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Oligodeoxynucleotides Expressing Polyguanosine Motifs Promote Antitumor Activity through the Upregulation of IL-2

Nobuaki Kobayashi,* Choongman Hong,* Dennis M. Klinman,* and Hidekazu Shirota*†

The primary goal of cancer immunotherapy is to elicit an immune response capable of eliminating the tumor. One approach toward accomplishing that goal uses general (rather than tumor-specific) immunomodulatory agents to boost the number and activity of pre-existing CTLs. We find that the intratumoral injection of polyguanosine (poly-G) oligonucleotides (ODN) has such an effect, boosting antitumor immunity and promoting tumor regression. The antitumor activity of poly-G ODN was mediated through CD8 T cells in a TLR9-independent manner. Mechanistically, poly-G ODN directly induced the phosphorylation of Lck (an essential element of the T cell–signaling pathway), thereby enhancing the production of IL-2 and CD8 T cell proliferation. These findings establish poly-G ODN as a novel type of cancer immunotherapy. The Journal of Immunology, 2013, 190: 000–000.

Numerous strategies designed to improve the generation and activity of tumoricidal CD8 T cells have been examined (1–4). This includes various forms of vaccination, the ex vivo expansion followed by adoptive transfer of tumor-specific CTLs, and the administration of immunostimulatory agents that promote the expansion of pre-existing CTLs. An example from the latter category involves the administration of immunostimulatory oligonucleotides (ODN) (5–9).

The rationale for studying such ODN is predicated on the ability of the innate immune system to recognize non–self-DNA expressed by viral and microbial pathogens (10, 11). This involves the TLR9-mediated recognition of unmethylated CpG motifs present at high frequency in bacterial DNA and the recognition of double-stranded viral DNA by cytosolic receptors (12). Both preclinical and clinical studies show that treatment with CpG-containing ODN can slow the rate of tumor growth and lead to clinical remission in cancer patients (13–15). The mechanism by which CpG ODN contribute to tumor regression has been linked to the activation of tumor-specific CD8+ CTLs and NK cells (16, 17). Those results were primarily derived from studies of conventional K-type CpG ODN (also referred to as B type), in which multiple CpG motifs are expressed on a linear phosphorothioate backbone (18). D-type ODN (also referred to as A type) differ structurally from K type in expressing a single CpG motif on a palindromic loop and having a run of polyguanosine (poly-G) nucleotides at the 3’ end (18). D and K ODN differ in their cellular specificity and functional activity, although both trigger via TLR9 (19). This has been largely attributed to the poly-G tail on D ODN, as D ODN lacking this tail are inactive.

In this context, poly-G motifs are recognized by scavenger receptors expressed on the surface of immune cells that improve the uptake of ODN containing such motifs (20). Evidence from murine studies suggests that poly-G ODN may have a role in the treatment of cancer. For example, ODN expressing TTAGGG motifs reduce carcinogen-induced inflammation and block tumor formation in the 7,12-dimethylbenz-A-anthracene/12-O-tetradecanoylphorbol 13-acetate skin carcinoma model (21).

This study initially sought to compare the antitumor activity of K versus D ODN. Unexpectedly, results showed that the poly-G tail of the D ODN manifests antitumor activity in a TLR9- and CpG-independent manner. Indeed, poly-G–containing ODNs lacking a CpG motif directly activated CD8 T cells to generate tumor-specific CTLs. Mechanistic studies established that poly-G ODN induced the phosphorylation of Lck (T cell–signaling pathway) on mouse and human CD8 T cells, thereby enhancing the production of IL-2 and the CD8 T cell proliferation. Thus, poly-G ODN enable the expansion of tumor-specific CTLs.

Materials and Methods

Animals and cell lines

BALB/c mice were obtained from the National Cancer Institute (NCI; Frederick, MD) and studied at 6–8 wk of age. CD8 TCR-transgenic (Tg) mice specific for peptide 518–526 of PR8 hemagglutinin (HA) were a gift from Dr. T. Sayers (NCI). TLR9 and MyD88 knockout (KO) mice were kindly provided by Dr. S. Akira (Osaka University, Osaka, Japan). All studies were approved by the Animal Care and Use Committee. The following cell lines were purchased from American Type Culture Collection (Manassas, VA): CT26, which is a colon carcinoma cell line; and LBRM-33 clone 4A2, which is a T cell lymphoma. MC38, which is a colon carcinoma cell line, was gifted by Dr. G. Trinchieri (NCI).

ODN and reagent

ODNs were synthesized at the Core Facility of the Center for Biologics Evaluation and Research, Food and Drug Administration (Bethesda, MD). The sequences of ODN used in this study are present in Table I. All ODN were free of detectable protein or endotoxin contamination. Endotoxin contamination was assessed using the Limulus amebocyte cell lysate assay (Cambrex Bioscience, Walkersville, MD; sensitivity 0.1 U endotoxin/mg) and protein contamination using the Pierce bicinchoninic acid protein assay kit (Thermo Scientific, Rockford, IL; sensitivity 2.5 μg/ml).

A770041, an Lck inhibitor, was purchased from Axon Medchem (Groningen, The Netherlands).

In vivo tumor studies

Mice were challenged with viable tumor cells s.c. (the number of cells varied with the tumor type as described in the figure legends). Tumor growth

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curves were generated using 6–14 mice per group, and all results were
derived from combining data from two to three independent experiments.
Tumor size was calculated by the formula: (length × width × height)/2.
Any animal for which the tumor exceeded a diameter of 2.0 cm was
immediately euthanized as per Animal Care and Use Committee protocol. To
deplete NK or CD8 T cell subsets, mice were injected i.p. with anti-mouse
Asialo GM1 Ab (Wako Pure Chemical Industries) or anti-mouse CD8 (53-
6.72) Ab (BioXCell, West Lebanon, NH), respectively, on days 2 and
0 and then twice per week post-tumor implantation. To neutralize IL-2,
mice were injected i.p. with anti-mouse IL-2 (JES6-1A12) Ab from Bio-
XCell on days 12 and 15 and then twice per week post-tumor implantation.

Preparation of mouse and human CD8 T cells
Single-cell suspensions of splenocytes were layered onto Histopaque-1.083
(Sigma-Aldrich, St. Louis, MO) and centrifuged for 20 min at 2000 rpm.
Cells at the interface were isolated and purified by negative selection using
MACS Abs against CD4, MHC class II–, CD11c–, NK1.1–, and B220-
positive cells (Miltenyi Biotec, Auburn, CA). The purity of CD8 T cells
was >90% as determined by flow cytometry. Human PBMCs were separated by Ficol-Hypaque centrifugation (Sigma-
Aldrich) from buffy coats obtained from healthy donors. CD8+ T lympho-
cytes were purified by magnetic cell sorting using CD8+ T cell isolation kit
(Miltenyi Biotec).

ELISPOT assay
Spleen cells were isolated from naive or CT26 tumor-bearing mice treated
with ODN. A total of 1.5–3.0 × 10^6 splenocytes/well were stimulated for
14 h with 1 ng/ml CT26 immunomodulator T cell epitope AH-1, SPSTDYQF
(22), in 96-well Immulon II plates (Thermo LabSystems, Franklin, MA)
previously coated with monoclonal rat anti–IFN-γ Ab (BD Biosciences, San
Jose, CA) or IL-2 (R&D Systems, Minneapolis, MN). The plates were
washed and treated with biotinylated polyclonal goat anti–IFN-γ or anti–IL-
2 Ab (R&D Systems) followed by streptavidin alkaline phosphatase. Spots
were visualized by the addition of a 5-bromo-4-chloro-3-indolyl phosphatase
solution (Sigma-Aldrich) in low-melt agarose (Sigma-Aldrich) and counted
manually under ×40 magnification. The number of cytokine-secreting cells
was determined by a single blind reader, and all data were generated by
analyzing three separate wells per sample.

Cell proliferation assays
In vitro cell proliferation was analyzed by stimulating cells in vitro for 24 h
with HA peptide, anti-CD3 Ab (BD Pharmingen, San Diego, CA), or
Dynabeads Human T-Activator CD3/CD28 (one bead per cell; Invitrogen,
Grand Island, NY). Various ODN were included during culture in 96-well
plates (Corning, Corning, NY). [3H]Thymidine was added during the last
10 h of culture. [3H]Thymidine incorporation was analyzed with a β-
scintillation counter.

ELISA
Cytokine levels in culture supernatants were measured by ELISA, as de-
scribed previously (23). Pairing anti–IL-2 and anti–IFN-γ mAbs were
purchased from BD Pharmingen. The 96-well Immulon H2B plates
(Thermo LabSystems) were coated with cytokine-specific Ab and then
blocked with PBS/1% BSA. Culture supernatants were added, and bound
cytokine was detected by the addition of biotin-labeled secondary Ab,
followed by phosphatase-conjugated avidin and a phosphatase-specific
colorimetric substrate (Pierce, Rockford, IL). Standard curves were gen-
erated using recombinant cytokines purchased from R&D Systems.

Western blotting
LBRM-33 A2 cells stimulated with anti-CD3 Ab were lysed in cold lysis
buffer containing protease and phosphatase inhibitors. This solution was
boiled for 5 min, size-separated on a 4–12% gradient SDS-PAGE, and
transferred onto a polyvinylidene difluoride membrane. Immunoblots were
probed with Abs specific to the phospho-Src family (Tyr^416) Ab, phospho-
p38, and phospho-ERK1/2 (Cell Signaling Technology, Danvers, MA),
followed by HRP-coupled donkey secondary Ab (GE Healthcare, Piscat-
away, NJ). Signals were visualized with the ECL Plus Western blotting
detection reagents (GE Healthcare).

Statistical analysis
A two-way ANOVA was used to analyze tumor growth. The Student t test
was used to analyze all other results. The p values <0.05 were considered
to be statistically significant.

Results
Antitumor activity of D ODN is CD8 T cell dependent but CpG
independent
Initial studies compared the ability of K versus D ODN to induce
tumor-specific immune responses. Earlier work showed that CpG
ODN were most effective when delivered intratumorally (17, 24).
Thus, CT26 colon cancer cells were inoculated into the flank of
BALB/c mice. Once the tumors became established (>5 mm in
diameter, typically on day 12), they were injected with 200 μg of
ODN. As seen in Fig. 1, both types of CpG ODN strongly inhibited
tumor growth (Fig. 1A, 1B). Yet although control K ODN (in
which the critical CpG motif was inverted to GpC) had no effect
on tumor growth, treatment with control D ODN inhibited the
development of CT26 tumors just as well as CpG-expressing D
ODN.

Previous studies attributed the tumor regression induced by K
ODN to CpG-dependent activation of the immune system via the
TLR9 pathway (6, 7, 25). Yet this mechanism could not explain
the activity of control D ODN, which lack a CpG motif. To ex-
amine this point, CT26 tumors were injected into syngeneic TLR9
KO mice and the effect of ODN administration examined. Treat-
ment with K or control K ODN had no effect on tumor growth,
consistent with previous reports. In contrast, both D and control

FIGURE 1. Effect of ODN treatment on the growth of established CT26
and MC38 tumors. Total of 10^3 CT26 (A–G) or 10^3 MC38 (H) colon cancer
cells were injected into the flank of BALB/c, C57BL/6, or TLR9 KO mice
on day 0. Once the tumors reached an average diameter of 5 mm (>day 12),
200 μg of ODN was injected intratumorally for 5 consecutive d. (C)
Mice were also treated i.p. with anti-CD8, anti-asialo GM1, or control Ab
on days 11, 13, and 15. Data represent the combined means ± SD of 7–14
mice/group from two to three independent experiments. The sequences of
the relevant ODNs are provided in Table I. *p < 0.05, **p < 0.01 when
compared with mice treated with PBS.
D ODN inhibited tumor growth in the TLR9-deficient mice (Fig. 1D, 1E). This finding suggests that a component of both D and control D ODNs was inducing tumor regression in a TLR9-independent manner. To gain further insight into the mechanism underlying this effect, neutralizing Abs were used to deplete either CD8+ T cells or NK cells from the tumor-bearing mice. D ODN-mediated protection was abrogated by depletion of CD8 T cells, whereas depletion of NK cells or the administration of control Ab had no effect on tumor regression–induced control D ODN (Fig. 1C). Similarly, the administration of anti-CD8 Abs without poly-G treatment had no effect on tumor development (Supplemental Fig. 2). These results suggest that CD8 T cells are essential for the in vivo antitumor effect following poly-G ODN administration.

Contribution of the poly-G motif to D ODN-mediated antitumor activity

D ODNs express both a 3’ poly-G tail (typically 6 Gs in length) and a central CpG dinucleotide arrayed on a palindromic hexamer that forms a stem-loop structure (18). Multiple sequence variants were synthesized in which nucleotides were substituted into either the stem-loop or poly-G tail (Table I). In vivo studies showed that alterations in the poly-G tail abrogated the antitumor activity of D-control ODN. For example, replacing the entire poly-G region with poly-A eliminated protection following tumor challenge (Fig. 1F). As the D-control poly-A ODN contained all other elements of the D-control ODN, this finding suggests that the poly-G tail was responsible for the antitumor activity. Consistent with this prediction, an ODN consisting solely of 12 Gs was fully protective ($p < 0.01$), whereas a 6-mer poly-G provided detectable but more limited protection ($p < 0.05$; Fig. 1G). In contrast, poly-A ODNs of similar length were nonprotective (Fig. 1G). The protection conferred by poly-G ODN was not limited to a single tumor type or mouse strain. Virtually identical findings were obtained using the MC38 colon cancer model in C57BL/6 mice (Fig. 1H).

Poly-G ODN enhance tumor-specific immunity

To clarify the mechanism by which ODN-containing poly-G motifs promote antitumor immunity, cells were isolated from the draining lymph nodes (LN) of CT26 tumor-bearing mice. The cells were cultured ex vivo with the AH-1 peptide, which is a CD8-restricted epitope expressed by CT26; cultured ex vivo with the AH-1 peptide, which is a CD8-restricted lymph nodes (LN) of CT26 tumor-bearing mice. D ODN-mediated protection was abrogated by depletion of CD8 T cells, whereas depletion of NK cells or the administration of control Ab had no effect on tumor regression–induced control D ODN (Fig. 1C). Similarly, the administration of anti-CD8 Abs without poly-G treatment had no effect on tumor development (Supplemental Fig. 2). These results suggest that CD8 T cells are essential for the in vivo antitumor effect following poly-G ODN administration.

Consistent with the ability of CpG ODN to induce antitumor immunity, the number of cells spontaneously secreting IFN-γ in the draining LN of tumor-bearing mice treated with either K or D ODN was significantly elevated when compared with animals with tumor alone (Fig. 2A). Restimulating these cells ex vivo with AH-1 peptide resulted in a further 7-fold increase in IFN-γ production. Tumor-bearing mice treated with control K ODN resembled PBS-treated mice in terms of both background and AH-1–dependent IFN-γ production.

The analysis of D-type ODN and associated controls yielded a very different outcome. LN cells from mice treated with control D ODN in which the CpG motif was inverted to an inactive GpC retained their ability to respond to AH-1 stimulation (Fig. 2A). This activity was lost when poly-A was substituted for the poly-G tail (Fig. 2B). Moreover, cells from tumor-bearing mice treated with a pure poly-G ODN responded strongly to AH-1 stimulation, whereas those treated with a pure poly-A ODN had no activity (Fig. 2B). This enhancement in tumor-specific IFN-γ–producing cells was observed from 3 to 7 d after poly-G treatment (Fig. 2C). However, no significant difference in total CD8 T cells was observed following poly-G ODN administration (Supplemental Fig. 3). These findings support the conclusion that a poly-G motif is necessary and sufficient to induce tumor-specific CD8 T cell activation and does not increase total CD8 T cell numbers.

Poly-G–containing ODN enhance the proliferation of activated T cells

The mechanism by which ODN-containing poly-G motifs enhance tumor-specific immunity was explored. Their effect on Ag presentation was investigated by using spleen cells from Tg mice in which CD8 T cells express the receptor for HA. When these cells were stimulated in vitro with a low concentration of HA peptide, their proliferation was minimal (Fig. 3A). Significantly greater proliferation was observed when poly-G or control D ODN were added to the peptide-stimulated cultures (Fig. 3A). Even more striking was the effect of poly-G–containing ODNs on the HA-dependent production of IFN-γ. Whereas peptide or ODN alone had no effect on IFN-γ levels, the combination of HA plus any poly-G–expressing ODN resulted in a significant elevation in IFN-γ concentration (Fig. 3B). In all of these studies, replacing the poly-G motif with a poly-A abrogated peptide-induced T cell proliferation and IFN-γ production.

Previous studies showed that control D ODN did not induce APCs to mature or secrete cytokines (18, 19 and data not shown). Thus, it seemed likely that T cells rather than APCs were the target of the poly-G ODN. To evaluate this hypothesis, CD8+ lymphocytes were purified from both TLR9 and MyD88 KO mice. Poly-G–expressing ODNs significantly enhanced CD8 T cell proliferation and IFN-γ production in these cultures despite the absence of APCs (Fig. 3C and data not shown). It should be noted that poly-G ODN had no effect on unstimulated CD8 T cells (Fig. 3C) but had a small effect when added to unseparated spleen cells (Fig. 3A). This may reflect either the interaction of APCs with T cells in the spleen or poly-G–induced proliferation of splenic macrophages (26). Pursuing these findings, we examined whether poly-G ODN could stimulate human CD8 T cells. As in mice, human T cells stimulated with anti-CD3 Ab and poly-G–expressing ODNs significantly enhanced CD8 T cell proliferation and IFN-γ production in these cultures despite the absence of APCs (Fig. 3C and data not shown). It should be noted that poly-G ODN had no effect on unstimulated CD8 T cells (Fig. 3C) but had a small effect when added to unseparated spleen cells (Fig. 3A). This may reflect either the interaction of APCs with T cells in the spleen or poly-G–induced proliferation of splenic macrophages (26). Pursuing these findings, we examined whether poly-G ODN could stimulate human CD8 T cells. As in mice, human T cells stimulated with anti-CD3 Ab and poly-G–expressing ODNs significantly enhanced CD8 T cell proliferation and IFN-γ production in these cultures despite the absence of APCs (Fig. 3C). These results suggested that poly-G ODN mediated tumor protection in a TLR9-independent manner. To confirm that observation, CD8 T cells were purified from both TLR9 and MyD88 KO mice. In the absence of stimulation, those cells neither proliferated nor secreted IFN-γ (Fig. 3E, 3F and data not shown).

The addition of anti-CD3 Ab induced a low level of proliferation.

Table I. Sequence of synthetic ODNs used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-CpG (1555)</td>
<td>GCTAGAAGCTTTAAGCCTT</td>
</tr>
<tr>
<td>K-Control (1612)</td>
<td>GCTAGAAGCTTTAAGCCTT</td>
</tr>
<tr>
<td>D-CpG (D35)</td>
<td>GGtgcattgatgcagGGGGGGGG</td>
</tr>
<tr>
<td>D-Control (D122)</td>
<td>GGtgcattgatgcagGGGGGGGG</td>
</tr>
<tr>
<td>D-Control poly A</td>
<td>GGtgcattgatgcagGGGGGGGG</td>
</tr>
<tr>
<td>Poly-G</td>
<td>GGGGGGGGGGGGGGGGGGGGGGG</td>
</tr>
<tr>
<td>Poly-A</td>
<td>GGGGGGGGGGGGGGGGGGGGGGG</td>
</tr>
<tr>
<td>G2 3’ tail</td>
<td>TCAAGCTTTAGGGGGGGGGGGGG</td>
</tr>
<tr>
<td>G3 3’ tail</td>
<td>TCAAGCTTTAGGGGGGGGGGGGG</td>
</tr>
<tr>
<td>G4 3’ tail</td>
<td>TCAAGCTTTAGGGGGGGGGGGGG</td>
</tr>
<tr>
<td>G6 3’ tail</td>
<td>TCAAGCTTTAGGGGGGGGGGGGG</td>
</tr>
<tr>
<td>G9 3’ tail</td>
<td>TCAAGCTTTAGGGGGGGGGGGGG</td>
</tr>
<tr>
<td>G6 5’ tail</td>
<td>GGGGGGTTGCGAGCTTGGAGAGAG</td>
</tr>
<tr>
<td>Center G6</td>
<td>TCAAGCTTTAGGGGGGGGGGGGG</td>
</tr>
</tbody>
</table>

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and cytokine production. Both measures of T cell activation were significantly enhanced by the addition of ODN-expressing poly-G motifs (Fig. 3E, 3F). As APCs were absent from these cultures, such results suggest that poly-G–containing ODN act directly on CD8 T cells to enhance their receptivity to activation in a TLR9- and MyD88-independent manner. ODN lacking poly-G motifs (including K CpG ODN) were unable to enhance the activation of CD8 T cells (Fig. 3E, 3F and data not shown).

**Effect of poly-G length and location on T cell activation**

Several sets of ODNs were synthesized to determine how the length and location of the poly-G motif influenced T cell stimulation.
Initial experiments examined the effect of pure poly-G ODN ranging from 2 to 12 nt in length. As seen in Fig. 3G, T cell proliferation rose significantly once the poly-G motif reached 6 nt in length, an effect that persisted in 9- and 12-mers.

Having established that a 6-mer poly-G was strongly stimulatory, the importance of location was probed by inserting that 6-mer at various sites in a control (nonstimulatory) TCAAGCTTGA ODN. Stimulation was greatest when the poly-G motif was located at the 3' end, modest when located centrally, and undetectable when placed at the 5' end (Fig. 3H).

To further clarify the importance of poly-G length on ODN activity, motifs ranging from 2 to 9 nt in length were inserted at the 3' end of the control ODN. Consistent with results from Fig. 3G, a 6-mer poly-G tail was required for optimal stimulation (Fig. 3I).

**Contribution of IL-2 to poly-G–mediated T cell activation and antitumor activity**

As IL-2 plays an important role in the proliferation of T cells, the effect of poly-G ODN on IL-2 levels was examined. Studies were performed on T cells from TLR9 KO mice to eliminate any possible influence of CpG motifs. When the T cells were stimulated with anti-CD3 Ab, the production of IL-2 was minimal (Fig. 4A). However, when poly-G-containing ODN were coadministered with the anti-CD3, IL-2 production by CD8+ T cells increased significantly. This effect was not observed with poly-G ODN alone or when D-control poly-A ODN was coadministered with the anti-CD3 (Fig. 4A).

To exclude the possibility that IL-2 production was influenced by non-T cells present in culture, this experiment was repeated using the IL-2–secreting LBRM33 CD8 T cell line (27). As above, the combination of poly-G ODN plus anti-CD3 Ab significantly enhanced IL-2 production (Fig. 4B). These results support the conclusion that poly-G ODN work directly on CD8 T cells, increasing their ability to proliferate and secrete cytokines (IL-2 and IFN-γ) in response to Ag or other forms of TCR stimulation.

This observation was pursued by studying the effect of poly-G ODN in vivo. Either naive or tumor-bearing mice were treated with ODN and IL-2 production by cells in the draining LN examined ex vivo by ELISPOT assay. No change in IL-2 production was observed when poly-G ODN was injected into naive mice or when poly-A ODN was injected into the tumor (Fig. 4C). In contrast, the number of IL-2–producing cells in tumor-bearing mice rose significantly after treatment with poly-G ODN. This effect peaked at 3 h, persisted through 12 h, and returned to baseline 24 h after ODN delivery (Fig. 4D).

To examine whether this upregulation of IL-2 by poly-G ODN contributed to antitumor immunity, neutralizing Ab was used to deplete IL-2 after poly-G treatment of tumor-bearing mice. As seen in Fig. 4E, depletion of IL-2 abrogated poly-G–dependent tumor protection.

**Poly-G ODN promote phosphorylation via the Lck pathway**

We sought to clarify the signaling pathway responsible for the poly-G–mediated upregulation of IL-2 transcription. Multiple molecules can induce the expression of IL-2 in T cells, most notably phosphorylated NF-κB, JNK, ERK, and/or p38 MAPK (28, 29). The phosphorylation of those molecules was therefore examined in LBRM33 cells costimulated with poly-G plus anti-CD3 Ab. Results showed that poly-G ODN in the absence of anti-CD3, and anti-CD3 in the absence of poly-G ODN, induced a modest, concentration-dependent phosphorylation of ERK and p38 (Fig. 5A, 5B). The magnitude of this phosphorylation was significantly increased by costimulating the cells with poly-G plus anti-CD3. In contrast, poly-A ODN had no effect on phosphorylation, and poly-G plus anti-CD3 did not enhance the phosphorylation of JNK or NF-κB (Fig. 5A and data not shown).
The upstream signaling pathway common to both ERK and p38 involves the Src family tyrosine kinase Lck. Consistent with that model, phosphorylation of Lck was observed when LBRM33 cells were costimulated with poly-G ODN plus anti-CD3. This effect was mediated by poly-G in a concentration-dependent manner and was not observed using poly-A ODN (Fig. 5A, 5B). Consistent with a model of cascading immune stimulation, the increase in IL-2 levels following poly-G ODN stimulation was inhibited by A770041, an inhibitor of Lck (30) (Fig. 5C).

**Discussion**

This work uses the well-established CT26 and MC38 colon cancer models to examine the effect of intratumoral administration of poly-G ODN on tumor growth. Interest in the activity of poly-G motifs grew from preliminary studies designed to compare the activity of K versus D CpG ODN. The latter, which are unique in expressing a poly-G tail, were found to manifest antitumor activity in a CpG- and TLR9-independent manner (Fig. 1A, 1B, 1D, 1E). Extensive studies of sequence-modified D ODNs revealed that the poly-G tail was both necessary and sufficient for this antitumor activity (Fig. 1F, 1G). Exploration of the mechanism underlying this effect indicates that poly-G ODNs act directly on CD8+ T cells, causing them to proliferate and secrete cytokines including IFN-γ and IL-2 (Figs. 3, 4).

IL-2 is a canonical T cell growth factor that plays a critical role in the expansion of CD8+ memory T cells (28, 29). Autocrine production of IL-2 enables the expansion of tumor-specific CTLs (31–34). Although IL-2 has been approved by the Food and Drug Administration for use in melanoma and renal cancers, its therapeutic utility is compromised by a short t1/2 and dose-limiting toxicity (35–38). These problems might be circumvented by intratumoral injection of poly-G ODN, as such treatment could drive the local production of IL-2 by tumor-specific T cells, thereby minimizing systemic side effects.

Current results demonstrate that poly-G ODN stimulate the expansion of CD8+ T cells both in vitro and in vivo (Figs. 2, 3). Preliminary studies further show that poly-G ODN directly stimulated CD4+ T cells. These effects required that the T cells also be triggered via their Ag receptor, as poly-G stimulation alone failed to induce T cell proliferation or IL-2 production (Figs. 3, 4). This series of findings is consistent with results from Bendigs et al. and Lipford et al. (39, 40), who showed that ODN containing both CpG and poly-G motifs were able to costimulate T cells activated via their TCR. Such ODN induced the expansion of T cells, and the effects were blocked by cyclosporin A or anti–IL-2 Ab. As T cells do not express TLR9 (Supplemental Fig. 1C), we interpret their results as showing that the poly-G component of the ODNs studied was responsible for the T cell expansion. In contrast, Jing et al. (41, 42) recently reported that poly-G ODN can suppress the proliferation of cancer cells by blocking STAT3 activation. Although we also observed an inhibitory effect of poly-G ODN on the proliferation of CT26 tumor cells, our in vivo studies do not support the conclusion that these ODN are directly tumoricidal. Specifically, there was no suppression of tumor growth in RAG1 KO mice or in mice depleted of CD8 T cells (Fig. 1C and data not shown). These findings suggest that poly-G–mediated tumor regression is CD8 T cell dependent rather than due to a direct effect on tumor cells.

Peng et al. (43) reported that poly-G ODN suppressed Treg cell function through a mechanism dependent upon the TLR8–MyD88 pathway. In our studies, poly-G ODN remained active in MyD88 KO mice (Fig. 3F). Because the TLR8 pathway is entirely MyD88 dependent, we conclude that the stimulatory activity of poly-G ODN differs from the suppressive activity observed by Peng et al. (43). Consistent with that interpretation, the LBRM33 T cell line is highly responsive to poly-G ODN-mediated activation but does not express TLR7 or TLR8. We also found that RAW 264.7 macrophages express TLR7/8 but are unresponsive to stimulation with poly-G ODN (19, 26) (Supplemental Fig. 1A–C). Rather, current findings indicate that poly-G ODN directly interact with CD8+ T cells to stimulate the Lck pathway, thereby triggering phosphorylation of the ERK and p38 components of the MAPK pathway (Fig. 5). This is consistent with evidence that Lck–activated ZAP-70 directly induces the phosphorylation of p38 and ERK, leading to the induction of IFN-γ/IL-2 and the proliferation/differentiation of T cells (44–46). The TCR–CD3 complex and the CD28 molecules that provide the costimulatory signal needed by T cells to initiate IL-2 production lie upstream of Lck (28, 29).

Poly-G ODN serve to enhance the effects of TCR stimulation, leading to a significant expansion in CD8+ T cell activation. In the context of tumor-specific immunity, we found that poly-G ODN improved the generation of tumoricidal T cells in the draining LN (Figs. 2, 4). The costimulation provided by poly-G administration also boosted IL-2 production in tumor-bearing mice through the enhancement of Lck phosphorylation (Fig. 4). Studies of the tu-
morb microenvironment suggest that T cells are tolerized or be-

come anergic owing either to the absence of critical costimulatory

signals or the presence of suppressive factors/cells (47–49). Yet

current results show that poly-G ODN contributed to the expa-
nation of CD8+ T cells in the tumor microenvironment.

Current results help clarify several intriguing observations made

in earlier studies of D ODN. Efforts to optimize the activity of that

class of CpG ODN revealed that inclusion of a 3′ poly-G tail was

critical. Although modest activity was observed when a string of 4

Gs was added to the ODN, maximal immune stimulation required

the presence of 6 Gs. This set of findings is consistent with results

shown in Fig. 3, in which the greatest stimulation was obtained

when the poly-G motif was at least 6 nt in length and located at

the 3′ end of the ODN.

The mechanism underlying the antitumor activity of poly-G ODN

includes the enhancement of IL-2 production, which drives T cell

proliferation. Indeed, current results are the first, to our knowl-

dge, to document that poly-G ODN can directly activate CD8+ T

cells, a finding equally observed in cells from both mice and hu-

mans (Fig. 3). This raises the possibility that poly-G ODN may be

of use in clinical situations that require strong T cell stimulation,

as in vaccine adjuvants and cancer immunotherapy. Such a con-

clusion is consistent with the observation that D-type CpG ODN,

which are characterized by their expression of poly-G motifs, are

better immune adjuvants than K CpG ODN (50). The novel fea-

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with other cancer treatments, including radiation and chemother-

apy. Ongoing efforts are directed toward identifying the receptor

involved in poly-G ODN recognition, as this should facilitate the

development of agents with greater activity and therapeutic utility.

Disclosures

The authors have no financial conflicts of interest.

References


integrating immunity’s roles in cancer suppression and promotion. Science

331: 1565–1570.


Rev. Drug Discov. 5: 471–484.


oligonucleotides induces the differentiation and reduces the immunosuppressive


669.


oligomer and its crosstalk with other classes of TLRs in human monocytes.

Eur. J. Immunol. 41: 11.9A.


P. G. Wijnands, J. B. Haanen, A. J. van der Eerdtwijk, R. J. Scheper, and T. D.

de Grujil. 2008. Local administration of PF-3512676 CpG-B instigates tumor-

specific CD8+ T-cell reactivity in melanoma patients. Clin. Cancer Res. 14:

3133–3142.

11. Liu, C., Y. Lou, G. Lizie, H. Qin, S. Liu, B. Rabinovich, G. J. Kim, Y. H. Wang,

Y. Ye, A. G. Sikora, et al. 2008. Plasmacytoid dendritic cells induce NK cell-

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References

Supplemental Figure 1

A. IL-6 (pg/ml)

B. TNFα (pg/ml)

C. Western blot analysis of TLR7, TLR8, TLR9, and GAPDH

D. IL-2 (pg/ml)
Supplemental Figure 2

**Days after inoculation**

<table>
<thead>
<tr>
<th>Tumor volume (mm³)</th>
</tr>
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<tbody>
<tr>
<td>15 19 23 27</td>
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</table>

- **PBS**
- D-Control ODN
- + anti-CD8 Ab
- anti-CD8 Ab
Supplemental Figure 3

Days after poly-G treatment

# of CD8 T cell in LN

PBS
Poly-G
Supplemental Figure Legends

Supplemental figure 1. Effect of ssRNA and poly-G ODN on CD8 T cell and macrophage cell lines

A and B) The RAW 264.7 macrophage cell line and the LBRM33 4A2 CD8 murine T cell line were cultured with 3 uM ssRNA or 3 uM poly-G ODN for 24 h. Culture supernatants were assayed for IL-6 and TNFα levels by ELISA. Data represent the mean + SD from three independent experiments. C) TLR7/8/9 mRNA levels were determined by RT-PCR in the RAW 264.7 macrophage cell line and LBRM33 CD8 T cell line. D) The LBRM33 cell line was cultured with anti-CD3 Ab (1 ng/ml) plus ssRNA or poly-G ODN (3 uM). IL-2 levels in culture supernatants were assessed after 24 hr by ELISA. Data represent the mean + SD from three independent experiments.

** p < 0.01 versus untreated group.

Supplemental figure 2. Effect of poly-G ODN and anti-CD8 Ab treatment on the growth of established CT26 tumors.

Mice were treated as described in Fig. 1. Once the tumors reached an average diameter of 5 mm (≈day 12) 200 ug of ODN was injected intra-tumorally for 5 consecutive days. Some groups of mice were also treated i.p. with anti-CD8 Ab on days 11, 13, and 15. Data represent the combined mean + SD of 5 - 12 mice/group from 2-3 independent experiments.

** p < 0.01 versus PBS treated group.
Supplemental figure 3. The number of CD8 T cells in tumor draining lymph node following poly-G treatment.

Mice were treated as described in Fig. 2C. 1, 3 or 7 days after ODN treatment, single cell suspensions were prepared from the draining lymph node of each animal. The number of CD8\(^+\) and CD3\(^+\) cells in the lymph node of PBS treated vs poly-G ODN treated mice were determined by FACS. Results represent the mean of 5-6 independently analyzed mice/group.