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TARGETING TUMOR MICROENVIRONMENT-ASSOCIATED IMMUNE CELLS WITH NANOPARTICLES-BASED STRATEGIES

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ABSTRACT

The propensity of cancer cells to escape the immune system and to initiate a complex balance environment is due to its ability to utilize immune cells via associated factors, including non-tumoral components such as blood vessels, fibroblasts, cells, and signaling molecules. Collectively, these are referred to as the tumor microenvironment (TME). TME is the tumor's surrounding environment and has a crucial role in promoting tumor progression, angiogenesis, metastasis, and increasing tumor resistance to several therapies. TME's immunosuppressive factors depress the immune response after altering the metabolism of immune cells within TME. Accordingly, targeting TME with nanoparticles may be a beneficial tool to regulate and enhance the efficacy of tumor immunotherapies. Here, we review distinct structural and functional properties of immune cells within TME, tumor-associated immunosuppressive factors, and the interaction between TME and immune cells. We finish with a summary of recent findings related to novel nanoparticle strategies to target TME.

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Introduction

Cancer is a major, global public health issue [1, 2]. In 2020, it was the second leading cause of death after cardiovascular diseases, with 19 million new cases diagnosed and approximately 10 million deaths [3]. Cancer occurs when a group of abnormal cells undergo uncontrolled divisions and spread through the blood and lymph nodes to destroy nearby tissues [4]. Even though conventional cancer treatments, such as chemotherapy, are widely used, they lack tumor specificity, which results in the elimination of both malignant and normal cells, thus reducing survival rates [5]. Alternative immunotherapies though are receiving increasing interest due to their ability to induce specific immune responses. However, the surrounding tumor environment is equipped with immunosuppressive factors to maintain and promote tumor growth. TME can suppress the functions of immune cells, such as antigen-presentation of dendritic cells (DCs), resulting in tumor progression. Moreover, recent evidence has shown both cellular and noncellular components of TME that promote growth, invasion, and metastasis of cancer [6]. Consequently, TME remains an obstacle in the field of combination therapies and immunotherapies [7]. Fortunately, different applications of nanotechnology have been reported to be more likely than more traditional therapies to overcome the barriers of TME and harness the immune system. To be more specific, recently, engineered nanoparticles have shown unique characteristics in improving the efficacy of cancer immunotherapies [8]. Those characteristics include reducing side effects and promoting survival rates; targeting specific tumor tissues; targeted drug delivery to tumor sites, such as anti-programmed death 1 (anti-PD-1); and antigen-presenting cell (APC) delivery to lymph nodes such as DCs [9]. Therefore, further studies of cancer biology and TME are needed. This review provides insight into the role of tumor-associated immune cells, the interaction of malignant cells with the immune system, and nanoparticle applications in the development of cancer therapies to overcome the challenges of TME.

Tumor Microenvironment

Even though cancer biology studies have been shifting towards a focus on genetic alterations of tumors, various work has also been directed to examining the functional level of TME due to its ability to secrete tumor-associated factors. Those factors include immunosuppressive and chronic inflammatory mechanisms, such as growth factors, cytokines, and chemokines [10]. Within TME, the distinct immune response has helped to distinguish tumor types. The three main types are

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as follows. First, inflammatory tumors or "hot tumors", where interaction with immune cells results in the inflammatory response. Second, immune inhibitory tumors, in which both TME and specific immune responses are dominated by inhibitory immune cells such as inactive DCs within TME. The third type, immune escape tumors or "cold tumor" immune cells, are prevented from entering TME due to a lack of major histocompatibility complex (MHC) class I and tumor-associated antigen loaded on MHC class I. As a result, cold tumors lead to the failure of immunotherapies, and many recent studies have aimed to convert cold tumors into hot tumors [11]. The immunosuppressive properties of different tumor types have been studied due to the immune system's interaction with tumor cells, which has a crucial role in tumor growth [6]. Hence, TME is a heterogeneous population that contains cancer cells, infiltrating immune cells, stromal cells, blood vessels, and cellular components that support the interaction, overlap, and cross-talk of malignant cells [12]. Tumor cells can evade immune system recognition and elimination via mechanisms that include downregulation of MHC class I molecules and the prevention of cytotoxic T lymphocytes (CTL) [13]. However, the accumulation of TME cells varies across the different stages of the tumor. In the early tumor development stage, there are hematopoietic progenitor cells (HPC), endothelial progenitor cells (EPC), fibroblasts, and inflammatory cells [14]. In early development, tumor infiltration into immune cells plays an essential role in restraining cancer progression. This includes macrophages, natural killer cells (NKs), and DCs [15]. Nevertheless, the associated factors impact the anti-tumor response by inhibiting immune cells. More specifically, TME contains various types of immunosuppressive factors and cells, which mainly include myeloid-derived suppressor cells (MDSCs), regulatory T cells (Tregs), tumor-associated dendritic cells (TDCs), and tumor-associated macrophages (TAMs). Immunosuppressive cells also include type 2-polarized macrophages (M2). The recruitment of different immunosuppressive cells is crucial in TME development and immune evasion [14, 16]. Furthermore, TME contains distinct cells – endothelial cells and immune cells representing the majority [13]. These tumor interacting cells are embedded in an extracellular matrix that facilitates cross-talk between cells and supports tumor growth **(Figure 1)** [17].

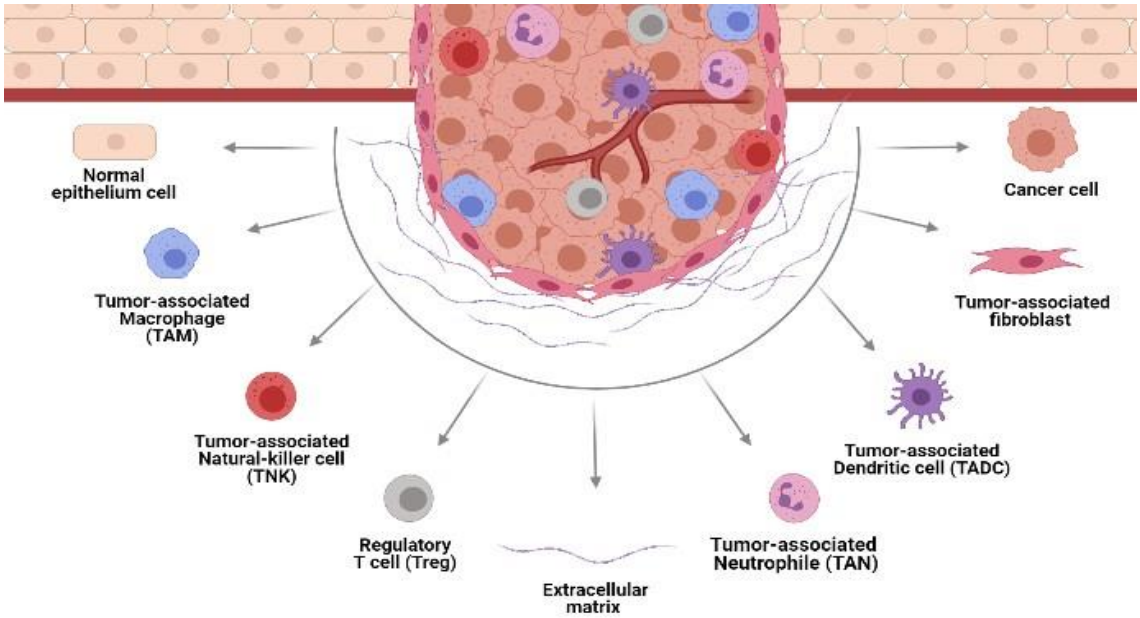

Figure 1. TME-associated immunosuppressive factors. The recruitment of different immune cell types and other immunosuppressive factors within the TME includes tumor-associated macrophages (TAMs); tumor-associated natural killer cells (TNKs); regulatory T cells (Tregs); tumor-associated neutrophils (TANs); the extracellular matrix and tumor-associated fibroblasts. Created with BioRender.com.

Myeloid-Derived Suppressor Cells (MDSCs)

MDSCs are recognized as the main immunosuppressive factor in TME, more so than in the lymphoid organs. These cells help form TME and suppress the immune response in melanoma and lung cancer patients [18, 19]. MDSCs are a heterogeneous population of immature myeloid cells that are recruited abnormally within TME. Notably, MDSCs play a crucial role in tumor escape, angiogenesis, and metastasis [19, 20]. In metastasis, the tumor cells require a favorable environment for growth and dissemination to nearby organs and tissues [21]. Accordingly, MDSCs enhance metastasis by forming the pre-metastatic niches to promote circulating tumor cells [22]. Within circulation, MDSCs are capable of surrounding and escorting the tumor cells inside the blood vessels. Thus, MDSCs contribute to metastasis development via different strategies, such as inhibiting the volume of immune cells in circulation (e.g., NKs) and increasing tumor cell extravasation [23]. Aside from their heterogeneity, the high plasticity of MDSCs in TME provides further mechanisms to suppress the immune response **(Figure 2)** [24]. For instance, MDSCs deplete the extracellular essential amino acids for T cells (e.g. cysteine) [25] via upregulation of certain enzymes such as Indoleamine 2,3-dioxygenase (IDO1) and nitric oxide synthase-2 (NOS2) [26]. IDO1 is one example of the released immunosuppressive enzymes that can increase Treg

production. Consequently, targeting this enzyme has shown great promise as a combination therapy in several pre-clinical studies [27]. Furthermore, MDSCs play a role in immune-checkpoint expression as a result of hypoxia and other immunological signals such as interferon-gamma (IFN-γ) [28, 29].

The released ATP from dying cells can be converted to adenosine by MDSCs, allowing it to proceed with its immunosuppressive activity. This includes controlling the differentiation of naïve $CD8^+$ T cells [30]. Extracellular adenosine can also block the cytotoxic activity of NKs and their ability to release cytokines and inhibit the anti-tumor response of CTL, which leads to tumor evasion [31]. Adenosine also facilitates the tumor's immune escape by enhancing tumor-associated macrophages (TAMs), which leads to them exerting their immunosuppressive functions [32]. Immunosuppressive molecules released by MDSCs include inflammatory cytokines such as Interleukin-10 (IL-10) and transforming growth factor-beta (TGF-β). Such cytokines can increase Treg levels and inhibit T effector cells [31]. Several potential therapeutic strategies aim to inhibit MDSC differentiation, migration, and recruitment via the blocking of C-X-C Motif Chemokine Receptor 1 (CXCR1) which has a key role in pre-metastatic formation [33, 34], with CXCR2 directing the migration of the MDSC to the tumor [35]. However, CXCR2 serves an essential role in the chemoresistance of different types of tumors, including breast and lung cancer [36, 37]. Therefore, a recent study aimed to target CXCR2 by IFN-γ improved clinical outcomes and proven the effectiveness of anti-PD1 therapy in pancreatic cancer [38].

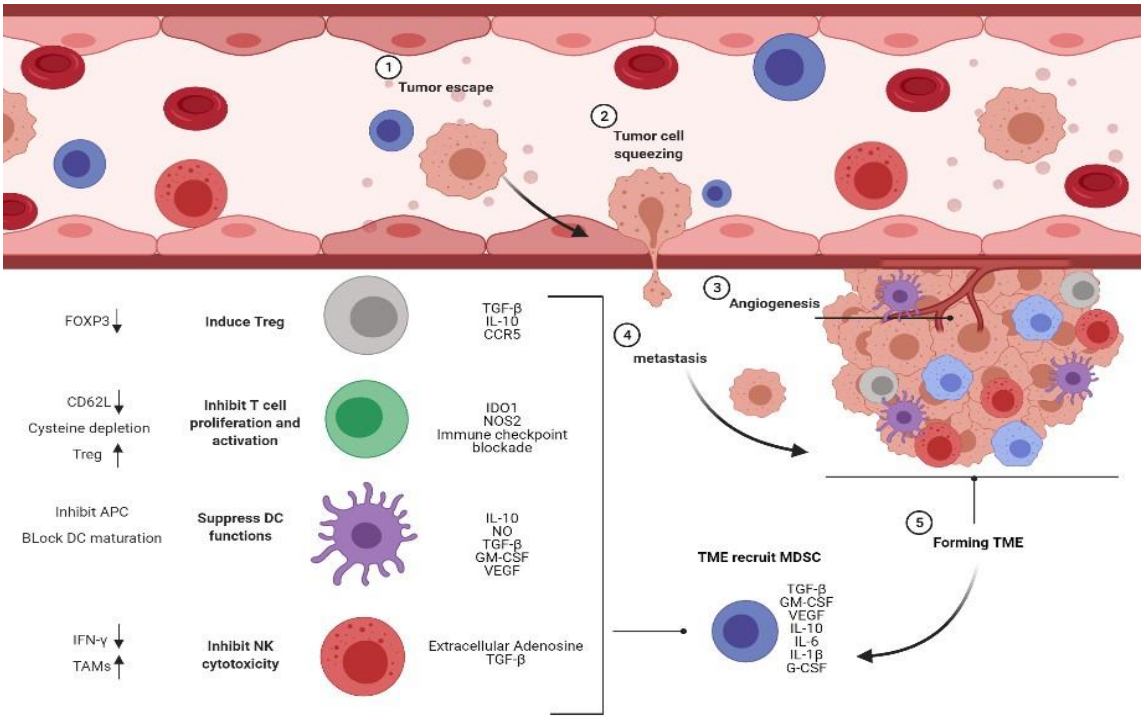

Figure 2. Immunosuppressive factors released by MDSC. Within TME, the released immunosuppressive factors of MDSCs collectively suppress the immune response and lead to tumor growth. MDSC: myeloid-derived suppressor cell; Treg: regulatory T cell; DC: dendritic cell; TAMs: tumor-associated macrophages; NKs: natural killer cells; TGF-β: transforming growth factor-beta; GM-CSF: granulocyte-macrophage colony-stimulating factor; VEGF: vascular endothelial growth factor; G-CSF:: granulocyte colony-stimulating factor; IL: interleukin; IFN-γ: interferon-gamma; NOS2: nitric oxide synthase-2; IDO1: indoleamine 2,3-dioxygenase; CCR5: CC-chemokine-receptor type 5; APC: antigen-presenting cell; TME: tumor microenvironment. Created with BioRender.com.

Regulatory T cells (Tregs)

Tregs are a subpopulation of T cells that have a role in suppressing an immune response. The growth and infiltration of Tregs are elevated by cancer-associated fibroblasts (CAFs) [39], with Tregs being a central player in regulating hemostasis and maintenance of immune tolerance. Thus, Tregs have long been recognized for their therapeutic potential in autoimmune diseases and neoplasms [40]. Nonetheless, depletion of Tregs from mouse models has been shown to increase anti-tumor immunity and reduce tumor growth [41]. Moreover, human colorectal adenomas have shown a high accumulation of Tregs surrounding the tumor [42], implying that tumor cells utilize Tregs to maintain tumor progression through inhibiting the anti-tumor immune response. Aside from $CD4^+$, Tregs express a transcription factor known as forkhead box P3 (FOXP3), which plays a crucial role in the production, differentiation, and functions of Tregs [43], including several suppressive mechanisms such as the release of inhibitory cytokines [44]. Treg-mediated metastasis and angiogenesis occur via the release of vascular endothelial growth factor (VEGF) and leads to reduced patient survival [45]. In addition, anti-tumor immunity can be downregulated by releasing several inhibitory cytokines, including TGF-β, IL-10, and IL-35 [44, 46]. Furthermore, TGF-β plays a crucial role in suppressing the immune response by inhibiting APCs such as DCs and macrophages [9]. In addition,

TGF-β secretes perforins and granzymes to target NKs and CTLs, which eventually prevents tumor elimination [47]. The complex interaction between Tregs and TME requires a more comprehensive investigation. Consequently, researchers have suggested controlling Tregs rather than eliminating them. For instance, targeting the PI3K signaling pathways of Tregs or blocking associated inhibitory cytokines [48]. At the same time, others have suggested depleting Tregs within TME as a novel immunotherapy strategy, thus preventing harm caused by Tregs outside the tumor environment [49].

Dendritic cells (DCs)

DCs are emerging regulatory cells characterized by their role as a bridge between the innate and adaptive immune systems. During infection, immature DCs differentiate into mature DCs to initiate adaptive immune response through their potent function as APC. In general, DCs can uptake antigen, process it into small peptides, and present it on their MHC class I. After maturation, DCs can migrate to a draining lymph node to activate CTLs. There are two main signals to activate CTLs: signal 1 is fully recognizing peptide-uploaded MHC class I via T cell receptors (TCR); signal 2 is the stimulation signal between CD80/CD86 and CD 28, referred to as "costimulatory molecules" [50]. Additionally, releasing cytokines is essential for the expansion and differentiation of T cells such as type I IFN and IL-12 [51]. However, TME remains a significant concern in the face of various cancer immunotherapies due to its impact on anti-tumor immunity. Furthermore, it has recently been shown that TDCs are associated with different cancer types, including breast, colorectal, gastric, and renal cancers [52]. Different suppressive factors of TME suppress the functions of DCs, such as recruitment of DCs, their maturation, and their mobilization [53, 54]. In terms of immunosuppressive factors inhibiting DC maturation, this includes IL-6, IL-10, macrophage colony-stimulating factor (M-CSF), VEGF, and TGF-β [52]. In addition to blocking maturation, IL-6 and M-CSF inhibit the differentiation of DCs to $CD14^+$ monocytes. IL-6 has also been shown to impact various functions of DC, such as inducing tolerogenic phenotypes of DCs and switching the differentiation of DCs into macrophages [55]. Preventing the recruitment of DCs is essentially associated with IL-10, which blocks DC maturation by preventing the release of IL-12 and inhibits antigen presentation [56]. IL-10 also switches differentiation from monocyte precursors to TAMs, the latter of which have a significant role in tumor angiogenesis. Additionally, they can inhibit the production of IL-12 with the secretion of a high level of IL-10 [57]. Consequently, the increasing level of TAMs reduces the monocyte and cDC1 levels which result in tumor progression [58]. The mobilization of mature DCs is another function that regulates TME-associated cytokines such as TGF-β and IL-8 [59]. Recent evidence suggests that TME impairs the APC ability of DCs within TME, which results in tumor progression [60] through the targeting of DC machinery that helps promote APC, including downregulation of the expression of MHC together with blocking of the production of cytokines [13].

Tumor-associated macrophages (TAMs)

Macrophages derived from monocytes are considered a crucial regulator of TME and are usually termed TAMs. The immunosuppressive cytokines released by these monocytes, such as TGF- β, can inhibit T cell proliferation and result in tumor growth. Among other types of infiltrating immune cells such as MDSCs and Tregs, TAMs association with poor prognosis means they are major players within TME. TAMs also support cancer cell proliferation, which leads to tumor metastasis through the production of proteolytic enzymes such as serine protease, cathepsins, and matrix metalloproteinases (MMPs) [61]. Metastasis begins with the ability of cancer cells to escape immune detection and spread through blood and lymph vessels to the surrounding stroma [62]. The characteristic of cancer cells to be able to attach to tissues is related to an event known as epithelial-mesenchymal transition (EMT). EMT shapes the cancer's biological characteristics (e.g. invasion) [63]. Recent studies report that TAMs are correlated with EMT regulation, finding that TAMs contribute to EMT by releasing multiple factors, including IL-1β, TNF-α, and TGF-β [64-66]. Notably, several recent studies report that TAMs are a major source of MDSCs, suggesting a loop of released immunosuppressive factors [67]. TAMs contribute to angiogenesis with a variety of strategies such as the interaction of $CD206^+$ with galectin-9 [68]. TAMs presence is also associated with increased vessel density for providing nutrients to cancer cells within TME [69]. And while VEGF and TGF-β are considered major players in the role of TAMs in tumor angiogenesis [70], other factors are also considered proangiogenic; notably IL-1, IL-8, CXCL8, and MMP-9 [71, 72]. It should be noted that since the differentiation and survival of TAMs depend on colony-stimulating factor-1 (CSF-1), potential therapies focused on CSF-1 are aimed at targeting TAMs. These therapies play an essential role in increasing survival rates through the inhibition of the CSF-1 receptor [73, 74]. There are two mains macrophages classified according to their differentiation status and role: classically activated macrophages (M1) and type 2-polarized macrophages (M2). M1, also known for their role as proinflammatory cells, release cytokines that help to eliminate tumor cells. For instance, M1 produces IFN-γ that enhances antitumor immunity [75]. Conversely, M2, which produces anti-inflammatory cytokines, plays a critical role in suppressing immunosurveillance and hampering T cells [76]. Accordingly, TAMs features are similar to M2 because, within TME, TAMs tend to differentiate to M2 [66]. Importantly, Arginase 1 and $CD206^+$ are classical markers of M2 [77]. Due to the correlation between M2 cells within TME and cyclooxygenase 2 (COX-2) in hepatocellular carcinoma patients, a recent study found that COX-2-blockade significantly suppresses tumor growth and invasion [78].

Natural killer (NKs)

NKs are a heterogeneous population of innate immune cells that can eliminate cancer cells without the existence of antigens. Therefore, they can kill cancers directly in the absence of MHC specificity. NKs, induce apoptosis via the release of granules

containing perforin and granzymes [79]. However, within TME, the cell-mediated function of NKs is impacted by immunosuppressive factors such as TGF-β [80]. Indeed, different studies have shown that NKs are involved in cross-talk with cancer cells [81], and facilitate tumor progression [82]. NKs in the existence of granulocyte-macrophage colony-stimulating factor (GM-CSF) can be converted into MDSCs, the latter of which have a role in metastasis as described previosley. NKs have also been associated with angiogenesis because of their increasingly high levels of angiogenic factors such as VEGF. Apart from these characteristics, NKs also restrain anti-tumor immune responses by reducing IFN-γ [82]. In general, within TME, NK phenotypes are altered to be anergy or reduced-cytotoxicity [80]. Thus, this evidence suggests that NKs are a suitable target for further development of the effectiveness of immunotherapies.

Targeting TME with nanoparticles

Many successful cancer immunotherapeutic strategies have been restricted by TME-associated factors. Taking together, the physiological and biological states of TME structure and function are closely related to cancer development and metastasis. This knowledge has helped the development of nanoparticles-based strategies for treating tumors [83-85]. Nanoparticles are small particles, with a size that ranges between 1 to 100nm, and have unique physical and chemical features; these particles can penetrate tumor tissue and blood vessels, spreading throughout the body, formerly one of the main hurdles in treating cancer [84]. Nanoparticles are used in immunotherapy for rapid and guaranteed transport of loaded material to tumor cells. The minute size of nanoparticles has helped to establish various applications in the medical field. Consequently, the concept of nanomedicine is now becoming well-established in both diagnostic and therapeutic applications. Nanoparticles mainly have a role in enhancing anti-tumor efficacy via encapsulating drugs, increasing their penetration depth, and targeting tissues [83]. In a recent study, nanoparticles were engineered by manipulating surface charge to enhance binding to proteins; resulting in what is known as “protein-based nanoparticles”, and thus improving cancer treatment [84]. Therefore, CTLs are activated by delivering antigens to DCs and then delivering DCs to the lymph node to initiate an antigen-specific immune response. In addition, the use of nanoparticles in encapsulating adjuvants or antigens is attracting considerable attention among researchers. For instance, several studies support the idea of using nanoparticles as vaccine adjuvants due to their role in low toxicity in treating and diagnosing different types of cancer [85]. In hypoxia, the rapid growth of the tumor cells leads to the distortion of the blood vessels. Thus, tumor cells recruit immunosuppressive cells, including Tregs and MDSCs, to support TME. The released factors by TME, such as VEGF and TGF-β, play multiple roles in suppressing the immune response. These include inhibiting the functions of DCs and transforming macrophages to their pro-tumorigenic state. Nanoparticles can also be used to target these components of TME and to alter the immunosuppressive environment of TME through several mechanisms. A previous study has reported targeting CD40, CD205, and CD11c; the common biomarkers of DCs. It has shown a high cellular uptake with a high IL-12 production [86]. It should also be noted that nanoparticles can harness immune responses by targeting DCs to enhance their antigen presentation and maturation processes. A recent study was aimed at encapsulating DCs with nanoparticles to penetrate physical barriers and deliver antigens to the lymph node. As a result, nanoparticles were significantly associated with increasing antigen presentation, which led to an antigen-specific immune response and IFN-γ production [87]. Additionally, a potential mechanism to induce more robust CTLs is the use of ultra-small nanoparticles such as Fe3 O4 [88]. Given that DCs are the most potent APC and capable of initiating antigen-specific immune responses, targeting DCs within TME is crucial in the development of DC-based therapeutic strategies. Remarkably, nanoparticles help the immune system utilize the DCs within TME, reactivate DCs, and inhibit tumor growth. However, much remains to be understood in terms of targeting different DCs subsets to determine the appropriate design of nanoparticles. Several studies have reported using iron oxide nanoparticles to target TAMs within TME, aiming to enhance the repolarization of macrophages from M2 to M1 [89, 90]. Consequently, the amount of TNF-α and CD86 is significantly increased along with IL-10. Targeting TAMs within TME can also inhibit tumor growth and metastasis [91]. In addition, Fe3 O4 has been used to initiate an immune-supportive environment by transforming TAMs from M2 to M1 and “cold tumor” to “hot tumor” [92]. As a result, this has helped researchers to develop a nanoparticle delivery system known as “dual-targeting”. This strategy enhances $CD4^+$ and $CD8^+$ T cell proliferation, releases antigens, suppresses Tregs, and improves the immune response of CTLs [93]. Several strategies have also aimed to enhance M1 polarization, such as chitosan and NK-loaded nanoparticles, and have shown high immune responses and low toxicity [94, 95]. In summary, targeting TAMs within TME has surpassed many obstacles of TAMs traditional therapies such as nucleic acid based-therapies. However, deeper investigation into the role of nanoparticles on TAMs will help the development of new therapeutic strategies. Targeting CAFs is one of many nanomedicine strategies for targeting the TME pathway. CAFs are associated with tumor progression and angiogenesis via the release of pro-angiogenic factors such as VEGF [96]. The formation of myofibroblasts depends on TGF-β and plays a role in facilitating tumor invasion [97]. However, Fe3 O4 nanoparticles have been found to significantly inhibit the formation of myofibroblasts [92]. Recently, a modification of ferritin-based nanoparticles has shown great promise in terms of increasing T cell infiltration [98]. Although CAFs are considered a major obstacle against cancer therapies owing to their ability to cross-talk with cancer cells, different CAFs subsets show anti-tumor effects. Thus, a recent study has suggested that, depending on the stage of tumorigenesis, CAFs have two different functional phenotypes – F1 and F2 – with a role in either supporting or suppressing TME, respectively [99]. It is now apparent then, that understanding CAF-based nanomedicine is crucial to overcoming such challenges to the development of more effective cancer immunotherapies. Notably, nanoparticles are now being used as a platform for delivering anti-angiogenic drugs, owing to their role in improving drug stability and increasing drug loading capacity. For instance, anti-angiogenic drugs, such as low

molecular weight heparin, block the VEGF signaling pathways [100]. It can thus be concluded that a combination of anti-angiogenic drugs and immunotherapy has synergic anti-tumor effects and can slow tumor growth.

Conclusion

TME complexity remains an obstacle in treating cancer and significantly affects tumor progression, angiogenesis, and metastasis. In addition, TME aims to inhibit the immune response by suppressing CTLs. In particular, TME is correlated with many of the failures of immunotherapies due to of a lack understanding of the role of TME immunosuppression. However, TME can manipulate the immune system via different strategies, such as reprogramming immune cells, releasing immunosuppressive factors, transforming TAMs from M1 to M2, and dysfunction of APC. Thus, targeting the immunosuppressive factors of TME with nanoparticles holds extreme promise for the future development of more promising anti-tumor therapies. That said, in terms of evaluating the efficacy of such cancer immunotherapies, much remains to be understood in the context of the interaction of immune cells with TME and designing the appropriate nanoparticles.

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