

Screening of novel immunostimulatory CpG ODNs and their anti-leukemic effects as immunoadjuvants of tumor vaccines in murine acute lymphoblastic leukemia

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Abstract. Acute lymphoblastic leukemia (ALL) is a common malignant disease and a major cause of mortality due to recurrent disease. Immunotherapy is a promising strategy for eradicating minimal residual disease and thus preventing the relapse of leukemia. Apart from stem cell transplantation, CpG oligodeoxynucleotides (ODNs) are excellent candidates for the immunotherapy of leukemia. However, the number of usable CpG ODNs is limited. In this study, we tested a panel of CpG ODNs and obtained three CpG ODN sequences with strong immunostimulatory activity by comparing their capacity to activate lymphocytes. The data revealed that the flanking bases, the spacing of individual CpG motifs and poly-guanosine ends, contribute to the immunostimulatory activity of a CpG ODN. In the immunotherapy of murine leukemia with the novel ODN as the adjuvant, we found that CpG Seqs 14 and 19 were effective in the treatment of a leukemia model by prolonging survival span, augmenting natural killer cell and CTL cytotoxicity, as well as increasing the number of long-term survivors. The ability of CpG ODN to induce both strong innate and adaptive anti-leukemic immune activity could render it an appropriate agent for therapeutic applications in acute leukemia. This study demonstrates the feasibility of active immunotherapy with CpG ODNs in patients with acute leukemia and thus represents a potential alternative therapeutic strategy for eradicating residual disease which is resistant to conventional cytoreductive treatment.

Introduction

Despite intensive chemotherapy regimens, long-term survival occurs in only a third of cases of adult acute leukemia.

Additional therapeutic strategies are required in order to further prolong remission duration and eradicate minimal residual disease, ideally with less toxicity than conventional chemotherapy. One of these approaches is active immunotherapy with novel vaccination regimens. We previously launched a phase-I clinical trial and evaluated the efficacy and toxicity of vaccination in patients with relapsed or refractory acute leukemia, which proved to be a feasible, safe, and capable way of eliciting anti-leukemic responses *in vivo* (1).

As tumor cells are considered to be poorly immunogenic, it is difficult to elicit an effective specific anti-tumor activity from these cells alone. In addition, tumor cells develop various immune mechanisms to escape from host immune surveillance, for example, down-regulating co-stimulatory signals essential for the activation of the immune system. An appropriate adjuvant could help break the immune tolerance in acute leukemia patients. To that end, tumor antigens need to be administered in combination with adjuvants. The most common adjuvant used so far in experimental models is Incomplete Freund's adjuvant (IFA). IFA has also been successfully used in human immunotherapy against melanoma involving gp 100 peptide immunization (2). However, it is not widely used in human vaccination protocols due to its undesirable severe side-effects, which include swelling and indurations at the injection site. IFA has also been reported to induce specific CTL tolerance as opposed to immunity against immunizing antigens (3). Cytokines, such as interleukin (IL)-2, are another type of adjuvant being increasingly used in hematological malignant disease. However, systemic therapy with cytokines is also associated with severe toxic side effects, including wheezing and difficulty in breathing, dizziness when changing position, rapid weight gain or sudden swelling, chest pain or irregular heartbeat, and so on. For these reasons, alternative potent and safe adjuvants need to be identified.

In the last few years, a type of synthetic oligonucleotide containing unmethylated cytosine and guanine dinucleotides within specific-sequence contexts (CpG motifs), has been shown to provoke strong immune responses (4) as well as remarkable T cell-dependent anti-tumor efficacy in a series of murine tumor models. CpG oligodeoxynucleotides (ODNs) are thought to mimic bacterial DNA and are recognized as a 'danger' signal by the innate immune system of mammals.

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As toll-like receptor (TLR)9 agonists, CpG ODNs boost innate immunocytes such as B-cells, dendritic and natural killer (NK) cells, as well as monocytes, causing them to proliferate, mature and produce various cytokines, including IL-6, and -12, tumor necrosis factor (TNF) and interferon (5). In addition, CpG ODN stimulation leads to the generation of Th1 type adaptive immune responses (6-8). These properties have led to considerable interest in the clinical use of CpG ODNs in vaccines, cancer and autoimmune disease therapy (9).

Despite these promising benefits of CpG ODNs, the numbers of usable CpG ODNs are limited in the current clinical setting of cancer immunotherapeutics. The clinical restriction of CpG ODNs is due to several reasons: The absence of convenient screening assays, difficulty to predict the structure of potent CpG ODNs and the differences between species of CpG motif recognition (10). While the primary focus for the medicinal application of CpG ODNs has been on cancer immunotherapy in pre-clinical and clinical trials against cancer (11-14), there have been no reports of the *in vivo* consequences of the immunotherapeutic effects of CpG ODN treatment as adjuvants in acute leukemia. In this study, in order to develop CpG ODNs for clinical use, we designed a panel of CpG ODNs (Table I) with different structural features following the characteristics which contribute to the potency of a human CpG ODN (15). We then compared their ability to induce cell proliferation and cytokine secretion, and selected the most potent ODN. Finally, we observed the treatment effect of vaccines with the formulation of a CpG ODN adjuvant and their ability to elicit anti-leukemic immune responses in a mouse model.

Materials and methods

Mice. Six-week-old female Balb/c mice were purchased from the Laboratory Animal Center, Medical School of Xi'an Jiaotong University. Female DBA/2 mice between 4 and 6 weeks of age were purchased from the Shanghai Laboratory Animal Center after approval by the Chinese Academy of Sciences. The animals were housed in a specific pathogen-free animal facility under isothermal conditions and were allowed access to food and water *ad libitum*.

Leukemia cell line and cell culture. L1210, which is a murine lymphoid leukemia cell line of DBA/2 origin, was a generous gift from Professor Yili Wang (Institute for Cancer Research, College of Life Science and Technology, Xi'an Jiaotong University, China) and was maintained in RPMI-1640 (Gibco, BRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37°C in a humidified atmosphere of 5% CO₂.

CpG ODN. CpG ODN were synthesized and purified by the Coley Pharmaceutical Group (San Diego, USA) under good manufacturing practice conditions. The sterile, endotoxin-free solution of CpG ODN was supplied in vials containing 1 mg in 1 ml TE buffer and stored at -20°C. For future use, CpG ODN was further reconstituted in sterile PBS (0.1 M PBS, pH 7.2) at a concentration of 500 μ g/ml and stored at 4°C. All dilutions were conducted using pyrogen-free reagents.

The sequences of CpG ODN used in this study are described in Table I.

The in vitro study

Cell preparation and culture. Human peripheral blood mononuclear cells (PBMCs) were collected and isolated from the peripheral blood of healthy volunteers by Ficoll-Paque density gradient centrifugation (Histopaque-1077, Sigma-Aldrich, St. Louis, MO, USA) as previously described (16), after receiving approval from the Institute for Cancer Research, College of Life Science and Technology, Xi'an Jiaotong University. Cells were suspended in RPMI-1640 culture medium supplemented with 10% (v/v) heat-inactivated (56°C, 30 min) fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin (all from Life Technologies, Grand Island, NY). All compounds were purchased endotoxin-tested. Viability was determined before and after incubation with ODN by trypan blue exclusion (conventional microscopy). In all experiments, >95% of PBMCs were viable. The cells (final concentration, 2.5x10⁶ cells/ml) were then cultured in complete medium in a 5% CO₂-humidified incubator at 37°C for 48 h. Different ODNs (Table I; final concentration, 1 μ g/ml) and LPS (from *Salmonella typhimurium*; Sigma Aldrich) were used as stimuli.

Spleens were removed from 6-week-old female Balb/c mice, dispersed and centrifuged at 1,000 rpm for 5 min. The supernatant was removed and the cell pellet was re-suspended in 0.84% NH₄Cl to eliminate red blood cells. Cells were washed twice with RPMI-1640 medium, re-suspended in the culture medium and cultured at 5x10⁶/ml with the different ODNs.

Cell proliferation assay. The proliferation of human PBMCs and mice splenocytes was assessed by the quantitative assay, Cell Counting Kit-8 (CCK-8) (Dojindo, Gaithersburg, MD) according to the manufacturer's instructions. Briefly, this cell-counting method is an optical density colorimetric assay that quantifies the number of viable cells per well based on the activity of cellular dehydrogenases. At 44 h, 20 μ l/well CCK-8 were added to the medium and cultured together for 4 h, and then the absorbance at 450 nm was determined.

Detection of cytokines. PBMCs and mouse splenocytes were plated in 24-well dishes using 2.5x10⁶ or 5x10⁶ cells/ml, respectively. The CpG ODNs were added to the cell cultures at a final concentration of 1 μ g/ml. The cells were then incubated at 37°C for 24 h and the supernatants were collected for ELISA assays. The experiments were performed twice for each CpG ODN in triplicate. The secretion of IL-6 and -12p70 was measured by ELISA according to the manufacturer's instructions. The required reagents, including cytokine antibodies and standards, were purchased from PharMingen.

The in vivo study

Leukemia model. DBA/2 mice received live L1210 cells on day 0 by intraperitoneal (i.p.) injection. The cell dose was 1x10⁵ cells per mouse. Cells were diluted in 200 μ l of PBS before injection. We then divided the animals into 7 groups, 10 mice in each. All treatments were initiated the following day.



SPANDIDOS the CpG ODN panel used.

PUBLICATIONS

ODN	Sequence (5' to 3')	Length
1826	TCCATGAC <u>CG</u> TTCCCTGAC <u>CG</u> TT	20 mer
2006	<u>TCG</u> TCGTTTTGT <u>CG</u> TTTTGT <u>CG</u> TT	24 mer
CpG 2006GC (2137)	TGCTGCTTTTGTGCTTTTTGTGCTT	24 mer
CpG 1826GC (2138)	TCCATGAGCTTCCTGAGCTT	20 mer
CpG Seq 1	TCGT <u>CG</u> TATTGT <u>CG</u> TATTGT <u>CG</u> TA	24 mer
CpG Seq 2	TCGT <u>CG</u> TTTTGT <u>CG</u> TATTGT <u>CG</u> TA	24 mer
CpG Seq 3	TCGT <u>CG</u> TTTTGT <u>CG</u> TTTTGT <u>CG</u> TA	24 mer
CpG Seq 4	TCGT <u>CG</u> TCTTGT <u>CG</u> TCTTGT <u>CG</u> TC	24 mer
CpG Seq 5	TCGT <u>CG</u> TTTTGT <u>CG</u> TCTTGT <u>CG</u> TC	24 mer
CpG Seq 6	TCGT <u>CG</u> TTTTGT <u>CG</u> TTTTGT <u>CG</u> TC	24 mer
CpG Seq 7	TCGT <u>CG</u> TTTTGT <u>CG</u> TCTTGT <u>CG</u> TA	24 mer
CpG Seq 10	TCGT <u>CG</u> TTGT <u>CG</u> TT	14 mer
CpG Seq 11	TCGT <u>CG</u> TTGT <u>CG</u> TT GGGGGG	20 mer
CpG Seq 12	TCGT <u>CG</u> TTGT <u>CG</u> TTGT <u>CG</u> TT GGGGGG	26 mer
CpG Seq 13	TCGT <u>CG</u> TTTTGT <u>CG</u> TTGT <u>CG</u> TT	22 mer
CpG Seq 14	TCGT <u>CG</u> TTTTGT <u>CG</u> TTGT <u>CG</u> TT	23 mer
CpG Seq 15	TCGT <u>CG</u> TTGT <u>CG</u> TTTTGT <u>CG</u> TT	22 mer
CpG Seq 16	TCGT <u>CG</u> TTGT <u>CG</u> TTGT <u>CG</u> TT	21 mer
CpG Seq 17	TCGT <u>CG</u> TTGT <u>CG</u> TTGT <u>CG</u> TT	20 mer
CpG Seq 18	TCGT <u>CG</u> TTTGT <u>CG</u> TTGT <u>CG</u> TT	21 mer
CpG Seq 19	TCGT <u>CG</u> TTTGT <u>CG</u> TTTGT <u>CG</u> TT	22 mer
CpG Seq 20	TCGT <u>CG</u> TTTGT <u>CG</u> TTTGT <u>CG</u> TT	23 mer

CG dinucleotides are underlined. Poly G motifs are in bold letters. All sequences are synthesized with a wholly phosphorothioate backbone.

Table II. Vaccine formulation of each group.

Group	Vaccine formulation
Control	PBS solution
Single	Inactivated L1210
Non-CpG	Inactivated L1210 + CpG 1826GC or CpG 2006GC
1826	Inactivated L1210 + CpG 1826
2006	Inactivated L1210 + CpG 2006
Seq 14	Inactivated L1210 + CpG Seq 14
Seq 19	Inactivated L1210 + CpG Seq 19

Vaccines were prepared as described in Materials and methods.

Vaccine preparation. Live L1210 cells were conditioned with 60 μ g/ml of mitomycin C (Sigma) at 37°C for 30 min. CpG ODN (50 μ g, 0.1 ml) was then added into the inactivated L1210 cells (1×10^5 cells, 0.1 ml). The volume of all vaccine injections was 200 μ l.

Therapeutics. Mice received the vaccines by subcutaneous injection on days 1, 4, 7, 10 and 14 after the i.p. inoculation

of live L1210 cells. The vaccine formulation is shown in Table II. The animals were observed daily for survival for a minimum of 60 days.

Cytotoxicity assays of CTL and NK cells. At 14 days after inoculation, spleens from the tumor-bearing mice were collected and single-cell suspensions were prepared in order to test CTL activity and NK cytotoxicity. The erythrocytes were depleted with ammonium chloride lysis buffer. The cells were directly used as NK effector cells. For the preparation of CTL effector cells, splenocytes (5×10^6) were co-cultured with inactivated L1210 cells (1×10^6) in 5 ml RPMI-1640 for 5 days in the presence of recombinant IL-2 (20 U/ml) and were then harvested as CTL effector cells. The CTL and NK cytotoxicities were determined using the CytoTox 96 assay kit (Promega, Madison, WI, USA) following the manufacturer's instructions. L1210 and YAC-1 cells were used as the target cells for the CTL and NK cytotoxicity, respectively, and were seeded into the microtiter plate at various effector to target (E/T) ratios. The amount of lactate dehydrogenase (LDH) released in the culture supernatant was then measured, and the percentage of cytotoxicity was calculated according to the following formula: % of specific lysis = [(experimental LDH release-spontaneous LDH release)/(maximum LDH release-spontaneous LDH release)] x 100.

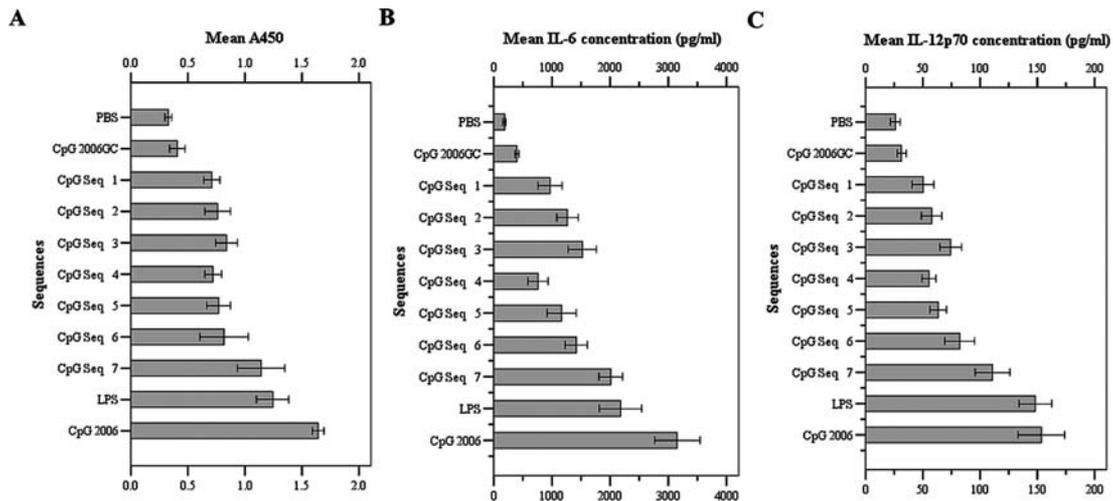


Figure 1. Effect of 3'-flanking bases of the CpG motif on the stimulatory activity of an ODN. (A) PBMCs were assayed for proliferation in response to the indicated ODN. Human PBMCs were incubated with ODN ($1 \mu\text{g/ml}$) with the sequences indicated in Table I. (B) IL-6 was determined in the supernatant. (C) PBMCs were stimulated and IL-12p70 was determined. Shown are the mean values and standard deviations from three independent experiments.

Statistical analysis. Data were expressed as the means \pm SD. The two-tailed Student's *t*-test was used for a two-group comparison. For a comparison of more than two groups the Kruskal-Wallis test (ANOVA) was used. Survival curves were plotted using Kaplan-Meier estimates. The log-rank test was used to analyze survival data. Statistical significance was set at <0.01 . All statistical tests were performed using SPSS 15.

Results

Differential immune activities of CpG ODN with different structural characteristics. The CpG motif is the essential element for the immunostimulation of bacterial DNA and synthetic ODNs. Other factors also contribute to the activity of CpG ODN, such as flanking bases, spacing, poly-guanosine (poly G) ends, length, number of CpG motifs, and so on. In this study, we examined a panel of synthetic B-class ODNs in order to investigate their structure-activity relationship.

In order to evaluate the impact of flanking bases on activity, we progressively replaced part or all of 3'TpT with 3'TpA or 3'TpC, and tested its ability to stimulate human PBMCs. The data indicate that substituting TpA (CpG Seqs 1-3 vs. CpG 2006, $P<0.01$) or TpC (CpG Seqs 4-6 vs. CpG 2006, $P<0.01$) for 3' TpT reduces ODN activity (Fig 1). In addition, there was no significant difference between 3'TpA and 3'TpC in stimulating activity (CpG Seq 1 vs. CpG Seq 4, CpG Seq 2 vs. CpG Seq 5, CpG Seq 3 vs. CpG Seq 6, $P>0.01$). Furthermore, the immunostimulating activity of CpG ODN decreased with the increment of substitution. For example, the activity of CpG Seq 1 and 4, with all of 3'TpT in the sequence replaced, was slightly lower than that of CpG Seq 2, 3, 5 and 6 ($P<0.01$). However, it should be noted that the activity of CpG Seq 7 with all of 3'TpT replaced in the sequence, was stronger than that of both CpG Seq 1 and 4 with the same level of substitution ($P<0.01$).

An important question is whether the immune effects of CpG motifs can be modified by the presence of other types of sequence motifs. Poly G runs consisting of 4-6 Gs, have previously been reported to increase the immunostimulatory

activity of phosphodiester ODNs (PE ODNs) (17-19). In order to analyze the effects of poly G strings on phosphorothioate ODNs (PTO-ODNs), CpG Seq 11 and 12, each of which contained a 3'-end poly G addition, were examined by proliferation assay and cytokine secretion (Fig. 2). The results showed that the 3'-end poly G addition did not enhance the activity of CpG Seq 11 (vs. CpG Seq 10, $P>0.01$). As for CpG Seq 12, the 3'-end poly G sequence addition greatly reduced the ability to induce cell proliferation and IL-6 secretion (vs. CpG Seq 17, $P<0.01$).

The spacing between single motifs is also critical, and the separation by TpT was preferable (CpG 2006) (15). However, the importance of isolated T spacing within CpG ODNs has not yet been studied in detail. Therefore, in this study, we designed a panel of CpG ODNs with different T spacing sequences. As shown in Fig. 3, CpG Seq 13 and 14, with T spacing adjacent to the 3'-end partly deleted, were not significantly different from CpG 2006 ($P>0.01$) concerning PBMC proliferation and IL-6 and IL-12p70 secretion. However, the immunostimulatory activity of CpG Seq 15 and 20, with the T spacing adjacent to the 5'-end deleted, was greatly decreased (vs. CpG 2006, $P<0.01$). CpG Seqs 16-19, whose 5'- and 3'-end T spacings were both changed, showed significantly decreased activity (vs. CpG 2006, $P<0.01$), except for CpG Seq 19. The immunostimulatory activity of CpG Seq 13, 14 and 19 was significantly higher than that of other ODNs, and was equivalent to that of CpG 2006 ($P>0.01$).

Screening of a panel of ODNs for most potent sequences. Previous studies have demonstrated that *in vitro* assays for PBMC activation, and the production of IL-6 and -12, could provide valuable information *in vitro* to predict the adjuvant activity of a given ODN *in vivo* (15). Based on these results, we screened a panel of CpG ODNs by comparing their ability to induce the proliferation of PBMCs and the production of IL-6 and IL-12p70, in order to find the most potent sequence for tumor vaccination.

As further statistical analysis showed (Table III), CpG Seqs 13, 14, and 19, were much more potent than other

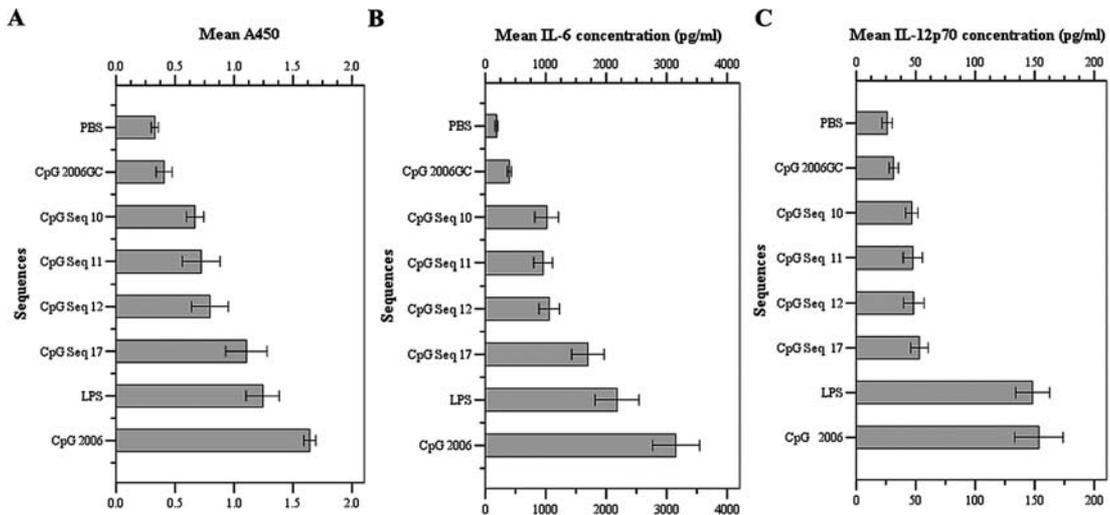


Figure 2. The 3'-end poly G addition cannot increase the immunostimulatory capacity of CpG ODNs which are completely phosphorothioate-modified. Shown are the mean values and standard deviations from three independent experiments.

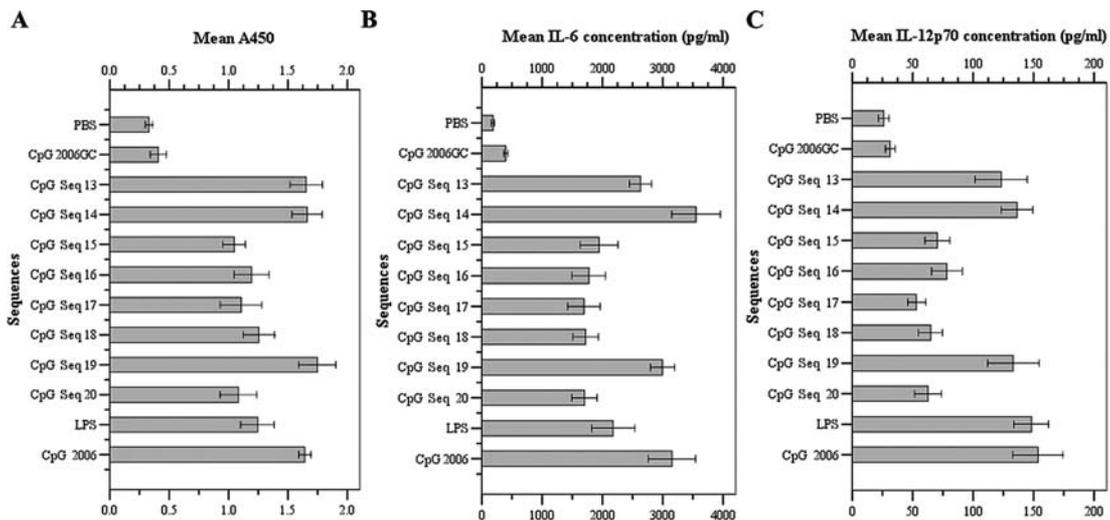


Figure 3. Effect of T spacing sequences on the stimulatory activity of CpG ODNs.

ODNs ($P < 0.01$). The maximum responses of CpG Seqs 14 and 19 were up to 93.4435 ± 9.8247 and $89.1638 \pm 12.2184\%$, respectively, almost equal to those of CpG 2006 ($P > 0.01$). In addition, there was no significant difference between CpG Seq 14 and 19. Moreover, CpG Seq 13 was a potent inducer of immunostimulation, and the maximum response was up to $80.7241 \pm 12.9917\%$. However, this ODN was slightly less active than CpG Seqs 14 and 19, and CpG 2006 as well ($P < 0.01$). In this primary screening, we obtained three B-class ODNs with strong immunostimulatory activity, Seqs 13, 14 and 19.

In vitro mouse splenocyte proliferation and cytokine secretion. Previous studies have demonstrated that CpG ODNs are effective vaccine adjuvants in mice (6,20-23). However, these mice stimulatory ODNs have shown more modest effects on human leukocytes (24). Due to the evolutionary divergence of TLR9 in CpG recognition between species, the precise

sequence motif that is optimal for stimulating immune cells of one species frequently differs from that which is optimal for stimulating cells of other species. In order to develop potent CpG ODNs for both humans and mice, we examined the immunostimulatory effect of the three sequences on mouse splenocytes. As seen in Fig. 4, CpG 1826, containing three '5'-GACGTT-3' motifs, showed the highest activity in inducing splenocyte proliferation and cytokine secretion ($P < 0.01$). In inducing IL-6 secretion, CpG 1826 showed activity as high as 1301 ± 110 pg/ml of IL-6. CpG Seqs 13, 14 and 19 showed similar activity as CpG 2006 in splenocyte proliferation ($P > 0.01$), while a little lower in IL-6 secretion ($P > 0.01$). However, CpG Seq 19 induced a markedly higher concentration of IL-12p70 compared to CpG 2006 ($P < 0.01$), while CpG Seq 13 and 14 showed the same activity with it ($P > 0.01$). On the whole, CpG Seqs 13, 14 and 19 showed strong immune activity in both humans and mice *in vitro* ($P < 0.01$).

Table III. Screening for the most potent phosphorothioate ODN sequences for the activation of human PBMCs.

ODN	% Maximim response			
	Proliferation	IL-6	IL-12	Average
CpG Seq 1	26.9445±4.3373	23.1652±5.3144	19.0271±6.4525	23.0456±5.8253
CpG Seq 2	30.3364±6.8520	32.1631±4.7968	24.6947±6.0327	29.0647±6.1609
CpG Seq 3	35.9401±5.7023	39.8247±6.2803	37.6304±6.4072	37.7984±5.5766
CpG Seq 4	27.6426±4.5795	17.1290±4.3516	22.8613±4.2521	22.5443±5.9396
CpG Seq 5	31.1050±6.4246	29.1197±6.3342	29.2978±4.7604	29.8408±5.1884
CpG Seq 6	34.4263±13.0492	36.6879±4.9666	44.0539±8.7219	38.3894±9.3146
CpG Seq 7	57.3490±12.5061	54.2744±5.2660	66.3868±10.2786	59.3367±10.1082
CpG Seq 10	24.0016±4.4947	24.6940±5.0527	16.0821±3.3152	21.5926±5.5992
CpG Seq 11	27.7249±9.8238	22.9662±3.9966	16.7804±5.5606	22.4905±7.6443
CpG Seq 12	32.8397±9.5352	25.9957±4.3898	17.1963±5.8695	25.3439±9.0710
CpG Seq 13	93.4208±8.2751	72.6642±4.6764	76.0874±14.6044	80.7241±12.9917 ^a
CpG Seq 14	93.9097±7.7033	100.0000±10.2961	86.4208±9.0534	93.4435±9.8247 ^a
CpG Seq 15	50.7604±5.8223	52.2112±7.9964	34.7457±6.9168	45.9058±10.3380
CpG Seq 16	61.0089±9.0953	47.3018±7.1550	40.8840±8.6249	49.7316±11.4602
CpG Seq 17	54.7235±10.7053	44.8600±6.8943	21.1272±4.9834	40.2369±16.4437
CpG Seq 18	65.3080±8.1215	45.6322±5.4850	30.3648±6.7056	47.1017±16.2914
CpG Seq 19	99.9976±9.6790	83.4871±5.1268	84.0068±14.5911	89.1638±12.2184 ^a
CpG Seq 20	53.2120±9.4316	45.0847±5.4055	28.6858±7.5261	42.3275±12.6795
CpG 2006	92.5346±3.1045	88.1764±10.1051	100.0000±13.9353	93.5703±10.1637

The level of immunostimulatory activity induced by 1 $\mu\text{g/ml}$ of 18 different ODNs was monitored in PBMCs from three donors. In order to facilitate the comparison between donors, the maximum response in each assay was set to 100, and the relative strength of each ODN was then calculated by the formula: $\frac{\text{Response to ODN} - \text{background}}{\text{Maximum response} - \text{background}} \times 10,000$

^aSignificantly greater than any other ODN ($P < 0.01$).

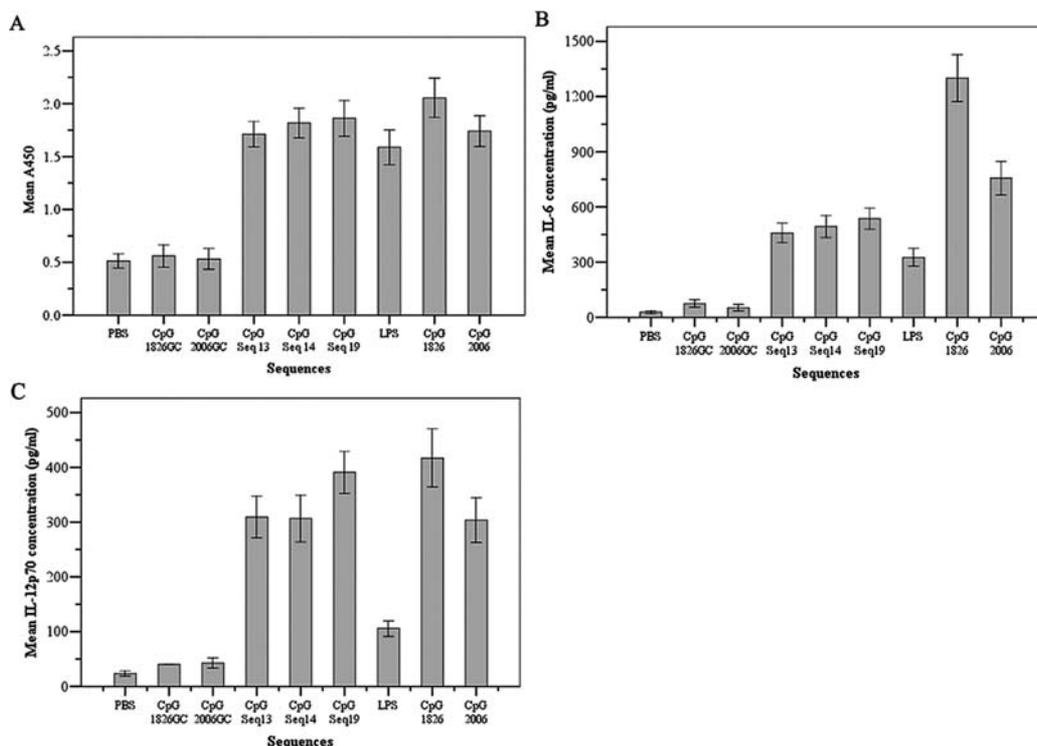


Figure 4. The immunostimulatory effects of CpG ODNs on mouse splenocytes. Peritoneal splenocytes (5×10^5) from Balb/c mice were treated with or without 1 $\mu\text{g/ml}$ of CpG ODNs for 48 h. The proliferation (A) of the splenocytes, as well as the secretion of IL-6 (B) and IL-12p70 (C), was determined. All tests were duplicated. Error bars indicate standard deviation.

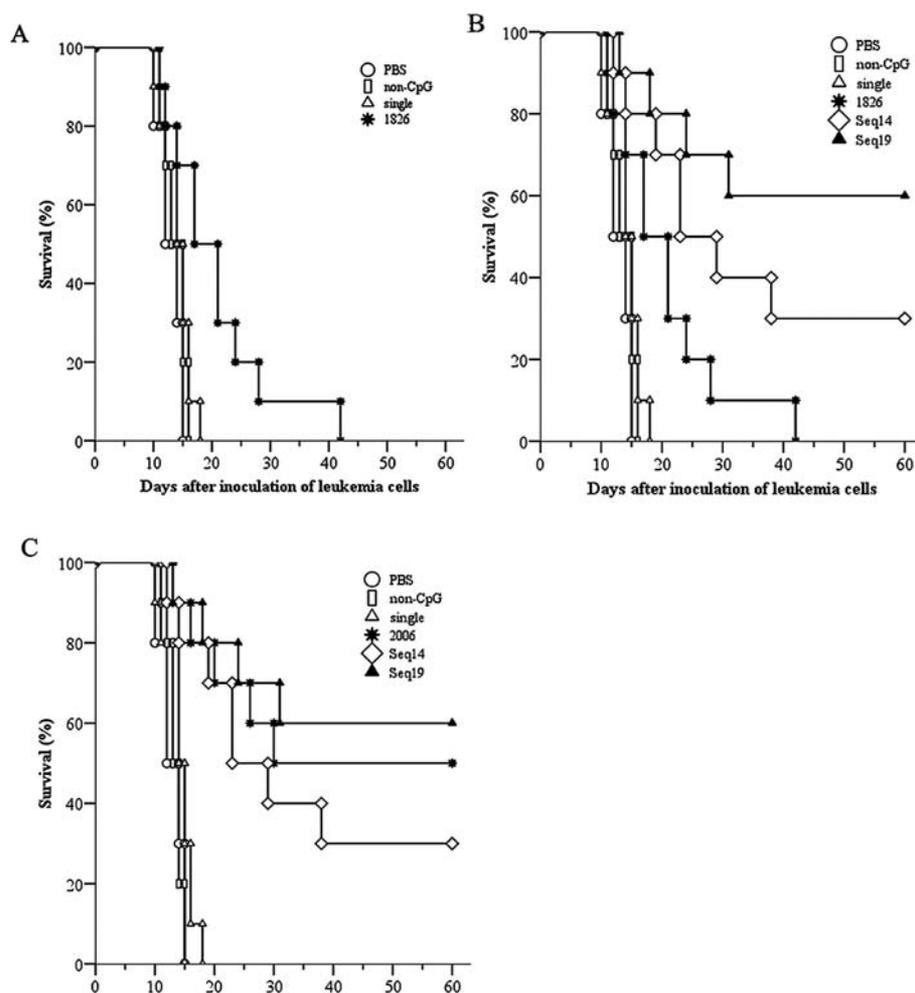


Figure 5. Injection of vaccines with or without CpG ODNs induces significant survival prolonging effects in DBA/2 mouse model. (A) The optimal mouse CpG ODN (CpG 1826) significantly improved the life-span compared to the single group only vaccinated with inactivated L1210 cells. Non-CpG here refers to CpG 1826GC as the ODN backbone control. (B) CpG Seq 14 and 19 compare to CpG 2006, which is optimal for humans. Non-CpG here refers to CpG 2006GC as the ODN backbone control. (C) CpG Seq 14 and 19 compared to the mouse motif, CpG 1826. Non-CpG here refers to CpG 1826GC. Analysis of each experiment yielded a value of $P < 0.05$.

In vivo anti-leukemic activity of CpG ODNs in mice with acute leukemia. In order to investigate whether CpG ODNs with strong *in vitro* stimulatory effects on both humans and mice had detectable anti-leukemic activity *in vivo*, groups of syngeneic DBA/2 mice ($n=10$) received 1×10^5 live L1210 cells on day 0 by i.p. injection. The active immunotherapy began the following day. Mice received the vaccines with or without CpG ODN adjuvants by subcutaneous injection. During the experiment, the leukemia-bearing mice were observed daily for survival for at least 60 days.

In our experiments, CpG ODNs conferred a longer survival span in the leukemia-bearing mice. As seen in Fig. 5, mice from the control group injected with PBS began to die on the 9th day after inoculation with live L1210 cells, and the median survival time (MST) was 12 ± 1.3 days. The single group injected with inactivated L1210 cells alone, whose MST was 14 ± 1.3 days, had no increase in survival time relative to the control group ($P > 0.01$). The non-CpG group, used as the control ODN, did not present an improvement of life-span compared to that of either the control or single groups ($P > 0.01$). However, the MST of animals treated with CpG 1826 as the vaccine adjuvant was prolonged to 17 ± 12.8

days, which was significantly improved when compared to that of the other groups ($P < 0.01$). Furthermore, the MST of the CpG 2006 group was as long as 30 days, and 50% of the mice survived for >60 days. Specifically, the long-term survival rate for mice treated with CpG 2006 was 50%. Above all, vaccines containing CpG Seq 14 or 19 showed surprisingly strong activity in prolonging survival span compared to the single group ($P < 0.001$). The activity of both CpG Seq 14 and 19 was comparable to that of the CpG 2006 group, and that of the CpG Seq 19 group was even higher than that of the CpG 1826 group ($P < 0.01$). Although the CpG Seq 14 group showed a moderate increase in survival span compared to the CpG 1826 group, there was no statistical difference. However, 40% of the mice survived long-term in the CpG Seq 14 group, while all the mice from the CpG 1826 group died before the 60th day after inoculation.

Potent anti-leukemic activity of CpG ODN with enhanced NK cell and CTL cytotoxicity. In order to examine the mechanism underlying the anti-leukemic effect of the vaccine with CpG ODN adjuvants, DBA/2 mice were inoculated by i.p. injection with 1×10^5 live L1210 cells. The same treatment was given

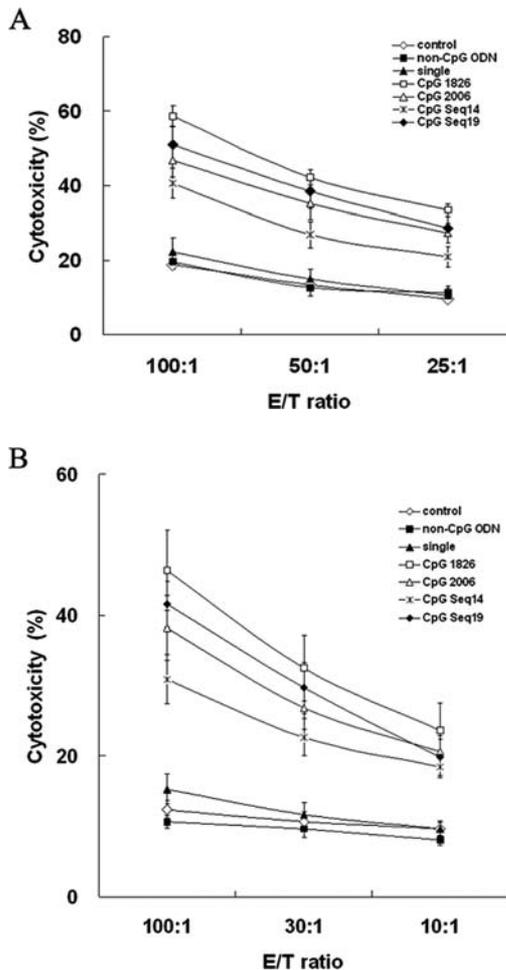


Figure 6. CpG ODN vaccination enhanced both the NK and CTL activities. (A) Splenocytes isolated from tumor-bearing mice were used in NK cytolytic assays E/T ratios of 100:1, 50:1 and 25:1. (B) Splenic lymphocytes of tumor-bearing mice from different groups were prepared as previously described and were then collected as CTL effector cells. L1210 cells were used as the target cells. The tumor-specific CTL cytotoxicity was determined at E/T ratios of 100:1, 30:1 and 10:1. Both the NK and CTL cytotoxicities were determined by a standard LDH release assay.

as described previously. Fourteen days after inoculation, the leukemia-bearing mice from the different groups were all sacrificed and the splenocytes were isolated to be used in cytolytic assays against YAC-1 cells by a standard LDH release assay. As shown in Fig. 6A, the NK activity of lymphocytes from mice treated with tumor vaccine containing CpG ODNs was significantly higher than that of the mice from the control or non-CpG groups ($P < 0.01$). The CpG 1826 group showed the highest NK lysis activity at each E/T ratio among these CpG ODN-treated groups ($P < 0.01$). As for the CpG Seq14 and 19 groups, there was no significant difference between them, and both of them were similar to the CpG 2006 group ($P > 0.01$) concerning NK activity.

Moreover, specific CTL cytotoxicity was observed in the isolated splenocytes after they were co-cultured with inactivated L1210 cells for 5 days in the presence of recombinant murine IL-2 (20 U/ml), and were then collected as CTL effector cells and the L1210 cells were used as the target cells. As seen in Fig. 6B, the specific CTL cytotoxicity of

mice from the control group was on the base line, and there was no significant difference between the single and non-CpG groups. This suggests that tumor antigens alone cannot induce effective specific CTL cytotoxicity. The splenic CTL activity of mice treated with CpG ODN plus inactivated L1210 cells was higher than that of the groups treated with PBS or inactivated L1210 cells alone ($P < 0.01$). The highest CTL activity was induced in the CpG 1826-treated group in comparison to the other groups ($P < 0.01$). There was no significant difference between the CpG Seq19 and CpG 2006 group in CTL activity. However, the activity of the CpG Seq14 group was lower than that in the groups treated with other stimulatory CpG ODNs ($P < 0.01$). This suggests that specific anti-leukemic responses are significantly enhanced by the addition of CpG ODN adjuvants.

Discussion

CpG motifs are highly specific in receptor recognition, and therefore, even a single base difference also impacts the magnitude of the resulting immune response, and subsequently influences their anti-tumor activity. A CpG motif, consists of an unmethylated CG dinucleotide within certain neighboring nucleobases, and is considered to be an essential element responsible for the immunostimulatory effects of bacterial DNA and synthetic ODNs, especially the unmethylated CG dinucleotide. The immune activity of CpG ODNs is completely lost if CG dinucleotides are deleted or methylated. In this study, the control non-CpG sequences, CpG 2006GC and CpG 1826GC, with transversion of CG to GC, did not show apparent activity. Aside from the essential element, other factors also contribute to this activity, such as flanking bases, spacing, poly G ends, length, number of CpG motifs, and so on.

Early studies have indicated that the flanking bases of CpG dinucleotides play a significant role in modulating the level of immune activity of CpG ODNs. An optimal CpG ODN for B-cell activation was considered to contain a CpG motif in which the CpG dinucleotide was flanked by two 5' purines (preferably a GpT or GpA dinucleotide) and two 3' pyrimidines (preferably a TpT dinucleotide) (25). It was then found that the exchange of the precise adenine on the 5' side of the CpG dinucleotide with thymidine, results in slightly higher activity, and the 6-mer motif, 5'-GTTCGTT-3', has been demonstrated to be optimal in activating the human immune system (24). PF-3512676, containing three such potent motifs, has been widely used in clinical trials. Apart from the preferable 3'TpT dinucleotide, 3'TpC or TpA were also considered to be the formula of the most active B-class CpG ODN. However, our study shows that such replacement of the flanking bases (CpG Seqs 1-7, replace 3'TpT with 3'TpC or 3'TpA) leads to a significant reduction in immunostimulatory activity when compared to CpG 2006. In addition, there were no significant differences between CpG Seq 3 and 6, CpG Seq 2 and 5 and CpG Seq 1 and 4 as shown by lymphocyte proliferation assay and the cytokine secretion test. The two flanking sequences made equivalent contributions to the activity of the CpG motif, yet their roles were less significant than that of 3'TpT. Of note, is the fact that the activity of CpG Seq 7 was markedly stronger than that of



Seq 2 and 4 with the same level of substitution had two 3'TpT replaced). Structurally, CpG Seq 7 contains three different motifs (5'-GTCGTT-3', 5'-GTCGTC-3' and 5'-GTCGTA-3') formed by switching 3'TpT with TpC and TpA, which are the most notable characteristics. Therefore, we speculated that synthesized single ODNs containing multiple different CpG motifs could help to enhance the immune stimulating activity. Detailed studies of large numbers of normal donors have indicated that the response of PBMCs from different individuals to CpG stimulation is heterogeneous (15,26). Also, mixtures containing three or more different CpG ODNs have been shown to be capable of activating immune cells from virtually all donors, and maximizing the immune response (27). Therefore, we hypothesized that complex ODNs containing multiple different CpG motifs could play the same role in activating a heterogeneous population.

In addition to the bases adjacent to the CpG dinucleotides, we wished to determine whether other more distal sequences in ODNs could also modulate the stimulatory effect. Specifically, poly G runs, consisting of 4-6 Gs, have previously been reported to increase the immunostimulatory activity of PE ODNs (17-19). The location of the poly G motif within ODNs also affects their ability to uptake CpG ODNs, and the 3'-end location proved to be better than the 5'-end. However, in our study, the addition of the 3'-poly G end to PTO-ODNs did not increase their immune activity. Instead, in certain sequences such as CpG Seq 12, the immunostimulation reduced, which was completely different from that of PE ODNs. The mechanisms of this are not quite clear. According to previous studies, poly G additions cause ODNs to be taken up more efficiently, and enhanced cellular uptake also results in increased immunostimulation correlated with increased IL-6 secretion and PBMC proliferation (18). However, the poly G string only increased the cellular uptake of the otherwise weak incorporated PE-ODNs, and yet was unable to additionally increase that of PTO-ODNs, which were already being taken up very efficiently and are resistant to enzymatic degradation. It has also been observed that TLR9 cells transfected with high concentrations of PTO-poly G ODNs, are still less effective. Thus, it could be speculated that poly G strings are beneficial for cellular internalization, although they interfere with subsequent signal induction (28). Our data also support the previous opinion that poly G motifs can exert suppressive or stimulating effects on the immunostimulatory activity of CpG ODNs according to the modification of the ODN backbone.

Spacing sequences between individual CpG motifs also contribute to the immunostimulatory activity of CpG ODNs, and the separation by TpT is preferable (CpG 2006) (15). In this study, we found that deletion of the T spacing adjacent to the 5'-end lead to a significantly reduced immune activity of CpG ODNs. However, that of the 3'-end TpT spacing did not have such an effect. CpG Seqs 15 and 20, with T spacing adjacent to the 5'-end deleted, showed a significant decrease in IL-6 / IL-12 secretion and PBMC proliferation. Moreover, CpG Seqs 16-19, with both the 5' and 3'-end T spacing deleted, showed markedly decreased activity except for CpG Seq 19. However, CpG Seq 13 and 14, with T spacing adjacent to the 3'-end partly deleted, showed similar activity

to their prototype, CpG 2006. Taking into account the results from a previous study that the CpG ODN bind to TLR9 is closely related to their 5'-end, we inferred that the deletion of the 5'-end T spacing changes the 5'-end structure of CpG ODN, and subsequently interferes with the affinity between CpG ODN and TLR9, thus decreasing the immunostimulatory activity. However, to our surprise, CpG Seq 19 proved to be an exception. This sequence showed stronger immunostimulation than all the other CpG ODNs with T spacing deleted and was equivalent to CpG 2006. The mechanisms of this are unclear. The most distinguishing feature of CpG Seq 19 compared to CpG 2006 is the deletion of one thymidine in the T spacing sequence of both the 5' and 3'-ends, respectively, which makes this ODN more symmetrical in structure than other CpG ODNs with one or more thymidines deleted. We speculated that the structural symmetry could have a beneficial effect for the CpG ODN on binding to its receptor, TLR9, and that it could offset the negative impact caused by the deletion of the 5'-end T spacing on ligand binding to a certain extent.

Acute leukemia is a common malignant disease in clinical practice. Despite the development of a myriad of novel therapies, a large number of patients afflicted with this disease, still succumb to it. Therapeutic interventions with measurable and sustainable anti-tumor effects are required. Immunotherapy represents such an intervention. Conventional allogeneic bone marrow transplantation has proven to be the most effective immunotherapy. The graft-versus-leukemia effect observed after allogeneic stem cell transplantation, suggests that an effective immune response can cure acute leukemia, but the application is limited by its toxicity and the difficulty of finding suitable donors. Therefore, vaccination strategy has considerable appeal. The leukemia vaccine has shown specific anti-leukemic effect and lower toxicity as has previously been observed. Antigens in the vaccine formula determine the specificity of anti-tumor immune response. In previous studies, we investigated and identified a type of leukemia-associated antigen in acute monocytic leukemia (29). However, tumor antigens alone may not be able to elicit effective specific anti-leukemic immunity, as leukemic blasts develop various escape mechanisms. In this study, all mice in the single group treated with inactivated L1210 cells alone died by the 18th day after i.p. inoculation with live L1210 cells. The MST (14 ± 1.3 days) was not significantly prolonged compared to the control group (12 ± 1.3 days) treated with PBS buffer, neither was the CTL cytotoxicity, although we used whole tumor cells as the antigen source to maximally maintain the spectrum of tumor antigens. An appropriate adjuvant could help to improve the immunogenicity of tumor antigens, break the immune tolerance in leukemia patients, and thereby enhance the efficacy of the tumor vaccination. Examples of adjuvants are the granulocyte-macrophage colony-stimulating factor and Freund's adjuvant, effective cytokines such as IL-12, and synthetic oligonucleotides comprising the CpG motif. Synthetic ODNs containing CpG motifs are excellent candidates for the combined treatment with tumor vaccines (30,31), and many studies have shown that they can also enhance adaptive immunity for monotherapy (32).

The L1210 murine lymphoid leukemia cells are highly aggressive. The i.p. injection of 1×10^5 cells can cause death

of all mice within 16 days. As few as 1×10^2 L1210 cells result in uniform mortality within 32 days. Mice that survive for >60 days after inoculation of L1210 cells could be regarded as long-term survivors (33). Our data showed that, compared to the control group, inactivated L1210 cells did not significantly prolong the survival span of mice, most likely due to their poor immunogenicity. However, the therapeutic effect was greatly enhanced by the CpG ODN adjuvant. The survival time of CpG ODN-treated mice was markedly prolonged, and 30-60% of mice achieved long-term survival. This suggests that the immunostimulatory CpG ODN adjuvant could act in synergy with tumor antigens to elicit a functional anti-leukemic effect and induce the immune protection against progressive leukemia.

Further studies on NK cytotoxicity and CTL activity for the uncovering of the immune mechanism, also support this result. In our present study, markedly elevated CTL activity against L1210 was observed in mice treated with the CpG ODN adjuvant compared to the single and control groups, which is in agreement with the survival time of mice. The potent CTL responses of the CpG ODN-treated mice strongly suggest that the leukemia vaccine containing the CpG ODN adjuvant induces specific anti-leukemic activity and effective immune protection with regressed ascites and prolonged survival span. The mechanism of enhanced CTL activity could be attributed to the antigen presentation being changed by CpG ODN activation. As far as we know, CpG ODN functions through the activation of TLR9 which is expressed mainly in B-cells and dendritic cells in humans. Both of these cells play an important role in antigen presenting, especially the dendritic cells. The activation of TLR9 leads to a Th1-like cytokine and chemokine environment, then it activates dendritic cells and up-regulates the expression of the co-stimulatory molecules in plasmacytoid dendritic cells (pDCs). The required co-stimulatory signals for T cell activation are provided, then T cells are shifted in response to tolerance and this results in enhanced CTL activity. If there is no TLR9 activation, tumor antigens are presented by immature dendritic cells, which create an environment that favors the maintenance of T cell tolerance, preventing the development of anti-tumor immunity. Apart from the specific anti-leukemic CTL response, we found that NK cells were also critical in the rejection of leukemia, and markedly augmented NK cell cytotoxicity was observed in mice injected with CpG ODNs, suggesting that non-specific immunity could be involved in the anti-leukemic responses of the active immunotherapy of the leukemia vaccine. On the whole, the vaccine containing the CpG ODN adjuvant has a strong *in vivo* anti-leukemic activity with augmented NK and CTL activity.

CpG ODN is considered safe and well tolerated in comparison to many other adjuvants, as previously described (34). The most common side-effects are mild to moderate injection-site reactions and transient flu-like symptoms. As we found in our study, all injections were safe and very well tolerated. There were no ulcerations, granulomas, or other injection site abnormalities. However, we found that some mice in the CpG 1826-treated group showed progressive weight loss and reduced motoricity after being injected and all mice died despite regressed ascites. Nevertheless, both the

NK and CTL activities in this group were much stronger than those in any other group. This indicates that CpG 1826 induces the most potent innate and adaptive anti-leukemic response *in vivo*, despite of no long-time survival in this group. We speculated that this phenomenon was most likely related to the excessive immune response induced by CpG 1826. As earlier studies have shown, rodents respond with high serum concentrations of pro-inflammatory cytokines, such as TNF- α , to CpG ODN administration, which can result in a lethal 'cytokine storm' (35). However, there was no change in serum TNF- α levels following CpG injection in humans and primates (36), suggesting that CpG ODN should be safe for and well tolerated by humans. This is possibly related to the different cellular distributions of TLR9 between primates and rodents. TLR9 is limitedly expressed in B-cells and pDCs in humans, whereas the opposite occurs in mice, monocytes, and possibly all DC subsets (37,38). As TLR9 is expressed in a broader range of immune cells in rodents, the immune responses could be more intense and broader than those in primates. Therefore, mice tend to be more susceptible to toxicities as opposed to primates. Moreover, we also found that mice treated with CpG 2006, CpG Seq 14 and 19, displayed less damage in the spleen and liver when compared to the CpG 1826-treated mice (data not shown). The reason for this could be the species-specific differences in the optimal CpG sequences for potency. The 6-mer motif, 5'-GACGTT-3', is considered to be the optimal CpG motif for rodents (39). For example, CpG 1826 containing the 5'-GACGTT-3' motif is potent in inducing immunostimulatory effects in mice. Whereas the 5'-GTC GTT-3' motif in CpG 2006, CpG Seq 14 and 19, represents the optimal CpG motif for primates (15), in mice, most of its potency in CpG 1826 is relatively weaker. As we observed in this study, these CpG ODN sequences showed good tolerance and no obvious immunotoxicity.

In conclusion, these data indicate that structural characteristics of immunostimulatory CpG ODNs, such as flanking bases, spacing, poly G ends, length, number of CpG motifs and so on, have a significant impact on their activity. These findings have important implications for the rational design of B-class ODNs. We then selected several CpG ODNs with high activity in human and mouse immune cells and used them as immune adjuvants, thus demonstrating that CpG ODNs are active against the highly malignant L1210 leukemia mouse model via innate and adaptive immune augmentation. Among the CpG ODN-treated groups, CpG Seq 19 led to the best therapeutic effect, with 60% of mice achieving long-term survival. The long-term survival of mice in the CpG Seq 14 group was 30%. The effective anti-leukemic activity validates these *in vitro* observations. Given the high efficiency and low toxicity, we believe that CpG Seq 14 and 19 could be applied in pre-clinical and clinical studies for the immunotherapy of acute leukemia. Towards improving the therapeutic efficacy of CpG ODN, further studies are currently underway.

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