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Intratumoral Injection of CpG Oligonucleotides Induces the Differentiation and Reduces the Immunosuppressive Activity of Myeloid-Derived Suppressor Cells

Yuko Shirota,* Hidekazu Shirota,*† and Dennis M. Klinman*

Immunostimulatory CpG oligonucleotides (ODN) activate cells that express TLR9 and have been shown to improve the host’s response to tumor Ags. Unfortunately, the immunosuppressive microenvironment that surrounds many cancers inhibits Ag-specific cellular responses and thus interferes with CpG-mediated immunotherapy. Myeloid-derived suppressor cells (MDSC) represent an important constituent of this immunosuppressive milieu. Large numbers of MDSC are present in and near tumor sites where they inhibit the activity of Ag-specific T and NK cells. Current studies indicate that the delivery of CpG ODN directly into the tumor bed reduces the immunosuppressive activity of monocytic (CD11b+, Ly6G−, Ly6Chigh) MDSC. Monocytic MDSC express TLR9 and respond to CpG stimulation by 1) losing their ability to suppress T cell function, 2) producing Th1 cytokines, and 3) differentiating into macrophages with tumoricidal capability. These findings provide insight into a novel mechanism by which CpG ODN contribute to tumor regression, and they support intratumoral injection as the optimal route for their delivery. The Journal of Immunology, 2012, 188: 1592–1599.

Synthetic oligodeoxynucleotides (ODN) containing unmethylated CpG motifs mimic the ability of bacterial DNA to stimulate the innate immune system. CpG ODN trigger cells that express TLR9, thereby promoting the maturation and improving the function of professional APCs while supporting the generation of Ag-specific B cells and CTL (1–3). Preclinical and clinical trials indicate that CpG ODN have potent immunostimulatory effects that enhance the host’s response to cancer (4, 5). Kawarada et al. (6) and Heckelsmiller et al. (7) showed that CpG ODN facilitated the induction of tumor-specific immunity and memory (6, 7). This involved both improved plasmacytoid dendritic cell (DC) entry into the tumor site and the activation of tumor-specific CD8+ CTL and NK cells. This activity was optimized by direct injection of CpG ODN into the tumor, as CpG DNA was far less effective when delivered systemically (6, 7). Virtually all studies to date have examined the effect of CpG ODN on nascent tumor foci and tumors <5 mm in diameter. This work extends those studies to better understand the effect of CpG ODN on tumors of clinically relevant size (>1 cm diameter).

Despite evidence that tumor-specific CTL are expanded in the periphery, immune-mediated tumor destruction is difficult to achieve by any form of immunotherapy. For example, CpG ODN administered alone or in combination with vaccines promote the induction of tumor-specific cellular and humoral immune responses yet rarely lead to prolonged tumor regression (4, 5, 8). Analysis of the tumor microenvironment indicates that the lytic activity of CTL and NK cells is suppressed by regulatory T lymphocytes, myeloid-derived suppressor cells (MDSC), and/or M2 macrophages surrounding the tumor (9, 10). Thus, it appears that successful immunotherapy will require both the amplification of tumor-specific immunity plus a means of reversing tumor-associated immune suppression.

MDSC are key contributors to the inhibitory microenvironment found at the tumor site. MDSC are a heterogeneous population of early myeloid progenitors that arise in the bone marrow (11, 12). Their numbers are expanded in the peripheral lymphoid organs of cancer patients and they frequently constitute a majority of tumor-infiltrating cells. Two distinct subpopulations of MDSC have been identified: both are Gr-1+ and CD11b+ with granulocytic MDSC being Gr-1high, Ly6G+, and Ly6Chigh, whereas monocytic MDSC (mMDSC) are Gr-1low, Ly6Glow, and Ly6Chigh. Although both subsets suppress T and NK cell responses through the production of arginase-1 and/or inducible NO synthase (iNOS), mMDSC show greater suppressive activity on a per cell basis (13–15). Additionally, mMDSC promote the generation and/or expansion of regulatory T lymphocytes (16). An agent capable of blocking the immunosuppressive activity of mMDSC might therefore improve the efficacy of tumor immunotherapy.

This study examines the effect of CpG ODN on mMDSC. Consistent with earlier work, intratumoral injection of CpG (but not control) ODN promoted tumor regression. Within the tumor microenvironment, CpG ODN treatment increased the number of tumor-infiltrating T and NK cells while decreasing the frequency and inhibitory activity of resident mMDSC. Further results showed that the effect of CpG ODN on TLR9-expressing mMDSC included 1) triggering their rapid production of Th1-type cytokines (including IL-6, IL-12, and TNF-α), 2) impairing their ability to secrete arginase-1 and NO (factors critical to their suppression of T cell activity), and 3) inducing their differentiation into tumoricidal macrophages. These results suggest additional mechanisms through which CpG ODN could promote tumor regression.
Materials and Methods

Animals and tumor cell lines

BALB/c and C57BL/6 mice were obtained from the National Cancer Institute (Frederick, MD) and studied at 6–10 wk of age. CD8 TCR transgenic (Tg) mice specific for peptide 518–526 of PR8 hemagglutinin (HA) were a gift from Dr. T. Sayers (National Cancer Institute). All studies were approved by the National Cancer Institute Frederick Animal Care and Use Committee. The CT26 colon cancer cell line was a gift from Dr. Z. Howard (National Cancer Institute).

ODN and reagents

Phosphorothioate ODN were synthesized at the Core Facility of the Center for Biologics Evaluation and Research, Food and Drug Administration (Bethesda, MD). The following ODN were used: CpG ODN 1555 (5’-GCTAGAGCTTACGGT-3’) and control ODN 1612 (5’-GCTAGACGT- TACGGT-3’). All ODN were free of detectable protein or endotoxin contamination.

Peptidoglycan, monophosphoryl lipid A, and imiquimod were purchased from InvivoGen (San Diego, CA). Polyinosinic-polycytidylic acid was purchased from Sigma-Aldrich (St. Louis, MO).

In vivo tumor studies

Mice were injected s.c. with 10^6 CT26 tumor cells. Solid tumors formed that reached a diameter of ~1 cm after 2 wk, at which time they were injected with 200 μg CpG or control ODN. Tumor size was calculated by the formula: (length × width × height)/2. Tumor growth curves were generated from three to five mice per group, and all results were derived by combining data from two to three independent experiments. Any animal whose tumor exceeded a diameter of 2.0 cm was immediately euthanized as per Institutional Animal Care and Use Committee protocol.

To deplete NK and CD8+ T cells, mice were injected i.p. with 500 μg rat anti-mouse CD8 (53.6.72) Ab, 25 μl ascites containing anti-asialo GM1 Ab (Wako Pure Chemical Industries, Osaka, Japan) or 500 μg control Ab (LTF-2) from BioXCell (West Lebanon, NH). These Abs were delivered i.p. on days 12, 14, 17, and 20 after tumor challenge.

Preparation of mMDSC

Two techniques were used to prepare mMDSC from the spleens of tumor-bearing mice. Single spleen-cell suspensions were layered onto Ficoll (density, 1.083 g/ml) and centrifuged for 20 min at 2000 rpm. Cells at the interface were isolated, stained, and FACS sorted to isolate CD11b+Gr-1- Ly6c+ mMDSC (purity by this method was ~98%). Alternatively, mMDSC were purified by magnetic cell sorting using the mouse MDSC isolation kit (density, 1.083 g/ml) and centrifuged for 20 min at 2000 rpm. Cells at the interface were isolated, stained, and FACS sorted to isolate CD11b+Ly6c+ mMDSC (purity by this method was 90–95% as determined by flow cytometry).

Flow cytometry

Cells were washed with PBS, fixed in 4% paraformaldehyde for 10 min, and stained with anti-CD11b, -CD8, -Dx5, –Gr-1, -Ly6c, -Ly6g, -F4/80, and/or -CD45 Abs for 30 min at 4˚C. All Abs were obtained from BD Pharmingen (San Diego, CA). Stained cells were washed, resuspended in PBS/0.1% BSA plus azide, and analyzed by FACSCalibur (BD Pharmingen).

Detection of intracytoplasmic arginase-1 and IL-12

Single-cell suspensions were prepared from the spleens of tumor-bearing mice. These cells were surface stained to detect CD11b, Gr-1, and Ly6c triple-positive cells. They were then treated with cell permeabilization solution (BD Pharmingen) followed by anti-arginase-1, anti-IL-12, or control Ab followed as needed by PE-conjugated anti-goat Ab. The frequency of intracellularly stained cells expressing the surface markers of mMDSC was determined by FACS.

RT-PCR and quantitative RT-PCR

Total RNA was extracted from target cells using TRIzol reagent (Life Technologies, Carlsbad, CA) as recommended by the manufacturer. Total RNA (1 μg) was reverse-transcribed in first strand buffer (50 mM Tris-HCl [pH 7.5], 75 mM KCl, and 25 mM MgCl₂) containing 25 μg/ml oligo(dT), 200 U Moloney leukemia virus reverse transcriptase, 2 mM dinucleotide triphosphate, and 10 mM DTT. The reaction was conducted at 42˚C for 1 h. A standard PCR was performed on 1 μl of the cDNA synthesis using TLR primer pairs (InvivoGen). Aliquots of the PCR reactions were separated on a 1.2% agarose gel and visualized with UV light after ethidium bromide staining.

Arginase-1 mRNA levels were examined using the Applied Biosystems StepOne RT-PCR system, in which primers obtained from the Gene Expression Assay set (Applied Biosystems, Foster City, CA) were amplified using the TaqMan Gene Expression Master Mix kit. mRNA expression levels were then calculated by StepOne software (Applied Biosystems) after correction for GAPDH expression independently for each sample.

T cell proliferation assay

HA or OVA-specific CD8 T cells were purified by magnetic cell sorting (MACS; Miltenyi Biotec) using mouse anti-CD8 beads and were labeled with CFSE (Invitrogen, Carlsbad, CA) as previously described (17). CD8 T cells (5 × 10^5) were cocultured with 10^6 mitomycin C-treated naive spleen cells plus 5 × 10^5 Ly6c+ mMDSC in the presence of peptide (0.1 μg/ml) for 3 d. Cell division was analyzed using a FACS caliber to monitor cellular CSFE content.

Nitrite assay

NO levels in culture supernatants were assessed using the Griess reagent (Sigma-Aldrich). Nitrite concentration was calculated by comparison with a standard curve generated by sequentially diluting sodium nitrite.

CTL assay

mMDSC were incubated with 1 μM CpG or control ODN for 48 h. Various numbers of these ODN-pulsed mMDSC were added to 10^6 CT26 target cells. After 4 h in culture, supernatants were recovered and released lactate dehydrogenase (LDH) assayed as recommended by the manufacturer (Roche Diagnostics, Indianapolis, IN). Mean percentage specific lysis of triplicate wells was determined by the formula: % cytotoxicity = [(experimental – spontaneous LDH release)/(maximum – spontaneous LDH release)] × 100.

Statistical analysis

A two-sided unpaired Student t test was used to analyze tumor growth and cellular responses. A p value of <0.05 was considered to be statistically significant.

Results

Intratumoral injection of CpG ODN reduces tumor growth

Previous studies established that intratumoral injection of CpG ODN could slow and in some cases reverse the growth of nascent tumors (<5 mm diameter) (6, 7, 18). However, tumors of such small size are difficult to detect clinically and can lack the immunosuppressive microenvironment that inhibits the efficacy of immunotherapy directed against larger tumors (≥1 cm diameter).

To evaluate the effect of CpG ODN on tumors of clinically relevant size, BALB/c mice were injected s.c. with CT26 colon tumor cells. These tumors grew to >1 cm in diameter within 2 wk, at which time they were injected twice with 200 μg CpG ODN. This treatment reduced the rate of tumor growth by >50%, whereas control ODN had no effect (p < 0.01; Fig. 1A). The efficacy of intratumoral CpG injection significantly exceeded that of systemic ODN delivery (p < 0.01; Fig. 1B). The impact of CpG treatment on established tumors involved both CD8 and NK cells, as coadministration of neutralizing Abs against either cell type abrogated CpG-dependent tumor regression (Fig. 1C). In addition to delaying tumor progression, CpG treatment significantly increased the fraction of DX5+ NK cells and CD8+ T cells infiltrating the tumor (data not shown).

mMDSC express TLR9 and produce cytokines in response to CpG stimulation

Most immune cells infiltrating established tumors are of myeloid lineage and bear the CD11b surface marker (Fig. 2A). This includes both mMDSC that are Gr-1+CD11b+Ly6c+ and granulocytic MDSC that are Gr-1−CD11b+Ly6g−. mMDSC are extremely potent suppressors of tumor-specific immune responses (15). To examine the effect of CpG ODN treatment on mMDSC, ODN were injected 14 d after the initiation of CT26 tumors (at which time tumor volumes ranged from 200 to 400 mm^3). CpG treatment had...
no effect on granulocytic MDSC or on the total number of CD45<sup>+</sup> cells infiltrating the tumor bed (Fig. 2A, Supplemental Fig. 1). However, CpG treatment reduced the frequency of mMDSC infiltrating the tumor site by >3-fold ($p < 0.01$; Fig. 2A, 2B). This reduction in mMDSC number persisted for 4 d (Fig. 2C) and was independent of tumor size (Supplemental Fig. 2A). To determine whether intratumoral injection of CpG ODN had systemic as well as local effects on mMDSC, mice were injected bilaterally with CT26 cells. The infiltration of mMDSC was reduced in tumors injected with CpG ODN but remained high in contralateral tumors treated with control ODN (Supplemental Fig. 2B).

CpG treatment also reduced the number of MDSC in the spleen (Supplemental Fig. 2D). However, this systemic effect was influenced by tumor volume, consistent with previous studies (19, 20). Thus, when spleens from mice with tumors of equal size were compared, no effect of CpG treatment on mMDSC frequency was observed (Supplemental Fig. 3A, B). This result indicates that the reduction of splenic mMDSC number is a consequence of CpG-induced reductions in tumor size rather than an independent effect on mMDSC.

To better understand the effect of local CpG ODN treatment on tumor-infiltrating mMDSC, the expression of TLR9 (the cognate receptor for CpG DNA) by mMDSC was examined. As seen in Fig. 3A, TLR9 mRNA was readily detected in Gr-1<sup>-</sup>CD11b<sup>+</sup>Ly6c<sup>+</sup> cells. Consistent with that observation, FACS-purified mMDSC response to in vitro stimulation with CpG ODN by secreting a variety of cytokines (including IL-6, TNF-α, and IL-12; Fig. 3B and data not shown). The mMDSC origin of these cytokines was confirmed by FACS analysis, in that Gr-1<sup>-</sup>CD11b<sup>+</sup>Ly6c<sup>+</sup> cells isolated from either the tumor or spleen of CpG-treated mice contained high levels of intracytoplasmic IL-12 (Fig. 3C). In the same studies, control ODN did not induce cytokine production. By comparison, the level of TLR9 mRNA expression by purified Gr-1<sup>-</sup>CD11b<sup>+</sup>Ly6g<sup>+</sup> cells was very low, and granulocytic MDSC failed to secrete cytokines in response to CpG ODN stimulation (Fig. 3A, Supplemental Fig. 3).

CpG-treated monocyteic MDSC fail to inhibit T cell activation
mMDSC suppress the proliferation and functional activity of Ag-stimulated T cells (11, 12). To examine the effect of CpG treatment on this inhibitory activity, mMDSC were isolated from tumor-bearing mice. The studies shown used splenic rather than tumor-infiltrating MDSC, as the former could be isolated at higher yield and purity. However, all results involving splenic mMDSC were confirmed in similar results. *$p < 0.01$ versus untreated group.

FIGURE 1. Intratumoral injection of CpG ODN impacts tumor immunity. CT26 colon cancer cells ($10^5$) were injected s.c. into BALB/c mice on day 0. These formed solid tumors that reached an average diameter of >1 cm by day 14. A–C. On days 14 and 15, mice were injected i.p. or intratumorally with 200 µg CpG or control ODN, and tumor size was monitored. C. Mice were also injected i.p. with anti-CD8, anti-asialo GM1 (NK cell), or control Ab on days 12, 14, 17, and 20. Data represent the mean ± SEM of five to eight mice per group from two independent experiments. *$p < 0.05$ compared with the untreated (tumor alone) group. **$p < 0.01$ compared with the untreated (tumor alone) group.

FIGURE 2. Effect of intratumoral CpG ODN on CD11<sup>+</sup>b cells. A. Mice were treated as described in Fig. 1. The number of tumor-infiltrating CD11<sup>+</sup>b cells that expressed Ly6c and Ly6g was determined on day 17 by FACS. A. Representative results from one mouse per group and (B) mean ± SD from five independently analyzed mice per group, showing Ly6c and Ly6g expressing cells as a percentage of all tumor-infiltrating CD45<sup>+</sup> lymphocytes. C. The frequency of CD11b<sup>+</sup>, Ly6c double-positive cells as a percentage of CD45<sup>+</sup> tumor-infiltrating cells was examined 2–5 d after CpG administration. Means ± SD from four to seven independently analyzed mice per group are shown. *$p < 0.01$ versus untreated group.

FIGURE 3. mMDSC express TLR9 and respond to CpG ODN. Spleens were removed from CT26 tumor-bearing mice and CD11b<sup>+</sup>Ly6c<sup>+</sup>Gr-1<sup>−</sup> or CD11b<sup>+</sup>Ly6g<sup>+</sup>Gr-1<sup>−</sup>MDSC were FACSorted to >98% purity. A. TLR9 mRNA levels were determined by RT-PCR in comparison with the RAW 264.7 macrophage cell line (positive control) and EL4 thymoma cell line (negative control). B. FACS-sorted mMDSC (10<sup>6</sup>) were cultured with 1 µM CpG or control ODN for 24 h. Culture supernatants were assayed for IL-12 levels by ELISA. Data represent the mean ± SD from three independent experiments. C. MDSC isolated independently from the spleen or tumor of CT26 tumor-bearing mice were cultured with 1 µM CpG or control ODN for 8 h. Brefeldin A was added during the final 4 h incubation. Cells were stained to identify CD11b<sup>+</sup>Ly6c<sup>+</sup>Gr-1<sup>−</sup> mMDSC for the presence of intracytoplasmic IL-12. All experiments were repeated three times with similar results. *$p < 0.01$ versus untreated group.
more limited studies of tumor-infiltrating cells (Supplemental Fig. 4), mMDSC were mixed with HA-specific CD8\(^+\) T cells (isolated from HA TCR Tg mice). The vast majority of these T cells proliferated when stimulated with HA peptide (Fig. 4A, 4B). This proliferation was reduced by >74% when mMDSC were added to the culture (p < 0.01; Fig. 4A, 4B). However, when the mMDSC were pretreated with CpG ODN, their ability to suppress T cell proliferation was abrogated (p < 0.01; Fig. 4A, 4B). In contrast, no reduction in suppressive activity was observed when MDSC were pretreated with control ODN (Fig. 4B).

This set of findings led us to examine the effect of CpG ODN treatment on the behavior of MDSC in vivo. Mice bearing CT26 tumors were injected with CpG or control ODN. mMDSC isolated from tumor-bearing mice treated with control ODN (or left untreated) significantly inhibited the proliferation of Ag-stimulated CD8\(^+\) T cells (p < 0.01; Fig. 4C). In contrast, MDSC isolated from tumor-bearing mice treated with CpG ODN failed to suppress T cell proliferation (p < 0.01). The same pattern was observed when using MDSC isolated from CpG-treated mice bearing EL4 tumors, establishing the consistency of this general finding (data not shown).

The ability of mMDSC to suppress T cell activation is linked to their metabolism of L-arginine via arginase-1 and release of iNOS (13, 14). The amount of iNOS and arginase-1 produced by MDSC was therefore evaluated. MDSC from tumor-bearing mice were stimulated to produce iNOS by culture with Ag-activated T cells (Fig. 5A). The addition of CpG ODN to these cultures reduced iNOS production by half (p < 0.01). Control ODN had no such effect (Fig. 5A). Similarly, CpG but not control ODN significantly reduced the level of arginase-1 present in culture supernatants of purified mMDSC (Fig. 5B). Intracytoplasmic staining of Ly6C\(^+\) cells confirmed that mMDSC were the source of this arginase-1 (Fig. 5C).

**CpG ODN induce mMDSC to differentiate into macrophages**

The mechanism by which CpG treatment reduced the ability of MDSC to suppress T cell activation was investigated. mMDSC were isolated from the spleens of tumor-bearing mice and cultured in vitro with CpG or control ODN for 48 h. Cell surface staining of CpG-treated cultures revealed that their expression of F4/80 increased by 3-fold whereas expression of Ly6c and Gr-1 decreased by 3-fold over this period (p < 0.01 for both parameters; Fig. 6A, 6B and data not shown). No such effects were observed when MDSC were cultured with control ODN. These results suggest that Ly6c\(^+\) MDSC were induced to differentiate into F4/80\(^+\) macrophages by CpG ODN, a finding consistent with the shared lineage of these two cell types.

We sought to determine whether CpG ODN were directly inducing TLR9-expressing mMDSC to differentiate into macrophages or were stimulating other cell types to produce factors that induced such differentiation. To distinguish between these alternatives, highly purified mMDSC were pulsed with CpG ODN, the ODN was then washed away, and the cells were then cultured in transwell plates with unstimulated MDSC. As seen in Fig. 6D, the CpG-pulsed mMDSC in the upper well downregulated Ly6c and increased F4/80 expression, demonstrating that even short-term exposure to CpG ODN triggered their differentiation into macrophages. In contrast, naive mMDSC in the lower well (exposed to factors secreted by the CpG-pulsed cells in the upper well) did not alter their expression of Ly6c or F4/80. Similarly, culture supernatants from CpG-activated MDSC had no effect on the differentiation of naive mMDSC (data not shown). These findings suggest that CpG ODN directly induce the differentiation of MDSC into macrophages.

The effect of other TLR ligands on mMDSC maturation was also investigated. Initial studies examined TLR mRNA levels in highly purified mMDSC. As seen in Fig. 7A, mRNAs encoding TLRs 2, 3, 4, 7, and 8 (in addition to TLR9; Fig. 3A) were expressed by these cells. When stimulated with ligands directed against each receptor, MDSC responded by proliferating and secreting TNF-\(\alpha\), IL-12, IL-6, and/or IL-10 (Fig. 7B). However, only ligands targeting TLR7 and TLR9 (imiquimod and CpG DNA) induced mMDSC to differentiate into macrophages (Fig. 7C).

**FIGURE 4.** CpG ODN treatment inhibits the suppressive activity of mMDSC. A and B, mMDSC from the spleen of CT26 tumor bearing mice were isolated by MACS (final purity, 90–95%). These cells were cultured for 3 h with 1 \(\mu\)M CpG or control ODN and then washed. ODN-pulsed MDSC (5 \(\times\) 10\(^5\)) were cultured for 3 d with 5 \(\times\) 10\(^5\) CFSE-labeled CD8\(^+\) HA-specific Tg T cells plus 10\(^6\) mitomycin C-treated spleen cells (as APCs) in the presence of 0.1 \(\mu\)g/ml HA peptide. CD8 \(T\) cell proliferation was monitored by CFSE dilution. A, Representative example and (B) mean ± SD (n = 5 independent MDSC preparations in two independent experiments) are shown. C, Mice bearing 1.5-cm-diameter CT26 tumors were injected i.p. with 300 \(\mu\)g CpG or control ODN. Three hours later, spleens were removed and Ly6c\(^+\)Gr-1\(^-\) MDSC were isolated by MACS sorting. These MDSC were cocultured with CFSE-labeled CD8\(^+\) HA-specific Tg T cells plus HA peptide for 3 d, as described above. The proliferation of CD8\(^+\) T cells was monitored by CFSE dilution. Results represent the mean ± SD of four to seven independent mice per group studied in three independent experiments. *p < 0.01 versus CpG-treated MDSC.
Macrophages derived from CpG-treated monocytic MDSC support tumor elimination

Macrophages are broadly classified into two distinct types that have opposite effects on tumor growth. M1 macrophages contribute to Th1-associated responses and thus improve host resistant to cancer (21). In contrast, M2 macrophages suppress the host’s inflammatory response and enhance angiogenesis, thereby supporting tumor development (22, 23). To determine which type of macrophage was generated by CpG ODN treatment, MDSC derived from tumor-bearing mice were cultured in vitro with ODN and then coinjected with CT26 tumor cells into BALB/c mice. As seen in Fig. 8A, tumor cell growth was significantly reduced when coadministered with macrophages derived from CpG-treated MDSC. Similarly, when mMDSC pulsed in vitro with CpG ODN were injected into established CT26 tumors, tumor growth was again significantly slowed ($p < 0.05$; Fig. 8B). In contrast, MDSC that were untreated or cultured with control ODN had no significant effect on tumor growth.

To investigate the mechanism underlying the protective effect of CpG-activated/differentiated mMDSC, their cytotoxic activity was examined. Tumor-derived mMDSC treated with control ODN manifest little cytotoxic activity against CT26 target cells (Fig. 8C).

In contrast, mMDSC incubated in vitro for 48 h with CpG ODN exhibited significant CTL activity against CT26 targets (Fig. 8C).

Discussion

It is well established that CpG ODN treatment can improve the host’s immune response to tumor challenge (5, 24). This activity was historically attributed to CpG-mediated activation of DC and macrophage, leading to improved NK and CD8 T cell killing. Current results suggest that CpG ODN can also act on mMDSC, further aiding tumor elimination, mMDSC express TLR9 (Fig. 3), and treating such cells with CpG DNA rapidly and significantly increased their production of Th1 cytokines (most notably IL-6, IL-12, and TNF-$\alpha$; Figs. 3, 7) while reducing their capacity to inhibit CTL activity. Subsequently, CpG-treated MDSC differentiated into macrophage with tumoricidal activity (Figs. 6, 8). These findings demonstrate that CpG ODN reduce the immunosuppressive activity of mMDSC. Whereas the experiments presented in this study used MDSC from CT26 tumor-bearing mice, the findings were reproduced in studies of MDSC generated in mice challenged with other tumor types (including 4T1 breast cancer, Renca renal tumor, EL4 thymoma, and TC-1 tumor lines; data not shown). The ability of MDSC to downregulate tumor-immunosuppression and support antitumor responses may represent a novel mechanism of action for CpG ODN.
CD11b, Ly6c+, Gr-1int) were FACS sorted to remove from CT26 tumor-bearing mice. mMDSC (identified as 23) were removed from other donor mice as described in Fig. 4, into BALB/c mice. mMDSC were MACS purified from three independently studied cell populations. Measurements of LDH. Results are the mean ± SD from three independent experiments. C, CD11b+ cells stimulated for 48 h were analyzed for the expression of Ly-6c and F4/80 by flow cytometry. Experiments were repeated three times with similar results.

**FIGURE 7.** Effect of TLR ligands on monocytic MDSC. A, Spleens were removed from CT26 tumor-bearing mice. mMDSC (identified as CD11b, Ly6c+, Gr-1123) were FACS sorted to >98% purity. TLRs 2, 3, 4, 7, and 8 mRNA levels were determined by RT-PCR in comparison with the RAW 264.7 macrophage cell line (positive control) and EL4 thymoma cell line (negative control). B and C, Purified mMDSC were cultured with 1 μM ODN, 1 μg/ml peptidoglycan (PGN), 30 μg/ml polyinosinic-polycytidylic acid, 1 μg/ml monophosphoryl lipid A (MPL), and 10 μg/ml imiquimod. B, Culture supernatants were assayed after 24 h for IL-6, TNF-α, IL-12, and IL-10 levels by ELISA (all values in ng/ml). Data represent the mean ± SD from three independent experiments. *p < 0.01 compared with the untreated treatment group or untreated MDSC. **FIGURE 8.** Effect of CpG-treated monocytic MDSC on tumor growth. CT26 colon cancer cells (106 A; or 3 × 106 B) were injected s.c. into BALB/c mice. mMDSC were MACS purified from other donor mice as described in Fig. 4, pulsed with CpG or control ODN for 3 h, and then washed extensively. ODN-treated MDSC (2 × 106) were injected into the tumor site on days 0 and 2 (A) or into established tumors on days 12 and 13 (B). Data show tumor size in six to seven mice per group from two independent experiments. C, ODN-pulsed MDSC were mixed with CT26 target cells at various E:T ratios in vitro. Specific lysis was determined by quantitative measurements of LDH. Results are the mean from three independently studied cell populations per group. *p < 0.01 compared with the untreated treatment group or untreated MDSC.
tumor injection was key to increasing the number of tumor infiltrating T and NK cells and reducing the number and suppressive activity of mMDSC (Figs. 1, 2, 4 and data not shown). In this context, two phase I clinical trials in which CpG ODN were delivered intratumorally to treat malignant skin tumors yielded promising results. Hofmann et al. (37) used this strategy to induce complete or partial tumor remission in half of all subjects, whereas Molenkamp et al. (38) and Brody et al. (39) showed that intratumoral CpG administration (alone or combined with radiation therapy) could induce systemic tumor regression by improving the generation of tumor-specific CD8 T cells.

Zoglei et al. (40) recently reported that CpG ODN treatment reduced the suppressive activity of granulocytic (CD11b+Ly6g+) MDSC. In those studies, CpG ODN had no direct on MDSC but instead stimulated plasmacytoid DC to produce type I IFN, which indirectly promoted MDSC differentiation (40). This contrasts to our results that show that CpG ODN directly induced mMDSC to differentiate into macrophages. Data provided in this study demonstrate that: 1) mMDSC express TLR9 (Fig. 3, 2) no other cells are required for such differentiation to occur (Fig. 6), and 3) CpG ODN, but not the factors produced by CpG-stimulated MDSC, are required for such differentiation to occur (Fig. 6), and 3) CpG ODN REDUCE MDSC-MEDIATED IMMUNOSUPPRESSION.

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### Disclosures

D.M.K. and members of his laboratory hold or have applied for patents concerning the activity of CpG ODN, including their use in preventing/treating tumors. The rights to all such patents have been transferred to the U.S. government. The other authors have no financial conflicts of interest.

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