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Harnessing the Immune Regulatory Balance of Toll-like Receptor 9 Agonists in Cancer Immunotherapy.

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Review Article

Abstract

Toll-like receptor 9 (TLR9), a naturally existing immune regulatory site, not only takes part in enhancing anti-tumor immunity but also promoting the immunosuppressive environment of the growing tumor. TLR9 has shown its potential activity as a therapeutic target in cancer immunotherapy. However, the increased pro-tumor inflammation mediated by TLR9 should also be considered. Therefore, it is important to develop a better understanding of its regulatory mechanisms to improve the therapeutic efficacy. This review will discuss the mechanisms of immune homeostasis regulated by TLR9 signaling pathways, and also introduce the strategies of how to harness the immune regulatory balance by TLR9 agonists, CpG oligodeoxynucleotides, in cancer immunotherapies.

Keywords: Toll-like receptor 9, CpG oligodeoxynucleotides, Plasmacytoid dendritic cells, Tumor microenvironment, Immunotherapy.

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Introduction

With improved understanding of the immune system, immunotherapies, such as immune checkpoint inhibitor and chimeric antigen receptor T cell therapies, have shown unprecedented efficacy in treating cancer [1]. The success of cancer immunotherapy reveals the power of host immunity on killing cancer cells and the feasibility of unleashing restraints on anti-tumor immunity. However, the immunosuppressive tumor microenvironment (TME) and the low immunogenicity of cancer cells restrict the therapeutic efficacy of cancer immunotherapies in a fraction of patients [2-4]. Therefore, deciphering the underlying mechanisms promoting the generation of an immunosuppressive TME is urgently needed to better harness host anti-tumor immunity.

Toll-like receptors (TLRs) serve as a bridge between innate and adaptive immunity [5-7], and are naturally existing immune regulatory sites that not only take part in the enhanced anti-tumor immunity but also influence the immunosuppressive environment of the growing tumor [8,9]. TLR9, one of 13 TLR types, has been widely studied as target molecular in relation to anti-tumor therapies in clinical

trials [10]. Chemically synthesized CpG oligodeoxynucleotides (ODNs) are confirmed TLR9 agonists that directly inhibit cancer cells, as well as induce anticancer immune responses, with acceptable toxicity [11,12]. Thus, TLR9 plays a key role in multiple anticancer effects mediated by CpG ODNs. Furthermore, CpG ODNs may be exploited as immunotherapeutics or immune adjuvants to improve the efficacy of current immunotherapies.

In this review, we discuss the results of available studies to develop an improved understanding of homeostasis of the TME and regulation of the immune system triggered by TLR9 agonists. Due to the pro-tumor inflammatory cytokines produced by CpG ODNs, we also discuss options to exaggerate the anticancer effects without causing excessive inflammation promoting the development of cancer in order to improve current immunotherapies.

TLR9-mediated TME in Immunotherapies

Homeostasis of the TME

The TME consists of stromal cells, inflammatory cells, extracellular matrices as well as tumor cells. The TME not only plays an important role in the processes of tumor initiation, progression, and metastasis, but also influences the efficacy of anti-tumor therapies [1,13]. Homeostasis of the immune system is maintained by a balance between the suppressed and enhanced immune responses. However, the TME is different from the normal tissue milieu. Tumor cells and tumor-infiltrating immune cells usually suppress activation of the immune system through changes in the expression or secretion of immunosuppressive factors. This maintains homeostasis of the TME via immune-tolerance [14,15].

Under steady state conditions, cytokines secreted by tumor cells, and tumor-infiltrating immune cells, can restrain the activation and maturation of pDCs. Non-activated pDCs play a critical role in the development and maintenance of the TME through decreasing the secretion of perforin and granzyme by CD8⁺ T cells, suppressing natural killer (NK) cell-mediated cytotoxicity, and promoting the differentiation of CD4⁺ T cells into activated regulatory T cells (Tregs) [16,17]. For example, interleukin(IL)-6 interferes with the maturation of pDCs, IL-8 blocks the migration of pDCs into lymph nodes, IL-10 inhibits the further expansion of T-cells mediated by pDCs, and elevated expression of cyclooxygenase-2 interferes with pDC functions in multiple ways [8].

Non-activated pDCs (such as pDCs within tumor-draining lymph nodes and tumor-infiltrating pDCs) express high levels of suppressive immunomodulators such as indoleamine 2,3-dioxygenase (IDO). The increased expression of IDO in pDCs results in activation of stress-response kinase GCN2, which can further inhibit T cell proliferation and promote the differentiation of CD4⁺ T cells into activated Tregs by inhibiting the activation of mTORC2 and Akt signaling [8,18-23]. Apart from its contribution to Treg activation, IDO can also alter the phenotype of macrophages and neighboring cells to increase the secretion of inhibitory cytokines such as IL-10 and transforming growth factor (TGF)- β . Moreover, these macrophages and neighboring cells can inhibit the production of the positive regulator, IL-12 by pDCs [24,25]. Thus, in the context of tumor progression, the TME exhibits a multifaceted ability to play a suppressive role in the immune balance.

Although checkpoint blockade therapies can improve endogenous immune responses against tumors significantly, immunosuppressive mechanisms reduce the curative effects in some patients. It is likely that the dynamic TME can avoid immune-mediated clearance when inhibiting a single signaling checkpoint due to compensatory enhanced immunosuppressive mechanisms [26,27]. Thus, it is difficult to improve immunotherapy by only enhancing the anti-tumor effects or inhibiting the

immunosuppressive mechanisms at a single signaling checkpoint. Reprogramming the immune response would be more beneficial.

Regulation of the TME by TLR9 agonists

TLR9 participates in both positive and negative immune regulation, thereby maintaining homeostasis of the immune system. TLR9 agonists can strengthen the recruitment or activation of activated effector cells to tumor tissue and overturn the pDCs induced immunosuppression mechanisms simultaneously. In other words, CpG ODNs harness the immune regulatory balance in cancer immunotherapy, especially the mechanisms related to maturation of pDCs, is key to this understanding.

Immunomodulatory effects of CpG ODNs

pDCs, the only DCs expressing TLR9, play a key role in initiating TLR9-mediated immune responses and balancing the TLR9-triggered immune system. The maturation of pDCs is affected by the presence of some diseases such as autoimmune diseases, viral infections, and tumors, and can then influence and further shift the immune balance (Figure 1) [16,20,28,29]. In addition, T cell types mediated by activated pDCs determine the suppression or activation of immune surveillance in the TME.

Through the pathway of interferon regulatory factor-7 (IRF-7) that is expressed constitutively at high levels in pDCs, CpG ODNs can induce pDCs to secrete type I interferons (IFNs), promote pDC maturation, increase inflammatory cytokine secretion by innate immune cells, and further promote adaptive immune responses to enhance immunotherapeutic effects [16,30-32]. Furthermore, IFN- α can block angiogenesis to reduce tumor growth [33,34]. In response to stimulation by CpG ODNs, pDCs differentiate into mature DCs with upregulated major histocompatibility complexes and co-stimulatory molecules. Such mature DCs have an increased ability of antigen cross-presentation to CD8⁺ T cells [20,35,36], and promote T-cell survival, type 1 T helper cell (Th1) polarization, CD8⁺ T cells activation, memory T cell differentiation, and enhance NK cell-mediated cytotoxicity and IFN- γ production [37-49]. In this situation, the immunosuppression state can be overturned and the immune system can further exert an anti-tumor effect.

Molecular regulation mechanism of CpG ODNs

Taking TLR9 downstream signaling pathways into consideration, CpG ODNs can not only promote the secretion of type I IFNs but also the production of some pro-inflammatory cytokines, such as IL-6. Therefore, TLR9 may contribute to anti-tumor effects as well as tumor-promoting signals [40-43]. Due to the dichotomous role of TLR9, it is important to develop a better understanding of its signaling pathway and the regulatory mechanisms of downstream molecules. This may provide new approaches to improve the efficacy of current immunotherapies and decrease tumor-promoting inflammatory effects.

According to current research, the expression of TLR9 can be divided into two parts. While full-length TLR9 is expressed predominantly on the cell membrane, multiple cleaved forms are expressed mainly in the endoplasmic reticulum of pDCs and B cells. TLR9 expressed on the surface of B-cell lymphocytes can inhibit the activation of endosomal TLR9 [44,45].

When stimulated by CpG ODNs, TLR9 initiates the activation of a family of IL-1 receptor-associated kinases (IRAKs), including IRAK-1, -2, -4 and -M, after inducing the recruitment of myeloid differentiation antigen 88 (MyD88). The sequential activation of IRAKs induces an interaction with tumor necrosis factor associated factor (TRAF)-6 resulting in activation of nuclear factor- κ B (NF- κ B)

and mitogen-activated protein kinase. This will cause the most important cellular responses of inflammation or apoptosis [46]. In addition, NF- κ B activation can upregulate many tumor-promoting inflammatory cytokines. These cytokines will promote NF- κ B activation in a positive feedback loop [41]. Therefore, identifying negative regulators of NF- κ B activation, such as the over-expression of TRIAD3A, an E3 ubiquitin-protein ligase, may provide a target to avoid excessive inflammation promoting the development of cancer [47].

On the other hand, direct interactions among MyD88, TRAF-6, and IRF-7 are required in TLR9-mediated pDC activation. Furthermore, IRAK-1 and -4 can activate IRF-7 which is required for the production of IFN- α [48]. Thus, inhibitors of these molecules may disrupt anti-cancer effects. For example, transmembrane protein single immunoglobulin interleukin-1-related receptor can suppress TLR9 activation and enhance sensitivity to endotoxin shock [47]. Over-expression of MyD88 can cause the formation of MyD88 homodimers and inhibit downstream MyD88-dependent signaling pathways by preventing IRAK-1 phosphorylation through IRAK-4 [49,50]. Similarly, suppressor of cytokine signaling 1 can inhibit the TLR9 signaling pathway by suppressing IRAK-1 expression [51]. IRAK-M, dependent on TGF- β , is also a negative regulator of TLR signaling that can promote the evasion of host immune surveillance by upregulating the rate of immunosuppressive M2 phenotype macrophages [52,53]. And IRAKs are deubiquitylated by A20 which blocks TLR9 signaling [54]. In addition, the interaction of p47 (phox) with TRAF4, which inhibits TRAF-6, plays a suppressive role in TLR9-mediated signaling [55].

Overall, owing to the biphasic modulation of TLR9 signals that affect the balance of the immune system, suppressing negative regulators related to the signals of IFN- α production, and simultaneously activating negative regulators related to pro-tumor inflammatory signals may have anti-cancer effects. Although some molecules that negatively regulate TLR9 signaling have been found, their roles in tumor immunotherapy are just starting to emerge.

Clinical applications of TLR9 agonists

Available data indicate that TLR9 activation can harness the immune regulatory balance in cancer immunotherapy. There are some pre-clinical and clinical trials using CpG ODNs as an immune adjuvant to enhance the anti-tumor effects in some cancers as follows. Furthermore, CpG ODNs in combination therapies to promote pDC maturation may provide new insights into cancer treatment. However, TLR9 activation can also cause pro-inflammatory reactions. Thus, the heterogeneity of TLR9 signaling pathways in various cell types needs to be further considered and the impact of TLR9 agonists on anti-tumor responses should be evaluated.

CpG ODNs as Immune adjuvants

Although CpG ODNs have no direct pro-apoptotic effect on multiple myeloma cells, in some cases they can cause immune activation. When co-cultured with pDCs, CpG ODNs induce the maturation of pDCs that then secrete IFN- α and IFN- λ which further induce G2-phase arrest in MM cells [56,57]. Besides, CpG ODNs can not only induce pDC maturation but also activate NK cells. Intravenous administration of CpG 7909 at doses of 0.01 to 0.64 mg/kg three times a week activates NK cells in patients with refractory non-Hodgkin lymphoma [58]. Because the activation of pDCs by CpG ODNs can enhance their antigen presentation activities, combination therapies with vaccines may improve the outcome in tumor patients. However, despite the fact that CpG ODNs treated with a vaccine-derived peptide can improve the production of peptide-specific T cells as well as cytokines (such as IFN- α ,

TNF- α , and IL-2) *in vitro*, there is no significant difference in the clinical outcomes between experimental and control group patients when comparing median progression-free and overall survival times with peptide vaccines including Melan-A/MART-1 peptide, gp100, MelQbG10, ISA-51, MAGE-A3, and Wilms' Tumor nuclear protein 1 [59-61]. Nevertheless, CpG ODNs can improve the killing effects of cytotoxic immune cells on tumor cells compared with using other therapies alone.

Many studies have shown that promoting the delivery of CpG ODNs to the tumor site may improve the anti-tumor effects. For example, some researchers found that intra- or peri-tumoral injection increased the infiltration of myeloid-derived suppressor cells, which will enhance the generation and activity of M1 macrophages. In that circumstance, the tumoricidal activity of M1 macrophages, and localization and the antigen-presenting ability of pDCs, would also be increased. Thus, compared to systemic injection of CpG ODNs, intra- or peri-tumoral injection was more effective [62,63]. Furthermore, compared with systemic administration, intrapulmonary delivery of CpG ODNs in non-small cell lung cancer decreased Treg- and M2 macrophage-mediated immunosuppression, increased the number of M1 macrophages, and prompted CD4⁺ T cells to differentiate into CD8⁺ T and Th1 cells [64].

CpG ODNs combined with other therapies

Chemotherapy, radiotherapy, and targeted therapy have shown powerful therapeutic effects on tumors. Combination therapies with CpG ODNs may improve these effects by activating immune cells. For example, combined therapy of CpG ODNs with cyclophosphamide (CTX) showed better outcomes in patients with lymphoma, compared with CpG ODN or CTX therapy alone. Moreover, CTX inhibited the infiltration of Tregs into tumor sites, which enhanced the Th1 and cytotoxic lymphocyte responses [65]. Similar synergistic effects have been discovered in unresectable stage III and IV melanoma, advanced non-small cell lung cancer, and glioma [66-68].

Otherwise, CpG ODNs combined with radiotherapy result in a high complete tumor remission rate by increasing tumor-reactive memory CD8⁺ T cells, protecting normal B cells from irradiation-induced cell death, and enhancing M1 macrophage viability [69-71]. Additionally, in a phase I/II study, CpG-based *in situ* tumor vaccination improved radiotherapy outcomes with a high complete tumor remission rate, and induced systemic anti-lymphoma clinical responses by exerting direct effects on human malignant B cells and increasing tumor-reactive memory CD8⁺ T cells [72]. When combined with targeted therapies, CpG ODNs can increase the efficacy of rituximab by promoting the activation and expansion of Fc receptor-bearing NK cells. The upregulated CD20 expression mediated by CpG ODNs might further promote the killing effects of rituximab [58,73].

Perspective

Despite the fact that TLR9 agonists have shown favorable activity in many clinical trials, the results of several trials are disappointing. In particular, clinical trials in recurrent or metastatic squamous cell carcinoma of the head and neck [74], and in non-small cell lung cancer [12], have yielded poor results. The magnitude and duration of responses to immunotherapies are affected by the tumor, the host, and the microenvironment. Because of these discouraging results, the application of CpG ODNs should be individualized, and more attention should be paid to the CpG ODN type, the treatment time point, and the application strategy of CpG ODN therapy. Furthermore, the heterogeneity of TLR9 expression, the distinctive responses in different cancers, and the influence of factors as well as mechanisms involved in immune homeostasis should be considered in cancer immunotherapies.

Although TLR9 activation can harness the immune regulatory balance in cancer immunotherapy, the

increased pro-tumor inflammation and the over production of type I IFNs with the risk of causing autoimmune disease mediated by TLR9 should also be considered. By exploring the mechanism of pDC maturation, clarifying pivotal molecules that promote pDC maturation will help enhance CpG ODN-induced anti-tumor effects, in order to perform an optimal cancer immunotherapy with limiting tumor-promoting inflammatory effects and enhancing cytotoxic lymphocyte responses related signals without causing autoimmune reaction.

The comprehensive control of the immunosuppressive TME will significantly increase the effectiveness of current immunotherapies. As the concept of “cocktail therapy” has evolved [75], studies show that TME can sensitize cancer cells to DNA-damaging chemotherapies [76], cancer radiotherapy may be immunogenic [77], and antibody–CpG conjugates can improve immune stimulatory activity [78]. Therefore, combination therapies will become a new trend and the complexity of identifying optimal dosing levels and schedules for each component should also be taken into account, with better harnessing the immune regulatory balance of Toll-like receptor 9 agonists in cancer immunotherapy.

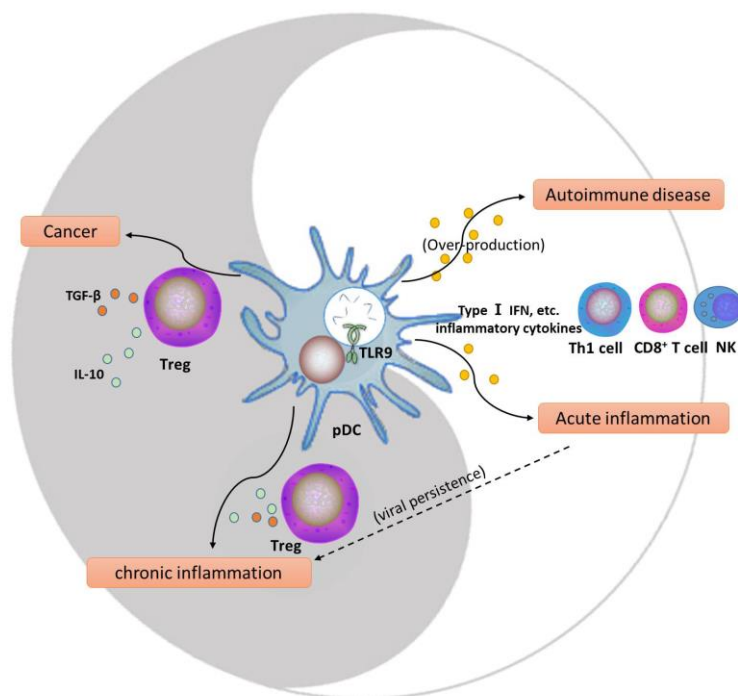


Figure 1. Toll-like receptor 9 agonists in cancer immunotherapy.

Roles of Toll-like Receptor (TLR) 9 in Disease Status

TLR9 plays a critical role in homeostasis of the immune system. Plasmacytoid dendritic cell (pDC) maturation shifts the balance from immunosuppression (dark segment) to immunoactivation (light segment). For example, in autoimmune diseases and acute inflammation, TLR9 agonists can induce mature pDCs to secrete inflammatory cytokines (such as type I IFNs) that further enhance the positive immune effects to promote type 1 T helper (Th1) cell and cytotoxic lymphocyte responses. On the other hand, pDCs may be deleted or become hypo-responsive to secrete type I IFNs, and promote CD4⁺ T cells to differentiate into activated Tregs in chronic inflammation and cancer.

List of Abbreviations

TLR: Toll-like receptor; ODNs: oligodeoxynucleotides; TME: Tumor Microenvironment; IFNs: Interferons; pDCs: Plasmacytoid Dendritic Cells; Tregs: Regulatory T Cells; IL: Interleukin; IRF-7: Interferon Regulatory Factor-7; NK: Natural Killer Cell; IDO: Indoleamine 2,3-Dioxygenase; TGF: Transforming Growth Factor; MyD88: Myeloid Differentiation Antigen 88; IRAKs: IL-1 Receptor-Associated Kinases; NF- κ B: Nuclear Factor- κ B; Th1: Type 1 T Helper; TRAF: Tumor Necrosis Factor Associated Factor; TRIF: Toll/IL-1 Receptor-Domain Containing Adaptor Protein Inducing IFN- β .

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

The datasets supporting the conclusions of this article are included within the article and its additional files.

Competing Interests

The authors declare that they have no competing interests.

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Author Contributions

LB carried out the primary literature search, drafted and revised the manuscript, and participated in discussions. LZ helped modify the manuscript. WC and JTC contributed to the coordination, and participated in discussions and literature searches. JWC carried out the design of the research and literature analysis, drafted and revised the manuscript, and participated in discussions. All authors read and approved the final version of the manuscript.

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