Tetrahedral DNA nanostructures synergize with MnO2 to enhance antitumor immunity via promoting STING activation and M1 polarization

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Abstract Stimulator of interferon genes (STING) is a cytosolic DNA sensor which is regarded as a potential target for antitumor immunotherapy. However, clinical trials of STING agonists display limited anti-tumor effects and dose-dependent side-effects like inflammatory damage and cell toxicity. Here, we showed that tetrahedral DNA nanostructures (TDNs) actively enter macrophages to promote STING activation and M1 polarization in a size-dependent manner, and synergized with Mn2+ to enhance the expressions of IFN-Î³ and iNOS, as well as the co-stimulatory molecules for antigen presentation. Moreover, to reduce the cytotoxicity of Mn2+, we constructed a TDN–MnO2 complex and found that it displayed a much higher efficacy than TDN plus Mn2+ to initiate macrophage activation and anti-tumor response both in vitro and in vivo. Together, our studies explored a novel immune activation effect of TDN in cancer therapy and its synergistic therapeutic outcomes with MnO2. These findings provide new therapeutic opportunities for cancer therapy.
1. Introduction

The host anti-tumor immunity is mainly dependent on the T cell-mediated adaptive immunity. However, innate immunity mediated by antigen-presenting cells (APCs) like macrophages and dendritic cells (DCs) as well as innate lymphocytes like natural killer (NK) cells, also plays a critical role in initiating the host defense against tumor cells1–3. Currently, PD-1/PD-L1 immune checkpoint blockade (ICB) therapy enhances the tumor-specific adaptive immunity mediated by tumor-infiltrating lymphocytes, and has been demonstrated as a powerful strategy for clinical treatment of various cancer types4–6. However, only 20%–40% of cancer patients respond to the treatments and even fewer patients achieve durable remission, due to inadequate innate immune activation7. In this regard, new strategies to effectively kill tumor cells are needed for tumor therapy.

The immunosuppressive tumor microenvironment (TME) is caused by a high density of immunosuppressive cells, including tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), regulatory T cells (Tregs) and exhausted cytotoxic lymphocytes (CTLs)8. TAMs largely express an alternatively activated (or M2) phenotype, which promote tumor progression. Therefore, it is of great significance to reprogram TAMs towards a tumor-killing M1 type. The strategy can facilitate the solution of low immune response of adaptive immunity. Clinical investigations also show that the accumulation of proinflammatory M1 TAMs is associated with longer survival in patients suffering from some solid tumors9.

Recent studies have confirmed the essential role of type I interferons for immune responses to eradicate tumor cells. Interferons trigger transcription of diverse genes, which results in both direct (on tumor cells) and indirect (through immune regulations of macrophages, DCs, T cells, NK cells) tumor-killing effects10. Stimulator of interferon genes (STING) is a cytosolic DNA sensor that is highly expressed in APCs, and functions to recognize certain types of DNA, such as the uptake of tumor-derived DNA by APCs. Activation of STING subsequently induced the production of IFN-β and interferon-stimulating genes (ISGs), as well as NF-κB-dependent pro-inflammatory cytokines11. It is well known that STING/type I IFN pathway is critical in anti-viral response and autoimmunity12. Recently, it is also reported that activation of STING/IFN-β promotes the host anti-tumor immunity13. Preclinical studies have demonstrated that treatment with the STING agonist cGAMP suppresses tumor growth by promoting antigen presentation and recruiting CD8+ T cells to the TME14. Interestingly, recent advances reveal that manganese (Mn2+) dramatically enhances the host anti-tumor immune responses mediated by STING/type I IFN pathway15. However, the dosage of manganese ions application in vivo needs attention due to its neurotoxicity16. To date, the results of clinical trials for STING agonists in cancer therapy are still unsatisfactory, due to the poor penetration efficiency of current STING agonists as well as the inflammatory side-effects prone to occur17. In this regard, scientists put many efforts to develop anti-tumor therapy based on optimal STING activation strategies, with higher efficiency and fewer side-effects.

DNA nanostructures were first introduced by Seeman18 in 1980s. Since then, numerous DNA nanostructures are constructed and applied in drug delivery or bioengineering. Among these nanostructures, tetrahedral DNA nanostructures (TDNs) have showed excellent potential in drug delivery and biomedical treatment. TDNs can easily enter cells without transfection agents or electronic transfection. In addition, TDNs display little cytotoxicity to various cell types19, and function to influence many cell biological events, such as promoting cell proliferation and migration20,21, maintaining the chondrocyte phenotype22, regulating anti-oxidative activity23. However, the function of TDNs on host immune response remains largely unknown.

In this study, we demonstrated that TDNs promoted the activation of STING/type I IFN pathway in macrophages, and induced M1 polarization. Then we explored the size effect of TDNs and found that T17 (TDN with 17 bp side length) activated macrophages with the best efficiency. Moreover, in vitro and in vivo studies showed a synergy of T17 and manganese in augmenting IFN-β production and M1 polarization as well as anti-tumor immune responses. Importantly, T17-MnO2 (release Mn2+ intracellularly) displayed much stronger effects than T17 plus Mn2+ in macrophage activation and anti-tumor immunity. These findings identify a novel biological function of TDNs, which may provide potential strategies for cancer immunotherapy.

2. Materials and methods

2.1. Reagents

All DNA single-strands of TDNs used in this work were synthesized by Sangon Biotech (Shanghai, China); TE and TM buffer were purchased from Sangon Biotech (Shanghai, China); Cell Counting Kit-8 was purchased from Glpbio (GK1001, Montclair, CA, USA); TRizol was purchased from Invitrogen (15596018, Carlsbad, CA, USA); Reverse Transcriptase, GoTag qPCR Master Mix and Lactate Dehydrogenase Assay Kit were purchased from Promega (M1705, A6002, G1780, Madison, WI, USA).

2.2. Studies in animals

Female C57BL/6J(B6) mice (age, 6–8 weeks; body weight, ~20 g) were purchased from the Animal Supply Center of Sun Yat-sen University. Tmem173+/− (STING knockout) mice were purchased from Model Animal Research Center of Nanjing University. All experiment protocols were approved by the Ethics Committee Board for Human and Animal Experiments in Zhongshan School of Medicine of Sun Yat-sen University. The procedure was performed in accordance to the National Commission for the Protection of Subjects of Biomedical and Behavioral Research guidelines for animal experiments. All efforts were made to minimize suffering.

2.3. Murine tumor models

Hepa1-6 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS and 100 IU/mL.
penicillin/streptomycin. Hepa-1-6 cells (2.5 × 10^5) resuspended in 100 μL of 0.9% NaCl were injected subcutaneously into C57BL/6J(B6) mice. To examine the antitumor function of different treatment strategies, tumors were treated via intratumoral injection with 100 μL of either vehicle control (0.9% NaCl), Mn^{2+} (50 mmol/L), MnO_2 (2 mmol/L), T17 (3 μmol/L), T17 plus Mn^{2+} or T17-MnO_2 (first treatment on Day 8, second treatment on Day 14, third treatment on Day 17). Tumor growth was monitored every other day after treatment. Tumor volume was calculated by the following Eq. (1):

\[
\text{Tumor volume} = 0.5 \times \text{Length} \times \text{Width}^2
\]

Tumor sections were harvested after 12 days’ treatment and stained with hematoxylin–eosin staining (H&E).

### 2.4. Tumor-infiltrating leukocytes analysis

Tumors were isolated and harvested on Day 12 after indicated treatments, and then processed with a tissue processor (Milenyi, Gentle MACS Octo, Bergisch Gladbach, Germany), and digested in a solution of 1 mg/mL Collagenase Type II (Gibco, 17101015, Carlsbad, CA, USA) in RPMI-1640 media (Gibco, A1049101), with a constant temperature shaker at 37 °C, with 100 μL of either vehicle control (0.9% NaCl), Mn^{2+} (50 mmol/L), MnO_2 (2 mmol/L), T17 (3 μmol/L), T17 plus Mn^{2+} or T17-MnO_2 (first treatment on Day 8, second treatment on Day 14, third treatment on Day 17). Tumor growth was monitored every other day after treatment. Tumor volume was calculated by the following Eq. (1):

\[
\text{Tumor volume} = 0.5 \times \text{Length} \times \text{Width}^2
\]

2.5. Preparation of TDNs

Specific DNA single strands of TDNs as shown in Table S1 were mixed in the same concentration in TM buffer (10 mmol/L Tris–HCl, pH 8.0, 50 mmol/L MgCl_2). The mixture was heated to 95 °C for 10 min and then rapidly cooled to 4 °C for 10 min. Agarose gel electrophoresis was used to demonstrate the successful synthesis of TDNs.

### 2.6. Assembly and characterization of T17-MnO_2

A mixed aqueous solution of 20 mL of tetrathylammonium hydroxide (0.6 mol/L) and H_2O_2 (3 wt%) was added to 10 mL of MnCl_2 (0.3 mol/L) solution. The mixture was stirred at room temperature overnight to obtain MnO_2. The prepared MnO_2 was centrifuged at 2000 rpm (Eppendorf, 5424, Hamburg, Germany) for 20 min and washed with a large amount of distilled water and methanol, and then dried at 60 °C. Then 10 mg of the sample was dispersed in 10 mL of water, and the MnO_2 nanosheets were obtained by ultrasonic treatment for 4 h. T17 was incubated with MnO_2 nanosheets at room temperature for 2 h to obtain T17-MnO_2. The synthesis of T17-MnO_2 was characterized by the nanoparticle size analyzer.

### 2.7. Cell isolation and culture

C57BL/6(B6) mice were sacrificed by cervical dislocation, and the femur and tibia were separated to expose the bone marrow cavity. Washed out the cells from the bone marrow cavity, and lysed the erythrocytes. Then collected the rest bone marrow cells by centrifugation. The collected cells were cultured in 10% FBS DMEM medium containing 30% L929 cell supernatant for 7 days to induce them differentiating into the bone marrow-derived macrophages (BMDMs).

### 2.8. Activation of T cells in vitro

C57BL/6(B6) mice were sacrificed, and the spleens were isolated and ground. The erythrocytes were lysed, and the rest cells were collected by centrifugation. Biotinylated CD3/CD4/CD8 antibody mixture was added into cells (per 1 × 10^6 cells in 5 μL) and incubated at room temperature for 30 min. Then the magnetic beads were added into cells (per 1 × 10^6 cells in 5 μL) and incubated at room temperature for 30 min. The cells were transferred into the flow tube and placed on the magnetic rack, incubated at room temperature for 8 min. The supernatant was carefully absorbed and removed from the magnetic rack. One time sorting buffer was added and gently blown for 10–15 times to resuspend the cells, then put back into the magnetic rack and incubated for 6 min and the CD3^+ /CD4^+ /CD8^+ T cells were obtained. Then T cells and BMDMs were added into 96-well U-bottom plate in a ratio of 2:1 and co-cultured for 3 days.

### 2.9. Real-time polymerase chain reaction analysis

Total RNA was isolated from cell lysates with TRizol. Revert aid first strand cDNA synthesis kit was used to prepare cDNA. Then, real-time PCR reactions were performed by CFX96 Real-Time PCR System (Bio-Rad, Hercules, CA, USA). Sequences of primer pairs are listed in Table S2.

### 2.10. Western blot

Cells were lysed in 1× RIPA lysis buffer with protease and phosphatase inhibitors. And then the proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to polyvinylidene difluoride membranes. The target membranes were incubated with primary antibodies: anti-β-actin (Sigma–Aldrich, A1978, St. Louis, MO, USA), GAPDH (Sigma–Aldrich, G9295), iNOS (CST, 13120, Danvers, MA, USA), Arginase-1 (CST, 93668), phospho-TBK1 (Ser172) (CST, 5483), TBK1 (CST, 38066), phospho-IRF3 (Ser386) (CST, 37829), IRF3 (CST, 11904) overnight at 4 °C, followed by incubation with secondary antibodies. The membranes were visualized with New-SUPER ECL (KeyGEN, KGP1128, Nanjing, China) according to the manufacturer’s instructions.

### 2.11. Flow cytometry analysis

Single-cell suspensions were prepared from BMDMs or fresh mouse tumor tissues. The cell suspension was filtered and stained with Alexa Fluor 488-labeled anti-CD3, PE-labeled anti-CD4, and Cy7-labeled anti-CD8 antibodies against following molecules were purchased from BioLegend (Beijing, China): anti-CD11b (101206), F4/80 (123116), CD11c (117318), Ly-6G/Ly-6C (108428), CD3 (100218), CD4 (100406), CD8 (100714), NK1.1 (108728), iNOS (696806), CD69 (104508), CD86 (105014), CD206 (14170), MHCII (107608), PD-1 (135216).

For intracellular cytokine analysis, 3 × 10^5 cells were seeded in a 96-well plate in DMEM containing 10% FBS and supplemented with PMA/Ionomycin/Brefeldin A cocktail. After 4 h, cells were washed, stained with surface antibodies for 30 min, and
then fixed with fixation buffer and subsequently stained intracellularly with antibodies against IL-1β (Invitrogen, 2016884), IFN-γ (BioLegend, 505808), TNF-α (BioLegend, 506306) and granzyme B (BioLegend, 515408). After 45 min, cells were washed and fixed in a solution of 1% paraformaldehyde.

2.12. Statistical analysis

The statistical analysis was conducted by GraphPad Prism/Excel software, and the data were shown as mean ± standard deviation. The difference between the two groups was analyzed by Student’s t-test, and the difference between multiple groups was analyzed by one-way ANOVA. P < 0.05 indicated a statistically significant difference.

3. Results

3.1. Synthesis and features of TDNs

As the scheme showed (Fig. 1A), TDNs were synthesized by four single-strand (ss) DNAs that are self-assembled by complementary pairing. The stabilities of assembled TDNs vs dsDNA in the presence of serum were examined, and the results showed that structured TDNs displayed more stabilities than dsDNA (Fig. 1C).

It has been reported that TDNs can enter the cell without transfection agents or electronic transfection, by virtue of their unique three-dimensional structure. To access whether the TDNs enter the cell in a size-dependent manner, we next assembled five TDNs in different sizes, namely T7, T13, T17, T26, and T37, respectively, based on the length of ss DNAs assembling the TDNs. Agarose gel electrophoresis data demonstrated the successful assembly of the indicated TDNs with different sizes (Fig. 1B, Fig. S1A–E). Then we synthesized TDNs with one Cy5-labelled ss DNA and three unlabelled ss DNAs using the same nuclear acid sequence as shown in Table S1, and traced their cellular uptake by BMDMs. At 1 and 3 h after incubating with Cy5-TDNs in different sizes, including T7, T13, T17, T26 and T37, the fluorescence of intracellular Cy5-TDNs in BMDMs were examined by flow cytometry. The cellular uptake efficiency of the indicated TDNs in different sizes was comparable, and more than 95% BMDMs were Cy5-TDN positive after 3 h incubation (Fig. 1D). To explore whether tetrahedral DNA actively or passively enter the macrophages, we synthesized Cy5-labelled ssDNA (Cy5-a) as well as Cy5-ab and Cy5-abc, which is composed of one Cy5-labelled ss DNA plus one or two unlabeled ss DNAs, respectively, under the same conditions as TDNs synthesis, and then traced their cellular uptake by BMDMs. The fluorescence of intracellular Cy5-a, Cy5-ab or Cy5-abc in BMDMs was examined by flow cytometry, and the data showed that less than 15% BMDMs were Cy5 positive after 3 h incubation of the unassembled DNAs above (Fig. 1E), indicating that it is TDNs rather than ssDNA that can actively enter the macrophages.

3.2. T17 activates the STING/IRF3/type I IFN pathway in macrophages and favors M1 polarization

So far, the biological functions of TDNs are still unclear. Considering TDN is a kind of DNA materials, we next examined whether TDNs can activate the cytosolic DNA sensor and downstream type I IFN pathway, and if so, whether there is a size-dependent effect. Primary mouse BMDMs were incubated with five TDNs with different sizes as mentioned above, and then analyzed for the STING activation and type I IFN production. Among the indicated TDNs, T17 showed the most prominent effects in up-regulating the mRNA expression of Ifnb as well as the protein levels of phosphorylated TBK1 and IRF3 (Fig. 2A and B). Therefore, T17 was selected for subsequent experiments. After treatment of T17, cell viability of BMDMs was examined and the results showed that T17 had no cytotoxicity to BMDMs (Fig. 2C). It is reported that TDNs up-regulated the mRNA level of Inos and Tnfa in mouse macrophage-like RAW264.7 cells. Our PCR data showed that T17 treatment in primary BMDMs increased the expression of M1-related genes, including Stat1, Inos, Tnfa, Il1b, Cd86 and Cd74, but decreased the expression of M2-related genes like Arg1 and Cd206. The expression of the genes relating to activate STING/type I IFN pathway was also enhanced in T17-treated vs control group (Fig. 2D). Western blot results further confirmed that TDN-enhanced INOS expression in macrophages in a size-dependent manner, and T17 showed the best effects in INOS induction (Fig. 2B). T17 up-regulated the protein level of INOS and down-regulated ARG-1 in BMDMs (Fig. 2E). Flow cytometry data also demonstrated that T17 increased the production of INOS, TNF-α and IL-1β by BMDMs (Fig. 2F, Fig. S2A–C). To further examine whether the T17-promoted M1 polarization depends on STING signaling, WT and STING KO (Tmem173−/−) BMDMs were treated with T17, and then analyzed for M1 polarization. Real-time PCR data showed that T17 still promoted Inos expression in STING KO BMDMs, although the
T17-mediated Inos increase was much lower than that in WT BMDMs (Fig. 2G), indicating that T17-induced M1 polarization partially depends on STING. Furthermore, we tested the efficiency of synthetic ss DNAs (a, ab, abc) vs TDNs in macrophage activation and found that only TDNs can induce the production of Ifnb and Inos, whereas other ss DNAs failed to activate macrophages (Fig. 2H and I). Since the synthetic TDNs contain little ssDNA, and displayed a high stability during 12 h in the serum (Fig. 1B and C), we speculate that it is TDNs that actively enter the macrophages and induce the macrophages activation. Collectively, these results suggested that T17 promotes the activation of STING/type I IFN pathway and M1 polarization.

### 3.3. T17 and manganese ion synergistically enhance the immune response

Recent studies have suggested that Mn^{2+} and Mg^{2+} promotes the activation of cGAS-STING/type I IFN pathway. Moreover, it is also reported that Ca^{2+} functions as second messenger in mediating signaling transduction. In this regard, we next tested whether T17 cooperates with distinct divalent metal ions to enhance the immune functions of macrophages. After treatment with these three divalent metal ions (Mn^{2+}, Mg^{2+}, Ca^{2+}), the cell cytotoxicity was examined. The results showed that within the concentration of 50 μmol/L, divalent metal ions had no obvious cytotoxicity to BMDMs, while at a concentration of 100 μmol/L, manganese ions exhibited a cytotoxic effect on BMDMs. Accordingly, BMDMs were treated with T17 and distinct metal ion solutions within 50 μmol/L. Real-time PCR data showed that only Mn^{2+} significantly up-regulated the mRNA levels of Ifnb, Isg15 and Inos in a concentration-dependent manner (Fig. 3A‒C). More importantly, the combination of T17 with Mn^{2+} acted synergistically to enhance the activation of STING/type I IFN pathway and M1 polarization (Fig. 3D). Also, the phosphorylation and dimerization of STING protein were detected by Western blot, and the data further confirmed that co-treatment of T17 and Mn^{2+} synergistically induced STING activation (Fig. 3E).
Moreover, the effects of T17 and Mn2⁺ on antigen presentation and T cell activation were investigated. T17 also rapidly entered CD4⁺ T cells (Fig. S3B) and CD8⁺ T cells (Fig. S3C). However, direct treatment of T17 and Mn2⁺ in T cells did not promote the expression of CD69 and IFN-γ in CD4⁺ T cells, or CD69 and granzyme B (GZMB) in CD8⁺ T cells, suggesting that T17 and Mn2⁺ did not directly influence the T cell function. While after co-culture for 3 days with BMDMs pretreated with Mn2⁺, T17 or T17 plus Mn2⁺ (pt-BMDMs), the expression levels of the above molecules were slightly increased in T cells when co-cultured with BMDMs pretreated with Mn2⁺ or T17, and dramatically increased in T cells co-cultured with T17 plus Mn2⁺-pretreated BMDMs (Fig. 3F–I). These results indicated that combination of T17 with Mn2⁺ synergistically improved the antigen presentation activities of BMDMs, thereby leading to the sequential activation of T cells co-cultured with macrophages.

3.4. **T17-MnO₂ enhances the immune response of BMDMs**

Since manganese ions exhibited a cytotoxic effect at a concentration more than 100 μmol/L, we next constructed a MnO₂ nanosheet which can react with intracellular GSH and slowly release Mn²⁺ in the cellular compartment. T17 was adsorbed onto the MnO₂ nanosheet to assemble T17-MnO₂ (Fig. 4A). We characterized the synthesis of T17-MnO₂ by nanoparticle size analyzer. The diameter of T17-MnO₂ was significantly larger than that of T17 and MnO₂ (Fig. 4B). And the electronegativity of T17-MnO₂ was much greater than that of T17 and MnO₂ (Fig. 4C), suggesting that the electronegative DNA structure successfully adsorbed onto the surface of MnO₂ nanosheets. These data indicated the successful synthesis of T17-MnO₂.

Next, the cytotoxicity of T17-MnO₂ was examined by detecting the release of LDH from BMDMs after treatment and the results...
showed that T17-MnO₂ displayed no cytotoxicity to BMDMs (Fig. 4D). Furthermore, we investigated the potential of T17-MnO₂ on macrophage activation and anti-tumor therapy. Real-time PCR and Western blot data showed that T17-MnO₂ remarkably enhanced the Ifnb mRNA expression (Fig. 4E) and TBK1 phosphorylation in BMDMs (Fig. 4F), suggesting that T17-MnO₂ activated the STING/type I IFN pathway in macrophages. In addition, after T17-MnO₂ treatment, the protein level (Fig. 4G and Fig. S4A) and mRNA levels (Fig. S4B) of M₁ marker INOS in BMDMs was significantly elevated. Besides, the expression of costimulatory molecules including CD86 (Fig. 4H) and MHCII (Fig. S4C) was increased, while the expression of CD206, a surface marker for M₂ macrophages, was significantly reduced (Fig. 4I). These results together confirmed that T17-MnO₂ promoted antigen presentation and favored M₁ polarization in macrophages.

3.5. T17-MnO₂ dramatically improves the anti-tumor response

Next, we wonder whether T17-MnO₂ play a role in anti-tumor immunity. Considering Hepa1-6 cells have the advantages of short time and high successful rate when used to establish tumor models. Besides, the pathological process of the tumor models is similar to that of its clinical counterpart²⁵,²⁶. C57BL/6J (B6) mice were subcutaneously inoculated with Hepa1-6 cells, and then received intratumor injections of vehicle control (0.9% NaCl), Mn²⁺ (50 mmol/L), MnO₂ (50 mmol/L), T17 (200 nmol/L), T17+MnCl₂ and T17-MnO₂ for 24 h. Compared with the control group, treatment with Mn²⁺, MnO₂ slightly reduced the tumor growth, while treatment with T17, T17 plus Mn²⁺ and T17-MnO₂ dramatically suppressed the tumor growth. Meanwhile, the weight change of the mice in each group after the treatment was comparable (Fig. 5D). Moreover, mice-bearing Hepa1-6 xenograft displayed a much stronger efficacy in suppressing tumor growth after treatment with T17-MnO₂, or T17 plus Mn²⁺, when compared with that in T17-treated group. To be noted, although T17-MnO₂ and T17 plus Mn²⁺ showed potent anti-tumor activities, the usage of Mn²⁺ in T17 plus Mn²⁺ group was twenty-five times more than that in T17-MnO₂ group, indicating that T17-MnO₂ complex had a higher anti-tumor efficacy and less cytotoxicity than free Mn²⁺ plus T17. H&E staining revealed tumor necrosis in the T17-MnO₂ group (Fig. 5E).

Next, we evaluated the role of T17-MnO₂ in host antitumor immune response. Tumor-infiltrating cells from each group were isolated and analyzed by flow cytometry. Intratumoral treatment with T17-MnO₂ slightly increased the percentage of CD11b⁺Gr1⁺ neutrophils (CD11b⁺Gr1⁺), but dramatically increased the percentages of antigen-presenting cells including dendritic cells (DCs, CD11b⁺CD11c⁺) and macrophages (CD11b⁺F4/80⁺) (Fig. 6A and Fig. S5A). Additionally, the expression levels of CD86 on the surface of DCs and macrophages were increased after T17-MnO₂ treatment, while the CD206 expression on the surface of macrophages was down-regulated (Fig. S5B–S5D).
T17-MnO₂ also up-regulated the mRNA expression of *Ifnb* and *Inos* in TILs (Fig. S5E and S5F). Moreover, tumor-infiltrating natural killer (NK) cells, CD4⁺ (helper) and CD8⁺ (cytotoxic) T cells increased the most in T17-MnO₂-treated mice, among all the indicated groups (Fig. 6B‒D and Fig. S6A). And the CD4⁺ T cells isolated from T17-MnO₂-treated mice contained a higher proportion of effector and memory T cell subsets (Fig. 6E). The proportion of activated lymphocytes among a subpopulation of helper T cells (CD3⁺CD4⁺/CD3⁺CD4⁺CD69⁺) and cytotoxic T cells (CD3⁺CD8⁺/CD3⁺CD8⁺CD69⁺) was also higher than that of other groups (Fig. S6B and S6C). More importantly, treatment with T17-MnO₂ increased the cytokine production in tumor-infiltrating T cells, including IFN-γ (Fig. 6 F) and TNF-α (Fig. S6D) in CD4⁺ T cells, as well as granzyme B in CD8⁺ T cells (Fig. 6G), and the expression levels of immune checkpoint programmed death-1 (PD-1) on tumor-infiltrating CD8⁺ T cells also decreased slightly (Fig. S6E). Taken together, our results demonstrated that T17-MnO₂ induced a potent host anti-tumor immune response and therefore inhibited the tumor growth with a high efficacy.

4. Discussion

Tumor-infiltrating immune cells play a critical role in regulating tumor growth. To date, a variety of anti-tumor immunotherapies have been developed and displayed a great potential in clinical cancer treatment27. However, most of the current anti-tumor immune therapies are mainly focused on tumor infiltrating lymphocytes (TILs) and only benefit a small percentage of cancer patients, particularly those with immune active (“hot”) tumors. Nonetheless, most of the immune therapies targeting TILs are failed in treating “cold” tumors, in which T cell infiltration is not prevalent and immunosuppressive immune cell infiltration is high28. Recent studies have demonstrated the critical role of cGAS-STING/type I IFN pathway in host anti-tumor immunity10,29,30. However, clinical trials of the STING agonists, cyclic dinucleotides (CDNs) in tumor therapy do not succeed, due to the poor penetration ability, low stability and inflammatory side effects of CDNs31. Thus, the present study was designed to develop a novel anti-tumor immune strategy based on highly efficient activation of STING/type I IFN signaling.

In the present study, we demonstrated that TDNs actively entered the macrophages within a short time period. Moreover, TDNs showed a stronger stability in serum and more resistance to DNase, when compared to classical dsDNAs. So far, a variety of DNA nanostructures have been designed and assembled, but only TDNs have been reported to be able to actively enter the cells without transfection, and display little cytotoxicity to various cells18. These features endow TDNs a great potential during *in vivo* application. However, most of the studies on TDNs are mainly focused on drug
delivery and biosensor detection, whereas the function of TDNs on host immune response remains largely unknown.

Our study demonstrated that TDNs induced STING activation and expression of \( \text{Ifnb} \) and \( \text{Isgs} \). It is well known that STING directly recognizes CDNs like cGAMP and c-di-GMP with a high affinity\(^{10}\). Recently studies demonstrated that cGAMP was transferred to bystander cells via VRACs\(^{32}\). However, the targetability and drug-loading capacity of cGAMP need to be improved. It has been reported that TDNs can be easily modified with aptamers or peptides to direct specific binding to the targets on tumor sites\(^{33,34}\). TDNs also display a potential in drug delivery system, by loading small molecule drugs or chemical inhibitors. In this regard, it is possible to develop TDNs loaded with certain inhibitors (e.g., inhibitors of the NF-\( \kappa \)B pathway) to reduce the side effects (e.g., inflammatory cytokine storm) and achieve the best anti-tumor effects. Therefore, using TDNs which can solve these problems is a promising, relative low-cost method to activate STING pathway. Moreover, STING also functions as a central molecule of various cytosolic DNA sensors to induce type I IFN production. In addition to CDNs, STING activation may also be triggered by many other kinds of DNAs, including viral or bacterial DNA, as well as DNA released from mitochondria or dead cells\(^{35}\). Nonetheless, these DNAs are not suitable for \textit{in vivo} STING activation, for their poor penetration ability and low efficacy.

![Figure 6](image-url)

**Figure 6**  
T17-MnO\(_2\) promotes anti-tumor responses by activating both innate and adaptive immunity. Hepa1-6 growth and mice treated with vehicle control (0.9% NaCl), Mn\(^{2+}\) (50 mmol/L), MnO\(_2\) (2 mmol/L), T17 (3 \( \mu \)mol/L), T17 plus Mn\(^{2+}\) or T17-MnO\(_2\). Mice were sacrificed and tumor-infiltrating leukocytes were analyzed by flow cytometry on Day 20. (A) Percentage of neutrophils (CD11b\(^+\)Gr1\(^+\)), dendritic cells (DCs, CD11b\(^-\)CD11c\(^+\)) and macrophages (CD11b\(^+\)F4/80\(^+\)) were analyzed. (B–D) Tumor-infiltrating CD4\(^+\)/CD8\(^+\) T cells and natural killer (NK) cells. (E) Frequency of naïve cells (CD44\(^-\)CD62L\(^+\)), effector/effector memory cells (CD44\(^+\)CD62L\(^-\)) and central memory cells (CD44\(^+\)CD62L\(^+\)) in tumor-infiltrating CD4\(^+\) T cells. (F) IFN-\( \gamma \) in CD4\(^+\) T cells and (G) granzyme B in CD8\(^+\) T cells. Data are presented as mean ± SD (\( n = 4 \)). *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \), ****\( P < 0.0001 \).
The activation of STING largely depends on the role of cGAS, an enzyme that catalyzes the formation of cGAMP from GMP and AMP. It is reported that dsDNA activates cGAS in a size-dependent manner, and only those dsDNA with a length more than 20 bp can induce the phase separation of cGAS and sequential activation. In the present study, we constructed five types of TDNs with distinct sizes and DNA sequences (T7, T13, T17, T26, and T37), and demonstrated that T17 displayed the highest efficiency in promoting STING activation and Iσβ production. These results indicated that TDNs activated STING in a size-dependent manner, but this effect of TDNs had nothing to do with their DNA sequence. Since the cell entrance efficacy of all the five TDNs was comparable, we speculated that the size of T17 may be the most suitable to the DNA binding site of STING.

Several divalent metal cations have been reported to participate in modulating immune response. For example, Ca²⁺ functions as an intracellular second messenger to enhance signal transduction. Mn²⁺ dramatically increases the sensitivity of cGAS to dsDNA and promoted STING activation. Whereas Mg²⁺ promotes the recruitment and polarization of monocytes/macrophages, and slightly up-regulates cGAS-STING activation. Our results demonstrated that at a concentration of 25 or 50 μmol/L, only Mn²⁺ displayed a synergy with TDN in promoting Iσβ, Iσg15 and Inos expression. A recent study by Chen et al. presented a Mn-cGAMP nano vaccine (NVs) to direct cytosolic co-delivery of cGAMP and Mn²⁺ to potentiate the anti-tumor immune response. Our studies demonstrated that Mn²⁺ and TDNs not only synergistically strengthened the activation of STING/type I IFN pathway, but also promoted the M1 polarization and antigen presentation in primary macrophages. The data showed that T17-induced M1 polarization partially depended on STING, indicating that T17 might activate other pathways leading to M1 polarization. It is reported that activation of other DNA sensors such as TLR9, AIM2, LRRFIP1, DAI, sometimes also contribute to NF-κB activation. Type I IFN production, or M1 polarization, and this process is independent of STING. Since STING is the central molecule of various DNA receptor pathways, we speculate that the T17-induced M1 polarization is mainly dependent on STING.

To be noted, studies have demonstrated that an excess or accumulation of manganese is harmful to the central nervous system. Our results also confirmed the cytotoxicity of Mn²⁺ at a concentration of 100 μmol/L in vitro. A recent study by Lv group demonstrated that manganese promotes anti-tumor response by increasing the cGAS/STING activation. In their study, mice received continuous treatment of Mn²⁺ (5 mg/kg) before tumor growth (which mimics a tumor vaccine model), and the results show that Mn²⁺ prevents tumor growth and promotes anti-tumor response. However, in the Hepal-6 tumor model of our study, mice were treated with Mn²⁺ (30 mg/kg) or MnO₂ (0.867 mg/kg) for three times after the tumors had grown to a certain size more than 50 mm³ (which mimics a tumor treatment model). Our results demonstrated that treatment with Mn²⁺ alone had no obvious effect in restricting tumor growth, which may correlate to the side effects of Mn²⁺ at a high concentration. Therefore, we constructed manganese dioxide (MnO₂) nanosheets, which release the Mn²⁺ in a GSH-enriched environment such as TME. Recently, Yang et al. developed an in situ STING activating vaccination (ISSAV), by using cancer cell membrane (CM) to encapsulate manganese dioxide nanoparticles and immobilize photothermal agent, and showed the design facilitated TAAs and DAMPs released from dying cells under laser irradiation, increased the pH value of TME and created a favorable environment for T cell infiltration. In the present study, T17 was incubated with MnO₂ nanosheet to assemble the T17-MnO₂ nanostructure, and both in vivo and in vitro studies suggested that T17-MnO₂ displayed a much higher efficiency in triggering STING/type I IFN signal activation, M1 polarization as well as antigen presentation. More importantly, in mice-bearing Hepal-6 xenograft, intratumor injection of T17-MnO₂ achieved more efficient anti-tumor effects than T17 plus Mn²⁺, although the concentration of manganese applied in the latter group was twenty-five times more than that in T17-MnO₂ group. Two possible mechanisms may explain the better synergy of T17-MnO₂ in anti-tumor immunity. Firstly, Zhao et al. demonstrated that Mn²⁺ bind to the active site of cGAS to induce STING activation. After the assembly of T17-MnO₂, T17 attached to the surface of MnO₂ nanosheet (a controlled release system of Mn²⁺), thereby increasing the local concentration of T17 and Mn²⁺ to promote a sustained and stronger activation of STING pathways. Secondly, Mn²⁺ displays a neurotoxic effect at a high concentration, while MnO₂ can react with glutathione (GSH) that is high in the tumor microenvironment, to produce Mn²⁺ in a sustained release manner. This advantage of MnO₂ can limit the side effects of in vivo application of manganese at a high concentration.

Consistently, among all the indicated treatment with TDN and/or manganese, T17-MnO₂ displayed the most potent ability to reprogram TAM polarization from the tumor-killing M1 type, thereby boosting both innate and adaptive anti-tumor immune responses.

5. Conclusions

Summarily, our study demonstrated a synergy of TDNs and manganese in triggering STING activation, Iσβ/Iσg15 production, M1 polarization and antigen presentation. This TDN-mediated macrophage activation was influenced by the TDN size, rather than DNA sequence. Moreover, compared to TDN plus Mn²⁺, MnO₂ nanosheet with TDN displayed a much stronger synergy to promoting macrophage activation and host anti-tumor response. Therefore, our findings offered a novel anti-tumor immunotherapy strategy based on STING-mediated TAM reprogramming.

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Author contributions

Minhao Wu, Yuanqing Zhang and Jiaping Li designed the research. Siping Liang and Jiaying Li carried out the experiments
and performed data analysis. Zhenyu Zou, Miao Mao, Siqi Ming, Fan Lin, Ziyan Zhang, Can Cao and Jinyu Zhou participated part of the experiments. Siping Liang and Minhao Wu wrote the manuscript. Siping Liang, Minhao Wu, Yuanqing Zhang and Jiapeng Li revised the manuscript. All of the authors have read and approved the final manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare.

Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2021.12.010.

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