Class I/II hybrid inhibitory oligodeoxynucleotide exerts Th1 and Th2 double immunosuppression

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Abstract

We designed class I/II hybrid inhibitory oligodeoxynucleotides (iODNs), called iSG, and found that the sequence 5′-TTAGGG-3′, which has a six-base loop head structure, and a 3′-oligo $(dG)_{3-5}$ tail sequence are important for potent immunosuppressive activity. Interestingly, splenocytes isolated from ovalbumin (OVA)-immunized mice and treated with iSG3 showed suppression of not only interleukin (IL)-6, IL-12p35, IL-12p40, and interferon (IFN) γ mRNA expression, but also IL-4 and IL-13 mRNA expression. Thus, both Th2 and Th1 immune responses can be strongly suppressed by iODNs in splenocytes from allergen-immunized mice, suggesting usefulness in the treatment of diseases induced by over-active immune activation.

Highlights

・Inhibitory oligodeoxynucleotides (iODNs) are potent suppressors of innate immune responses induced by CpG ODNs.

・Class I iODNs are short, G-rich, and produce sequence-specific inhibition of all TLR9 responses.

・Class II iODNs include telomeric repeat motifs that inhibit STAT1, 3, and 4 signaling.

・Class I/II hybrid iODNs require a six-base secondary loop structure with a $3'\text{-}(G)_{3\text{-}5}$ polytail sequence for potent immunosuppressive activity.

・Class I/II hybrid iODNs exert a novel effect of Th1 and Th2 double immunosuppression.

Keywords:

iODN; class I iODN; class II iODN; class I/II hybrid iODN; immunosuppression.

Abbreviations: ODN, oligodeoxynucleotide; IL, interleukin; TLR, Toll-like receptor; PS, phosphorothioate; PO, phosphodiester; OVA, ovalbumin; ELISA, enzyme-linked immunosorbent assay; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IFN, interferon; Th1 cell, Type 1 helper T cell; Th2 cell, Type 2 helper T cell; STAT, signal transducer and activator of transcription.

1. Introduction

Diseases characterized by persistent immune activation can be prevented or treated with inhibitory/suppressive oligodeoxynucleotides (iODNs) in animal models. Immune cells respond to bacterial DNA containing unmethylated CpG motifs via Toll-like receptor 9 (TLR9) (1). Synthetic oligodeoxynucleotides (ODNs) containing unmethylated CpG motifs stimulate a strong innate immune response (2). This stimulation can be abrogated by either removing the CpG ODNs or adding iODNs (3). Trieu et al. have provisionally categorized iODNs into four classes (Class I, II, III, and IV) based on sequence and probable mode of action (4). Class I iODNs (such as H154) are G-stretch ODNs and are TLR9-specific competitors (3, 5). Class II iODNs (such as A151) have telomeric repeats, are TLR9-independent inhibitors of signal transducer and activator of transcription (STAT) 1, 3, and 4 signaling (6), and are potent suppressors of immune responses. Because Class II ODNs are generally synthesized as 24-base phosphorothioate-modified ODNs (PS-ODNs), they also fall into the category of Class IV iODNs, which are defined as long PS-ODNs that inhibit TLR9 responses in a non-sequence-specific manner. Class III iODNs include oligo-(dG), which forms a four-stranded structure and inhibits DNA uptake. The class I G-rich motifs show the most promise as selective and potent TLR9 inhibitors for therapeutic applications (3). However, there are no reports of an anti-Th2 immune response following treatment with iODNs. In this study, we designed a class I (H154) and class II (A151) hybrid iODN, which we named iSG and that includes the telomeric motif 5′-TTAGGG-3′. We analyzed the immune activity of iSG 3′-oligo $(dG)_{0-7}$ tail variants (iSG0, 1, 2, 3, 4, 5, 6, and 7) in mouse splenocytes. We identified iSG3, 4, and 5 as inducing strong immunosuppression and requiring a particular secondary structure. Finally, we investigated the effects of Th2 immunosuppressive activity by iSG3 in immune cells from ovalbumin (OVA)-immunized mice.

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2. Materials and methods

2.1. Reagents

Endotoxin-free desalted PS or phosphodiester (PO) bond ODNs were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, USA). The ODNs were reconstituted in endotoxin-free water and passed through a 0.22-μm pore microfilter (Nihon Millipore K.K., Tokyo, Japan). Mouse splenocytes were treated with an equimolar mixture containing mouse prototype CpG ODN 1555 (ODN_{1555}) (7, 8) plus negative control GpC ODN 1612 (ODN_{1612}) (7, 8), iODN class I H154 (3), iODN class II A151 (6, 9), or iODN class I/II hybrid iSG0, 1, 2, 3, 4, 5, 6, or 7 (Table 1). ODN_{1555} previously shown to be a potent stimulator of innate and adaptive immunity in mouse (7, 8).

2.2. Mice and OVA-immunized mice

Pathogen-free male C57BL/6 mice (5 weeks of age) and BALB/c mice (5 weeks of age) were purchased from Japan SLC (Shizuoka, Japan) and kept under temperature- and light-controlled conditions. Mice were given a standard diet of Labo MR Breeder (Nihon Nosan Co., Kanagawa, Japan) and sterile water *ad libitum*. After a preliminary period of 1 week, BALB/c mice (6 weeks of age, *n = 4*) were intraperitoneally sensitized once weekly for 3 weeks with 100 μg OVA (Sigma, St. Louis, MO, USA) and alum adjuvant (allergen/adjuvant ratio of 1/20). Mice were used for the study at 8 weeks of age. All experimental procedures were carried out in accordance with the Regulations for Animal Experimentation of Shinshu University, and the animal protocol was approved by the Committee for Animal Experiments of Shinshu University. Based on national regulations and guidelines, all experimental procedures were reviewed by the Committee for Animal Experiments and finally approved by the president of Shinshu University.

2.3. Cells and cell culture

Mouse splenocytes were prepared using standard methods (10, 11). Cells were cultured in triplicate wells of a 24-well plate (Nalge Nunc International K.K., Tokyo, Japan) at a final concentration of 2 x 10^6 cells/well (total 1 mL/well) in complete RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% fetal calf serum (Sigma), 100 U/mL penicillin, 100 mg/mL streptomycin, 25 mmol/L HEPES, 1.0 mmol/L sodium pyruvate, nonessential amino acids, and 0.0035% 2-mercaptoethanol.

2.4. Real-time quantitative PCR analysis

Total RNA was isolated from ODN-stimulated mouse splenocytes, treated with RNase-free DNase I (Roche, Lewes, UK) for 10 min at 37°C, and then heat inactivated at 70°C for 15 min (10, 11, 12). The cDNAs were prepared by reverse transcription from 1 μg total RNA using a PrimeScript® RT reagent kit (TaKaRa Bio Inc., Tokyo, Japan). An equivalent volume of cDNA was used for quantification of various cytokine cDNAs with real-time quantitative PCR using a Thermal Cycler Dice® Real Time System (TaKaRa Bio Inc., Tokyo, Japan). Fluorescent real-time quantitative PCR reactions were performed with SYBR Premix Ex Taq (TaKaRa Bio Inc.) using specific primers, with each reaction containing 5 ng cDNA in 25 μL. Primers for β-actin, interleukin (IL)-4, IL-6, IL-12p35, IL-12p40, IL-13, and interferon gamma (IFNγ) were purchased from TaKaRa Bio Inc. The PCR cycling conditions were 10 s at 95°C, followed by 40 cycles of 5 s at 95°C and 30 s at 60°C. As a control, poly (A)$^+$RNA samples were used as templates to check for the presence of contaminating genomic DNA. The sensitivity of the reaction and amplification of contaminating products such as the extension of self-annealed primers were evaluated by amplifying serial dilutions of cDNA. For cross-sample comparison of results obtained following various treatments, cytokine

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mRNA levels were first normalized to mRNA levels for β-actin. The results represent the means ± SD of three independent experiments.

2.5. Cytokine enzyme-linked immunosorbent assay (ELISA)

IL-6 levels in cell culture supernatants 48 h after various treatments were quantified using a commercially available ELISA kit (Quantikine mouse IL-6; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.6. Secondary structural analysis of iODNs

The secondary structures of iODNs (H154, A151, and iSG variants) were analyzed with CentroidFold software (13, 14). The detailed relationship between their secondary structure and immunosuppressive activity was further analyzed in this study.

2.7. Cell proliferation assay

ODN-mediated suppression was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT: Sigma-Aldrich) assay. Cells were seeded in 96-well plates at a density of 2×10^5 cells/well. The cultures were then exposed to 3.0 μM ODN_{1555} + 3.0 μM control ODN_{1612} or iODNs for 48 h. As a positive control, the cultures were exposed to 100 μg/mL Blasticidin S HCl (Invitrogen, Grand Island, NY, USA), which is a nucleoside antibiotic isolated from *Streptomyces griseochromogenes* that inhibits protein synthesis in both prokaryotic and eukaryotic cells, and is thus cytotoxic. The MTT assay was performed as described (9). Briefly, 100 μL medium containing MTT (0.5 mg/mL) was added to the washed cells for 2 h. Non-internalized MTT was then washed away, and the cells were lysed by adding 50 μL dimethylsulfoxide (Sigma-Aldrich), which released the MTT internalized by viable cells. The MTT concentration was measured colorimetrically, and cell proliferation

was determined as the optical density at 565 nm (OD_{565}) of treated/untreated cultures (medium control = 100%).

2.8. Statistical analysis

Statistical analyses were performed using MedCalc, version 9.3.7.0 (MedCalc Software, Mariakerke, Belgium) and S-Plus (Version 7, Insightful Corp, Seattle, WA, USA). All tests were two-sided; probability values less than 0.01 were considered significant. All values are expressed as means ± SD unless otherwise noted.

3. Results

3.1. Immunosuppressive activity of class I/II hybrid iODN in mouse immune cells.

To investigate the immunosuppressive activity of the class I/II hybrid iODN iSG3, we first examined the inhibition of IL-6 mRNA expression in mouse splenocytes stimulated with ODN_{1555} (Figure 1). The activity of iSG3 in suppressing IL-6 mRNA expression in splenocytes was compared with the activity of H154 and A151, both of which are known for their strong immunosuppressive activity. Splenocytes were treated with immunostimulatory ODN_{1555} (3.0 µM) plus different concentrations of ODN_{1612} (negative control), H154, A151, or iSG3. The iODNs (H154, A151, and iSG3) suppressed IL-6 mRNA expression in a dose-dependent manner, with high immunosuppressive activity at a concentration of 3.0 µM. Figure 1A shows that iSG3 induced significantly stronger suppression of IL-6 mRNA than H154 or A151 (0.1 to 3.0 µM) in this experimental system (Figure 1A). To further examine the suppressive effect of iSG3, we stimulated the cells with ODN_{1555} and also added ODN_{1612}, iSG3 (Phosphorothioated bond, PS), or iSG3 (Phosphodiester bond, PO). As shown in Figure 1B, IL-6 mRNA expression was also strongly suppressed by iSG3 (PS). On the other hand, no suppressive effect was seen with iSG3 (PO). Essentially the same observation was seen when

ELISA was used to measure the induction of IL-6 secretion (data not shown). Thus, iSG3 was more potent at suppressing IL-6 expression than A151 and H154 in CpG ODN-stimulated splenocytes.

We next analyzed the relationship between the number of bases in the 3′-oligo $(dG)_n$ tail of iSG and IL-6 suppressive activity and cell proliferation in cells stimulated with ODN_{1555} (Figure 1C, Supplemental Figure 1). The cultures were exposed to 3.0 μM ODN_{1555} + 0.1 or 3.0 μM iODNs or control ODN_{1612}. The concentrations of IL-6 in the supernatants were measured with ELISA (Supplemental Figure 1B). Interestingly, a 3′-oligo $(dG)_{3,4,5}$ tail (iSG3, iSG4 and iSG5) produced strong immunosuppression of IL-6 mRNA (Figure 1C). Results of IL-6 secretion were similar when measured with ELISA (Supplemental Figure 1A, B). We also examined the cell proliferation of splenocytes treated with 3.0 μM control ODN (ODN_{1612}) or iODNs (A151, H154, and iSG3) that were stimulated with 3.0 μM ODN_{1555} (Supplemental Figure 2). The cell proliferation (%, medium control = 100%) indices of cells treated with H154 (167.0% ± 10.1%), A151 (224.6% ± 14.9%), and iSG3 (154.7% ± 8.6%) were significantly lower than that of cells treated with $ODN_{1555}+ODN_{1612}$ (488.3% ± 19.4%) (Supplemental Figure 2). These indices were higher than the medium control (100%). These results suggest that iODNs did not kill cells, although iSG3 did inhibit the cell proliferation seen with CpG ODN stimulation.

3.2. Strong immunosuppression by iSG3 requires a specific secondary structure

We determined the secondary structure of the iODNs using CentroidFold software (13, 14). To evaluate the importance of iSG sequences, we used the software to draw the secondary structure of A151 and H154 (Figure 2). The iSG variants contain six bases that form a loop structure similar to A151 and H154 (Figure 2). In the iSG from class I/II hybrid iODNs, 5′-TTAGGG-3′ sequences are specifically found in the loop similar to A151 (Figure 2). To

identify the iSG3 core motifs responsible for the immunosuppressive activities, we examined the suppressive effect of mutated iODNs of H154, A151, and iSG3, which were named H154M, A151M, and iSG3M, respectively. The TTTAGGG sequence(s) in the A151 and iSG3 sequence(s) was/were changed to TTA*CCC* (Table 1, Figure 2). The 3′-G tail sequence inside the H154 and iSG3 sequence was changed to a 3′-*C* tail (Table 1, Figure 2). We examined the ability of mutated iODNs to suppress IL-6 mRNA expression in mice splenocytes (Figure 3). The mutated iODNs (H154M, A151M, and iSG3M) did not suppress IL-6 mRNA expression when TTAGGG and the 3′-G tail sequences were changed to TTA*CCC* and a 3′-*C* tail (Figure 3). The large hairpin loop structure formed by TTA*CCC* in iSG3M did not show a potent suppressive effect (Figure 2 and 3). H154M and A151M without TTAGGG and the 3′-G tail structure also showed low suppression. This revealed that a TTAGGG loop and/or a 3′-G tail structure are necessary for strong immunosuppression.

3.3. Th1 and Th2 double immunosuppression by iSG3 in immune cells from OVA-immunized mice

We examined the ability of iSG3 to suppress Th1- and Th2-type cytokine expression in splenocytes isolated from OVA-immunized mice (Figure 4). The cultures were exposed to 10 μg/mL OVA + 3.0 μM negative control ODN_{1612}, ODN_{1555}, or iODNs (A151, H154, iSG3) for 72 h. OVA + ODN_{1612} strongly induced mRNA expression of Th2-type cytokines such as IL-4 (Figure 4A) and IL-13 (Figure 4B), inflammatory cytokines such as IL-6 (Figure 4C), and Th1 cytokines such as IL-12p35, IL-12p40 (Figure 4D, E), and IFNγ (Figure 4F). OVA + ODN_{1555} also strongly induced mRNA expression of Th1 cytokines such as IL-12p35 and IL-12p40 (Figure 4D, E). In our preliminary study, ODN_{1555} strongly induced IFNγ mRNA after 6 to 12 h of stimulation. IFNγ was downregulated after 24 h (data not shown) in the mouse immune cells. Interestingly, cells stimulated with OVA + iSG3 for 72 h showed significant

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suppression of IL-4 and IL-13 mRNA compared with OVA + ODN_{1612}, A151, or H154 (Figure 4A, B). In particular, A151 showed negligible suppression of IL-13 mRNA (Figure 4B). Moreover, OVA + iSG3 or A151 significantly suppressed IL-12p40 and IFNγ mRNA compared with OVA + H154 (Figure 4E, F). Cells treated with OVA + A151 showed similarly suppressed levels of IL-12p40 and IFNγ mRNA as cells treated with iSG3 (Figure 4E, F).

To monitor the immunosuppressive activity after iSG3 removal, mouse splenocytes were exposed to 3.0 μM ODN_{1612} or iSG3 for 24 h. Cells were then washed with medium to remove the iSG3. Cells were resuspended in medium with 3.0 μM ODN_{1555} for 6 h or 48 h. Interestingly, immunosuppressive activity and inhibition of cell proliferation were maintained in splenocytes after iSG3 was removed from the culture (Supplemental Figure 3).

4. Discussion

Previous studies revealed that iODNs inhibit the immune response by blocking the stimulatory effects of CpG motifs (4, 15). In animal models, iODNs can be used to prevent or treat diseases characterized by persistent immune activation, including collagen-induced arthritis (16), inflammatory arthritis (5), silicosis (9), autoimmune neuritis (17), papillomas (18), ocular inflammation (19), and toxic shock (20). In this study, we examined the immune effect of iODNs in mouse immune cells. The results indicated that the class I/II hybrid iODN, iSG3, showed significantly higher suppression of proinflammatory cytokine mRNAs than H154 or A151. To the best of our knowledge, there have been no previous reports of suppression of Th2 immune responses by iODNs. In splenocytes from OVA allergic model mice, treatment with iSG3 reduced the expression of IL-4, IL-13, IL-12p40, and IFNγ mRNA in 10 μg/mL OVA-treated cells. This class I/II hybrid iODN suppressed not only mRNA levels of several Th1- and Th2-type cytokines, but also cell proliferation. iODNs can also

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inhibit several signal transduction cascades related to the production of Th1 cytokines such as IFNγ and IL-12 by binding and inhibiting the phosphorylation of STAT1, 3, and 4 proteins (20, 21). No effect was observed on STAT5 and 6. This effect is independent of TLR9 and leads to the absence of a response to IL-12 and IFNγ in T cells and a subsequent transition to the Th2 phenotype rather than Th1 (21). However, we have shown that iSG3 downregulated the expression levels of Th2 cytokines in allergen-treated cells from allergic model mice. Our study demonstrated that iSG3 can block both Th1 and Th2 immune responses mediated by allergen in immune cells from allergic model mice.

The molecular mechanism by which class I/II hybrid iODNs such as iSG3, 4, and 5 suppress immune responses remains unclear. No information has been reported on the relationship between the secondary structure and immunosuppression by iODNs. Therefore, in the present study, we focused on the role of the secondary structure using CentroidFold software (13, 14). CentroidFold is a standard software for predicting secondary structures and is useful for the functional analysis of RNA, for helping design PCR primers and siRNA, and leads to higher quality microarray probes (13, 14). We found that 3′-oligo $(dG)_{3-5}$ tail sequences are optimal for immunosuppression and that 5′-TTAGGG-3′ with a six-base loop head structure is important and possibly critical for potent immunosuppression. These structures may be required for immunosuppression of the Th1- and Th2-type immune responses.

Current studies show that iSG3 had little effect on cell proliferation in splenocytes, similar to the medium control, as assessed with the MTT assay. Because we also established that splenocytes pretreated with iSG3 maintain the ability to suppress the immune response after the iSG3 is washed out, the immunosuppression by iSG3 may be due to inhibition of cell signaling. Although additional studies on the relationship between iODNs and the iODN binding pocket of TLR9 or other receptors are needed to elucidate the molecular mechanism of iODN recognition, the current study emphasizes an important role in immunosuppression

for class I and II hybrid iODNs. Further detailed studies are needed to elucidate the mechanism of this iODN-based immunosuppressive drug candidate.

In summary, we found that 3′-oligo $(dG)_{3-5}$ tail sequences are optimal for immunosuppression and that a 5′-TTAGGG-3′ loop structure is important and possibly critical for potent immunosuppression. These structures may be required for immunosuppression of the Th1 and Th2 immune responses.

Acknowledgements

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Figure legends

Figure 1. Analysis of IL-6 mRNA expression in mouse splenocytes with real-time quantitative PCR. Mouse splenocytes (2×10^6 cells/mL) were pre-incubated in medium for 3 h prior to exposure to 3.0 μM ODN_{1555} plus ODN_{1612} or iODN for 6 h (A, B, C). H154, A151, and iSG3 (0.0001 to 3.0 μM) were used to determine the optimal concentration for the suppressive activity of iODNs (A). ODN_{1555} (3 μM) plus 3.0 μM ODN_{1612}, iSG3 (PS), or iSG3 (PO) were added to examine the suppressive effect of their phosphorothioate (PS) bond or phosphodiester (PO) bond subtypes (B). The suppressive activity of iSG 3′-oligo $(dG)_{0-7}$ tail variants (iSG0, 1, 2, 3, 4, 5, 6, and 7) in mouse splenocytes (C). IL-6 mRNA expression in mouse splenocytes treated with 3.0 μM ODN_{1555} and 3.0 μM iODNs. Results are shown as the ratio of IL-6 mRNA levels (normalized to β-actin; see Materials and Methods) in stimulated versus non-treated cells, as well as the IL-6 level in the medium control (C). The results represent the means ± SD of three independent experiments. Values with different letters (*i.e.,* a, b, c, d, e) were significantly different ($P < 0.01$). All assays were carried out at least three independent times in triplicate.

Figure 2. Secondary structural analysis of iODNs. Secondary structures were predicted with CentroidFold software. Pairs shown in red have a high probability of forming, pairs in green have a medium probability of forming, and pairs in blue have a low probability of forming. The normalized scale showing a probability from zero (dark blue) to one (red) is shown on the lower right.

Figure 3. Influence of the TTAGGG loop and/or 3′-G tail in the iODNs. The 3′-G tail of the iODNs was changed to a 3′-*C* tail. The TTAGGG sequence was changed to TTA*CCC*. Results are shown as the ratio of IL-6 mRNA levels (normalized to β-actin; see Materials and

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Methods) in stimulated versus non-treated cells, as well as the IL-6 level in the medium control. Values represent means, and error bars indicate the SD. The closed bars correspond to the ODN_{1555} plus control or optimal iODN (ODN_{1612}, H154, A151, and iSG3), and the open bars show ODN_{1555} plus mutated iODNs (H154M, A151M, and iSG3M). Values with different letters (*i.e.,* a, b, c, d, e) were significantly different ($P < 0.01$). All assays were carried out at least three independent times in triplicate.

Figure 4. Real-time quantitative PCR analysis of IL-4, IL-13, IL-6, IL-12p35, IL-12p40, and IFNγ mRNA levels in splenocytes isolated from OVA-immunized mice that were subsequently treated with 10 μg/mL OVA + 3.0 μM ODN_{1612}, ODN_{1555}, A151, H154, or iSG3. Mouse splenocytes were pre-incubated in medium for 3 h prior to exposure to OVA and ODNs for 72 h. Expression of IL-4 (A), IL-13 (B), IL-6 (C), IL-12p35 (D), IL-12p40 (E), and IFNγ (F) mRNAs were determined with real-time quantitative PCR. Results are shown as the ratio of cytokine mRNA levels (normalized to β-actin; see Materials and Methods) in stimulated versus non-treated cells, as well as the level in the medium control. Values represent means, and error bars indicate the SD of three independent experiments. Values with different letters (*i.e.,* a, b, c, d, e) were significantly different ($P < 0.01$).

Supplemental Figure 1. The suppressive activity of A151 and iSG variants. IL-6 mRNA expression (A) and IL-6 protein production (B) in mouse splenocytes treated with 3.0 μM ODN$_{1555}$ and 0.1 μM iODNs. Supernatants from stimulated cells were collected, and the concentration of IL-6 was measured with ELISA (B). Columns represent the mean concentrations (ng/mL). Values represent means, and error bars indicate the SD of three independent experiments. Values with different letters (*i.e.,* a, b, c, d) were significantly different ($P < 0.01$) (A, B). ND, not detected.

Supplemental Figure 2. Cell viability in mouse splenocytes. As a positive control, the cultures were exposed to Blasticidin S HCl. Splenocytes were treated with 3.0 μM ODN$_{1555}$ plus 100 μg/mL Blasticidin S HCl, 3.0 μM ODN$_{1612}$, H154, A151, or iSG3. Values represent means, and error bars indicate the SD of three independent experiments. Values with different letters (*i.e.,* a, b, c, d, e, f) were significantly different ($P < 0.01$). The assays were carried out at least three independent times in triplicate.

Supplemental Figure 3. Mouse splenocytes (2 x 10^6 cells /mL) were pre-incubated in medium for 3 h prior to exposure to 3.0 μM ODN$_{1612}$ or iSG3 for 24 h. Cells were washed with medium to remove the ODNs. Cells were resuspended in medium with 3.0 μM ODN$_{1555}$ for 6 h (A) or 48 h (B, C). Results are shown as the ratio of IL-6 mRNA levels in stimulated versus non-treated cells (A, B) and cell proliferation in mouse splenocytes (C). Values represent means, and error bars indicate the SD. Values with different letters (*i.e.,* a, b, c, d) were significantly different ($P < 0.01$). All assays were carried out at least three independent times in triplicate.
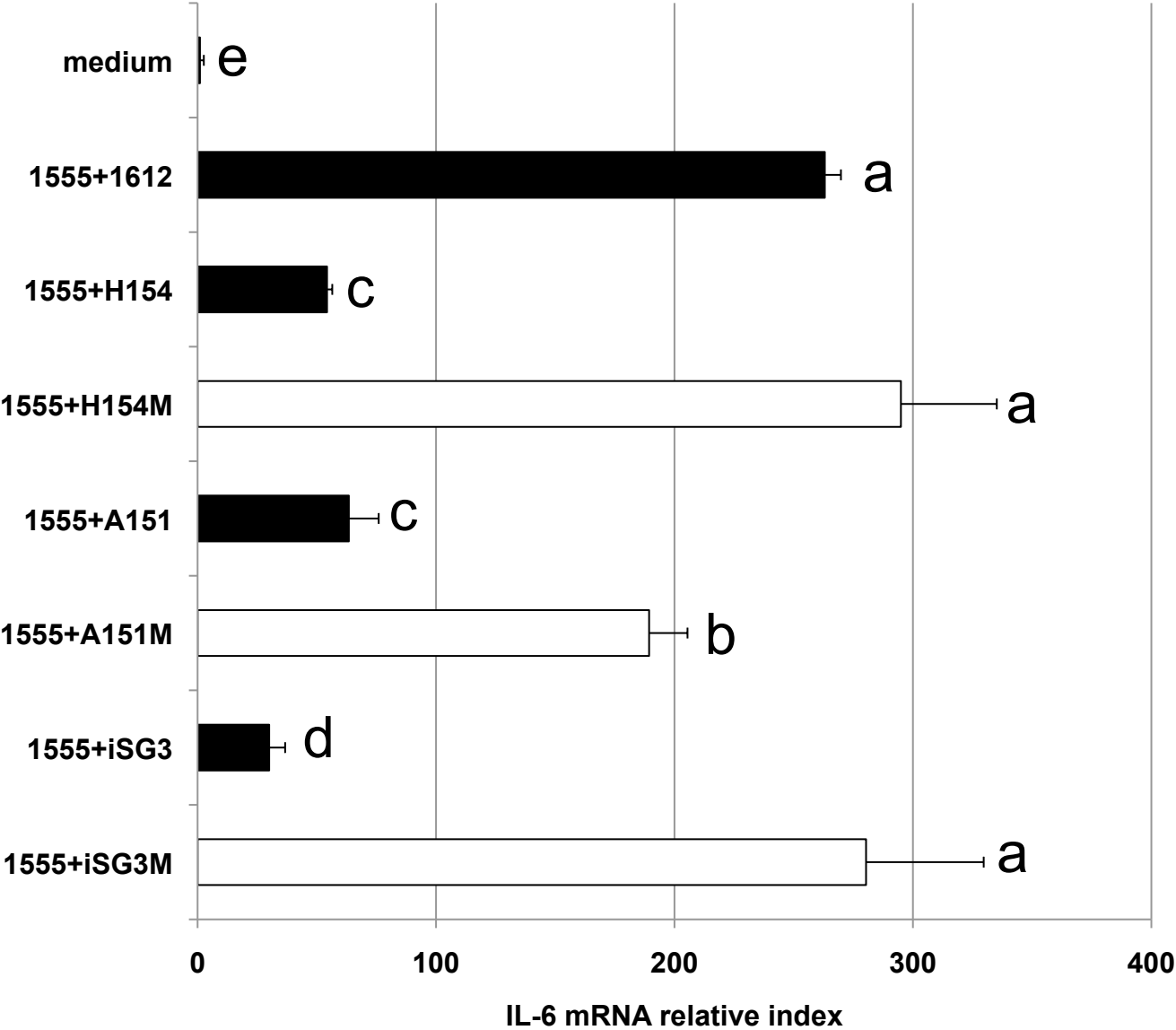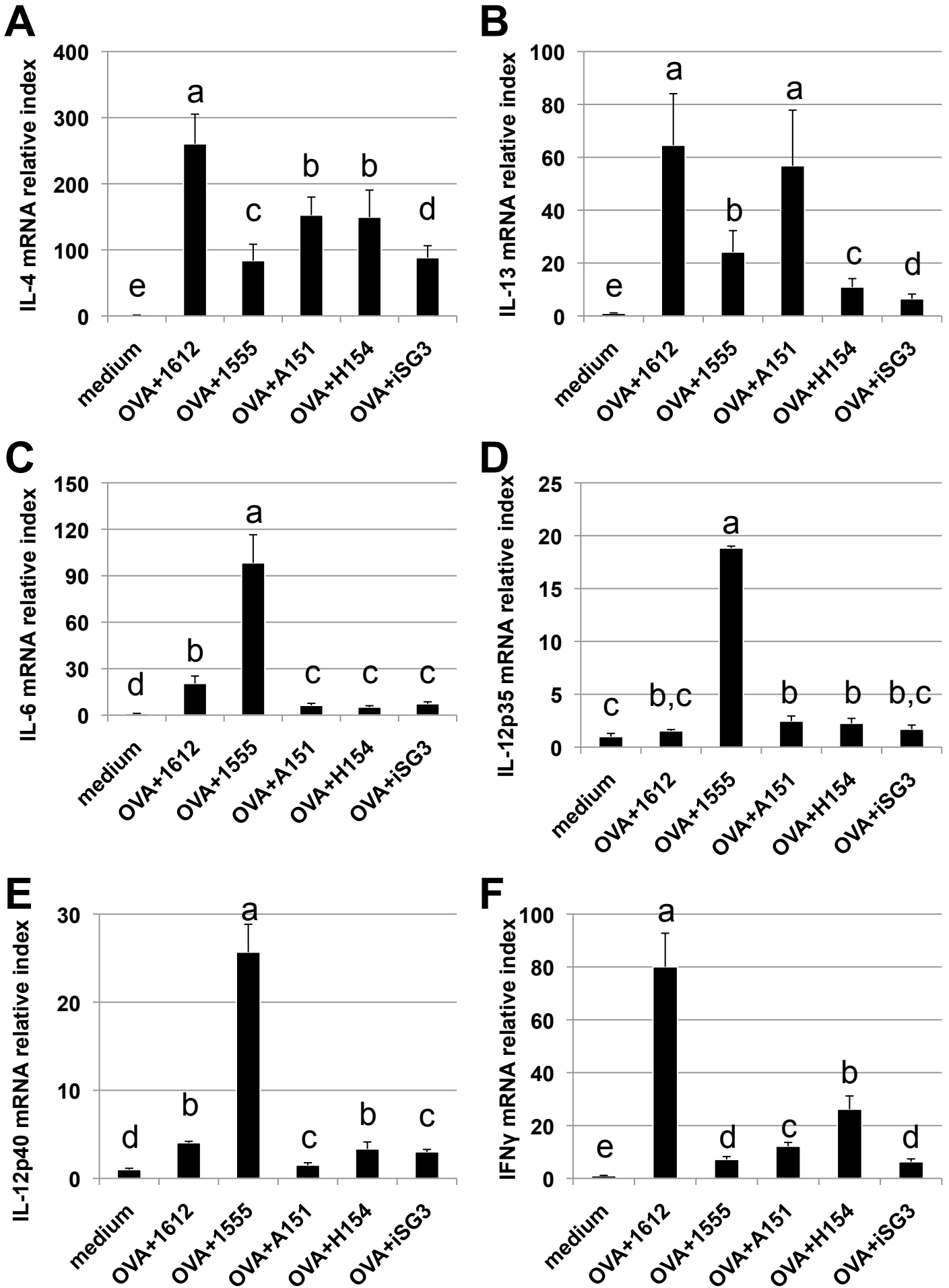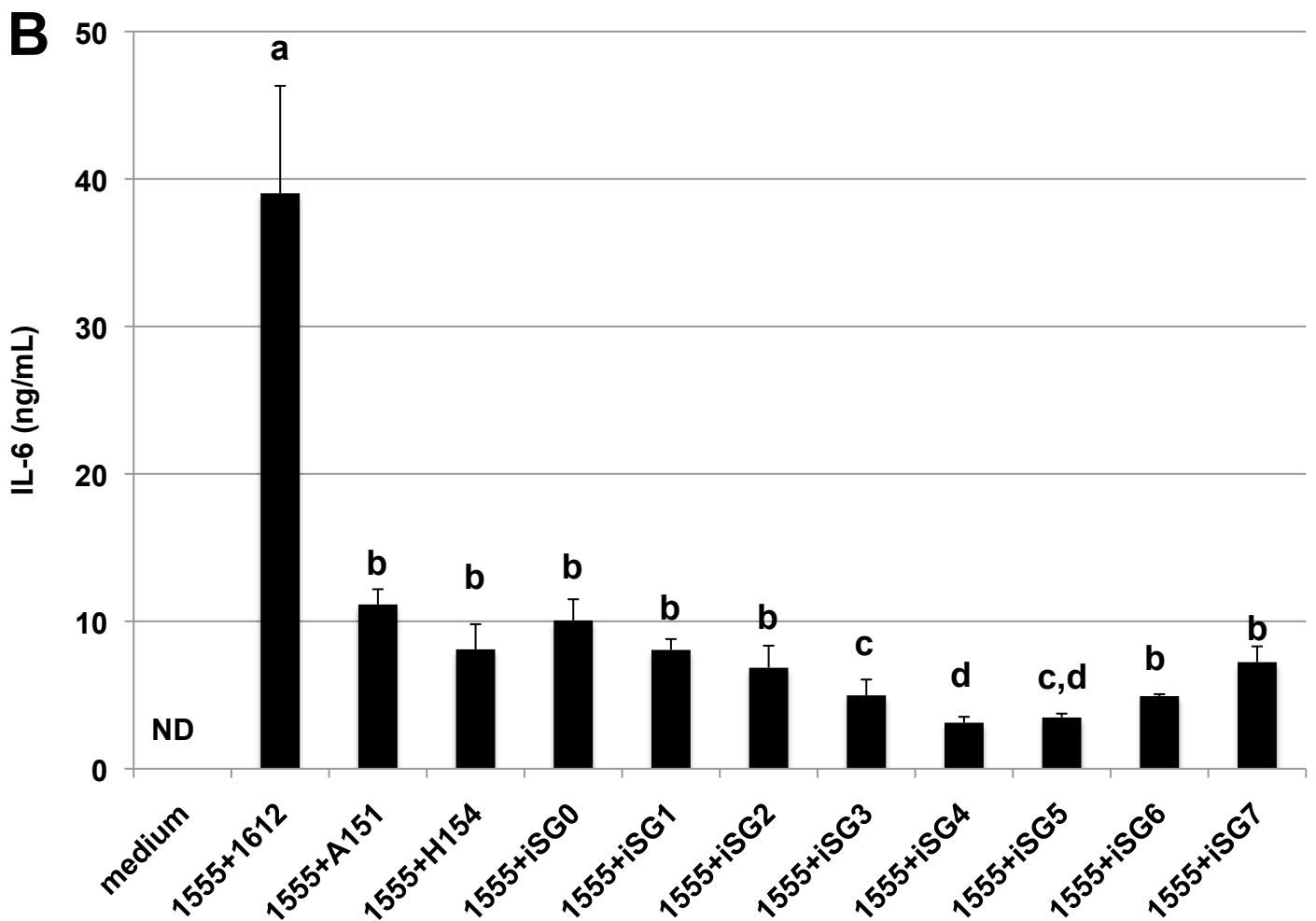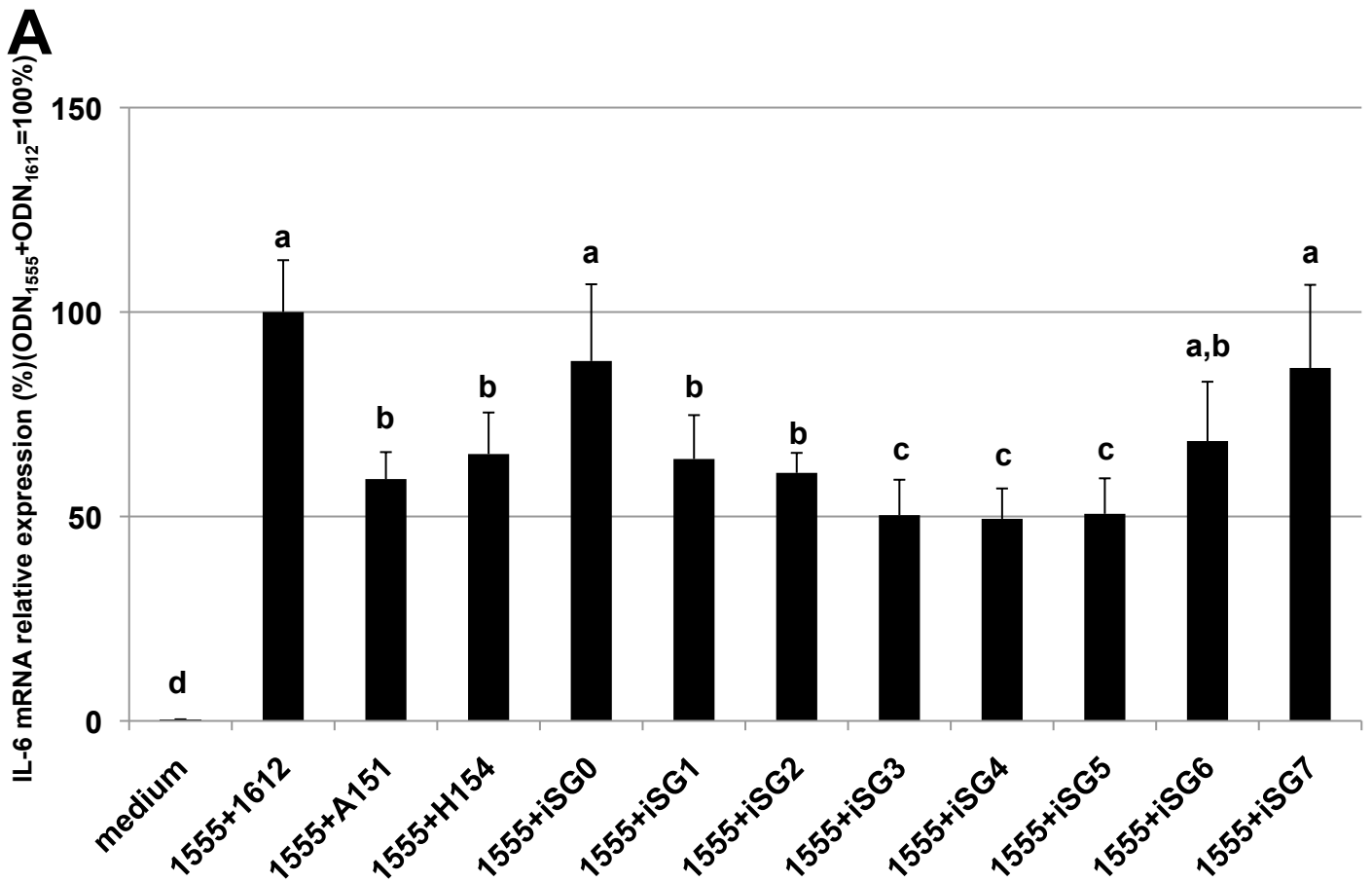
Figure 1
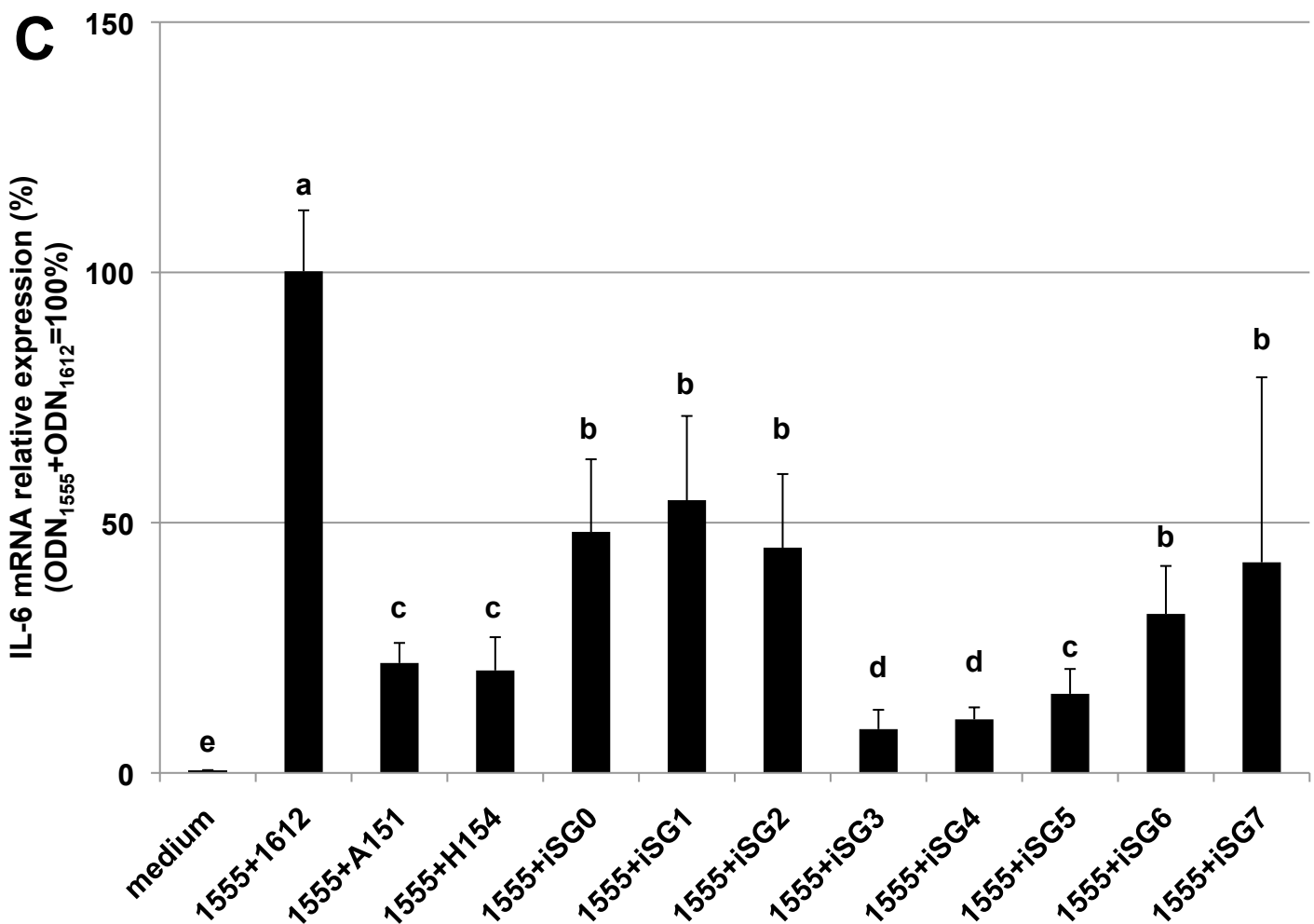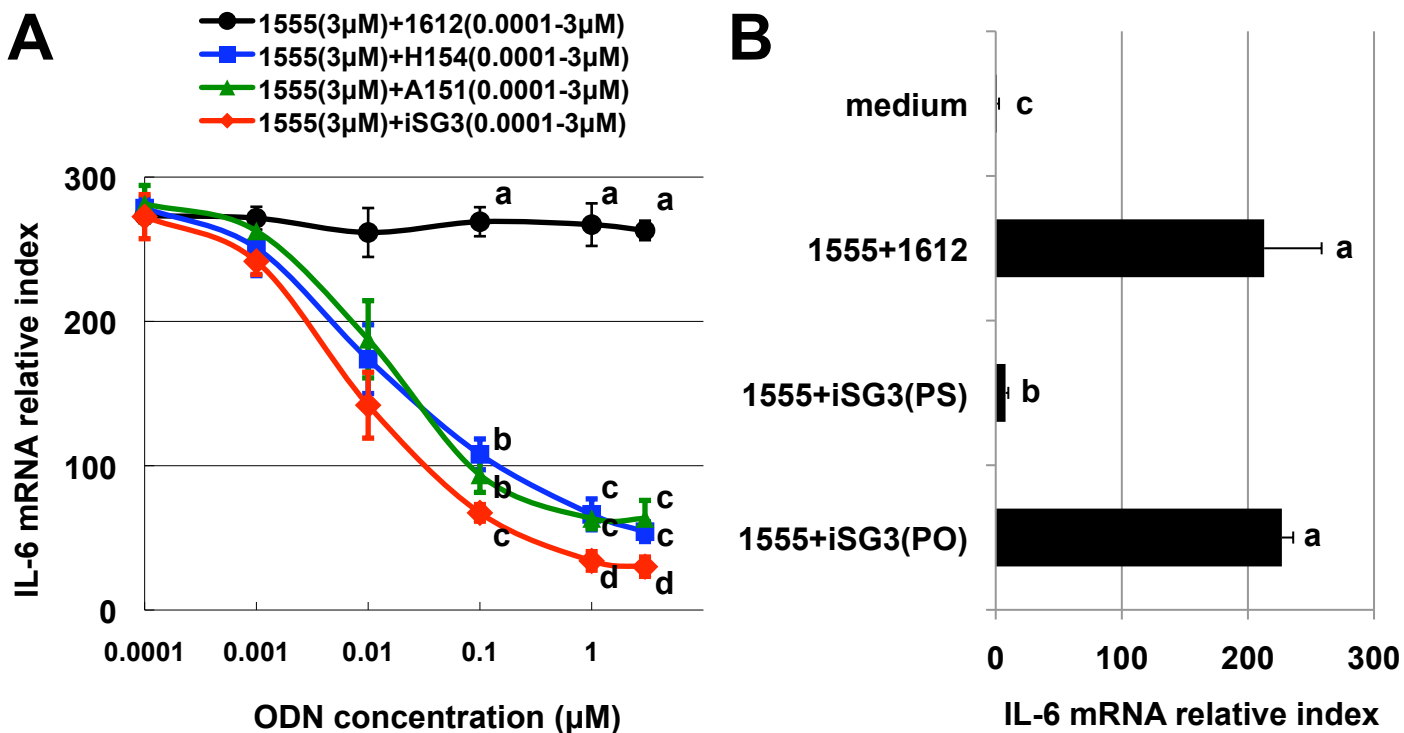
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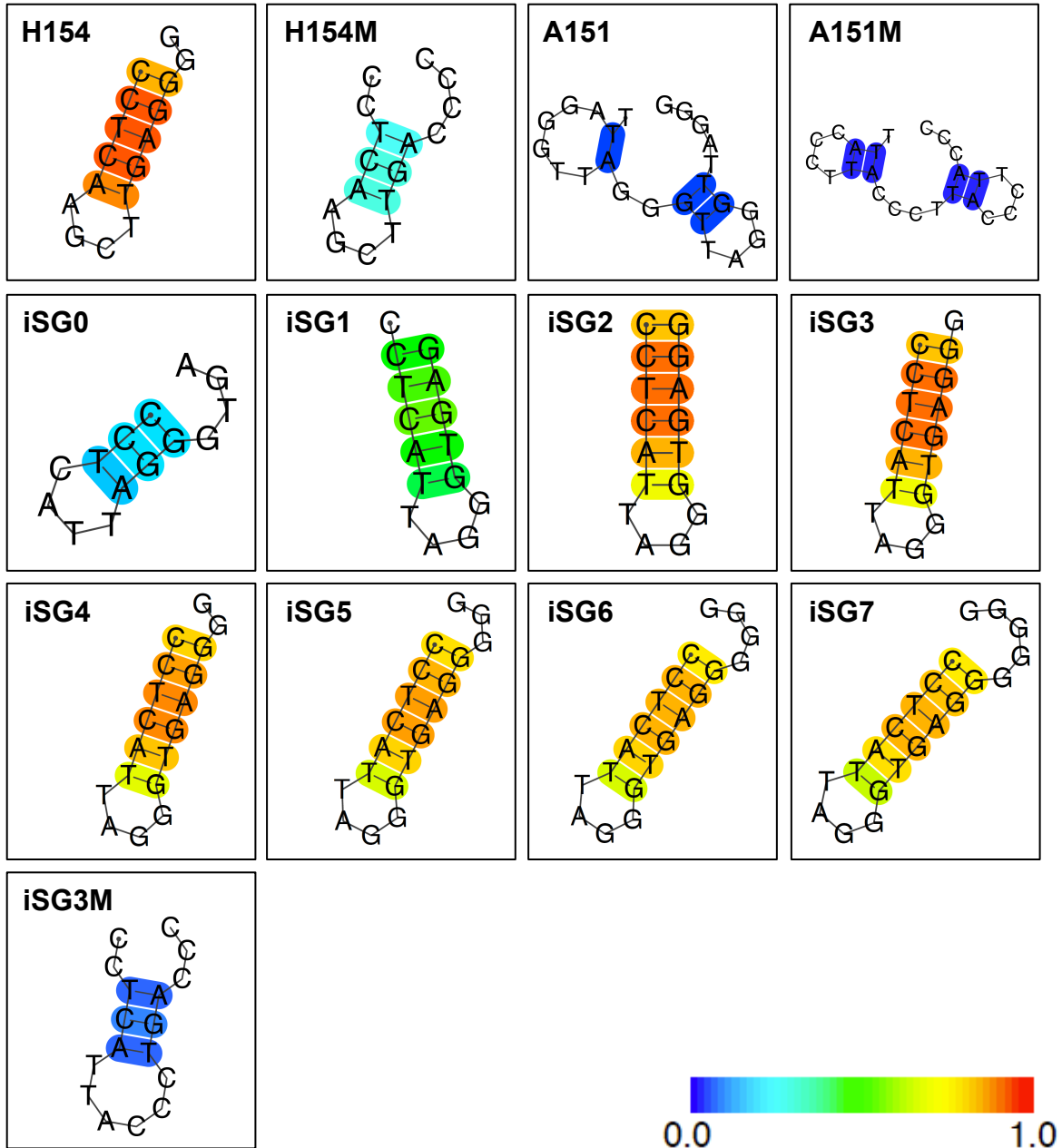
Figure 3

Figure 4

Table 1

Table 1. ODN sequences
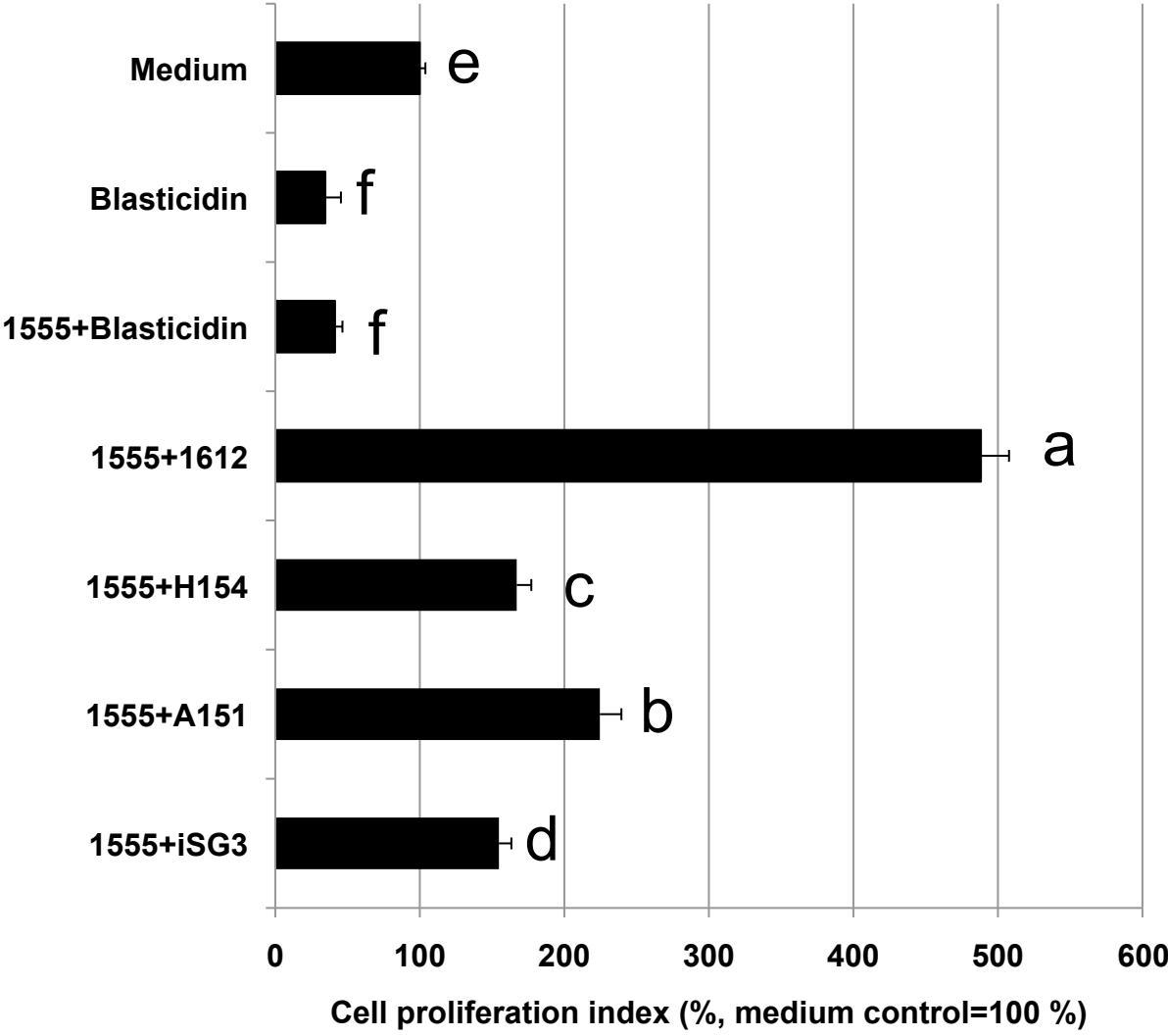
Name	5'-sequence-3'	Ref.
ODN$_{1555}$(CpG ODN)	GCTAGA**_CG_**TTAGCGT	7
ODN$_{1612}$(GpC ODN)	GCTAGA**_GC_**TTAGGCT	7
H154(Class I iODN)	**_CCTCA_**AGCTTGA**_GGGG_**	3
H154M(H154 mutation)	CCTCAAGCTTGA**_CCCC_**	This study
A151(Class II iODN)	TTAGGG**_TTAGGG_**TTAGGGTTAGGG	6
A151M(A151 mutation)	TTA**_CCC_**TTA**_CCC_**TTA**_CCC_**TTA**_CCC_**	This study
Class I/II hybrid iODNs iSG variants — iSG0	**_CCTCATTAGGG_**TGA	This study
iSG1	**_CCTCATTAGGG_**TGA**_G_**	This study
iSG2	**_CCTCATTAGGG_**TGA**_GG_**	This study
iSG3	**_CCTCATTAGGG_**TGA**_GGG_**	This study
iSG4	**_CCTCATTAGGG_**TGA**_GGGG_**	This study
iSG5	**_CCTCATTAGGG_**TGA**_GGGGG_**	This study
iSG6	**_CCTCATTAGGG_**TGA**_GGGGGG_**	This study
iSG7	**_CCTCATTAGGG_**TGA**_GGGGGGG_**	This study
iSG3M(iSG3 mutation)	CCTCATTA**_CCC_**TGA**_CCC_**	This study

Supplemental Figure 1

Supplemental Figure 2

Supplemental Figure 3

A — IL-6 mRNA relative index

category	label
medium	c
1612→removed→+1555	a
iSG3→removed→+1555	b
iSG3→+1555	b

B — IL-6 mRNA relative index

category	label
medium	d
1612→removed→+1555	a
iSG3→removed→+1555	b
iSG3+1555	c

C — Cell proliferation index (%, medium control=100 %)

category	label
medium	d
1612→removed→+1555	a
iSG3→removed→+1555	b
iSG3→+1555	c