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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−, Ly6C high) 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; 000–000.

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 Ly6clow, whereas monocytic MDSC (mMDSC) are Gr-1int, 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’-GCTAGACGTTAGCGT-3’) and control ODN 1612 (5’-GCTAGACGTTAGCCT-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-polyribydidylic acid was purchased from Sigma-Aldrich (St. Louis, MO).

In vivo tumor studies

Mice were injected s.c. with 10^5 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 from 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 μg ascites containing anti-asialo GM1 Abs (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 FACSorted to isolate CD11b+Gr-1(int) mMDSC (purity by this method was >98%). Alternatively, mMDSC were purified by magnetic cell sorting using the mouse mMDSC isolation kit according to the manufacturer’s instructions (MACS; Miltenyi Biotec, Auburn, CA). The purity of CD11b+Ly6c- cells 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, -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 FACS Calibur (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. These 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 intracellular 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 dNTPs, trypolysine, 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 ethidi um bromide staining.

Arginase-1 mRNA levels were examined using the Applied Biosystems Assay-on-Demand M-Mark QPCR kit for mice M-Mark (Applied Biosystems, Foster City, CA) as recommended by the manufacturer (TaqMan Gene Expression Master Mix kit). mRNA expression levels were then calculated using the StepOne software (Applied Biosystems) after correction for GAPDH expression independently for each sample.

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 MDCS 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 mm3). CpG treatment had
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.

no effect on granulocytic MDSC or on the total number of CD45^+ 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. 3C, 3E). This result indicates that the reduction of splenic MDSC 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^+CD11b^+Ly6c^+ cells. Consistent with that observation, FACS-purified mMDSC responded 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^+CD11b^+Ly6c^+ 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^+CD11b^+Ly6c^+ cells was very low, and granulocytic MDSC failed to secrete cytokines in response to CpG ODN stimulation (Fig. 3A, Supplemental Fig. 3).

CpG-treated monocyotic 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 mMDSC, as the former could be isolated at higher yield and purity. However, all results involving splenic mMDSC were confirmed in

FIGURE 2. Effect of intratumoral CpG ODN on CD11b^+ cells. A, Mice were treated as described in Fig. 1. The number of tumor-infiltrating CD11b^+ 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^+ lymphocytes. C, The frequency of CD11b, Ly6c double-positive cells as a percentage of CD45^+ 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^+Ly6c^+Gr-1^hi or CD11b^+Ly6g^+Gr-1^int MDSC were FACS sorted 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 MDSC (10^5) 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^+Ly6c^+Gr-1^hi 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 mMDSC 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 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 mMDSC. 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 MDSC 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-α, 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 μM CpG or control ODN and then washed. ODN-pulsed MDSC (5 × 10^6) were cultured for 3 d with 5 × 10^3 CFSE-labeled CD8+ HA-specific Tg T cells plus 10^6 mitomycin C-treated spleen cells (as APCs) in the presence of 0.1 μg/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 μg CpG or control ODN. Three hours later, spleens were removed and Ly6c<sup>hi</sup>Gr-1<sup>hi</sup> 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 TNFα; 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-

![Figure 5](http://www.jimmunol.org/)

CpG ODN treatment downregulates the production of NO and arginase by mMDSC. mMDSC purified by MACS sorting were cultured with HA-specific Tg CD8+ T cells plus 10^6 mitomycin C-treated spleen cells (as APCs) in the presence of HA peptide for 1 d as described in Fig. 4. NO levels in culture supernatants were quantified using the Griess reagent. mMDSC purified by MACS were pulsed with 1 μM CpG or control ODN for 36–48 h and analyzed (B) for arginase-1 mRNA levels by real-time quantitative PCR and (C) for expression of Ly6c and arginase-1 by flow cytometry. Results represent the mean ± SD of results from five independent MDSC preparations. *p < 0.01 versus untreated MDSC group.

![Figure 6](http://www.jimmunol.org/)

Differentiation of mMDSC. mMDSC were MACS purified as described in Fig. 4. The cells were incubated with 1 μM CpG or control ODN for 48 h and then analyzed for the expression of Ly6c and F4/80 by flow cytometry. Representative results are shown by dot plot (A) and histogram (B) (shadow, untreated; dotted line, control ODN; solid line, CpG ODN). The means ± SD in MFI from four independent experiments are shown in C. D, MDSC were pulsed with CpG or control ODN for 3 h, washed extensively, and then transferred to the upper well of a transwell plate. Untreated MDSC were added to the lower well of the same plate. After 48 h, both cell populations were analyzed for the expression of Ly6c and F4/80 by flow cytometry. Experiments were repeated three times with similar results. *p < 0.01 versus untreated group.
specific immune responses is well established (11, 12). mMDSC are particularly potent, as they suppress the activity of tumor-infiltrating CTL (15). In this context, MDSC are considered a major impediment to immunotherapy, and multiple strategies are being pursued to reduce their activity (25). The successful reduction in MDSC function by treatment with phosphodiesterase-5, all-ODN, Lpse, 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-poly-cytidylic 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. 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 monocylic MDSC. A, Spleens were removed from CT26 tumor-bearing mice. mMDSC (identified as CD11b, Ly6c−, Gr-1−) 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-poly-cytidylic 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. 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 8. Effect of CpG-treated monocylic MDSC on tumor growth. CT26 colon cancer cells (10⁴, A; or 3 × 10⁴, 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.

CpG ODN REDUCE MDSC-MEDIATED IMMUNOSUPPRESSION

An additional effect of CpG ODN treatment was to induce the differentiation of mMDSC into M1-like macrophages (Fig. 6). This process was characterized by the loss of Ly6c and Gr-1 (markers expressed by immature monocytes and mMDSC) and the acquisition of F4/80 (Fig. 6). Macrophages are typically categorized as being of either the M1 or M2 type. M2 macrophages are generated in a Th2-polarized environment (found in many tumor sites) and are characterized by their production of arginase-1 and IL-10. In contrast, M1 macrophages 1) express TLRs, 2) produce proinflammatory cytokines (such as IL-12) when stimulated, 3) express diminished levels of arginase-1, and 4) help protect the host from infectious pathogens and tumors (21, 24). This constellation of activities was exhibited by CpG-treated mMDSC (Figs. 3, 5). Such activity is consistent with the findings of Colombo et al. (31), who reported that coadministering CpG ODN plus IL-10 receptor Ab reprogramed M2-like macrophages and DC toward an M1-like phenotype, thereby improving anti-tumor immunity. The mechanism underlying this MDSC differentiation and the signals that control M1 versus M2 commitment are poorly defined and require further study.

Previous studies demonstrated that intratumoral injection of CpG ODN could slow and in some cases reverse the growth of small/nascent tumors (6, 7, 18, 32). In those experiments, CpG treatment led to a significant increase in the fraction of NK cells and CD8+ T cells infiltrating the tumor (6, 7, 18, 32). The current work confirms and extends those findings by showing that CpG treatment impacts mMDSC at the tumor site: reducing their number and blocking their suppressive activity. This, in turn, facilitated the expansion and infiltration of cytotoxic CD8+ T cells and thus supported immune-mediated tumor regression. Of note, our ability to characterize the effect of CpG ODN on mMDSC was facilitated by the study of large, established tumors, where the number and immunosuppressive activity of MDSC was enhanced.

The results of human clinical trials in which CpG ODN were administered systemically for cancer therapy were somewhat disappointing (33–35). Preliminary studies from our laboratory and elsewhere indicate that the route of ODN administration critically affects outcome, in that local but not systemic delivery of CpG DNA altered the tumor microenvironment (6, 7, 36). Improvements show that CpG treatment reduces the expression of NO and arginase-1 by mMDSC, allowing tumor-specific CTL to remain active (Fig. 5). In support of the potential clinical utility of this approach, the number of tumor-infiltrating CTL and NK cells rose after CpG treatment, coincident with a significant reduction in the rate of growth of large, established tumors.

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tumoral 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.

Zoglmeier 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 presented in this study demonstrate that: 1) mMDSCs 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, mediate this differentiation (Fig. 6). In contrast to the work of Zoglmeier et al. (40), we did not detect the proliferation of granulocytic MDSC following intratumoral injection of CpG ODN (Fig. 2), and we were unable to induce mMDSC to differentiate into macrophages by adding type I or type II IFNs (data not shown).

Of interest, we found that mMDSC expressed multiple TLRs (TLRs 2, 3, 4, 7, and 8; Fig. 7). When cultured with ligands directing against each of those receptors, MDSC responded by producing type I IFN, which elicits a coordinated response of CD8 T cells and innate effectors to cure established tumors in a murine colon carcinoma model. J. Immunol. 169: 3892–3899.


Supplemental Figure 1

UnRx'd  | Day 17  | Day 19  | Day 19
---------|---------|---------|---------
Untreated | 1.0     | 1.2     | 1.1     
CpG treated | 1.5     | 1.4     | 1.3     
CpG ODN   | 1.5     | 1.4     | 1.3     
Control   | 1.0     | 1.2     | 1.1     

Relative density of DC45+ cells
Supplemental Figure 2

A) Tumor infiltrate mMDSC

B) % of CD45 cells

C) Splenic mMDSC

D) All mice

E) Mice with tumors of equivalent size
Supplemental Figure 3

Untreated CpG ODN

CpG ODN
Supplemental Figure 4

A

Tumor infiltrate MDSC

% proliferating CD8 T cells

<table>
<thead>
<tr>
<th>Condition</th>
<th>No peptide</th>
<th>No MDSC</th>
<th>MDSC</th>
<th>CpG MDSC</th>
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<tr>
<td>Peptide</td>
<td>No</td>
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B

Nitrate (uM)

- MDSC
- CpG MDSC
- Control MDSC

C

Relative frequency of arginase 1 + cells

- (-)
- CpG
Supplementary Figure legends

Supplemental Figure 1. Frequency of CD45⁺ cells infiltrating the tumor site.

10⁵ CT26 colon cancer cells were injected s.c. into BALB/c mice on day 0. 200 ug of CpG or control ODN was injected intra-tumorally on days 14 and 15. A comparison of the density of CD45⁺ staining in treated vs untreated animals as determined by NIH ImageJ software (which correlates with cell number) is shown. Results represent the mean of three independent regions from four different tumors/group. Note there was no significant difference in CD45 cell frequency between groups.

Supplemental Figure 2. Frequency of monocytic MDSC at the tumor site and in the spleen.

10⁵ CT26 colon cancer cells were injected s.c. into the flank BALB/c mice on day 0. These formed solid tumors that reached an average volume of ≈200 mm³ by day 14. 200 ug of CpG ODN were injected intra-tumorally on days 14 and 15. Mice were sacrificed on days 17 - 20. The number of CD45⁺, CD11b⁺, Ly6c hi (mMDSC) or CD11b⁺, Ly6g⁺ (gMDSC) cells was determined by FACS. A) The percentage of mMDSC in the CD45⁺ cell pool isolated from tumors of mice treated with CpG (open circles) vs untreated (solid diamonds) mice is shown as a function of tumor size. B) 10⁵ CT26 colon cancer cells were injected s.c. into both flanks of BALB/c mice. 200 ug of CpG ODN was injected into the tumor on one side and control ODN into the contralateral tumor on days 14 and 15. The percentage of gMDSC and mMDSC in the CD45⁺ leukocyte pool from tumors treated with CpG (solid bar) vs control ODN (open bar) is shown. C) Splenic mMDSC frequency of CpG (open circles) vs control ODN (solid
diamonds) treated animals plotted as a function of tumor size. D) Total number of mMDSC and gMDSC in the spleen of untreated (open bar) vs CpG treated (closed bar) mice is shown. E) Frequency of MDSC in the spleens of mice with tumors 200-300 mm³ in size. Results represent the mean of 5-6 independently analyzed mice/group. * p < 0.01 versus untreated group.

Supplemental Figure 3. IL-12 production by mMDSC.

Spleen cells isolated from CT26 tumor bearing mice were cultured with 1 uM CpG or control ODN for 8 h. Brefeldin A was added during the final 4 h of incubation. Cells were surface stained to identify CD11b⁺, Gr-1⁺ mMDSC, fixed, permeabilized, and then stained for the presence of intra-cytoplasmic IL-12.

Supplemental Figure 4. CpG ODN treatment reduces the activity of mMDSC.

10⁵ CT26 colon cancer cells were injected s.c. into BALB/c mice. The tumors were removed 18 days later. Monocytic MDSC were isolated from tumor cell suspensions by MACS sorting (final purity 30-50%) A) These mMDSC were cultured for 3 h with 1 uM of CpG or control ODN and then washed. 5 x 10⁵ ODN-pulsed MDSC were cultured for 3 days with 5 x 10⁵ CFSE-labeled CD8⁺ HA-specific Tg T cells plus 10⁶ mitomycin C treated spleen cells (as APCs) in the presence of 0.1 ug/ml HA peptide. CD8 T cell proliferation was monitored by CFSE dilution. B,C) The MDSC were cultured for 2 days with CpG or control ODN. Nitrate levels in culture supernatants were quantified using the Griess reagent (B) while the level of arginase I mRNA was determined by real time qPCR (C). Results represent the mean + SD of results from 4 independent MDSC preparations.

* p < 0.01 versus CpG-treated MDSC. ** p < 0.05 versus untreated group.