

Full-length article

Structure-efficacy relationships of immunostimulatory activity of CpG-containing oligodeoxynucleotides on mouse spleen cells¹

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Key words

CpG; oligodeoxynucleotides; immunostimulation; structure-activity relationship

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Abstract

Aim: To study the relationship between primary structures of oligodeoxynucleotides (ODN) containing unmethylated deoxycytidyl-deoxyguanosine (CpG) dinucleotide motifs and their immunostimulatory activities in mouse spleen cells. **Methods:** A series of CpG ODN with different primary structures were synthesized. Their capabilities to stimulate mouse spleen cell proliferation were determined by [³H]thymidine incorporation assay. Cytokine (interleukin [IL]-6, IL-12, and IFN- α) secretion spectra induced by CpG ODN were assessed by ELISA. The ability of CpG ODN to activate natural killer cells was evaluated by standard 4 h ⁵¹Cr-release assay. Flow cytometry was utilized to examine the expressions of various lymphocyte surface molecules on diverse immunocytes. An effective CpG ODN for murine, ODN1826, was set as the template of modification and the positive control. **Results:** The immunostimulatory activities of CpG ODN with different sequences and compositions varied markedly, both in character and in extent. It was useless for improving the immunostimulatory activity of ODN1826 by simply increasing the functional hexameric CpG motif number, modifying the site of CpG motifs, or changing the distance between multi-CpG motifs. However, an addition of a self-complementary palindrome structure at the 3'-end, but not the 5'-end of CpG ODN, aroused marked improvement in its activity. Several designed ODN had superior comprehensive immunostimulatory properties compared to ODN1826. **Conclusion:** The immunostimulatory activity of a CpG ODN was relevant to its primary structure. It was useless for promoting immunostimulatory activity to simply change CpG motif number, space, or distance. The 3'-end palindrome structure of CpG ODN is associated with enhanced immunostimulatory activity.

Introduction

Certain molecular structures that are present in pathogens (pathogen-associated molecular patterns [PAMP]) are recognized by innate immune cells via pattern recognition receptors. The cells are activated upon recognition of PAMP and trigger the generation of optimal adaptive immune responses. The bacterial genome, compared to vertebrate DNA, contains a higher frequency of unmethylated deoxycytidyl-deoxyguanosine (CpG) dinucleotides. Small

oligodeoxynucleotides (ODN) with unmethylated CpG dinucleotides (CpG ODN) are able to perfectly mimic the immunostimulatory activity of bDNA^[1,2]. CpG ODN are known to stimulate innate and adaptive immunity because of their interesting immunostimulatory properties in a number of vertebrate, such as stimulating B-cell proliferation^[3–5], enhancing the expression and synthesis of cytokines^[6], and promoting natural killer (NK) cell cytotoxicity^[7]. Multiple studies have shown that CpG ODN-induced activation is presumably a 2-step process. First, CpG ODN, independent

of their sequence, are recognized by different receptors^[1,2,8-10]; Mac-1 surface immunoglobulins and scavenger receptors are possibly involved in this process. The cellular uptake of ODN is sequence independent, but can be influenced dramatically by backbone modifications. Phosphorothioated modifications can enhance cellular uptake and increase immunostimulatory activity and half-time of CpG ODN^[9,11]. In addition, runs of at least 4 guanines have been reported to enhance the cellular uptake of ODN due to the fact that base-quartet-stabilized, 4-stranded helices called tetraplexes are formed^[10,12]. Second, CpG ODN trigger the motif-dependent recognition of Toll-like receptor 9 (TLR-9) within the endosome and initiate immunocellular activation.

Immunostimulatory activities of CpG ODN depend on their structural and chemical characteristics. A number of researchers have studied the relationship between the CpG ODN structure and their immunostimulatory properties. CpG ODN were structurally and functionally divided into 3 types in previous studies for humans: A type (also known as D type), B type (also known as K type), and C type. A-type CpG ODN is capable of activating human plasmacytoid dendritic cells (pDC) to produce large amounts of type I IFN (α/β) and strongly activates NK cells. It has mixed phosphodiester/phosphorothioate backbones and contains a single hexameric purine/pyrimidine/CG/purine/pyrimidine motif flanked by self-complementary bases that form a stem-loop structure capped at the 3'-end by a poly G tail^[13]. B-type CpG ODN primarily activates B cells, resulting in their proliferation and antibody secretion. It has phosphorothioate backbones and encodes multiple TCGTT and/or TCGTA motifs^[13,14]. C-type CpG ODN optimally consist of a stimulatory hexameric CpG motif (5'-TCGTCGTT-3') linked by a T spacer to GC-rich palindromic sequences (5'-CGGCGCGCG-CCG-3')^[15]. The C-type CpG ODN very strongly stimulates B cells as well as type I IFN secretion *in vitro* in human peripheral blood mononuclear cells. *In vivo*, it is a strong, Th1-inducing adjuvant. In addition, another C-type CpG ODN is reported to display a B-type CpG ODN structure feature at the 5'-end and A-type CpG ODN structure feature at the 3'-end and shares the activities of both A- and B-type CpG ODN^[16]. Furthermore, the sequence of the optimal human hexamer motif was reported to be 5'-GTCGTT-3'^[17] and the optimal mouse motif was 5'-GACGTT-3'^[1].

Despite the previously mentioned reports, more detailed and systematic structure-activity relationship (SAR) studies of CpG ODN are still lacking. In this study, we tried to reveal some changes of the immunostimulatory properties of CpG ODN induced by the modifications of the ODN structure, for instance, additions and deletions of the func-

tional CpG motifs, modification of the distance and the sites of CpG motifs, and the addition of self-complementary sequences. We recruited CpG 1826, an effective immunostimulatory B-type CpG ODN for murine, as the positive control and the template of the SAR study. This ODN was a strong B-cell stimulator, but a weaker inducer of IFN- α ^[1,18,19]. Various modifications on the first-class structural level were performed on the basis of ODN1826, and the immunostimulatory activities of the structurally-modified CpG ODN were comprehensively investigated, including the ability to stimulate mouse B cell proliferation, the ability to induce cytokines (interleukin [IL]-6, IL-12, and IFN- α) secretion and the ability to induce NK cell killing activity, and the changes of the expression of various lymphocyte surface molecules. Finally, the relationship between the CpG ODN structures and their immunostimulatory properties were primarily concluded.

Materials and methods

CpG ODN Purified, single-stranded, phosphorothioated ODN containing CpG motifs were synthesized by Sangon Biotech Company (Shanghai, China). The sequences used in this study are listed in Table 1, where ODN1 was the intact 1826, positive control, and the template; ODN 2–12 were the sequences modified based on the structure of 1826; and ODN13–15 were negative controls.

Cells and cell culture Spleen cells from 4- to 8-week-old C57BL/6 mice were cultured in RPMI-1640 medium supplemented with 10% (*v/v*) fetal bovine serum (GIBCO, Carlsbad, CA, USA) and antibiotics (100 IU/mL penicillin and 100 μ g/mL streptomycin). Mouse B cells were obtained by centrifugation over Lympholyte M (CEDARLANE Laboratories, Hornby, Ontario, Canada) as described previously^[1]. Yeast artificial chromosome-1 (YAC-1) cells were kindly provided by Prof Wen-xia ZHOU (Beijing Institute of Pharmacology and Toxicology, Beijing, China).

Cytokine ELISA The mouse spleen cells were plated onto 24-well dishes (3×10^6 per well). The CpG ODN, dissolved in TE buffer, were added to the cultured cells at a final concentration of 0.05, 0.25, and 1.0 μ mol/L, respectively. The cells were cultured at 37 °C in a humidified incubator with 5% CO₂, and the culture supernatants (SN) were collected at the appointed time-points. If not used immediately, the SN were stored at -20 °C until the assay. The amounts of murine IL-6, IL-12, and IFN- α in the SN were measured using commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA). The experiments were performed 2 or 3 times for each CpG ODN in triplicate for each concentration.

Proliferation assays The proliferation of mouse B cells was determined by a [³H]thymidine incorporation assay as described previously^[1]. Briefly, the spleen cells (5×10^5 /well) were plated onto Costar 96-well plates (Corning Incorporated Corning, New York, NY, USA) and stimulated with CpG ODN at various concentrations (0.05, 0.25, and 1.0 $\mu\text{mol/L}$) or controls for 56 h, and then 0.5 μCi of [³H] thymidine was added to each well. The cells were harvested after incubation for another 16 h and the radioactivity was measured using a MicroBeta liquid scintillation counter (PerkinElmer, Boston, MA, USA). All assays were performed 4 times.

Measurement of NK-mediated cytotoxicity The NK cytotoxicity of mouse spleen cells stimulated by CpG ODN was assessed by standard 4 h ⁵¹Cr-release assays as previously described^[7]. Briefly, the mouse spleen cells were incubated with CpG ODN (0.25 $\mu\text{mol/L}$) for 36 h and then harvested as effector cells. One million YAC-1 cells, used as target cells, were incubated with 50 μCi of ⁵¹Cr for 1 h at 37 °C, then washed several times and incubated for 4 h with the effector cells (E:T ratio, 50:1). Thereafter, the SN were harvested and the radioactivity was measured using a MicroBeta liquid scintillation counter (PE, Boston, MA, USA). The results were expressed as the percentage of specific lysis in terms of mean \pm SD of the results read in triplet wells. The following formula was used: percent specific lysis (%)=(experimental counts-target cell spontaneous release counts)/(maximal release counts-target cell spontaneous release counts) \times 100. Spontaneous lysis was measured from wells containing only target cells, whereas maximum lysis was measured from the wells containing target cells incubated with 10% SDS.

The cytotoxicity of mouse spleen cells stimulated by CpG ODN to the B16 cells was measured using B16 cells as target cells as described earlier.

Abs and flow cytometry The mouse spleen cells were incubated with or without CpG ODN (0.25 $\mu\text{mol/L}$) for 48 h, and then the B cells, pDC, and NK cells were analyzed by two-color staining on a FACS Calibur flow cytometer (BD Pharmingen, Franklin lakes, New Jersey, USA). For B cells, FITC-labeled anti-CD19 and PE-labeled anti-CD80 mAbs were used. For pDC, FITC-labeled anti-CD11c and PE-labeled anti-Ia^b mAbs were used. For the NK cells, FITC-labeled anti-CD94 fmAbs were used (BD Pharmingen, Franklin lakes, NJ, USA).

Animals Female BALB/c mice (6–8 weeks old) were used for all the experiments and were purchased from Experimental Animal Center of Academy of Military Medical Sciences (Beijing, China; certificate No SYKX2002-001).

Statistical analysis Data are shown as mean \pm SD. Sta-

tistically significant differences were determined by Student's *t*-test. Differences were considered statistically significant when $P < 0.05$.

Results

Production of cytokines induced by CpG ODN In the present study, we investigated the production of several cytokines, including murine IL-6, IL-12 (P40), and IFN- α in immunocytes stimulated by CpG ODN. The positive control ODN1826 (ODN1) is a typical B-class CpG ODN. It consists of 2 stimulatory hexameric CpG motifs (5'-TCGACGTT-3') linked by a CCT spacer and a TpC dinucleotide at the 5'-end. ODN1826 induced strong IL-6 secretion in a dose-dependent manner. When its concentration reached 0.25 $\mu\text{mol/L}$, the induced IL-6 reached 4651 ± 301 pg/mL (Figure 1A). ODN2, ODN4, ODN5, and ODN7–12 also stimulated IL-6 production to a different extent, but the IL-6-inducing activity of all those sequences were weaker than that of ODN1826. ODN10, which had the most obvious effect on the upregulation of IL-6, can modulate the IL-6 level to 4579 ± 334 pg/mL at a higher concentration of 1.0 $\mu\text{mol/L}$. ODN3, which had a 5'-end, self-complementary palindrome and lost almost all IL-6-inducing activity, indicating that a free 5'-end was necessary for a CpG ODN to possess immunostimulatory activity. There was no obvious IL-6-inducing activity in the negative control sequences, ODN12–15.

With regards to IL-12, the tendency of sequence-dependent stimulatory activity was similar to that of IL-6. ODN4, ODN5, ODN7, ODN10, ODN11, as well as ODN1826, induced relatively high levels of IL-12 P40 (Figure 1B). However, ODN1826 was no longer the strongest stimulator for IL-12 production. At the highest studied concentration of 1.0 $\mu\text{mol/L}$, ODN1826 treatment upregulated IL-12 P40 secretion to 145 ± 12 pg/mL. At the same dose, ODN4, ODN7, ODN10, and ODN11 stimulated IL-12 P40 production to 172 ± 14 , 188 ± 14 , 229 ± 17 , and 184 ± 16 pg/mL, respectively ($P < 0.05$).

ODN1826 was a relatively weaker IFN- α stimulator (90 ± 7 pg/mL at the dose of 1.0 $\mu\text{mol/L}$) compared to its IL-6-inducing activity in this study. Our findings were consistent with those reported in other studies (Figure 1C)^[15]. Among all the ODN studied, the strongest IFN- α stimulators (the ODN were at a dose of 1.0 $\mu\text{mol/L}$) were partly similar to the induction of IL-6 and IL-12 production. The IFN- α stimulators were ODN4 (14 ± 15 pg/mL), ODN5 (97 ± 10 pg/mL), ODN7 (180 ± 14 pg/mL), ODN8 (115 ± 13 pg/mL), ODN10 (135 ± 6 pg/mL) and ODN11 (150 ± 14 pg/mL). Structural information (Table 1) demonstrated that, ODN4, ODN7, ODN10, and ODN11 all had a 3'-end, self-complementary palindrome motif, indicat-

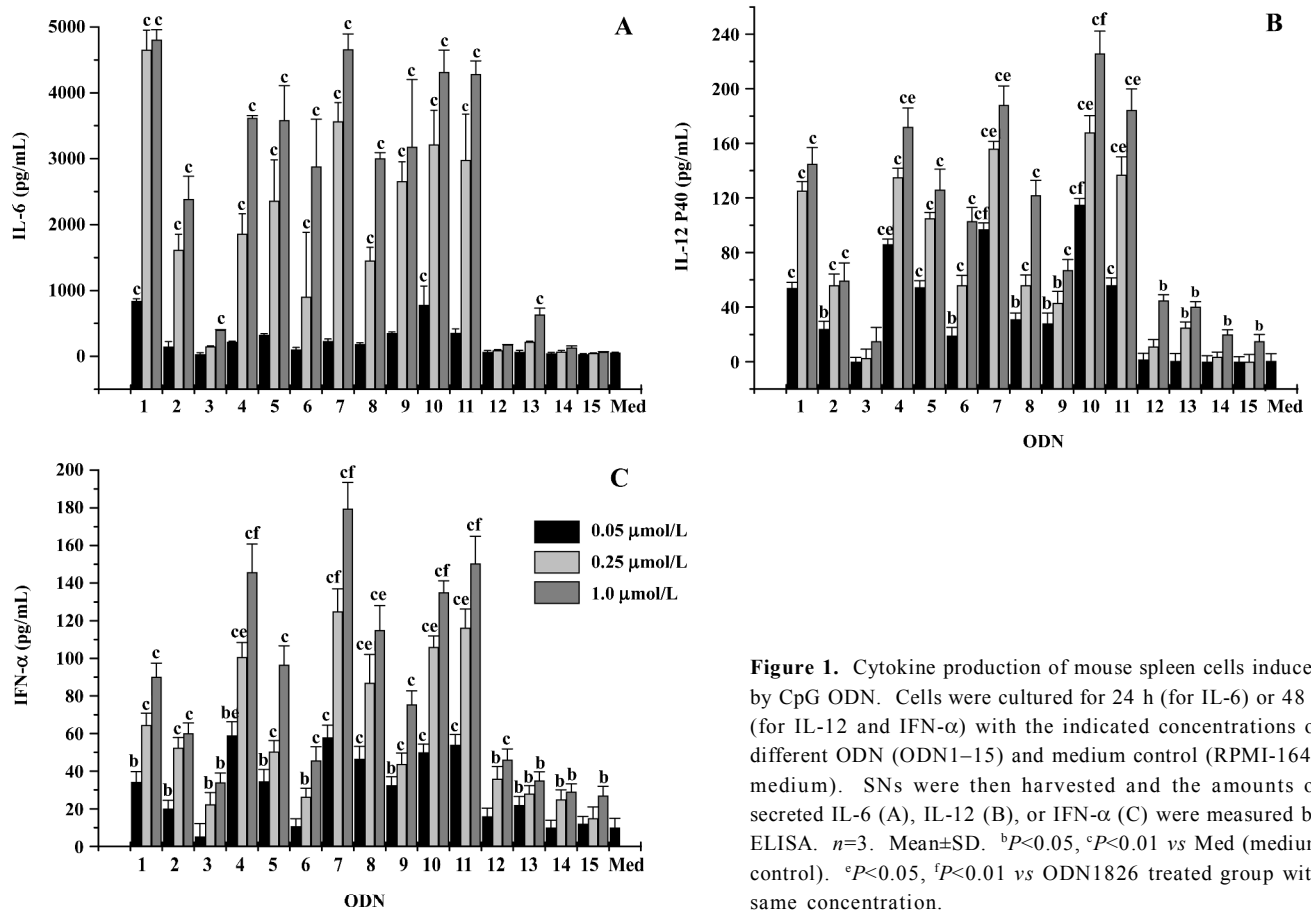


Figure 1. Cytokine production of mouse spleen cells induced by CpG ODN. Cells were cultured for 24 h (for IL-6) or 48 h (for IL-12 and IFN- α) with the indicated concentrations of different ODN (ODN1–15) and medium control (RPMI-1640 medium). SNs were then harvested and the amounts of secreted IL-6 (A), IL-12 (B), or IFN- α (C) were measured by ELISA. $n=3$. Mean \pm SD. ^b $P<0.05$, ^c $P<0.01$ vs Med (medium control). ^e $P<0.05$, ^f $P<0.01$ vs ODN1826 treated group with same concentration.

Table 1. Synthesized CpG ODN. All sequences had phosphorothioate backbone modification.

ODN	Sequence 5'–3'	Description
1	TCCATGACGTTCTGACGTT	ODN1826
2	TCCATGTCGTTCTGTCGTT	Hexameric motif from 5'-GACGTT-3' to 5'-GTCGTT-3'
3	TGCAGCTCTTTTGACGTCGAGGGGGG	5'-end, self-complementary palindrome and poly G tail
4	TGCAGTTCCGACGTTCCGCGCGCCG	C type one
5	TGCAGTTGACGTTTCATCCT	Same composition as ODN1826. Shorter distance between the 2 CpG motifs
6	GACGTTCCATCCTGACGTT	Same composition as ODN1826. Longer distance between the 2 CpG motifs
7	TGCAGTTTTGACGTTCCGCGCGCGCCG	Link from CC to TT
8	GACGTTCCGACGTTCCGCGCGCGCCG	5'-end lack TC
9	TGCAGTTCCGACGTTCCGACGTTCC	Addition of an extra CpG motif
10	TCCATGACGTTTTAAAACGTCATGGA	Totally self-complementary palindrome
11	TCCATGACGTTCCGCGCGCGCCG	Decrease of a CpG motif and the addition of a self-complementary CG tail
12	TCCGCGCGCCGCGGTCATGACGTT	5'-end, self-complementary palindrome CG
13	TCCATGACGAAGTACAGCCG	A random negative control
14	TGCTGCTTTTGTGCTTTTGTGCTT	Negative control ODN2137 ^[15]
15	CGGCGCGCGCGCGGCGCGCGCCG	Palindrome negative control

ing that a 3'-end palindrome structure is important for CpG ODN-induced IL-12 and IFN- α production.

Effect of CpG ODN on B cell proliferation According to Krug *et al*, A-class ODN were poor stimulators of human B

cell proliferation. In contrast, B- and C-class ODN efficiently stimulated B cell proliferation^[20]. In our study, B-class ODN1826 induced the upregulation of B cells to a moderate extent. ODN5, ODN6, ODN8, and ODN9 stimulated B cell proliferation with comparable efficiency and in a dose-dependent manner. However, ODN4, ODN7, ODN10, and ODN11 had a more obvious effect on stimulating the proliferation of B cells than ODN1826 (Figure 2).

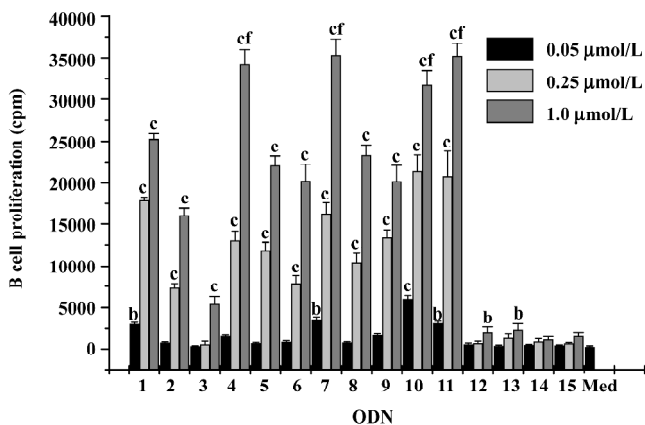


Figure 2. Mouse B cell proliferation induced by various CpG ODN. Proliferation was measured by [³H]thymidine uptake. *n*=4. Mean±SD. ^b*P*<0.05, ^c*P*<0.01, vs Med (medium control), ^f*P*<0.01 vs ODN1826 treated group with same concentration.

NK cell activation Another important activity of CpG ODN is the activation of NK cells^[7]. ODN with a 3'-end, self-complementary palindrome, such as ODN4, ODN7, ODN10, and ODN11 induced more efficient NK cytotoxicity (Figure 3).

Flow cytometry analysis CD19 is the characteristic surface marker of B cells, and the activation of B cell proliferation is characterized by the upregulation of cell surface molecules, such as costimulatory molecules CD80. CpG ODN can also activate pDC and promote its maturation, which can be determined by the upregulation of MHC II molecules (Ia^b) on the membrane of pDC. CD11c can be detected as the characteristic surface marker of pDC. In the present study, we determined the effect of CpG ODN on different immune cells by flow cytometry analysis on the basis of those marker molecules (Figure 4). The results showed that all CpG ODN, with the exception of ODN3, upregulated the expression level of CD80 on CD19-positive cells, in which ODN1826, ODN4, ODN7, ODN10, and ODN11 were especially efficient. As to the expression of Ia^b on CD11c-positive cells and CD94 expression on NK cells, the status was similar; the most potent CpG ODN were still ODN4, ODN7, ODN10, and ODN11.

SAR analysis The results showed that the optimal stimu-

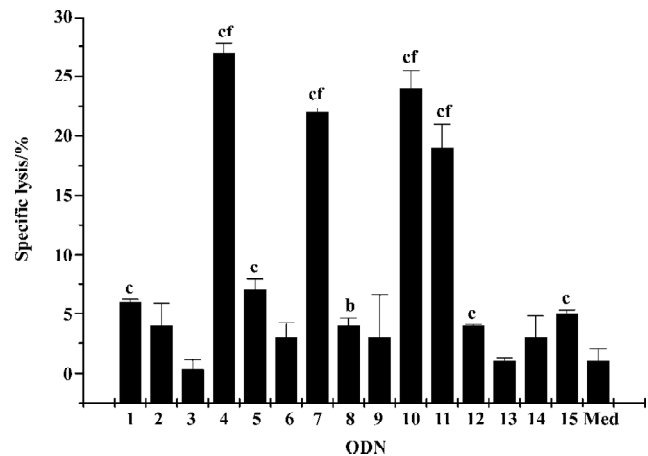


Figure 3. NK cytotoxicity of mouse spleen cells stimulated by CpG ODN. In the NK cytotoxicity assay, CpG ODN-treated mouse spleen cells at the concentration of 0.25 μmol/L were used as effector cells and ⁵¹Cr-labeled YAC-1 cells were used as target cells. Result was detected after the co-incubation of effector cells and target cells for 4 h at 37 °C. *n*=4. Mean±SD. ^b*P*<0.05, ^c*P*<0.01 vs Med (medium control). ^f*P*<0.01 vs ODN1826 treated group.

latory hexameric CpG motif for murine was 5'-TCGACGTT-3', which was consistent with previously published studies^[15]. Changing the hexameric motif from GACGTT to GTCGTT weakened the immunostimulatory activity of ODN. The CpG island without the flank of dinucleotide seemed to have no immunostimulatory efficiency, as was the case with ODN15. The number of CpG motifs was not a vital factor for immunostimulatory activity. Adding a hexameric CpG motif (ODN9) contributed no significance to immunostimulatory activity. In ODN11, the number of hexameric CpG motifs decreased to 1, but the activity of this ODN improved. The improvement of the activity might be derived from the 3'-end palindrome tail.

Owing to lack of a TpC dinucleotide^[21] at the 5'-end, the immune-stimulatory effects of ODN6 and ODN8 were weakened. The optimal distance between 2 hexameric CpG motifs seemed to be 3 nucleotides. Modification of the distance to a shorter (ODN5, the 2 motifs were connective) or a longer (ODN6, the 2 motifs spaced by an octanucleotide) one both weakened the comprehensive immunostimulatory activity.

The palindrome was essential to the immunostimulatory potency of a CpG ODN. The results showed that all the ODN with the best general immunostimulatory activity were that with self-complementary palindrome sequences, such as ODN4, ODN7, ODN10, and ODN11. However, the palindrome structures had to be at the 3'-end of the sequence.

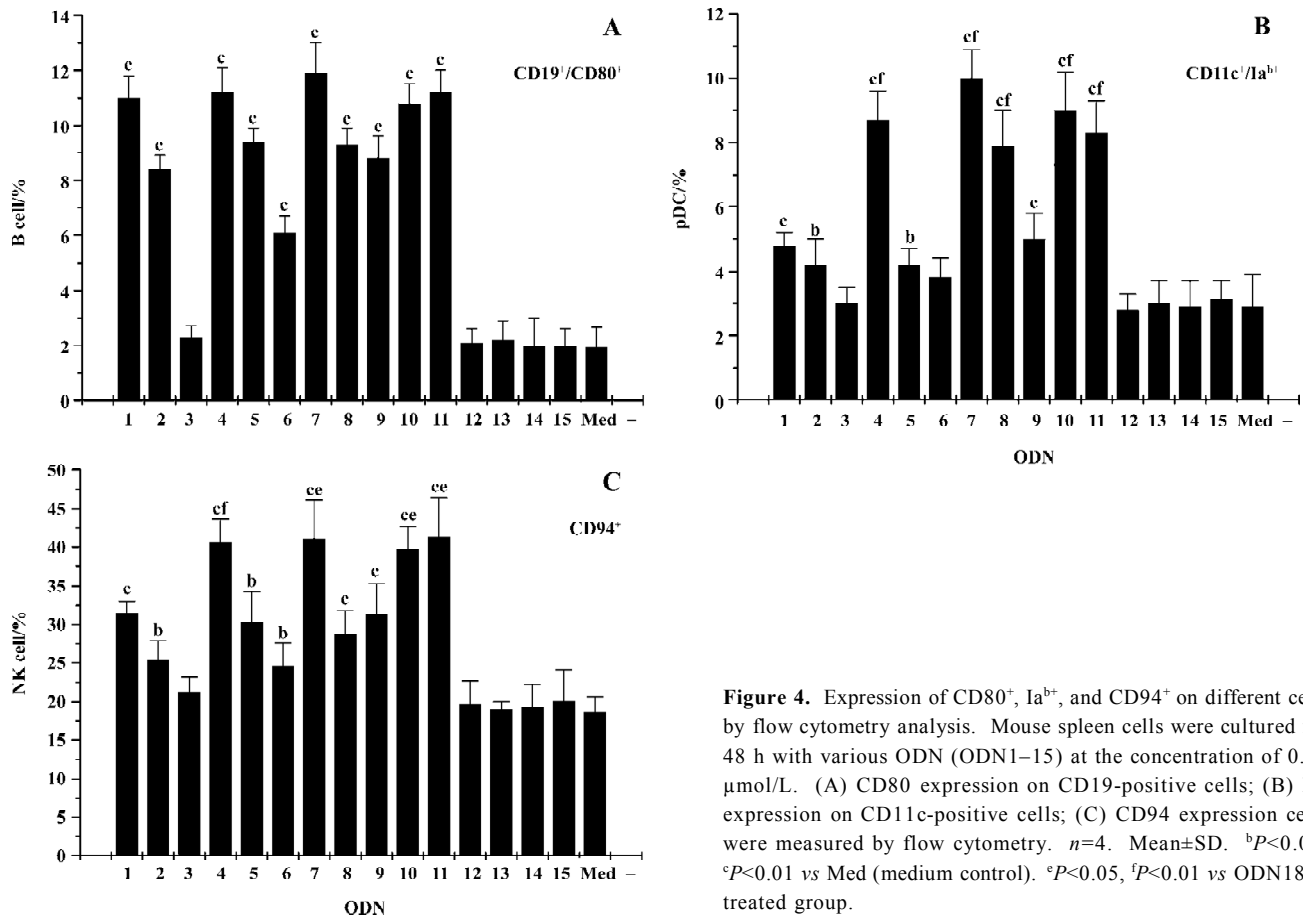


Figure 4. Expression of CD80⁺, Ia⁺, and CD94⁺ on different cells by flow cytometry analysis. Mouse spleen cells were cultured for 48 h with various ODN (ODN1–15) at the concentration of 0.25 μ mol/L. (A) CD80 expression on CD19-positive cells; (B) Ia^b expression on CD11c-positive cells; (C) CD94 expression cells were measured by flow cytometry. $n=4$. Mean \pm SD. ^b $P<0.05$, ^c $P<0.01$ vs Med (medium control). ^e $P<0.05$, ^f $P<0.01$ vs ODN1826 treated group.

Once the palindrome moved to the 5'-end, like ODN3, ODN12, and ODN15, immunostimulatory activity was almost totally lost.

Discussion

In the present study, we tried to explore a new role of the SAR of CpG ODN and find the optimal structure for mouse spleen cells. The positive control and the template ODN1826 was the optimal B-class CpG ODN reported in previously published studies. It consists of 2 stimulatory hexameric CpG motifs (5'-TCGACGTT-3') linked by a CCT spacer and a TpC dinucleotide at the 5'-end. ODN1826 was a strong stimulator for IL-6 and IL-12 production and a relatively weak inducer for IFN- α secretion. ODN1826 efficiently promoted B cell proliferation and partially promoted the maturation of pDC, but it lacked NK cell activation.

Various structural modifications were preformed on the basis of the template ODN1826. Some modifications introduced remarkable activity promotion to the CpG ODN, for instance, adding the palindrome to the sequence. However,

some structural changes failed to improve the immunostimulatory potency, such as modifications of the CpG number and the distance between functional CpG motifs or simply changing the motif sites. Previous studies^[17,19,21], in which conclusions was drawn that a TpC dinucleotide at the 5'-end was important for the potency of a CpG ODN, was partly supported in our study. ODN6 lacked the 5'-TpC dinucleotide, which had weaker stimulatory potency than ODN1826, but the lack of potency could be compensated by an addition of a 3'-end CpG-rich palindrome tail, this is why ODN8 possess comparable immunostimulatory activity vs 1826.

All ODN with a palindrome sequence could be classified as C-class CpG ODN^[15]. Our results indicated that C-class ODN possessed combined immune effects of A- and B-class CpG ODN^[7,13,20]. C-class ODN strongly stimulated B cell proliferation, NK cell activation, and IFN- α production. However, not all C-class CpG ODN had immunostimulatory potency. It was very important to arrange the site of the palindromes; self-complementary palindrome at the 3'-end normally accompanied with satisfactory immunostimulatory potency. In contrast, a 5'-end, self-complementary palin-

drome is usually useless, like ODN3 and ODN12. A previous study^[22] showed that the free 5'-end of the CpG ODN sequence was important for immunostimulatory potency. The 5'-end palindrome destroyed the free end; this may have been responsible for the lost of the immunostimulatory activity of ODN3 and ODN12.

However, ODN10, one of the most potent ODN involved in our study, possessed a holistic, self-complementary palindrome structure (5'-TCCATGACGTTTTAAAACGTCATGGA-3'). In theory, the sequence would form a large hairpin structure, so it was hard to keep a single chain 5'-end. Despite this, the comprehensive immunostimulatory activity of ODN10 was distinguished. Furthermore, in another study, a cyclic CpG ODN without any free ends had potent immunostimulatory efficiency (data not shown). This result challenged the hypothesis that the free 5'-end was essential for retaining the immunostimulatory activity for a CpG ODN. Undoubtedly, more investigations still needed to be carried out in order to elucidate this.

The analysis of cytokine secretion is a traditional method for the SAR of CpG ODN. Generally, a certain cytokine can be secreted by several immune cells, for example IFN- α secretion by pDC or cells of the mononuclear phagocyte system, so we could not identify which cells were influenced by CpG ODN. Thus, the more efficient and accurate method of flow cytometry was used as a powerful tool in the study for the determination of the cell-specific activation of ODN. As we known, CD19 was the characteristic surface marker of B cells, and the activation of B cell was characterized by the upregulation of cell-surface molecules, such as costimulatory molecules CD80. CD11c was the characteristic surface marker of pDC, and one of the mature markers of pDC was the upregulation of Ia^b expression on pDC. The results showed that both B-class CpG ODN and C-class CpG ODN could upregulate the level of CD80 expression on CD19-positive cells. C-class CpG ODN induced a much higher level of Ia^b expression on CD11c-positive cells than B-class CpG ODN. The CD94 expression on NK cells was similar to the Ia^b expression on pDC. The results obtained via flow cytometry and cytokine analysis were consistent.

All of the cellular immune effects of CpG ODN in murine were believed to result directly and indirectly from activating TLR-9-expressing pDC and B cells^[23-28]. Nevertheless, B cell and pDC activation seemed to need different structure characteristics^[28,29]. An explanation for this is that pDC activation was associated with ODN that can form secondary structures, such as the dimeric C-class and the multimeric A-class. These higher-ordered structures might induce TLR-9 cross-linking, promote the recruitment of one

or more additional cofactors or adaptor proteins into the TLR-9-signalling complex, and/or alter the intracellular compartmentalization of the ODN^[30,31]. Thus, the relationship between the primary and higher-level structure of CpG ODN and its accessibility, recognition, and combination with TLR-9 still need further study.

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