Chapter 7

Potential Anticancer Drugs Targeting Immune Pathways

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Abstract

Studies on the tumor microenvironment reveal that infiltration or induction of tumor-associated immune regulatory populations at the local tumor site is strongly associated with severe immunosuppression as well as worse prognosis of patients. Despite major advances in cancer immunotherapy, most of the therapeutic agents often fail to break negative immunosuppressive network to trigger anticancer immunity, leading to tumor progression and metastasis. Therefore, emergence of potent immunostimulatory agents is of great clinical importance. Emerging evidence suggests that metal chelates of Schiff bases hold the promise to overcome tumor-associated immunosuppression by inhibiting or subverting suppressive immune population toward pro-immunogenic type and thus can be used clinically for immunotherapy of different types of cancers.

Keywords: immunosuppression, tumor microenvironment, antitumor response, reactive oxygen species (ROS), tumor-associated macrophage (TAM), myeloid-derived suppressor cells (MDSC), regulatory T cell (Treg)

1. Introduction

To attain malignant phenotype, the tumor cells overcome the deadly encounter of immune system through a process known as immune editing and thus create an immunosuppressive environment [1]. This phenomenon is evident in clinical scenarios where patients with

malignant tumor quite often possess a blunt immune system [2]. Thus the immune system has an impact in the progression and severity of the disease. For most of advanced malignancies where chemotherapy is considered the treatment modality of choice often rely on the strategy to target and destroy rapidly dividing cancer cells, neglecting the possibility that immune system may contribute to the efficacy of treatment. In recent years, extensive research in the context of cancer immune system has led to the development of cancer immunotherapy field that may prove to be a powerful weapon in the treatment of cancer through modulating the immune system. The suppressive roles played by immune cells of tumor microenvironment in promoting tumor progression indicate that these cells can serve as novel therapeutic targets in the treatment of cancer [3]. Emerging evidence in recent time indicates that metal chelates of Schiff base can be used as a potent anticancer agent that imparts their cytotoxic effect through generation of reactive oxygen species (ROS) in cancer cells [4]. Recent study also highlights the role of those metal chelates as immune-stimulating agents [4] that possess the ability to inhibit or subvert immunosuppressive phenotype toward proimmunogenic type and thus associated with tumor regression. In search of novel immune-stimulatory or modulatory agents, we had synthesized a number of metal chelates of a Schiff base namely N-(2-hydroxy acetophenone) glycinate (NG). Among these, copper and manganese complexes of NG, e.g., copper N-(2-hydroxy acetophenone) glycinate (CuNG) and manganese N-(2-hydroxy acetophenone) glycinate (MnNG) exhibit immunomodulatory properties [4]. Other such metal chelates of iron, nickel, and zinc formed with the same organic moiety (NG) lack the property of immunomodulation [4]. This chapter deals with the major negative regulator of immune system during cancer, as well as the key aspects of how the immune system can be controlled or manipulated by the application of novel Schiff base metal chelates to enhance anticancer immunity.

2. Negative regulator of immune system and cancer

The failure of the immune system to completely eliminate the tumor cells results in the selection of tumor cell variants that are able to resist, avoid, or suppress the antitumor immune response, leading to the escape phase [5]. The escape phase of cancer immunoediting process is activated by the simultaneous alteration of both tumor intrinsic mechanisms enabling them to hide from immune attack to avoid recognition and extrinsic mechanisms that rely on disabling or eliminating immune attack by creating suppressive environment [1, 2]. The mechanisms, at the level of tumor, that evades immunity includes the loss of tumor antigen expression, downregulation of antigen presentation through major histocompatibility complex (MHC), defects in IFN-γ receptor signaling pathway, upregulation of antiapoptotic molecules (e.g., Bcl-XL, FLIP), expression of inhibitory cell surface molecules (e.g., PD-L1, FasL), and secretion of various tumor-derived soluble factors that encourage tumor outgrowth [1, 2]. The extrinsic factor of tumor immune escape mechanism is generated by the interferences of the progressing tumor with the host immune system through the induction and/or recruitment of potent immunosuppressive cells in tumor microenvironment that ultimately hinder the induction of protective antitumor immune activity [2]. In recent years, such immune cells have gained special attention, possibly due to their ubiquitous appearance and clear association with disease progression.

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Despite being considered as a major weapon of antitumor immunity, the activation, phenotype, and optimal function of T cells are regulated by a complex immunosuppressive network of tumor microenvironment [6]. Due to the weak immunogenic nature of tumor antigen, most of the tumor-specific T cells are deleted in thymic selection, resulting in low frequency of T cells with a low TCR affinity [3]. Furthermore, the presence of immunosuppressive cells in the tumor site often renders insufficient priming and boosting of T cells [3]. Over past decades, evidence implicated the fact that tumor microenvironment is dominated by Th2-type immune response than homeostatic tissue where Th1 type is prominent. Th1-type immunity promotes the expression of type 1 cytokines (TNFα, IFN-γ, and IL-2) and activates cell-mediated responses that are antitumor in nature [7]. On the other hand, Th2-type microenvironment favors the expression of type 2 cytokines (IL-4, IL-10, and TGF-β) that initiate tissue remodeling, angiogenesis, and occasional humoral immunity fostering a protumorigenic state [8]. The Th2 programming in the tumor microenvironment can be attributed by the presence of various immunosuppressive cells notably tumor-associated macrophages (TAMs), regulatory T cells (Tregs), and immature myeloid cells including the myeloid-derived suppressor cells (MDSCs) and immature dendritic cells (iDCs) that form an inhibitory network which suppresses local immunity [3]. Thus strategies involving the inhibition or subversion of suppressive immune population in tumor microenvironment hold the premise to effectively blunt Th2 programming and thereby, tilt the balance back toward Th1-based immune programs that would eventually impede tumorigenesis.

2.1. Tumor-associated macrophages (TAMs) and cancer

Macrophages are well distributed innate immune cells, known for their versatility and plasticity by exerting different functional properties in response to diverse environment stimuli. Originating from blood monocytes, macrophages are recruited into peripheral tissue where they differentiate into distinct mature macrophages by the expression of particular markers [9]. In mice, macrophages are known to exhibit phagocytic activity and express CD11b, F4/80, and colony-stimulating factor-1 receptor (CSF-1R; CD115) marker [9]. In humans, phagocytosis, CD68, CD163, CD16, CD312, and CD115 are the major characteristic features of this lineage [9]. Diverse immunological signals in tumor microenvironment lead macrophages to undergo polarized activation [10]. Such a polarization includes the "activated" macrophage (M1 macrophages) and "alternatively activated" macrophages (M2 macrophages). M1 macrophages that are activated following stimulation with Th1 cytokine IFN-γ alone or in combination with LPS or TNF-α or through engagement of Toll-like receptors (TLRs) are characterized by elevated expression of class II major histocompatibility complex (MHC), expression of interleukin (IL-12) and tumor necrosis factor α (TNF-α), generation of reactive oxygen species and nitric oxide (NO), and the ability to kill pathogens and cells [11]. Early in tumorigenesis M1 macrophages, generally characterized by an IL-12^{high} IL-10^{low} phenotype, support immune response to the nascent tumor by the secretion of large amounts of IL-12, IL-1α, IL-1β, IL-6, and TNF-α, and IFN-γ by Th1 lymphocytes. Furthermore, the induction of nitric oxide (NO) and ARG1 expression by M1 macrophages increases CTL cytotoxicity on tumor cells. In contrast, the "alternatively activated" macrophages (M2 macrophages) that differentiate in response to IL-4, IL-13, M-CSF/CSF-1, IL-10, and TGF-β1 are associated with inducing Th2-type responses, including

humoral immunity and wound healing [11]. Interestingly in the context of tumor immune response, reports highlights that macrophages in tumor microenvironment are biased away from the M1 to M2 type macrophages and known as tumor-associated macrophages (TAMs) [12]. During late-stage tumor progression, TAMs exhibit an IL-12^{low} IL-10^{high} phenotype with low tumoricidal activity [12] in TME. Growing body of literature has well established the fact that TAMs have been known to provide a favorable microenvironment for tumor growth, tumor survival, and angiogenesis [11].

2.1.1. Tumor-associated macrophages (TAMs) and immunosuppression

TAMs are the major components of infiltrating immune cells in the tumor site of virtually all types of malignancies. The abundant nature of TAMs within the tumor microenvironment enables them to maintain severe immunosuppression and has been often associated with worst prognosis. During the process of tumor progression, the secretion of tumor-derived soluble factors that support the development of TAM in the local tumor sites includes M-CSF, IL-10, IL-4, and IL-13. Several studies have reported that TAM-derived secreted mediators such as TGF-β, IL-10, arginase 1, prostaglandins, and indoleamine dioxygenase (IDO) make a significant contribution to immunosuppression [13]. Poor antigen presenting capacity of TAMs reduces the tumor-specific T-cell proliferation and response by releasing the immunosuppressive factors, IL-10 and TGF-β [10]. Several studies have reported that the maturation of dendritic cells (DCs) in situ is halted by IL-10 derived from TAM but increases the differentiation of macrophages toward TAM with defective antigen presenting machinery [9, 14]. In contrast, proinflammatory cytokine IL-12 is crucial for the development of CD4+ Th1 response as CD4+ Th1 cells are a major source of IFN-γ. However, autocrine production of IL-10 by TAM is, in part, responsible for the defective LPS/IFN-γ response and reduced expression of IL-12; thereby impaired the cell-mediated immunity in tumor site [15]. Furthermore, TAMs also severely downregulate the expression of other proinflammatory cytokines such as TNF-α, IL-6, CCL3, and IL-1β, upon activation with lipopolysaccharides (LPS) [12]. Immunosuppressive cytokine TGF-β derived from TAMs promotes the development of Th2 cells, and induction and infiltration of $CD4^+CD25^+FoxP3^+$ T cells (Treg) at the tumor site [13] are also regulated by TAMs through secretion of TGF-β and IL-10. Alteration of nutrient starvation of tumor microenvironment is another strategy of tumor-associated myeloid population. Depletion of L-arginine in the tumor microenvironment is also an important mechanism of TAM-mediated T-cell suppression [12]. Metabolization of L-arginine that depends on the activity of two crucial enzymes, nitric-oxide synthase (NOS) and arginase I (ARGI), has been shown to be differentially regulated by macrophages. The expression of iNOS is upregulated by Th1 cytokines, whereas induction of arginase depends on the Th2 cytokines. Thus, in late stage of tumor, high expression of arginase is found to be associated with TAMs. Metabolism of L-arginine by ARGI to urea and L-ornithine is necessary for tumor as L-ornithine is the precursor molecules of many tumor growth factors. Furthermore, depletion of L-arginine from extracellular space inhibits the re-expression of the CD3 ζ chain, which is required for a proper T-cell activity. TAMs display a defective and delayed NFkB activation signaling, which probably provides a molecular mechanism for altered TAM functions, including defective iNOS expression [12].

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2.1.2. TAMs as promising therapeutic targets in cancer therapy

Accumulated evidence has demonstrated that immunosuppressive nature of TAM favors tumor cells during tumor development and invasion, suggesting TAM as a target for clinical therapy. TAM-targeting cancer therapy involves on the strategy either by inhibition of TAM recruitment at local tumor site or by modulation of their behavior from protumorigenic M2 to antitumor M1 phenotype. The inhibition of recruitment of TAMs at tumor site can be achieved by targeting chemokine and their receptors as well as selective killing of TAM. Treatment of Met-CCL5 (receptor antagonist of CCL5) significantly reduces the number of macrophage infiltration at tumor site and promotes tumor regression [16]. The pharmacological inhibition of CCL2 with bindarit significantly reduces infiltration of macrophage and suppresses tumor growth [17]. Some studies have also shown that antitumor drug trabectedin exerts cytotoxicity on TAMs without hampering lymphoid subsets [13]. Combination of zoledronic acid with sorafenib dramatically decreases macrophage population, resulting in the enhancement of antitumor effect [16]. A number of other drugs that have also known to inhibit macrophage infiltration include thalidomide, pentoxifylline, and genistein [16]. Furthermore, inhibition of CSF-1R signaling by antibody is also associated with TAM infiltration and tumor regression [18]. Reprogramming of M2 like TAMs toward M1 type macrophage is another potential therapeutic approach. Several studies have shown that activation of TLR signaling stimulates M1 polarized macrophage response [19]. Restoration of IKKβ/NF-kβ pathway is another promising strategy to restore M1 macrophage-mediated intratumoral cytotoxicity [20]. Studies in this direction reveal that combination of CpG plus an anti-IL-10 receptor antibody switched infiltrating macrophages to M1 through restoration of NF-kβ activation thereby promote inflammatory functions [19]. These data suggest that switching the TAM phenotype from M2 to M1 during tumor progression may promote antitumor activities.

2.1.3. Novel approach for modulation of TAM behavior by CuNG

The first study that reveals CuNG as strong immunomodulator is associated with almost complete regression of drug-resistant tumor [21, 22]. CuNG treatment induces gradual reversal of immunosuppression as evident by restoration of lymphoproliferative response in drug-resistant tumor-bearing animal model. CuNG treatment in vivo increased the number of IFN-γ producing $CD4^+$ and $CD8^+$ cells but decreased the number of T regulatory marker-expressing T cells in tumor sites. Although no direct cell-mediated cytotoxicity was observed, robust expression of apoptogenic cytokines viz. IFN-γ and TNF-α at tumor site as well as peripheral and spleen mononuclear cells were sufficient enough to resolve many drug- and radiation-resistant tumors [21, 22]. This is particularly substantial as local cytokine milieu in tumor microenvironment profoundly affects the functional plasticity of macrophages, which plays a key role in skewing suppressive Th2 response toward Th1 type [11, 23]. The evocation of Th1 response largely depends on the critical level of the two regulatory cytokines, IL-10 and IL-12. IL-10 inhibits important aspects of cell-mediated immunity, whereas IL-12 induces type 1 cytokine production and effective antitumor cell-mediated response [3, 6].

Numerous therapeutic strategies involving TAM relies on reeducation or polarization of TAM suggesting that it could be possible to convert them toward nonsuppressive and

antitumorigenic type by creating appropriate cytokine microenvironment [23]. Previously it was shown that in the presence of IL-12, TAMs rapidly alter their functional phenotype from tumor supportive and immunosuppressive to antitumorigenic type [24]. Our study in this direction showed that CuNG owns the potential to alter the immunosuppressive phenotype of TAMs toward proinflammatory by evoking a robust IL-12 and decreased IL-10 and TGF-β production by TAMs and thereby polarize its functional phenotype toward inflammatory both in vitro and in vivo [25]. CuNG-mediated cytokine alteration in TAMs is associated with the establishment of beneficial cytokine in tumor microenvironment that skewed the unresponsive $CD4^+$ T-cell population toward Th1 type in contact in an independent manner. The critical balance between elevated IL-12 and reduced IL-10 in CuNG treated TAMs is significant; because in one hand presence of IL-12 results in hugely elevated levels of IFN-γ [3], which limits T-cell survival and shortens Th1 response [26], on the other hand small amount of IL-10 limits the self-killing mechanism of Th1 cells and thereby prolonged its persistence [27]. Interestingly, CuNG-treated TAMs maintained a stable balance between IL-10 and IL-12 production, where IL-12 levels were higher than IL-10. This critical balance between these two cytokines was sufficient enough to induce Th1 response. Further study disclosed that in vitro treatment of CuNG significantly reduces the immunosuppressive cytokines and augments IL-12 generation in the blood monocytes of patients with metastatic cancers. Therefore, abrogation of immune suppression in tumor microenvironment through CuNG is principally due to the reprogramming of TAM in terms of their cytokine profile [25].

To decipher the underlying mechanisms of phenotypic conversion of TAM immunosuppressive ($IL\text{-}12^{low}$, $TGF\text{-}\beta^{high}$) to proimmunogenic ($IL\text{-}12^{high}$, $TGF\text{-}\beta^{low}$) type, our study in this direction reveals that CuNG induces ROS generation by TAMs, which is associated with the activation of two mitogen-activated protein kinases (MAPKs) (p38MAPK and ERK1/2), and also causes upregulation of intracellular GSH in TAMs [28]. Earlier reports showed that LPS-induced IL-12 production in normal macrophages is regulated by the activation of p38MAPK signaling [29], and activation of ERK1/2 in macrophages is associated with the production of IL-10 and TGF-β and negatively regulates IL-12 production [30–32]. CuNG-mediated ROS generation leads to the activation of p38MAPK that upregulated the initial IL-12 production and to the activation of ERK1/2 pathway along with GSH upregulation separately, found responsible for IFN-γ production by TAMs. It is important to note that GSH/oxidized GSH plays a crucial role in regulating IFN-γ-mediated augmentation of IL-12 production by macrophages and DCs. CuNG-mediated activation of ERK1/2 signaling and GSH upregulation are independently associated with IFN-γ augmentation in TAMs. This IFN-γ, further, increased the GSH production that, in turn, prolonged IL-12 production and downregulated TGF-β production, thereby plays the decisive role in CuNG-mediated reprogramming of regulatory cytokine production by TAMs. Although ROS-mediated cytokine modulation in TAMs toward proimmunogenic ($IL\text{-}12^{high}$, $TGF\text{-}\beta^{low}$) type has also been found by application of other Schiff base metal, e.g., ZnNG and FeNG [28], unlike CuNG, their effect were found to be temporary. Therefore, inclusion of copper within NG scaffold provides the compound with a unique characteristic to skew and maintain a sustained proimmunogenic phenotype in TAM.

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2.2. Role of myeloid-derived suppressor cells (MDSCs) in immune regulation in cancer

2.2.1. History, origin, and phenotype

The descriptions of myeloid suppressor cells were first reported more than 20 years back in normal and tumor-bearing mice as well as in patients with cancer. Initially they were known as natural suppressor (NS) cells due to induction of tolerance to foreign antigens by inhibiting various activities of the immune system [33]. Recent evidence has disclosed that these suppressive cell populations significantly differ from normal myeloid precursors. In healthy individuals, normal hematopoiesis give rise to common myeloid progenitor cells in bone marrow, which, in turn, convert to immature myeloid cells (IMC) that ultimately differentiated into mature granulocytes, macrophages, or dendritic cells (DCs) [34]. However, in pathological conditions such as cancer, sepsis, trauma, and various infectious diseases, the differentiation of IMCs are partially blocked and thus resulting in the expansion of MDSCs [34]. Based on the origin and biological function, the term MDSCs has been suggested [35].

MDSCs are a heterogeneous group of myeloid population comprising granulocyte, monocyte, and dendritic cells, which have been prevented from fully differentiating into mature cells and are capable of suppressing the immune response [34]. They do not express the cell-surface markers that are exclusive for monocytes, macrophages, or DCs and are comprised of a mixture of myeloid cells with a morphology similar to both granulocytes and monocytes. In healthy mice, around 20–30 of cells represent this phenotype and approximately 2–4% of cells are present in the spleen, although the frequency of these cells in tumor-bearing mice is largely enhanced. In mice, MDSCs are characterized by the co-expression of the myeloid lineage differentiation antigens Gr1 and CD11b (also known as αM-integrin) [34]. Since Gr-1 antigen consists of two separate epitopes, Ly6G and Ly6C, the establishment of Ly-6C- and Ly-6G-specific mAbs has led to the identification of two MDSC subsets in the spleens of tumor-bearing mice: $CD11b^{+}Ly\text{-}6C^{low}Ly\text{-}6G^{+}$ MDSCs with granulocytic morphology (PMN-MDSCs) and $CD11b^{+}Ly\text{-}6C^{high}Ly\text{-}6G^{-}$ MDSCs with a monocytic phenotype (MO-MDSCs) [36]. The Ly6G molecule is expressed primarily by granulocytes, whereas Ly6C is highly expressed by monocytes. Although the pattern of PMN-MDSC and MO-MDSC subsets differs between tumors and organs, over 80% of MDSCs are PMN-MDSCs, whereas less than 10% of MDSCs are MO-MDSCs in most of experimental models [36]. However, it is difficult to discriminate PMN-MDSCs from neutrophils as neutrophils also express both CD11b and Ly6G.

In humans, identification of MDSCs is very difficult due to the absence of a good marker such as Gr-1 and fewer opportunities to obtain samples. However, several studies have identified PMN-MDSCs ($CD11b^{+}CD14^{-}CD15^{+}$ cells with a PMN morphology) and MO-MDSCs (being $CD11b^{+}CD14^{+}HLA\text{-}DR^{low/-}$ and secreting TGF-β) [34] in the peripheral blood of patients with cancer. Other studies have shown that PMN-MDSCs are usually defined as $CD14^{-}CD11b^{+}$ or, more narrowly, as cells expressing the common myeloid marker CD33, but lack expression of markers of mature myeloid and lymphoid cells and of the MHC class II molecule HLA-DR [34, 37]. MO-MDSCs have also been identified within a $CD15^{+}$ population in human peripheral blood [34]. In healthy individuals, IMCs represents about 0.5% of peripheral blood mononuclear cells [37]. Nevertheless, information on each human MDSC subset is much less specific than it is for mice and warrants further investigations.

2.2.2. MDSC-mediated immunosuppression in cancer

MDSC suppresses immunity by perturbing both innate and adaptive immune responses. MDSC exerts its suppressive activity against T cells through diverse mechanisms [34]. One of such mechanisms is associated with L-arginine metabolism. Expression of inducible nitric oxide synthase (iNOS) and arginase 1 (Arg1) in MDSCs is dependent on the substrate L-arginine. Arg1 and reactive oxygen species (ROS) are upregulated in activated PMN-MDSCs [36, 38], whereas Arg1 and iNOS are highly expressed in activated MO-MDSCs [39]. The upregulation of either Arg1 or iNOS results in L-arginine shortage from the tumor microenvironment, leading to consequent inhibition of T-cell proliferation through multiple mechanisms such as reduction of CD3 ζ-chain expression and IFN-γ/IL-2 secretion by T cells [40]. High levels of ROS in PMN-MDSCs can induce nitrosylation of the T-cell receptor (TCR) during direct cell-to-cell communication, which contributes to the inhibition of antigen-specific T-cell activation [34]. ROS production by PMN-MDSCs is known to be induced by several tumor-derived factors such as TGF-β, IL-6, IL-10, and GM-CSF [41]. The suppressive function of PMN-MDSCs depends on Arg1 and ROS [36, 38], whereas that of MO-MDSCs requires a signal transducer and activator of transcription 1 (STAT1) and iNOS [38]. In activated PMN-MDSCs, STAT3 is highly activated, which results in increased expression levels of ROS through the upregulation of NADPH oxidase (NOX2) but not NO production [38, 39]. On the other hand, STAT1 and iNOS are highly upregulated in MO-MDSCs, resulting in increased levels of NO but not ROS production [34]. In addition, STAT6 signaling pathway is involved in the upregulation of Arg1 and TGF-β through activation of IL-4 and IL-13, leading to immunosuppressive activity [40]. However, the immunosuppressive mechanisms overlap between G-MDSCs and M-MDSCs in human cancers. iNOS is also upregulated in PMN-MDSCs in a variety of human cancers [42–44]. $CD14^{+}HLA\text{-}DR^{-/low}$ MO-MDSCs express NADPH oxidase component gp91 (phox) and produce high level of ROS in human non-small cell lung cancers [45]. These MO-MDSCs inhibit T-cell proliferation and IFN-γ secretion in a cell-contact-dependent manner.

Another mechanism of MDSC-mediated T-cell suppression is associated with cysteine deprivation from local environment [46]. Cysteine is the essential component required for T-cell activation, differentiation, and proliferation, which they cannot synthesize; rather dependent on antigen-presenting cells. Dendritic cells and macrophages can deliver cysteine to T cells by converting methionine and cystine to cysteine [47]. Like the APCs, MDSCs also import extracellular cystine for converting it to cysteine, but unlike APCs, they do not export cysteine, leading to lack of cystine from local environment for dendritic cells and macrophage [46].

The potential suppressive property of MDSCs can also be reflected on the innate immunity. Studies have shown that MDSCs impair NK-cell development, IFN-γ production, and cytotoxicity against tumor cells. This suppression is mediated by membrane-bound TGF-β1 and through downregulation of NKG2D (the primary activating receptor for NK cells) [48]. Cytotoxic activity of NK cell and their apoptogenic cytokine secretion is also disrupted by $CD14^{+}HLA\text{-}DR^{-/lo}$ MO-MDSCs secretion in a cell-contact-dependent manner in human hepatocellular carcinoma [48]. The inhibition of NK cells is independent on arginase activity. The

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similar inhibitory mechanism of NK-cell development and functions by IL-1β-induced PMN-MDSCs have been also demonstrated in a mouse model [49]. Furthermore, MDSCs can skew macrophage-derived cytokine profiles from type 1 to type 2 putatively through Toll-like receptor 4 signaling pathway [49]. This effect is amplified by macrophages that increase the MDSC production of IL-10. Indeed, increased MDSC levels in peripheral blood and tumor are closely associated with the infiltration of $CD163^+$ M2 macrophages in human esophageal cancer [50].

2.2.3. Therapeutic strategies against MDSCs

Several strategies that are currently being tested against MDSCs to break the immunosuppressive network can broadly be categorized into four groups.

A first strategy would be promoting the differentiation of MDSCs into mature, non-suppressive cells. A number of agents, e.g., all-trans retinoic acid (ATRA), have been identified as a candidate agent that possesses this ability, which favors MDSC differentiation into mature DC, macrophages, and granulocytes [51]. Treatment of ATRA on mouse or human MDSCs results in the induction of myeloid cell differentiation both in vitro and in vivo and thereby improves antitumor immune responses [51]. However, ATRA was also shown to induce the development of $CD4^+$ regulatory T cells (Tregs), by upregulating expression of the T cell cell-fate regulatory transcription factor FoxP3 [52]. Thus, ATRA is not an ideal candidate for MDSC depletion, as simultaneous Treg induction induced by ATRA treatment could further contribute to tumor development. Another promoter of MDSCs differentiation is 25-dihydroxyvitamin D3, which has been reported to drive myeloid progenitor cell differentiation both in vitro and in vivo [52, 53]. Treatment in patients with head and neck squamous cell carcinoma resulted in reduction of the circulating $CD34^+$ MDSCs with increased levels of plasma IL-12 and IFN-γ and T-cell proliferation. However, in another study, 25-hydroxyvitamin D_3 alone has failed to improve the clinical outcome [52, 53].

Second promising approach is to inhibit or block the expansion of MDSCs. Many tumor-derived factors can induce the development and expansion of MDSCs from hematopoietic precursors. Several neutralizing antibodies or inhibitors against tumor-derived factors or those receptors such as GM-CSF, GMCSF receptor (GM-CSFR), M-CSF, M-CSF receptor (MCSFR), G-CSF, VEGF-A, or stem cell factor (SCF or KI) [52–54], and MMPs have been reported to inhibit MDSC expansion or mobilization. However, anti-VEGFA monoclonal antibody, bevacizumab, could not reduce the accumulation of MDSCs in human renal cell cancer [53].

Thirdly, MDSCs can be selectively depleted in pathological settings by employing certain chemotherapeutic agents such as sunitinib, cimetidine, gemcitabine, and 5-fluorouracil (5-FU) [53]. Application of such drugs in tumor-bearing hosts resulted in a dramatic decrease in the number of MDSCs and a marked improvement in the antitumor response. Treatment with 5-fluorouracil (5-FU) selectively induced apoptosis in MDSCs, resulting in delayed tumor growth with concurrent T-cell-dependent antitumor responses. As compared to gemcitabine, 5-FU induced a more potent apoptosis-mediated MDSC depletion in vitro and in vivo. However, one study shows that a combination of gemcitabine and capecitabine does not affect the levels of MDSCs in patients with advanced pancreatic cancer [52]. Furthermore, 5-FU treatment was not curative in this tumor model because of Nlrp3 inflammasome induction, which

led to MDSC-derived IL-1β secretion and angiogenesis [53]. Attempts have also been made to deplete Gr-1$^+$ MDSCs by using anti-Gr-1 antibody that result in delayed tumor growth in mice [53]. However, this antibody also targets neutrophils, thus lacking the necessary specificity for clinical use.

Fourthly, another attractive way to control MDSC function would be interrupting the underlying signaling pathways that are responsible for the production of suppressive factors by these cells. The molecules that can be effectively targeted for this purpose include cyclooxygenase 2, arginase 1, iNOS, and indoleamine 2,3-dioxygenase [52]. Since ARG1 and iNOS are the primary enzymes responsible for MDSC immunosuppression, these enzymes are the most likely targets for novel therapeutic interventions. Various different drugs including nitro-aspirin, COX-2 inhibitors, and phosphodiesterase-5 (PDE5) inhibitors have been shown to profoundly inhibit both ARG1 and iNOS activity in MDSC. By removing MDSC suppressive mediators, these drugs exhibited a potent ability to restore antitumor immune responses and delayed tumor progression in several mouse models [52–54]. Interestingly, in addition to inhibiting MDSC function, COX2 inhibitors also blocked the systemic development of MDSC as well as CCL2-mediated accumulation of these cells in the tumor microenvironment in a mouse model of glioma [52, 54].

2.2.4. Novel therapeutic strategies against MDSCs

Although various agents such as chemotherapeutic drugs, vitamin derivative, and mAb are widely applied against MDSCs, only few of them are known to exhibit their inhibitory effect directly on MDSCs. Moreover, restricted use of these drugs on certain conditions or cancer patients often opens the possibility to apply emerging agents that bear direct inhibitory effect on MDSCs.

Earlier investigations reveal CuNG as a strong immunomodulator that successfully induce the conversion of immunosuppressive TAMs toward proimmunogenic type, enhancing Th1 response sufficient to resolve drug-resistant cancer in animal model. Interestingly, the effect of CuNG is not exclusive to TAMs; rather it also targets MDSCs which happens to be another strong negative regulator of tumor immunity [55]. Treatment with CuNG in drug-resistant mice severely abrogates the accumulation of MDSCs in spleen as well as tumor site. Further study discloses that this reduction was associated with enhanced Th1 responses (evident from increase in IFN-γ production) and diminished Th2 responses (decrease in IL-4 production) as well as decline in Foxp3$^+$Treg numbers [55]. Detailed investigation reveals the underlying mechanism that is associated with Fas-FasL interaction between MDSCs and CD4$^+$T cells. Treatment of CuNG increases the FasR expression in MDSCs and FasL in CD4$^+$T cells. Although FasL expression is enhanced, FasR expression in CD4$^+$ T cells was not observed following the treatment that determines the specificity of CuNG [55].

Another study that deals with metal chelate of Schiff's base is associated with the immunomodulation of tumor microenvironment. Metal chelate of manganese (MnNG) can have a number of valence states and possess the ability to modulate the immunosuppressive TME by reducing the number of tumor-associated MDSCs in drug-resistant tumor-bearing mice. A closer look at MnNG-mediated action reveals that it promotes MDSC differentiation into dendritic cells but not toward macrophages [56]. Additionally, it helps in the proliferation of T cells that ultimately

skew Th2 immunity toward protective Th1 response. Thus MnNG may help in the differentiation of immunosuppressive MDSCs toward dendritic cells and with the upregulation of costimulatory molecules, namely CD80 and CD86, enabling them to act as better antigen-presenting cells (APCs) that ultimately generate protective antitumor immunity [56]. However, the plausible molecular mechanism underlying MnNG-mediated differentiation of MDSCs in TME is still elusive and warrants further investigations.

2.3. T-regulatory cells in cancer

The tumor escape of immune surveillance is also achieved by the recruitment of immunosuppressive $CD4^+CD25^+FoxP3^+$ Tregs into the tumor microenvironment. The phenotype and functional properties of Tregs are similar in mice and human. They include both natural Treg (nTreg) and locally induced Treg (iTreg) cells [57, 58]. nTreg cells are derived from thymus without specific antigenic stimulation and represents a small fraction (5–6%) of total $CD4^+$ T cells. They are characterized by the expression of CD25, FoxP3, and GITR [3]. $CD4^+$ or IL-10 expressing iTreg cells are also known to express high levels of Foxp3 and GITR [58]. Induced Tregs are emerged from naive T cells by specific modes of antigenic stimulation, especially in a particular cytokine milieu [57, 58]. Both subsets possess a strong capacity to suppress the immune system, although they differ in a distinct suppressive mechanism. A large number of $CD4^+CD25^+FoxP3^+$ Treg cells are found in both the circulation and tumor site of different cancer-bearing patients and their number inversely proportional to the survival of the patients. They are either naturally occurring or locally induced Treg population [58]. Experimental observation reveals that chemokine CCL2 produced by tumor and its associated macrophages facilitate the recruitment of thymus-derived Treg cells in tumor microenvironment [3]. Defective myeloid DCs also induced IL-10^+ regulatory T cells in vitro and in vivo in patients with cancer. Conversion of Treg from $CD4^+CD25^-$ T cells depends on local cytokine milieu. Immunosuppressive cytokines such as TGF-β and IL-10, which are present at high levels in the tumor microenvironment, might mediate induction and differentiation of iTreg cells [58]. Recent studies also highlighted the role of MDSCs to facilitate the de novo development of Tregs through TGF-β dependent and independent pathways [34].

2.3.1. Tregs and immunosuppression

Tumor-associated Tregs suppress both the innate and the adaptive immune responses. The suppressive function of tumor-induced Tregs depends on the antigen-specific stimulation of these cells. Once activated, they efficiently suppress $CD4^+$ and $CD8^+$ T cells in an antigen non-specific manner [58]. This suppressive potential of Tregs depends on the cell-cell contact and soluble mediators (e.g., TGF-β and IL-10) released by Tregs. Recent evidence suggests that Treg exerts immunosuppressive function by lowering the endogenous TAA-specific T-cell immunity, thereby contributing to tumor progression [3]. Furthermore, the progressive infiltration and induction of Treg tilt the balance between Treg cells and effector T cells toward immunosuppression. This is, in part, mediated by competition for IL-2 between Tregs and conventional T cells. IL-2 promotes the differentiation of T cells into effector T cells. Interestingly, it also promotes the differentiation of immature T cells into regulatory T cells. Therefore, competitive consumption of IL-2 is also indirectly related to suppressive mechanism for Tregs in established tumors [59]. Another effective way to suppress T-cell activation and promote tolerance is to reduce the

availability of tryptophan, which is performed by the IDO+ APC in the tumor microenvironment. CTLA4+ Tregs help inducing the expression of IDO in APCs through CTLA4 to mediate their suppressive activity [60]. Furthermore, Tregs also downregulate the expression of CD80 and CD86 through CTLA-4 and induce immunosuppressive costimulatory molecule B7-H4 on DCs by IL-10 and thus hamper activation of other T cells by DCs [61]. Additionally, Treg-mediated suppression of natural killer (NK)-cell function is associated with TGF-β in tumor-bearing mice [62].

2.3.2. Therapy against Tregs

Tregs within tumor site are crucial components for tumor immunosuppression. Therefore depletion or functional inhibition could be a promising strategy for inducing antitumor immunity. Selective depletion of Tregs can be achieved by anti-CD25 antibody, which significantly improves T cell-mediated tumor regression [58, 62]. CTLA4 is another potential target. Administration of CTLA4-specific antibody with cancer vaccine effectively reduces Tregs and improves immunity [58, 62]. However, blocking of CTLA4 is strongly associated with the induction of autoimmune manifestation [58, 62]. Furthermore, this antibody fails to induce depletion of peripheral Tregls. An alternative therapeutic approach for the depletion of Tregs is the use of denileukin diftitox (Ontak®), which is a ligand toxin fusion consisting of full-length IL-2 fused to the enzymatically active and translocating domains of diphtheria toxin. The complex inhibits protein synthesis, leading to apoptosis of Tregs [3]. A recent study indicates that treatment with this complex significantly reduces the absolute number of peripheral Tregs and increases effector T-cell activation in different cancer patients. However, this requires further detailed investigation.

2.3.3. CuNG-mediated Treg modulation

Another promising approach for blocking the infiltration or functional modulation of Treg includes the application of novel metal containing drug, e.g., CuNG that might have therapeutic advantages. Previously, it was found that a sizable proportion of CD4+ cells of drug-resistant tumor-bearing mice exhibited T-regulatory markers (CD25 and Foxp3). After in vivo treatment of CuNG, only a few CD25+Foxp3+ cells were observed in tumor-bearing mice [22, 25]. Moreover, CuNG-treated TAMs could modulate TGF-β producing CD4+CD25+ T cells toward IFN-γ producing T cells with concomitant decrease in the level of FoxP3 expression, indicating that Treg can be reprogrammed toward Th1 phenotype. However, this approach requires further investigations.

3. Conclusion

Cancer is a major problem worldwide and is the most common cause of death in many countries. Although treatment with conventional chemotherapy is widely successful, it fails to induce potent antitumor immune response. Emerging evidence indicates that treatment with metal-containing agents destroy cancer cells through ROS generation and also shows the

way to cure cancer through inhibition or subvertion of immunosuppressive network toward proimmunogenic type. Strategies to target these molecular pathways by novel, cost-effective, redox-active molecules may usher a new era in immune therapy.

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