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Newly Identified CpG ODNs, M5-30 and M6-395, Stimulate Mouse Immune Cells to Secrete TNF-α and Enhance Th1-Mediated Immunity

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Bacterial CpG motifs are known to induce both innate and adaptive immunity in infected hosts via toll-like receptor 9 (TLR9). Because small oligonucleotides (ODNs) mimicking bacterial CpG motifs are easily synthesized, they have found use as immunomodulatory agents in a number of disease models. We have developed a novel bioinformatics approach to identify effective CpG ODN sequences and evaluate their function as TLR9 ligands in a murine system. Among the CpG ODNs we identified, M5-30 and M6-395 showed significant ability to stimulate TNF-α and IFN-γ production in a mouse macrophage cell line and mouse splenocytes, respectively. We also found that these CpG ODNs activated cells through the canonical NF-κB signaling pathway. Moreover, both CpG ODNs were able to induce Th1-mediated immunity in *Mycobacterium tuberculosis* (Mtb)-infected mice. Our results demonstrate that M5-30 and M6-395 function as TLR9-specific ligands, making them useful in the study of TLR9 functionality and signaling in mice.

Keywords: TLR, CpG ODN, murine immune system, TNF-α, IFN-γ

Bacterial DNA contains short repeated unmethylated CpG motifs which activate the innate immune system in infected hosts in a TLR9-dependent manner (Hemmi *et al*., 2000; Akira and Hemmi, 2003). Bacterial genomic DNA is cleaved into small fragments in phagosomes or endosomes (Leifer *et al*., 2004), producing short, distinctive fragments containing multiple unmethylated CpG motifs that are identified as a pathogen-associated molecular pattern (PAMP) by TLR9. TLR9 is expressed within endosomes and transfers signals to down-stream effectors through the adaptor molecules, MyD88 (Hemmi *et al*., 2003) or TRAF (Akira and Hemmi, 2003). TLR9 signaling results in the activation of the transcription factors NF-κB, and AP-1 as well as IRFs, which promote transcription of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6, effector cytokines such as IL-12 and IFN-γ, type I interferons (Chuang *et al*., 2002; Matsushima *et al*., 2004).

Because TLR ligands are capable of bridging the gap between innate and adaptive immunity, synthetic CpG ODNs have seen use as immunomodulatory agents (Bradford *et al*., 2002; Wagner *et al*., 2004). CpG ODNs would be ingested by phagocytes via endocytosis and ligated with TLR9 inside endosomes. In this study, we developed a method to test the ability of synthetic CpG ODNs to stimulate TLR9 signaling in murine immune cells. We also evaluated their ability to stimulate TNF-α and IFN-γ production in macrophages and $CD4^+$ T cells, respectively. Our data indicate that the M5-30 and M6-395 synthetic CpG ODNs constitute effective TLR9 ligands in mice, having a stimulatory capacity similar to ODN1826, the best-described CpG ODN.

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Materials and Methods

Cells and animals

The Raw 264.7 mouse macrophage cell line, WEH1-164 murine fibrosarcoma cell line, and L929 cell line were originally purchased from ATCC and maintained in RPMI1640 containing 10% FBS (Gibco BRL, USA) in a 37°C humidfied atmosphere with 5% CO_2. Virulent *Mycobacterium tuberculosis* (Mtb) H37Rv-infected C57BL/6 mice (6-8 weeks old) were a gift from Dr. Robert J. North (Trudeau Institute Inc., USA). Splenocytes from Mtb-infected C57BL/6 mice were isolated from minced 4 spleens collected 10 days post-infection and passed through 70 μm stainless mesh screens. Erythrocytes were removed by treatment with red blood cell lysis buffer (Sigma-Aldrich, USA) for 1.5 min and the remaining cells were washed with RPMI1640 containing 10% FBS.

Construction and synthesis of CpG ODN library

Six different factors were chosen for subroutines in the computer program used to design CpG ODNs as follows; (1) the presence of optimal motifs (GTCGTT), (2) the presence of TpC dinucleotides on the 5′ end, (3) the presence of pyrimidine rich regions on the 3′ end, (4) palindrome-containing sequences, (5) the presence of a poly (G) track, (6) the presence of 5′-TGAT-3′ on the 3′ end. While the subroutines for the optimal motifs, TpC dinucleotides, and pyrimidine rich regions were used for every mixing step, the subroutines for palindrome content, poly (G) tracks, and 5′-TGAT-3′ were used randomly. As a result, six different types of CpG-ODNs were generated as follows.
Type 1: TpC dinucleotides+optimal motifs+pyrimidine rich,
Type 2: TpC dinucleotides+optimal motifs+palindrome-containing+pyrimidine rich,
Type 3: TpC dinucleotides+optimal motifs+palindrome-containing+poly (G) track+pyrimidine rich+5′-TGAT-3′,

Type 4: TpC dinucleotides+optimal motifs+poly (G) track+pyrimidine rich,
Type 5: TpC dinucleotides+optimal motifs+palindromes+pyrimidine rich+5′-TGAT-3′,
Type 6: TpC dinucleotides+optimal motifs+pyrimidine rich+5′-TGAT-3′.

Well-characterized CpG-ODNs such as ODN1826 were removed from the CpG-ODN library.

Two CpG ODNs in each type of group were randomly selected, therefore twelve CpG ODNs were chosen and synthesized with a phosphorothioate backbone to prevent breakage by intracellular endonucleases. All oligonucleotides were synthesized by Integrated DNA Technology, Inc. (USA) or Genotech (Korea). All CpG ODNs were dissolved in endotoxin-free water as 100 μM stock solutions and diluted to 3 μM for use in experiments. The sequence of M5-30, M6-395, and ODN1826 are 5′-TCTTTCGTCGTTAACGACGTCTGAT-3′, 5′-TCGTCGTTCTGTCGTTTGGTTGAT-3′, and 5′-TCCATGACGTT CCTGACG′T-3′, respectively.

Detection of TLR9 expression

Total RNA was extracted from Raw 264.7 cells using RNeasy Mini kits (QIAGEN, USA) and quantified with a UV spectrophotometer. cDNA was synthesized with Reverse Transcriptase using an RT-PCR kit (TaKaRa, Japan) and amplified in a Palm Cycler (Cobett, USA) using specific primers for 35 cycles. The sequences of forward and reverse primers used to amplify TLR9 and β-actin cDNAs were 5′-CCCTCCTGGTACAGGCTGC-3′, 5′-CTGCAGCCTCTTGGTACAC A-3′. 5′-AGGCTGTGCTGTCCCTGTATGC-3′, 5′-ACCCAAGAAGG AAGGCTGGAAA-3′, respectively.

Measurement of TNF-α secretion

Raw 274.7 cells were seeded in 96 well plates at densities ranging between 5×10^2 and 1×10^4. Cells were incubated with each CpG ODN at a concentration of 0.5 to 6 μM for 16 h. After stimulation, supernatants were transferred to anti-TNF-α coated plates. Each supernatant was diluted 1- to 1,000-fold using 10-fold serial dilutions. All procedures were performed using the Murine TNF-α ELISA Development kit (PeproTech, USA) according to the manufacturer's instructions. The concentration of TNF-α protein was measured at 401 nm.

Quantifying IFNγ-producing splenocytes

Changes in the numbers of IFNγ-producing splenocytes were determined by ELISPOT assay. Assays were performed with a commercially available ELISPOT kit (Mouse IFN-γ ELISPOT Set, BD Biosciences, USA) according to the manufacturer's instructions using cells from 3 mice, as described previously (Jung *et al*., 2008).

Flow cytometry

Mtb-infected splenocytes were prepared as described previously (Jung *et al*., 2009) and incubated in the presence of 10 g/ml brefeldinA (Epicenter Technologies, USA) with or without 3 μM CpG ODN for 6 h at 37°C. The cells were washed and resuspended in medium containing R-phycoerythrin (PE)-conjugated anti-CD4, and Peridinin chlorophyll protein (PerCP)-conjugated anti-CD8 mAbs. Anti–mouse CD16/CD32 was added to block nonspecific binding, and the cells were incubated for 1 h at 4°C. Cells were then washed and fixed overnight in 0.5% paraformaldehyde, washed again, and stained for intracellular IFN-γ with allophycocyanin (APC)-conjugated anti-IFN-γ mAb. All monoclonal antibody reagents were purchased from BD Biosciences. After washing, the cells were analyzed by flow cytometry (FACSCalibur model; BD Biosciences, USA) using FlowJo software (Tree Star, USA).

Western blotting

Total protein was extracted with Tris buffer (Tris-Cl, EDTA, SDS, Sodium Orthovant, proteinase inhibitor) at the indicated time points. Twenty microgram of protein was resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes (Amersham Biosciences, USA). Membranes were probed sequentially with anti-IkBα and anti-β-actin antibodies (Santa Cruz Biotechnology, USA). The membranes were washed and incubated with HRP-conjugated goat anti-rabbit IgG Ab (Santa Cruz Biotechnology). Blots were developed using the ECL Plus system (Amersham Biosciences).

Statistical analysis

Data were expressed as Mean±SD of three independent experiments performed with similar results. The data were analyzed using student's *t* test wherever applicable. $p<0.05$ was considered to be significant.

Results

Development of a computer program for constructing an electronic CpG ODN library

In an effort to identify novel TLR9 ligands, we constructed an electronic CpG ODNs library based on the criteria mentioned above. We further investigated the ability of several of these computer-identified CpG ODNs to function as TLR9 ligands. Several factors affecting of the ability of CpG ODNs to function as TLR9 ligands have been reported (Yi *et al*., 1998; Hartmann and Krieg, 2000; Hartmann *et al*., 2003). For example, early studies showed that a species-specific difference in optimal CpG motif sequences with *GTCGTT* being optimal for human (Bauer *et al*., 2001) and *GACGTT* being optimal for mice (He *et al*., 2007). The immunostimulatory efficiency of CpG motifs is enhanced by the number and spacing of CpG motifs. As described above, we chose six factors important for TLR9 stimulatory activity to develop a computer program capable of designing a library of CpG ODNs. We subjectively decided on an optimal length of CpG ODNs as 24 nucleotides. Figure 1 shows partial lists of six different types of CpG ODNs in the library.

Detection of TLR9 expression in mouse splenocytes and Raw 264.7 cells

Most antigen-presenting cells in the immune system are believed to express TLRs. Raw 264.7 macrophages and splenocytes were used to confirm TLR9 expression by RT-PCR. Splenocytes isolated from C57BL/6 mice and Raw 264.7 cells showed strong TLR9 expression, while WEH1-164 cells showed moderate TLR9 expression. TLR9 expression was not detected in L929 cells (Fig. 2).

Measurement of TNF-α secretion in Raw 264.7 cells

The release of TNF-α is an early cytokine in the inflammatory process and occurs, when TLR pathways are activated by specific ligands (Lee *et al*., 2004). Raw 264.7 cells were stimulated with each CpG ODN for 16 h, and the amount of TNF-α secreted was measured by ELISA. In Raw 264.7 cells, newly designed M5-30 and M6-395 CpG ODNs stimulated

Type 1	Type 2	Type 3
TCGTCGTTAGGTCGTTTCCCCGCT	TCGTCACTTGTCGACAAGTGATTT	TCAGAGTCGTTAATTAACGACTGG
TCCGTCGTTAGGTCGTTTTGTTTT	TCGGAATACTGCAGTATTCCCGTT	TCAATCGTCGTTAACGACGAGGGG
TCCAGTCGTTAGGTCGTTACTCGG	TCGTCGTTGGGTCGACCCAACGTT	TCTGCCAGTCGACTGGCAGGGGGG
TCGTTGTCGTTAGGTCGTTGCGTT	TCGGTCGTTGGCCAACGACCGCGT	TCCTCGGTGCACCGAGGTCGGGGG
TCTAGTGTCGTTAGGTCGTTCGGT	TCCAGTCGTTGGGCCCAACGACGT	TCGGGCTCAGTACTGAGCCGGGGG
TCATATCGTCGTTAGGTCGTTGCG	TCCACGTCGCGACGTCGTTCTTCA	TCGAGTAACGGCCGTTACGGGGGG
TCCGACCTGTCGTTAGGTCGTTTC	TCCTTCGTCGTTAACGACGATTCC	TCGTCGTTAGCTAACGAACGGGGG
TCCTATGATGTCGTTAGGTCGTTT	TCATTGAGTCGTACGACTCATCCC	TCCGTCGTTAGGCCTAACGAGGGG
TCATCTGAGAGTCGTTAGGTCGTT	TCTGCATAGTCGACTATGCGTTGG	TCTAGTCGTTAACGACTAGGGGGG
TCGTCGTTATGTCGTTGTCGTTCT	TCCCTACCGGTAGGTGGGTCGTTA	TCTTTGTCGTTATAACGACAAGGG
TCGGTCGTTATGTCGTTGGGCCTC	TCTTCCGTTAACGGAAGGGTCGTT	TCGACTGTCGACAGTCCGTGGGGG
TCTTGTCGTTATGTCGTTGCGCTG	TCGTCGTTGTACAACGACCTCGCG	TCGTGGTGTCGTACGACACCAGGG
TCTGGGTCGTTATGTCGTTCTCTT	TCGGTCGTTGTGTACACAACGAGT	TCGGGTCAGTACTGACCTCGGGGG
TCACGGGTCGTTATGTCGTTGTTT	TCGTGTCGTTAACGACATGCTGCG	TCGATCTTTGTCGACAAAGATGGG
TCAAATGGTCGTTATGTCGTTCCC	TCTCTGTCGTACGACAGTTGGGTA	TCTACGGCACGCGTGCCGTGGGGG
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Type 4	Type 5	Type 6
TCGTCGTCGTTAGGTCGGGGGGGG	TCTCCGTCGTTTAAACGACGTGAT	TCCATGGGTCGTTATGTCGTTGAT
TCACCCGTCGTTAGGTCGGGGGGG	TCCAGTGTCGTACGACACTTTGAT	TCAGGTGAGTCGTTATGTCGTGAT
TCGGGACGTCGTTAGGTCGGGGGG	TCTGACAGTCGTACGACTGTTGAT	TCCAAATGTGTCGTTATGTCTGAT
TCGTCAACGTCGTTAGGTGGGGGG	TCTATGGAGTACTCCATACGTGAT	TCTACACCGAGTCGTTATGTTGAT
TCCCGAGTCGTCGTTAGGGGGGGG	TCCGTTACATGTAACTTGTCTGAT	TCGTCGTTACGTCGTTTCCATGAT
TCAGAATTTAGTCGTTAGGTGGGG	TCGAAGATTTGTACAAATCTTGAT	TCGGTCGTTACGTCGTTCCGTGAT
TCGTCGTTATGTCGTTGCGGGGGG	TCGTCGTTTAAACGATTCTGTGAT	TCTTGTCGTTACGTCGTTGTTGAT
TCCGTCGTTATGTCGTTCGGGGGG	TCGGTCGTTTCGCGAAACGATGAT	TCATTGTCGTTACGTCGTTCTGAT
TCCCGTCGTTATGTCGTTACGGGG	TCACGTCGTTTCGAAACGACTGAT	TCATGCGTCGTTACGTCGTTTGAT
TCCGTGTCGTTATGTCGTGGGGGG	TCACAGTCGTACGACTGTTCTGAT	TCGCTTAGTCGTTACGTCGTTGAT
TCGTCTGTCGTTATGTCGGGGGGG	TCTGGTGTCGACACCACGTTTGAT	TCCGTTCCGTCGTTACGTCGTGAT
TCCCAGAGTCGTTATGTCGTGGGG	TCGTTGCGTCGTACGACGCATGAT	TCTTCGTGTGTCGTTACGTCTGAT
TCGCAGTCGTCGTTATGTCGGGGG	TCCTGCTGGTACCAGCAGCGTGAT	TCAGTCCGGGGTCGTTACGTTGAT
TCATACTAAGTCGTTATGTGGGGG	TCGAAGGGAGTACTCCCTTCTGAT	TCGTCGTTGAGTCGTTTTTGTGAT
TCTACGGCCGGTCGTTATGGGGGG	TCAAACACGCGTGTTTTCGTTGAT	TCTGTCGTTGAGTCGTTCTCTGAT
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Fig. 1. Partial lists of six different classes of CpG ODNs sequences. Fifteen sequences from each class are shown at random. The strategy used for ODN construction is described in 'Materials and Methods'.

levels of TNF-α similar to those induced by ODN1826, a known TLR9 ligand (Fig. 3A), while the others did lower level of TNF-α secretion (data not shown). All three TLR-stimulating CpG ODNs, M5-30, M6-395, and ODN1826, induced four-fold or larger increases in TNF-α production. In addition, we found that the response to M5-30 and M6-395 occurred in a dose-dependent manner, an observation that yielded a clue as to the optimal concentration for TLR9 stimulation (Figs. 3B and C).

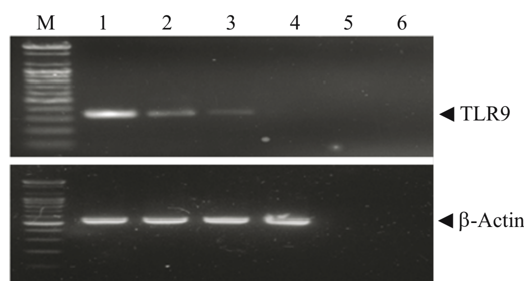

Fig. 2. Expression of TLR9 in mouse cell lines and tissue. TLR9 expression was detected by RT-PCR in Raw 264.7 and WEH1-164 cell lines and spleens C57BL/6 mice, but not in L929 cells. Lanes: M, molecular size marker; 1, C57BL/6 spleen; 2, Raw 264.7; 3, WEH1-164; 4, L929; 5, no RTase negative control; 6, no Taq polymerase negative control.

Identifying the signaling pathway activated by novel CpG ODNs

To confirm that M5-30 and M6-395 stimulate the canonical NF-κB signaling pathway via TLR9, expression of IkBα was determined by western blotting (Fig. 4). The essential step in this pathway is the degradation of IkBα, which bind NF-κB and prevents its translocation to the nucleus and subsequent transcription of proinflammatory cytokine genes, including TNF-α, IL-1β, and type I IFNs (Chuang *et al*., 2002). The expression of IκBα protein was decreased within 30 min after treatment with each CpG ODN, assuming that NF-κB was rapidly phosphorylated at the same time. Therefore, M5-30 and M6-395 were examined as specific TLR9 ligands in mouse macrophages.

Quantification of IFNγ-producing cells in splenocytes

IFN-γ is the most critical cytokine in Th1-mediated

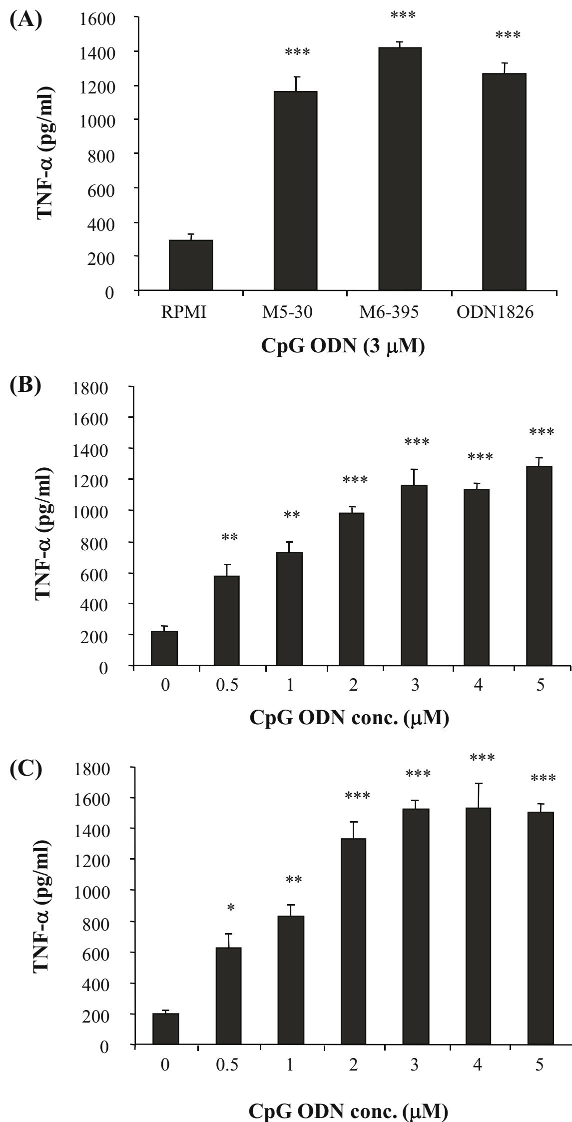

Fig. 3. Ability of M5-30 and M6-395 to induce secreting TNF-α production in Raw 264.7 cells. Newly synthesized M5-30 and M6-395 CpG ODNs were able to induce TNF-α production in Raw 264.7 cells (A). Treatment with M5-30 (B) and M6-395 (C) induced TNF-α production in Raw 264.7 cells in a dose-dependent manner. Each CpG ODN was administered to 5×10^5 Raw 264.7 cells at a concentration of 3 μM for 16 h. Then cell culture media were collected and used in TNF-α ELISA assays. Data are presented as Means±SD of results from triplicate wells per time points. Triplicate experiments yielded similar results. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; compared with the unstimulated cells.

et al., 2003; Harris *et al*., 2007), anti-viral and anti-tumor cytotoxic activity of $CD8^+$ T cells and expression of MHC molecules (Heit *et al*., 2003). Splenocytes from C57BL/6 mice were incubated with each CpG ODN overnight and the number of IFN-γ-producing cells was estimated by ELISPOT assay. As seen in Fig. 5, 7 to 9×10^4 cells produced IFN-γ after incubation with each CpG ODN, while IFN-γ-producing cells were rare in control groups. It appears that the IFN-γ-producing ability of mouse splenocytes stimulated with M6-395 was slightly superior to the other two CpG ODNs, however, the difference was not significant. Similar results were obtained in triplicate experiments.

Measurement of Th1-mediated immunity

To verify that M5-30 and M6-395 CpG ODNs can enhance Th1-mediated immunity against infection, we infected C57BL/6 mice with virulent *M. tuberculosis* stain, H37R via airborne route and isolated splenocytes on day 10 post-infection. Splenocytes were stimulated with CpG ODNs for 6 h, and then stained with anti-CD3, anti-CD4, anti-CD8, and anti-IFN-γ to analyze cell populations by flow cytometry. In T lymphocytes, production of IFN-γ is the unique sign to be fully developed into effector cells, especially the Th1 subtype. In unstimulated control splenocytes, less than 1% of total cells produced IFN-γ, but more than 5% of total cells produced IFN-γ after stimulation with CpG ODNs for 6 h (Fig. 5). Moreover, the proportion of IFN-γ-producing $CD4^+$ T cells and $CD8^+$ T cells was significantly increased from about 0.1% to a range of 0.88 to 1.5% after stimulation. All three CpG ODNs seemed to promote Th1-mediated immunity to a similar degree. Our results indicate that the ability to stimulate Th1-mediated immunity with novel CpG ODNs would be applicable to the development of vaccine adjuvants to protect infectious diseases.

Discussion

CpG ODNs are synthetic oligonucleotides that contain unmethylated CpG dinucleotides in particular sequence contexts (CpG motifs) (Krieg *et al*., 1995). These CpG motifs are present at a 20-fold greater frequency in bacterial DNA compared to mammalian DNA. They have been shown to induce a coordinated set of immune responses, activating cells of the innate immune system via receptors which recognize these molecular patterns (Bauer *et al.*, 2001). Two types of CpG ODNs have been identified based on their distinct capacities to stimulate plasmacytoid dendritic cells (pDC), key sensors of CpG motifs (Krug *et al*., 2001). CpG type A induces

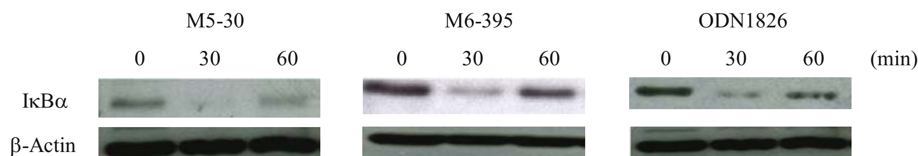

Fig. 4. CpG ODNs stimulate the canonical NF-kB signaling pathway. Raw 264.7 cells were treated with each CpG ODN at a concentration of 3 μM for 30 min or 1 h. Expression IkBα protein was diminished within 30 min after stimulation and recovered within 1 h.

immunity. It stimulates phagocytosis in macrophages (Hayashi

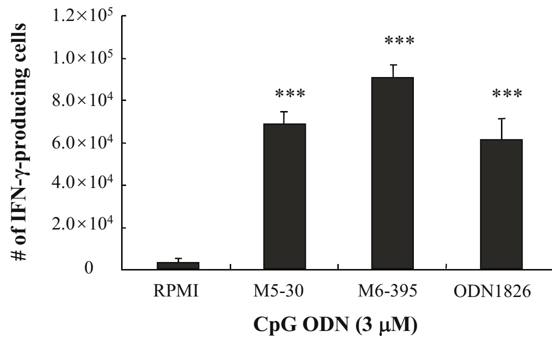

Fig. 5. M5-30 and M6-395 induce IFN-γ production in C57BL/6 mouse splenocytes. Mouse splenocytes were incubated with CpG ODNs at a concentration of 3 μM for 16 h. IFN-γ-producing splenocytes were detected by ELISPOT assay, and spots were counted by microscopy. Data are presented as Means±SD of results from three independent experiments. *** $p<0.001$ compared with the unstimulated cells (RPMI).

large amounts of IFN-α in pDCs whereas CpG type B does not but rather promotes survival, activation and maturation of pDCs (Guiducci *et al*., 2008). Although the sequences of CpG motifs differ between mice and humans, the recognition of CpG ODNs is primarily mediated by TLR9 in both species (Bauer *et al*., 2001). Ligation of TLR9 with CpG ODNs triggers a signal transduction pathway via two primary adaptor molecules, MyD88 and TRIF, which go on to activate several transcription factors. Finally, transcription factors, including NF-κB, AP-1, and IRFs stimulate the transcription of proinflammatory cytokines and type I interferons (Hemmi *et al*., 2000 and 2003). These cytokines activate innate immunity to eliminate pathogens and simultaneously induce adaptive immunity.

Recently, the therapeutic application of CpG ODNs in human maladies such as infectious diseases (Harris *et al*., 2007), cancers (Krieg, 2007), and autoimmunity (Krieg and Vollme, 2006) has been the subject of intense research; studies detailing the design and evaluation of human disease-specific CpG ODNs are readily available (Ichikawa *et al*., 2002; Wagner *et al*., 2004; Jiang *et al*., 2006). However, evaluations of experimental mouse CpG ODNs are comparatively rare. Here we introduce tools for finding and evaluating experimental mouse CpG ODNs *in silico*. We divided ODN sequences into 6 categories, each containing optimal core motifs, *GTCGTT*, randomly chose 12 ODNs and evaluated their ability to induce TNF-α and IFN-γ production in mouse macrophages and splenocytes, respectively. All 12 ODNs were able to stimulate the murine immune system, albeit to varying degrees.

Two ODNs, M5-35 and M6-395, possessed a potent ability to activate murine macrophages and splenocytes. Both trigger the TLR9 signaling cascade inside the cell within 30 min ; IκBα protein is efficiently degraded, freeing NF-κB to translocate into the nucleus. ODN1826 is the best-characterized commercial CpG ODN capable of stimulating the murine immune system (Dowling *et al*., 2008). Our results indicate that M5-35 and M6-395 ODNs possess equal or greater immunostimulatory capacity than ODN1826. The selection diversity in a sequence pool of CpG ODNs would be helpful to those who study molecular signaling pathway or application of therapeutic efficacy of TLR9 ligands in mouse system. Presumably most of CpG ODNs in the list of Table 1 were believed to have some degree of immune activation. On the other hand, some of them might have weak stimulatory effect or otherwise immune regulatory function. The development of another searching tool which can categorize effective CpG ODNs according to their immunologic function will be the next topic of us.

This study provides a useful method for the development of novel TLR9 ligands in a murine system. We believe this method will be useful in the construction of novel ligands to

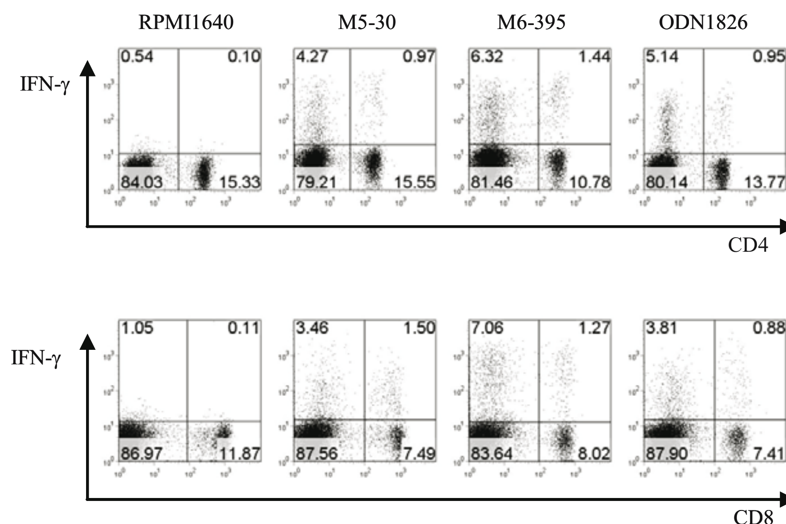

Fig. 6. Enhanced Th1-mediated immunity in Mtb-infected C57BL/6 splenocytes following treatment with M5-30 and M6-395. Mice were infected Mtb via airborne route and four spleens were pooled on day 10 post-infection. Cells were treated with or without CpG ODN for 6 h. Cells were stained with anti-CD4-PE, anti-CD8-PerCP, and anti-IFNγ-APC and then analyzed by flow cytometry.

study innate and adaptive immunity via the TLR9 signaling pathway.

Acknowledgements

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