated that Gr-1⁺CD11b⁺ cells could be incorporated into the vascular endothelium, promoting tumor vascularization and tumor progression. Furthermore, Gr-1⁺CD11b⁺ cells derived from tumor-bearing mice produced high levels of metalloproteinase 9 (MMP-9), which is involved in regulation of angiogenesis. Authors suggested that MMP-9 produced by those immature cells regulates bioavailability of VEGF in tumors and promotes tumor angiogenesis and vascular

stability. Selective deletion of MMP-9 in Gr-1⁺CD11b⁺ cells eliminated their ability to promote tumor growth, and led to inhibition of tumor formation. Authors observed that Gr-1⁺CD11b⁺ cells constituted about 5% of the total cells in tumor tissues and could represent a significant source of endothelial cells inside the tumor. A recent study of M.R. Young [60] demonstrated that tumors could skew differentiation of CD34⁺ progenitor cells into endothelial cells. CD34⁺ cells cultured in the presence of LLC tumor conditioned medium under conditions that support myeloid lineage cells skewed the differentiation of these precursor cells toward endothelial cells expressing CD31 and CD144. *In vitro* differentiation of CD34⁺ cells into endothelial cells was dependent on angiopoietin-1 in the tumor-conditioned medium. Adoptive transfer of LacZ⁺ CD34⁺ cells into tumor-bearing mice resulted in accumulation of LacZ⁺ cells within tumor mass. Differentiation of CD34⁺ cells into endothelial cells was confirmed by co-expression of CD31 and CD144 by donor's LacZ⁺ cells [60]. iMC may contribute to tumor growth directly by differentiating toward endothelial cells and by producing pro-angiogenic factors.

It is clear that iMC could differentiate in both lymphoid organs as well as inside tumor bed. In lymphoid organs these cells differentiate predominantly into antigen-presenting cells including dendritic cells and macrophages, whereas in a tumor microenvironment iMC become tumor-associated macrophages and/or endothelial cells.

Role of reactive oxygen species in regulation of myeloid cell differentiation

iMC derived from tumor-bearing mice as well as from cancer patients produce high levels of reactive oxygen species (ROS) [25;27;38;48]. In tumor-bearing mice Gr-1⁺CD11b⁺ cells were found to be a major producer of ROS [25]. Since the pool of ROS may include different types of molecules, ranging from singlet oxygen to hydrogen peroxide, we examined the type of ROS produced by iMC derived from tumor-bearing host. Gr-1⁺ cells were isolated from tumor-bearing mice, and different oxygen species were neutralized using specific inhibitors or scavengers. Catalase reduced ROS levels in iMCs >4-fold, indicating that H₂O₂ contributed greatly to the overall level of ROS in these cells. Increased levels of ROS also interfered with differentiation of myeloid cells in cancer [25]. Neutralization of H₂O₂ with catalase inhibited GM-CSF-induced proliferation of iMC and stimulated their differentiation. After a 4-day culture of iMC from tumor-bearing mice in presence of GM-CSF and catalase the proportion of undifferentiated Gr-1⁺CD11b⁺F4/80⁻ iMC decreased more than 3-fold, and the proportion of F4/80⁺ macrophages increased more than 3-fold [25]. These results suggest that a high level of hydrogen peroxide production by myeloid cells maintains its highly proliferative and undifferentiated status, whereas scavenging of ROS could diminish proliferation and stimulate differentiation of iMC.

How can tumor activate ROS in myeloid cells? A number of cytokines and growth factors may induce ROS production. Hyper-production of some of these factors by tumor cells may result in constant stimulation of ROS in myeloid cells, which may prevent their effective differentiation. ROS regulate transcription of many genes via their effect on several transcription factors including NF-κB, AP-1, c-myc, SP-1 and others [47;49]. Hyper-production of ROS may alter the balance of expression of different genes, which may affect differentiation of myeloid cells. These observations suggest that accumulation of iMC in tumor-bearing hosts is in part caused by the inability of these cells to differentiate into mature myeloid cells. Increased production of ROS, specifically hydrogen peroxide, induced by tumor-derived factors, may be responsible for this phenomenon.

Expansion of iMC in tumor-bearing hosts is associated with impaired T cell responses

Dramatic expansion of iMC in tumor-bearing hosts raises the question about their role in cancer. The accumulation of Gr-1⁺CD11b⁺ myeloid cells often is associated with a large tumor burden and a state of immune suppression [8;20;26;33;53]. Several groups have demonstrated this phenomenon is associated with T cell dysfunction. It was demonstrated that iMC derived from tumor bearing mice can: i) induce loss or significant decrease of the expression of the T cell receptor ζ chain (CD3 ζ), which is the principal part of TCR complex [38]; (ii) inhibit CD3/CD28-induced T cell activation/proliferation by production of reactive nitrogen and oxygen intermediates [26]; (iii) inhibit interferon- γ (IFN- γ) production by CD8⁺ T cells in response to the specific peptide presented by MHC class I molecules [20]; (iv) prevent development of CTL *in vitro* [12;31].

Recently Liu and colleagues [31] demonstrated an inverse correlation between the presence of myeloid immune suppressive cells in spleen and tumor progression. Tumor regression of transplantable immunogenic lymphoma has been associated with strong CTL response and low numbers of immune suppressive myeloid cells in spleen. At the same time, tumor progression has been associated with loss of CTL activity and domination of the immune suppressive myeloid cell population. *In vitro* studies demonstrated that CTL response against tumor could be restored after depletion of myeloid immune suppressive cell population [31].

iMCs obtained from cancer patients also inhibit CD8 T cells response [1]. An addition of iMC isolated from peripheral blood of HLA-A2-positive cancer patients inhibited production of IFN- γ by CD8⁺ T cells re-stimulated with specific peptide-pulsed DCs [1]. Thus, iMC isolated from cancer patients and tumor-bearing mice were able to inhibit CD8⁺ T cell responses in an antigen-specific manner. Since cancer progression is associated with increased production of iMC, this could be one of the mechanisms by which a growing tumor may induce an antigen specific CD8⁺ T cell unresponsiveness.

Mechanisms of immune suppression mediated by iMC

Several potential mechanisms for tumor-induced immune suppression mediated by iMC have been described. iMC cells have been linked to the induction of T-cell dysfunction in cancer through the production of *TGF- β* [5;54;63], *reactive oxygen species* [27;38;48], *L-arginine metabolism* [9;12;26;31;40;42], [Zea, 2005 #4276; 63; 64] and *peroxynitrites* [12;16;26].

TGF- β

Early studies of Young and colleagues [63] have demonstrated that myeloid progenitor cells derived from tumor-bearing mice produced increased amounts of TGF- β . These iMCs were immune suppressive and inhibited *in vitro* T cell proliferation, induced by anti-CD3 antibodies. Nitric oxide (NO), IL-10, and prostaglandin E₂ (PGE₂) also were among the factors produced by iMCs. However, it was TGF- β and NO that mediated the suppression of T cell proliferation by iMC. Beck and colleagues [5] suggested that iMC derived from a tumor-bearing host acquire immune suppressive features and prevent CTL response after contact with TGF- β present in blood serum. Recent work from Terabe and colleagues [54] described a pathway that might negatively regulate tumor immunity through Gr-1⁺ iMC produced TGF- β . These cells were found to be a major source of TGF- β in tumor-bearing mice. Authors proposed that tumor inoculation in mice induced IL-13 production by CD4⁺ CD1d-restricted T cells. iMC express IL-13 receptor, which is required for iMC to produce TGF- β that inhibits CTL induction.

L-Arginine metabolism

It appears that arginine metabolism in myeloid cells can be linked to tumor-associated T cell dysfunction. L-Arginine serves as a substrate for two enzymes: nitric oxide synthase, which generate NO and citrulline, and arginase, which converts L-Arg into urea and L-ornithine. Recent publications of Ochoa's group suggested a close correlation between the availability of arginine and regulation of T cell proliferation [43;44]. They demonstrated that increased activity of arginase I in myeloid cells lead to enhanced L-arginine catabolism. The shortage of the non-essential amino acid L-arginine regulates T-cell function through the modulation of CD3 ζ expression [43]. Tumor growth is associated with up-regulated expression and increased activity of arginase I in splenic myeloid cells [12;31;42], and especially, in tumor associated-macrophages (TAM) that are particularly effective in inhibition of T cell response including CTL and antigen-induced T cell proliferation [24]. This mechanism of T cell inhibition that involves both arginase and iNOS activation has recently described for a tumor microenvironment [9;24]. Human prostate cancer [9] and various murine tumors [24] have been shown to employ this mechanism to avoid T cell attack. As shown, in murine tumor model, T cell deletion in tumor site could be mediated by tumor-associated macrophages (TAM) [24;45]. This effect was dependent on STAT1 signaling, which controls iNOS and arginase I activity in TAM [24].

Reactive oxygen species (ROS)

As described above myeloid cells in tumor hosts produce high levels of reactive oxygen species. Oxidative stress, caused by iMC derived from tumor-bearing mice, inhibited ζ -chain expression in T cells and inhibited antigen-induced cell proliferation [38]. Recent studies demonstrated that iMC freshly isolated from tumor-bearing mice but not their control counterparts were able to inhibit antigen-specific response of CD8⁺ T cells [27]. iMC did not produce nitric oxide, however, iMC obtained from tumor-bearing mice had significantly higher levels of ROS than iMC isolated from tumor-free animals. Since ROS production by iMC can be blocked by arginase inhibitors, it appears that arginase I activity played an important role in ROS accumulation in these cell [27]. This suggests that arginase could be involved in mechanisms of T cell inhibition through generation of ROS, and also may be linked with the role of arginase I in T cell deletion observed in tumor site (see previous section on role of L-arginine metabolism in immune suppression). What could be the potential mechanism of the link between arginase I activity and ROS production? Arginase catalyzes the hydrolysis of L-arginine to urea and L-ornithine. L-arginine is used by NO synthase as a substrate for generation of NO [58]. However, low concentrations of L-arginine results in low NO formation and high generation of superoxide ($O_2^{\cdot-}$) (rev. in [6]. Thus, it is possible that high arginase activity in tumor-bearing mice-derived ImCs may have lowered the level of L-arginine and resulted in increased production of $O_2^{\cdot-}$ instead of NO. Superoxide itself is very unstable and is converted to H_2O_2 and oxygen. This is consistent with our data showing that in ImC ROS accumulates primarily in form of H_2O_2 , but not $O_2^{\cdot-}$ [27].

Inhibition of ROS in iMC completely abrogated the negative effect of these cells on T cells. This suggested that iMC generated in tumor-bearing hosts could suppress CD8⁺ T cell response via release of ROS. Interaction of iMC with antigen-specific T cells in the presence of specific but not control antigens, resulted in a significant increase of ROS production. That increase was independent of IFN- γ production by T cells, but was mediated by integrins CD11b, CD18, and CD29 [27]. Blockage of these integrins abrogated ROS production and iMC mediated suppression of CD8⁺ T-cell responses. Importantly, no T cell apoptosis or T cell deletion has been observed [27].

Schmielau and Finn [48] observed that, in peripheral-blood samples from cancer patients, an unusually large number of myeloid cells with a granulocyte phenotype co-purified with low-

density peripheral-blood mononuclear cells (PBMCs). They found that reduced CD3 ζ expression and decreased cytokine production by T cells correlated with the presence of activated myeloid cells in the PBMC population. Freshly isolated granulocytes from healthy donors, if activated, could also inhibit cytokine production by T cells. This action was abrogated by addition of an H₂O₂ scavenger, catalase, implicating H₂O₂ as the effector molecule.

Peroxynitrites

Peroxynitrite (ONOO⁻) is a reaction product of NO and superoxide (O₂⁻). Peroxynitrite is a powerful oxidant that can inhibit T cell activation and proliferation by impairment of tyrosine phosphorylation and apoptotic death. The involvement of ONOO⁻ in mechanisms of T cell inhibition by Gr-1⁺ iMC derived from tumor-bearing mice has been demonstrated first by Kusmartsev and colleagues [26]. It has been demonstrated that peroxynitrite production by myeloid cells could play a major role in preventing an antigen-stimulated T cell expansion in tumor-bearing hosts. Bronte and colleagues [9] recently reported that human prostatic adenocarcinomas are infiltrated by terminally differentiated cytotoxic T lymphocytes. These lymphocytes, however, are in an unresponsive status. Authors demonstrated the presence of high levels of nitrotyrosines in prostatic TIL, suggesting a local production of peroxynitrites. Restoration of TIL responsiveness to tumor could be achieved by simultaneous inhibition of iNOS and arginase activity. Thus, local peroxynitrite production could represent one of the important mechanisms by which tumor escape immune response.

Complex role of Jak/Stat signaling in myeloid cell differentiation and function in cancer

Soluble growth factors and cytokines produced by tumors interact with appropriated receptors on myeloid cells thus inducing intracellular signaling cascade. Janus family of tyrosine kinases (Jak) and signal transducer and activator of transcription (STAT) are critical components of diverse signal transduction pathway that are actively involved in cellular survival, proliferation, differentiation and apoptosis. Four members of Jak family have been identified in mammals: Jak1, Jak2, Jak3, and Tyk2. Jaks are constitutively associated with many cytokine and growth factor receptors. Phosphorylation of Jak generates docking sites for Stats. Subsequently recruited Stats are phosphorylated by activated Jaks and dimerized followed by their translocation into the nucleus, where they modulate expression of target genes. Presently, seven mammalian Stat proteins, Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b, and Stat6 have been described.

Stat1

It is known, that Stat1 negatively regulates angiogenesis, tumorigenicity and metastasis of tumor cells [7]. Stat1 is essential for IFN-mediated signaling, suggesting that Stat1 pathway is an important mediator for immune anti-tumor signals [51]. However, activation of Stat1 in tumor-associated macrophages (TAM) could also contribute to the mechanisms of T cell unresponsiveness [24]. It has been shown that the tumor microenvironment promotes differentiation of Gr-1⁺ myeloid cells into F4/80⁺ TAM, and in these cells up-regulates arginase I and iNOS expression through Stat 1-dependent mechanism [24]. Freshly isolated F4/80⁺ TAM from tumors displayed strong T cell inhibitory features and induced deletion of T cells. Since TAM derived from Stat1^{-/-} mice were not able to inhibit T cell response it suggested that tumor-activated Stat1 signaling in F4/80 tumor-associated macrophages could be responsible for T-cell deletion.

Stat3

Recent studies suggested that activation of Stat3 in tumor might play an important role in tumor escape from immune system [56]. The Stat3 signaling pathway in tumor cells can inhibit production of pro-inflammatory danger signals and induce expression of factors that inhibit DC functional maturation. Stat3 expression in macrophages has been associated with their ability to induce T cell tolerance, whereas targeted disruption of *Stat3* gene in these cells stimulated production of pro-inflammatory cytokines and abrogated their tolerogenic features [15]. It has tumor cell conditioned medium resulted in activation of Jak2 and STAT3 and was associated with an accumulation of iMC. Importantly, iMC derived from tumor-bearing mice demonstrated activated Jak2/Stat3 pathway [36]. Inhibition of STAT3 activation in HPC via dominant-negative STAT3D retroviral vector completely abrogated the effect of tumor-derived factors on the production of iMC.

Stat6

Stat6 is another member of Stat family, which has attracted attention since mice deficient for the Stat6 gene have enhanced immunosurveillance against primary and metastatic tumors. STAT6 is a downstream transcription factor for IL-4R, and IL-13R. More than 60% of Stat6^{-/-} mice immunologically reject spontaneous metastatic mammary carcinoma and survive indefinitely if their primary tumors are removed, whereas 95% of Stat6-competent BALB/c mice succumb to metastatic disease [37;52]. Authors suggested that Stat6 deficiency prevents signaling through the type 2 IL-4R α , thereby blocking the production of arginase I and promoting the synthesis of NO by myeloid cells.

Overall, obtained results suggest that Jak/Stat signaling could have a crucial role in regulation of tumor immunity through modulation differentiation/maturation as well as function of myeloid cells. Targeting of different members of Jak/Stat pathway could represent a promising new approach for regulation of anti-tumor immune response as well for development of new modalities for cancer treatment.

Improvement of anti-tumor immunity through iMC elimination or stimulation of its differentiation

Since expansion of iMC in tumor-bearing mice is associated with profound inhibition of anti-tumor immune response, it seems logical that elimination of those immune suppressive cells may help to enhance the immune defense mechanisms. Indeed, earlier experiments from H. Schreiber group [50] demonstrated that depletion of Gr-1⁺ cells significantly improved CD8⁺ T cell immune response and allowed for eradication of the tumor. More recent work of J. Berzofsky group [54] demonstrated that depleting of Gr-1⁺ myeloid cells or blocking TGF- β *in vivo* prevented the tumor recurrence, implying that TGF- β made by iMC is necessary for down-regulation of tumor immunosurveillance [54].

Another promising approach to reduce the proportion of iMC in tumor-bearing hosts might be use of agents that promote the differentiation of myeloid progenitors.

1-alpha 25-dihydroxyvitamin D (3)(1,25(OH)(2), a biologically active metabolite of vitamin-D3 is known as a stimulator of myeloid cell differentiation. In series of publications, Young MR and colleagues demonstrated that in both clinical and experimental settings vitamin D3 is effective in diminishing the levels of immune suppressive myeloid cells and increases the effectiveness of tumor immunotherapy [29;57;62]. When DCs were generated from the CD34⁺ cells *in vitro* in the presence of 1-alpha, 25-dihydroxyvitamin D3, their antigen presenting ability was enhanced [62]. Treatment of LLC tumor-bearing mice with vitamin D3 led to reduced tumor production of GM-CSF and lowered proportion of myeloid immune

suppressive cells [61]. Administration of vitamin D3 in combination with adoptive immunotherapy significantly reduced metastases in mice with established tumors, and also reduced metastases and recurrence after surgical excision of the primary tumors [57]. Studies in cancer patients with head and neck cancer also demonstrated the ability of 1- α , 25-dihydroxyvitamin D3 to promote differentiation of myeloid cells and reduce the proportion of immature myeloid immune suppressive cell population in peripheral blood [29].

Another compound, which is able to stimulate differentiation of the myeloid progenitors into myeloid DCs is a vitamin A or retinoic acid [20;21]. Mice with vitamin A deficiency [28] and mice treated with a pan-RAR antagonist [55] show accumulation of CD11b⁺Gr-1⁺ myeloid cells similar to the iMC that accumulate in cancer patients. Physiologic concentrations of all-trans retinoic acid (ATRA) induced *in vitro* differentiation of iMC from tumor-bearing mice into CD11c⁺MHC class II⁺ myeloid DC [20]. *In vivo* administration of ATRA significantly reduced the presence of iMCs in two different tested tumor models [23]. This was not caused by direct anti-tumor effect of ATRA or decreased production of growth factors by tumor cells. Experiments with adoptive transfer demonstrated that ATRA differentiated iMC *in vivo* into mature DC, macrophages, and granulocytes. Decreased presence of iMC in tumor-bearing mice noticeably improved CD4- and CD8-mediated tumor-specific immune response. Combination of ATRA with two different types of cancer vaccines in two different tumor models significantly prolonged the anti-tumor effect of the treatment [23]. These data suggest that elimination of iMC with ATRA may open an opportunity to improve the effect of cancer vaccines.

Concluding remarks

Increased number of immature myeloid cells in tumor-bearing hosts is associated with inhibition of T cell responses immune suppression, which substantially limits therapeutic effect of cancer vaccination and immune therapy. As evident from recent publications, cancer-associated immune suppression takes place at two different sites: I) in lymphoid organs; II) in tumor site (see Figure 1).

Growing tumors affect myelopoiesis by inhibiting DC differentiation and promoting accumulation of iMC. Those iMCs are precursors of antigen-presenting cells (DC and macrophages) and they have distinct immune suppressive features that allow them to inhibit development of anti-tumor T cell response in antigen-specific manner in lymphoid organs. One of the prominent characteristics of iMC is increased production of reactive oxygen species (ROS), which are involved in mechanism of immune suppression. Since ROS exert their effect only on very short distance immunosuppressive effect of iMC on T cells requires direct cell-cell contact. Antigen-specific interaction between cells is much more stable than antigen non-specific one. This may explain the fact that immunosuppressive effect of iMC in peripheral lymphoid organs (lymph nodes, spleens) and blood is antigen-specific. This also can explain the apparent paradox of why accumulation of large numbers of iMC in tumor-bearing hosts doesn't result in profound systemic immune suppression. Whether inhibitory effect of iMC is limited to only CD8⁺ T cells or extended to CD4⁺ T cells probably depends on the expression of MHC on the iMC surface. The population of iMC is a heterogeneous mix of precursor of different myeloid cells and early progenitors. The composition of this population varies from one tumor model to another and largely depends on cytokine profile produced by tumor cells. In many experimental tumor models iMC express MHC class I but very little or no MHC class II. In that case iMC have much more profound inhibitory effect on CD8⁺ than on CD4⁺ T cells. In some tumor models, like mammary adenocarcinomas DA3 (Gabrilovich, not published observation) or 4T1 [52] a substantial proportion of iMC may express MHC class II. In that case inhibition of CD4⁺ T cells is clearly evident [52]. More studies are needed to clarify the exact mechanism of CD4⁺ and CD8⁺ T-cell specific suppression mediated by iMC. Immune

suppression mediated by iMC requires the presence of three factors: iMC, activated antigen-specific CD8⁺ or CD4⁺ T cells, and tumor-associated antigen. This hypothesis may also explain the difficulties in generating tumor-specific immune response to vaccination in cancer patients. Immature myeloid cells present in tumor-bearing hosts have full access to tumor associated antigens used for vaccination and are able to inhibit the very same tumor-specific immune response such a vaccination is trying to induce. This underscores the necessity of combining cancer vaccines with strategies to eliminate iMC.

In addition, iMC can be actively recruited to tumor site, and then inside tumor these cells may differentiate toward tumor-associated macrophages and/or endothelial cells, thus promoting angiogenesis and tumor growth. Tumor-associated macrophages play significant role in local immune suppression. Importantly, their phenotype is quite different from that of macrophages in peripheral lymphoid organs. TAM produce much higher levels of NO and arginase I and are able not only to suppress CD8⁺ T cells but also induce apoptosis of CD4⁺ and CD8⁺ T cells. Understanding of precise mechanisms, which tumors use to affect differentiation of APC from myeloid precursors and inhibit T cell responses, could help to develop new approaches to cancer therapy and substantially improve the efficiency of existing cancer vaccination strategies.

References

1. Almand B, Clark JI, Nikitina E, English NR, Knight SC, Carbone DP, Gabrilovich DI. "Increased production of immature myeloid cells in cancer patients. A mechanism of immunosuppression in cancer". *J Immunol* 2001;166:678–689. [PubMed: 11123353]
2. Almand B, Resser J, Lindman B, Nadaf S, Clark J, Kwon E, Carbone D, Gabrilovich D. "Clinical significance of defective dendritic cell differentiation in cancer". *Clin Cancer Res* 2000;6:1755–1766. [PubMed: 10815894]
3. Atochina O, Daly-Angel T, Piskorska D, Harn D. "A shistosoma expressed immunomodulatory glycoconjugate expand peritoneal Gr1+ macrophages that suppress naïve CD4+ T cell proliferation via an interferon-gamma and nitric oxide dependent mechanism". *J Immunol* 2001;167:4293–302. [PubMed: 11591752]
4. Balkwill F, Mantovani A. "Inflammation and cancer: back to Virchow?". *Lancet* 2001;357(9255):539–45. [PubMed: 11229684]
5. Beck C, Schreiber K, Schreiber H, Rowley D. "C-kit+ FcR+ myelocytes are increased in cancer and prevent the proliferation of fully cytolytic T cells in the presence of immune serum". *Eur J Immunol* 2001;33(1):19–28. [PubMed: 12594828]
6. Boucher JL, Moali C, Tenu JP. "Nitric oxide biosynthesis, nitric oxide synthase inhibitors and arginase competition for L-arginine utilization". *Cell Mol Life Sci* 1999;55:1015–1028. [PubMed: 10484661]
7. Bromberg J. "Stat proteins and oncogenesis". *J Clin Invest* 2002;109:1139–1142. [PubMed: 11994401]
8. Bronte V, Apolloni E, Cabrelle A, Ronca R, Serafini P, Zamboni P, Restifo N, Zanovello P. "Identification of a CD11b(+)/Gr-1(+)/CD31(+) myeloid progenitor capable of activating or suppressing CD8(+) T cells". *Blood* 2000;96:3838. [PubMed: 11090068]
9. Bronte V, Casic T, Gri G, Gallana K, Borsellino G, Marrigo I, Battistini L, Iafrate M, Prayer-Galletti U, Pagano F, Viola A. "Boosting antitumor responses of T lymphocytes infiltrating human prostate cancers". *J Exp Med* 2005;201(8):1257–68. [PubMed: 15824085]
10. Bronte V, Chappell DB, Apolloni E, Cabrelle A, Wang M, Hwu P, Restifo NP. "Unopposed Production of Granulocyte-Macrophage Colony-Stimulating Factor by Tumors Inhibits CD8+ T Cell Responses by Dysregulating Antigen-Presenting Cell Maturation". *J Immunol* 1999;162:5728–5737. [PubMed: 10229805]
11. Bronte V, Serafini P, Apolloni E, Zanovello P. "Tumor-induced immune dysfunctions caused by myeloid suppressor cells". *J Immunol* 2001;24:431–446.
12. Bronte V, Serafini P, De Santo C, Marigo I, Tosello V, Mazzoni A, Segal DM, Staib C, Lowel M, Sutter G, Colombo MP, Zanovello P. "IL-4-induced arginase 1 suppresses alloreactive T cells in tumor-bearing mice". *J Immunol* 2003;170(1):270–8. [PubMed: 12496409]

13. Bronte V, Wang M, Overwijk W, Surman D, Pericle F, Rosenberg SA, Restifo NP. "Apoptotic death of CD8+ T lymphocytes after immunization: induction of a suppressive population of Mac-1+/Gr-1+ cells". *J Immunol* 1998;161:5313–5320. [PubMed: 9820504]
14. Cauley L, Miller E, Yen M, Swain S. "Superantigen-induced CD4 T cell tolerance mediated by myeloid cells and IFN-gamma". *J Immunol* 2000;165:6056. [PubMed: 11086037]
15. Cheng F, Wang HW, Cuenca A, Huang M, Ghansah T, Brayer J, Kerr WG, Takeda K, Akira S, Schoenberger SP, Yu H, Jove R, Sotomayor EM. "A critical role for Stat3 signaling in immune tolerance". *Immunity* 2003;19:425–436. [PubMed: 14499117]
16. De Santo C, Serafini P, Marigo I, Dolcetti L, Bolla M, P DS, Melani C, Guiducci C, Colombo M, Iezzi M, Musiani P, Zanovello P, Bronte V. "Nitroaspirin corrects immune dysfunction in tumor-bearing hosts and promotes tumor eradication by cancer vaccination". *Proc Natl Acad Sci U S A* 2005;102(11):4185–90. [PubMed: 15753302]
17. Gabrilovich D. "The mechanisms and functional significance of tumour-induced dendritic-cell defects". *Nat Rev Immunol* 2004;4:941–952. [PubMed: 15573129]
18. Gabrilovich D, Cheng P, Fan Y, Yu B, Nikitina E, Sirotkin A, Shurin M, Oyama T, Adachi Y, Nadaf S, Carbone DP, Skoultschi A. "H1o Histone and Differentiation of Dendritic Cells. A Molecular Target for Tumor-Derived Factors". *J Leuk Biol* 2002;72:285–296.
19. Gabrilovich D, Ishida T, Oyama T, Ran S, Kravtsov V, Nadaf S, Carbone DP. "Vascular endothelial growth factor inhibits the development of dendritic cells and dramatically affects the differentiation of multiple hematopoietic lineages in vivo". *Blood* 1998;92(11):4150–66. [PubMed: 9834220]
20. Gabrilovich DI, Velders M, Sotomayor E, Kast WM. "Mechanism of immune dysfunction in cancer mediated by immature Gr-1+ myeloid cells". *J Immunol* 2001;166:5398–5406. [PubMed: 11313376]
21. Hengesbach L, Hoag K. "Physiological concentrations of retinoic acid favor myeloid dendritic cell development over granulocyte development in cultures of bone marrow cells from mice". *J Nutr* 2004;134(10):2653–9. [PubMed: 15465762]
22. Hestdal K, Ruscetti F, Ihle J, Jacobsen S, Dubois C, Kopp W, Longo D, Keller J. "Characterization and regulation of RB6-8C5 antigen expression on murine bone marrow cells". *J Immunol* 1991;147(1):22–28. [PubMed: 1711076]
23. Kusmartsev S, Cheng F, Yu B, Nefedova Y, Sotomayor E, Lush R, Gabrilovich DI. "All-trans-retinoic acid eliminates immature myeloid cells from tumor-bearing mice and improves the effect of vaccination". *Cancer Res* 2003;63:4441–4449. [PubMed: 12907617]
24. Kusmartsev S, Gabrilovich D. "STAT1 signaling regulates tumor-associated macrophage-mediated T cell deletion". *J Immunol* 2005;174(8):4880–91. [PubMed: 15814715]
25. Kusmartsev S, Gabrilovich DI. "Inhibition of myeloid cell differentiation in cancer: The role of reactive oxygen species". *J Leukoc Biol* 2003;74(2):186–196. [PubMed: 12885935]
26. Kusmartsev S, Li Y, Chen S-H. "Gr-1+ myeloid cells derived from tumor-bearing mice inhibit primary T cell activation induced through CD3/CD28 costimulation". *J Immunol* 2000;165:779–785. [PubMed: 10878351]
27. Kusmartsev S, Nefedova Y, Yoder D, Gabrilovich DI. "Antigen-specific inhibition of CD8+ T cell response by immature myeloid cells in cancer is mediated by reactive oxygen species". *J Immunol* 2004;172(2):989–99. [PubMed: 14707072]
28. Kuwata T, Wang I, Tamura T, Ponnamperna R, Levine R, Holmes K, Morse H, De Luca L, Ozato K. "Vitamin A deficiency in mice causes a systemic expansion of myeloid cells". *Blood* 2000;95(11):3349–56. [PubMed: 10828015]
29. Lathers D, Clark J, Achille N, Young M. "Phase 1B study to improve immune responses in head and neck cancer patients using escalating doses of 25-hydroxyvitamin D3". *Cancer Immunol Immunother* 2004;53(5):422–30. [PubMed: 14648070]
30. Li Q, Pan PY, Gu P, Xu D, Chen SH. "Role of immature myeloid Gr-1+ cells in the development of antitumor immunity". *Cancer Res* 2004;64(3):1130–9. [PubMed: 14871848]
31. Liu Y, Van Ginderachter J, Brys L, De Baetselier P, Raes G, Geldhof A. "Nitric oxide-independent CTL suppression during tumor progression: association with arginase-producing (M2) myeloid cells". *J Immunol* 2003;170(10):5064–74. [PubMed: 12734351]

32. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. "Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes". *Trends Immunol* 2002;23(11):549–55. [PubMed: 12401408]
33. Melani C, Chiodoni C, Forni G, Colombo MP. "Myeloid cell expansion elicited by the progression of spontaneous mammary carcinomas in c-erbB-2 transgenic BALB/c mice suppresses immune reactivity". *Blood* 2003;102(6):2138–45. [PubMed: 12750171]
34. Mencacci A, Montagnoli C, Bacci A, Cenci E, Pitzurra L, Spreca A, Kopf M, Sharpe A, Romani L. "CD80+Gr-1+ myeloid cells inhibit development of antifungal Th1 immunity in mice with candidiasis". *J Immunol* 2002;169(6):3180–90. [PubMed: 12218136]
35. Menetrier-Caux C, Thomachot MC, Alberti L, Montmain G, Blay JY. "IL-4 prevents the blockade of dendritic cell differentiation induced by tumor cells". *Cancer Res* 2001;61(7):3096–104. [PubMed: 11306493]
36. Nefedova Y, Huang M, Kusmartsev S, Bhattacharya R, Cheng P, Salup R, Jove R, Gabrilovich D. "Hyperactivation of STAT3 is involved in abnormal differentiation of dendritic cells in cancer". *J Immunol* 2004;172(1):464–74. [PubMed: 14688356]
37. Ostrand-Rosenberg S, Clements VK, Terabe M, Park JM, Berzofsky JA, Dissanayake SK. "Resistance to metastatic disease in STAT6-deficient mice requires hemopoietic and nonhemopoietic cells and is IFN-gamma dependent". *J Immunol* 2002;169(10):5796–804. [PubMed: 12421960]
38. Otsuji M, Kimura Y, Aoe T, Okamoto Y, Saito T. "Oxidative stress by tumor-derived macrophages suppresses the expression of CD3 zeta chain of T-cell receptor complex and antigen-specific T-cell responses". *Proc Natl Acad Sci USA* 1996;93:13119–13124. [PubMed: 8917554]
39. Pandit R, Lathers D, Beal N, Garrity T, Young M. "CD34+ immune suppressive cells in the peripheral blood of patients with head and neck cancer". *Ann Otol Rhinol Laryngol* 2000;109:749–754. [PubMed: 10961808]
40. Pelaez B, Campillo J, Lopez-Asenjo J, Subiza J. "Cyclophosphamide induces the development of early myeloid cells suppressing tumor growth by a nitric oxide-dependent mechanism". *J Immunol* 2001;166:6608. [PubMed: 11359814]
41. Pollard J. "Tumour-educated macrophages promote tumour progression and metastasis". *Nat Rev Cancer* 2004;4(1):71–8. [PubMed: 14708027]
42. Rodriguez PC, Quiceno DG, Zabaleta J, Ortiz B, Zea AH, Piazuelo MB, Delgado A, Correa, Brayer J, Sotomayor EM, Antonia S, Ochoa JB, Ochoa AC. "Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses". *Cancer Res* 2004;64(16):5839–49. [PubMed: 15313928]
43. Rodriguez PC, Zea AH, Culotta KS, Zabaleta J, Ochoa JB, Ochoa AC. "Regulation of T cell receptor CD3zeta chain expression by L-arginine". *J Biol Chem* 2002;277:21123–21129. [PubMed: 11950832]
44. Rodriguez PC, Zea AH, DeSalvo J, Culotta KS, Zabaleta J, Quiceno DG, Ochoa JB, Ochoa AC. "L-arginine consumption by macrophages modulates the expression of CD3 zeta chain in T lymphocytes". *J Immunol* 2003;171(3):1232–9. [PubMed: 12874210]
45. Saio M, Radoja S, Marino M, Frey AB. "Tumor-infiltrating macrophages induce apoptosis in activated CD8(+) T cells by a mechanism requiring cell contact and mediated by both the cell-associated form of TNF and nitric oxide". *J Immunol* 2001;167(10):5583–93. [PubMed: 11698429]
46. Salvadori S, Martinelli G, Zier K. "Resection of solid tumors reverses T cell defects and restores protective immunity". *J Immunol* 2000;164:2214. [PubMed: 10657677]
47. Sauer H, Wartenberg M, Hescheler J. "Reactive oxygen species as intracellular messengers during cell growth and differentiation". *Cell Physiol Biochem* 2001;11:173–186. [PubMed: 11509825]
48. Schmielau J, Finn OJ. "Activated granulocytes and granulocyte-derived hydrogen peroxide are the underlying mechanism of suppression of t-cell function in advanced cancer patients". *Cancer Res* 2001;61:4756–4760. [PubMed: 11406548]
49. Schreck R, Albermann K, Baeuerle PA. "Nuclear factor kappa B: an oxidative stress-responsive transcription factor of eukaryotic cells (a review)". *Free Radic Res Commun* 1992;17:221–237. [PubMed: 1473734]

50. Seung L, Rowley D, Dubeym P, Schreiber H. "Synergy between T-cell immunity and inhibition of paracrine stimulation causes tumor rejection". *Proc Natl Acad Sci U S A* 1995;92(14):6254–8. [PubMed: 7603979]
51. Shankaran V, Ikeda H, Bruce A, White J, Swanson P, Old L, Schreiber R. "IFN-gamma and lymphocytes prevent primary tumor development and shape tumor immunogenicity". *Nature* 2001;410:1107–1111. [PubMed: 11323675]
52. Sinha P, Clements V, Ostrand-Rosenberg S. "Reduction of myeloid-derived suppressor cells and induction of M1 macrophages facilitate the rejection of established metastatic disease". *J Immunol* 2005;174(2):636–45. [PubMed: 15634881]
53. Subiza J, Vinuela J, Rodriguez R, De la Concha E. "Development of splenic natural suppressor (NS) cells in Ehrlich tumor-bearing mice". *Int J Cancer* 1989;44:307–314. [PubMed: 2527208]
54. Terabe M, Matsui S, Park JM, Mamura M, Noben-Trauth N, Donaldson DD, Chen W, Wahl SM, Ledbetter S, Pratt B, Letterio JJ, Paul WE, Berzofsky JA. "Transforming growth factor-beta production and myeloid cells are an effector mechanism through which CD1d-restricted T cells block cytotoxic T lymphocyte-mediated tumor immunosurveillance: abrogation prevents tumor recurrence". *J Exp Med* 2003;198(11):1741–52. [PubMed: 14657224]
55. Walkley C, Yuan Y, Chandraratna R, McArthur G. "Retinoic acid receptor antagonism in vivo expands the numbers of precursor cells during granulopoiesis". *Leukemia* 2002;16(9):1763–72. [PubMed: 12200692]
56. Wang T, Niu G, Kortylewski M, Burdelya L, Shain K, Zhang S, Bhattacharya R, Gabrilovich D, Heller R, Coppola D, Dalton W, Jove R, Pardoll D, Yu H. "Regulation of the innate and adaptive immune responses by Stat-3 signaling in tumor cells". *Nat Med* 2004;10(1):48–54. [PubMed: 14702634]
57. Wiers K, Lathers D, Wright M, Young M. "Vitamin D3 treatment to diminish the levels of immune suppressive CD34+ cells increases the effectiveness of adoptive immunotherapy". *J Immunother* 2000;23(1):115–24. [PubMed: 10687144]
58. Wu G, Morris SM. "Arginine metabolism: nitric oxide and beyond". *Biochem J* 1998;336:1–17. [PubMed: 9806879]
59. Yang L, DeBusk L, Fukuda K, Fingleton B, Green-Jarvis B, Shyr Y, Matrisian L, Carbone D, Lin P. "Expansion of myeloid immune suppressor Gr+CD11b+ cells in tumor-bearing host directly promotes tumor angiogenesis". *Cancer Cell* 2004;6(4):409–21. [PubMed: 15488763]
60. Young M. "Tumor skewing of CD34+ progenitor cell differentiation into endothelial cells". *Int J Cancer* 2004;109(4):516–24. [PubMed: 14991572]
61. Young M, Ihm J, Lozano Y, Wright M, Prechel M. "Treating tumor-bearing mice with vitamin D3 diminishes tumor-induced myelopoiesis and associated immunosuppression, and reduces tumor metastasis and recurrence". *Cancer Immunol Immunother* 1995;41(1):37–45. [PubMed: 7641218]
62. Young MR, Lathers DM. "Myeloid progenitor cells mediate immune suppression in patients with head and neck cancers". *Int J Immunopharmacol* 1999;21:241–252. [PubMed: 10408632]
63. Young MRI, Wright MA, Matthews JP, Malik I, Pandit R. "Suppression of T cell proliferation by tumor-induced granulocyte-macrophage progenitor cells producing transforming growth factor- b and nitric oxide". *J Immunol* 1996;156:1916–1921. [PubMed: 8596044]
64. Zea AH, Rodriguez PC, Atkins MB, Hernandez C, Signoretti S, Zabaleta J, McDermott D, Quiceno D, Youmans A, O'Neill A, Mier J, Ochoa AC. "Arginase-producing myeloid suppressor cells in renal cell carcinoma patients: a mechanism of tumor evasion". *Cancer Res* 2005;65(8):3044–8. [PubMed: 15833831]

Fig.1. Contribution of immature myeloid cells to mechanisms of immune evasion in tumor host
Growing tumors affect the process of myeloid cell differentiation and maturation through production of soluble factors. This results in accumulation of immature myeloid cells (iMC) that have increased Stat3 activity, increased ROS production and high MMP-9 expression. These cells migrate in peripheral lymphoid organs and inhibit CD8 T cell immune responses. iMC also can be recruited into tumor site where they differentiate into tumor-associated macrophages (TAM) or endothelial cells. TAM have high Stat1 as well arginase I activities and induce locally T cell apoptosis or anergy.