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Suppressive Oligodeoxynucleotides Inhibit Th1 Differentiation by Blocking IFN-γ- and IL-12-Mediated Signaling

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Repetitive TTAGGG motifs present at high frequency in mammalian telomeres can suppress Th1-mediated immune responses. Synthetic oligonucleotides (ODN) containing TTAGGG motifs mimic this activity and have proven effective in the prevention/treatment of certain Th1-dependent autoimmune diseases. This work explores the mechanism by which suppressive ODN block the induction of Th1 immunity. Findings indicate that these ODN inhibit IFN-γ-induced STAT1 phosphorylation and IL-12-induced STAT3 and STAT4 phosphorylation. As a result, T-bet expression is reduced as is the maturation of naive CD4+ T cells into Th1 effectors. These changes indirectly support the generation of Th2-dominated immune responses. Suppressive ODN may thus represent a novel approach to influence the Th1:Th2 balance in vivo.


Deoxyribonucleic acid has multiple and complex effects on the immune system. Bacterial DNA contains “CpG motifs” that trigger an immune response through TLR9 (1–3), while the telomeric ends of mammalian DNA contain large numbers of TTAGGG repeats that inhibit these immune responses (4). Synthetic oligonucleotides (ODN) containing TTAGGG motifs mimic the activity of telomeric DNA, reducing Th1 and proinflammatory cytokine production (4, 5). In murine models, suppressive TTAGGG ODN have been effective in the prevention/treatment of a variety of organ-specific and systemic autoimmune diseases (5–8).

Previous studies showed that ODN containing poly(G) and to a lesser extent poly(GC) motifs could suppress Th1 responses (5, 8). Yet little is known about the cellular targets of suppressive ODN, the molecular mechanism by which they inhibit Th1 responses, or their effect on Th2 immunity. Thus, studies were undertaken to explore the ability of suppressive ODN to modulate T cell maturation and activation. Experiments focused on CD4+ T cells, since they play a key role in both Ag-specific protective responses and pathologic autoimmune responses (9).

Stimulating the TCR of naive CD4+ T cells triggers them to differentiate into either Th1 or Th2 effectors (10). Th1 cells contribute to the development of delayed-type hypersensitivity reactions and cell-mediated immunity, whereas Th2 cells facilitate the induction of humoral immune responses (11). These two cell types form an interactive and mutually inhibitory network in which type 1 cytokines promote the maturation of Th1 and inhibit the maturation of Th2 cells and vice versa (12, 13).

Current findings indicate that suppressive ODN selectively reduce Th1 cytokine production, while enhancing Th2 immunity. These effects derive from the ability of suppressive ODN to inhibit the IFN-γ and IL-12 signaling pathways, thereby blocking the differentiation of naive CD4+ T cells into Th1 effectors. Although suppressive ODN do not directly promote Th2 differentiation, the resulting decrease in IFN-γ production facilitates the development of strong Th2 responses.

Materials and Methods

Animals

Female BALB/c (6–8 wk old) were obtained from the National Cancer Institute (Frederick, MD). DO11.10 OVA-TCR-transgenic (Tg) mice (14) (on BALB/c background) were obtained from The Jackson Laboratory (Bar Harbor, ME). TLR9-deficient mice (1) (on BALB/c background) were kindly provided by Dr. S. Akira (Osaka University, Osaka, Japan). All studies were approved by the Center for Biologics Evaluation and Research Animal Care and Use Committee.

T cells

CD4+ T cells were isolated from BALB/c splenocytes by negative selection using a combination of anti-MHC class II, anti-CD8, anti-DX5, anti-CD11c, and anti-CD11b microbeads (Miltenyi Biotec, Auburn, CA). The resulting cell population contained ≥90% CD4+ cells and ≤0.5% CD8+ I-A+, or DX5+ cells, as measured by flow cytometry.

Con A-activated T cells were prepared as described previously (15). Briefly, BALB/c spleen cells (2 × 10^6/ml) were stimulated with 2 μg/ml Con A (Pharmacia Biotech, Uppsala, Sweden). After 3 days of culture, the cells were harvested and cultured in medium containing 0.5% FBS for an additional 16 h to synchronize their cell cycle at G1. T cell blasts were isolated by centrifugation over Histopaque (Sigma-Aldrich, St. Louis, MO) at 1500 × g for 15 min. This population of cells normally contained ≥98% T cell blasts as measured by flow cytometry. The IL-12-responsive murine T cell clone (16) (2D6 cell, kindly provided by Dr. H. Fujiwara, Osaka University, Osaka, Japan) was maintained by stimulation with IL-12 (250 pg/ml; R&D Systems, Minneapolis, MN) and used 12–24 h after IL-12 depletion.

Oligodeoxynucleotides

Endotoxin-free phosphorothioate ODN were synthesized at the Center for Biologics Evaluation and Research core facility. The following ODN were used: suppressive ODN \( \text{ODN}_{1515} \) (TTAGGTTAGGTTAGGTTAGG) and control ODN \( \text{ODN}_{1612} \) (GCTAGACCTTACGCT).

Cytokine ELISAs

Cytokine levels in culture supernatants were measured by ELISA, as described previously (17). Paired anti-IL-4 and anti-IFN-γ mAbs were purchased from BD Pharmingen (San Diego, CA). Ninety-six-well Immulon
H2B plates (Thermo LabSystems, Franklin, MA) were coated with first-stage cytokine-specific Abs and then blocked with PBS/1% BSA. Culture supernatants were added, and bound cytokine was detected by the addition of biotin-labeled secondary Ab, followed by phosphatase-conjugated avidin and a phosphatase-specific colorimetric substrate (PNPP, Pierce, Rockford, IL). Standard curves were generated using recombinant cytokines purchased from R&D Systems. The detection limit for these assays was <15 pg/ml for IFN-γ and <3.8 pg/ml for IL-4. All assays were performed in triplicate.

In vitro stimulation of spleen cells to monitor Th differentiation

Spleen cells (2.5 \times 10^7/ml) from immunized DO11.10 mice were stimulated in vitro for 3 days with 100 μg/ml OVA (Sigma-Aldrich) with or without 5 μM ODN, 10 μg/ml anti-IFN-γ mAb (R&D Systems) and, in some cases, with 500 pg/ml recombinant mouse IL-12. The cells were washed and incubated for 2 more days in medium alone. Briefly, 3 \times 10^4 viable lymphocytes recovered by density gradient centrifugation over Ficoll-Paque (Pharmacia Biotech) were restimulated with 2 \times 10^5 APCs in the presence of 100 μg/ml OVA in 96-well plates. APCs were prepared by treating spleen cells from unimmunized BALB/c mice with 50 μg/ml mitomycin C (Wako Pure Chemical, Osaka, Japan) for 30 min at 37°C. After 2 days, the culture supernatants were assessed for IFN-γ and IL-4 levels (18, 19). All studies were performed in triplicate.

To examine Ag-independent T cell differentiation in the absence of APCs, naïve CD4^+ T cells (2.5 \times 10^6/ml) were stimulated in vitro with 0.1 μg/ml immobilized anti-CD3ε Ab (BD Pharmingen), 1 μg/ml soluble anti-CD28 mAb (BD Pharmingen) with or without 5 μM ODN, 10 μg/ml anti-IFN-γ mAb, and/or 300 pg/ml IL-12. After 3 days, cells were washed and cultured in fresh medium for 2 more days. Briefly, 3 \times 10^5 viable lymphocytes were restimulated with immobilized anti-CD3ε mAb (0.1 μg/ml) in 96-well plates. After 24 h, the culture supernatants were assessed for IFN-γ and IL-4 (20).

In vivo induction of effector Th cells detected by in vitro restimulation

To examine the activity of in vivo-activated T cells, BALB/c mice were primed i.p. with 100 μg of OVA emulsified in IFA. They were injected with 400 μg of ODN on the same day or 2 days after vaccination. On day 14, spleen cells (3 \times 10^7/well) were isolated and restimulated in vitro with 100 μg/ml OVA for 48 h in anti-CD3ε mAb for 16 h in triplicate in 96-well plates.

RT-PCR analysis

Total RNA was extracted from target cells using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) as recommended by the manufacturer. Five micrograms of total RNA was reverse-transcribed in first-strand buffer (50 mM Tris-HCl (pH 7.5), 75 mM KCl, and 2.5 mM MgCl2 containing 25 μg/ml oligo(dT)_{12-18}, 200 U Moloney leukemia virus reverse transcriptase, 2 mM dNTP, and 10 mM DTT. The reaction was conducted at 42°C for 1 h. A standard PCR was performed on 1 μl of the cDNA synthesized using the following primer pairs: the cells were CACC and mouse H2B plates (Thermo LabSystems, Franklin, MA) were coated with first-stage cytokine-specific Abs and then blocked with PBS/1% BSA. Culture supernatants were added, and bound cytokine was detected by the addition of biotin-labeled secondary Ab, followed by phosphatase-conjugated avidin and a phosphatase-specific colorimetric substrate (PNPP, Pierce, Rockford, IL). Standard curves were generated using recombinant cytokines purchased from R&D Systems. The detection limit for these assays was <15 pg/ml for IFN-γ and <3.8 pg/ml for IL-4. All assays were performed in triplicate.

Results

Suppressive ODN modulates Ag-specific T cell differentiation in vitro

To examine the effect of suppressive ODN on the activation of Ag-specific T cells in vitro, splenocytes from OVA TCR Tg mice were cultured with optimized concentrations of OVA with or without ODN (18, 19). When restimulated with Ag-pulsed APCs, cells cultured in the presence of suppressive ODN produced 5-fold more IL-4 (p < 0.05) but significantly less IFN-γ than cells cultured in OVA alone (p < 0.01, Fig. 1, A and B). These differences in cytokine production were selectively mediated by suppressive ODN since control ODN had no effect on cytokine levels (Fig. 1, A and B). The magnitude of the changes in cytokine production mediated by suppressive ODN was similar to those elicited by coculture with anti-IFN-γ, an immune modulator known to strongly inhibit Th1 differentiation (Fig. 1, A and B, and Refs. 20 and 21).

To further explore the effect of suppressive ODN on cytokine production, these studies were repeated in the presence of IL-12. IL-12 promotes Th1 immune responses, characterized by a significant increase in IFN-γ and decrease in IL-4 production (Fig. 1, A and B, and Ref. 22). Yet even in the presence of IL-12, suppressive ODN significantly increased IL-4 while decreasing IFN-γ production (Fig. 1, C and D). These effects were dose related (IFN-γ, R = −0.75; IL-4, R = 0.94), consistent with suppressive ODN promoting Th2 while inhibiting Th1 differentiation in vitro.

To examine whether suppressive ODN were directly inhibiting the differentiation of naïve T cells, purified CD4^+ lymphocytes were cultured with ODN plus a combination of anti-CD3ε plus anti-CD28 mAbs. Under these conditions (in which APCs were absent), suppressive ODN again increased IL-4 while decreasing IFN-γ production (Fig. 2, A and B). Adding IL-12 during culture by calculating the fold change vs the control group in each individual experiment. Correlation analysis is computed by linear correlation analysis between cytokine production vs concentration of suppressive ODN.

Statistical analysis

Student’s t test was used to analyze all results. To facilitate comparisons when an experiment was repeated multiple times, results were standardized by calculating the fold change vs the control group in each individual experiment. Correlation analysis is computed by linear correlation analysis between cytokine production vs concentration of suppressive ODN.

![FIGURE 1. Effect of suppressive ODN on the differentiation of Ag-stimulated T cells.](http://www.jimmunol.org/DownloadedFrom/152017003)
Inhibition of Th1 differentiation by suppressive ODN

Enhanced Th1 differentiation, yet this effect was again reduced by inclusion of suppressive (but not control) ODN (Fig. 2, C and D).

Although originally identified by their ability to suppress CpG-induced immune activation, the class of suppressive ODN typified by ODN A151 has been shown to down-modulate Th1 responses induced by other immune stimuli (5). To confirm that the effects observed on CD4+ T cells were not CpG related, these experiments were repeated using cells from TLR9 KO mice. As seen in Fig. 2, ODN A151 had the same effect on the activation of CD4+ T cells from wild-type (WT) and TLR9 knockout (KO) mice. Since neither Ags nor APCs contributed to T cell maturation in this model system, these findings suggest that suppressive ODN directly promote the differentiation of naive T cells into Th2 rather than Th1 effectors in a TLR9-independent manner.

Suppressive ODN modulate Ag-specific T cell differentiation in vivo

To determine whether suppressive ODN can influence T cell differentiation in vivo, the effect of administering A151 with Ag was examined. Normal BALB/c mice were immunized with OVA in IFA and injected at the same site (i.p.) with 300 µg of ODN on days 0 and 2. This dose and frequency of ODN administration was previously shown to be effective in vivo (5–7). The cytokine profile of the resultant immune response was monitored by restimulating spleen cells with Ag in vitro. As seen in Fig. 3, A and B, splenocytes from mice immunized with OVA plus suppressive ODN produced significantly more IL-4 and less IFN-γ than cells from animals immunized with OVA alone (or OVA plus control ODN, p < 0.05). These findings were confirmed by monitoring the number of spleen cells secreting IL-4 and IFN-γ by ELISPOT assay (data not shown).

To examine the effect of suppressive ODN on the broader T cell pool, spleen cells from OVA-immunized mice were stimulated in vitro with anti-CD3ε mAb. This brief Ag-independent activation

Figure 2. Suppressive ODN act directly on naive T cells. Purified CD4+ T cells from BALB/c (A–D) and TLR9 KO (E–H) mice were stimulated with anti-CD3ε and anti-CD28 mAb with or without ODN or anti-IFN-γ mAb in the absence (A, B, E, and F) or presence (C, D, G, and H) of IL-12. These cells were restimulated with anti-CD3ε and assayed for IFN-γ (A, C, E, and G) or IL-4 (B, D, F, and H) production. In three to four independent experiments, average IFN-γ and IL-4 production following Ab stimulation alone was similar in WT and KO mice, ranging from 1070 ± 177 pg/ml (A) and 2140 ± 1230 pg/ml (C), while IL-4 production was 113 ± 26 pg/ml (B) and 52 ± 46 pg/ml (D). To facilitate comparisons between experiments, the fold change in cytokine production in each treatment group was calculated relative to the Abs alone group.

Figure 3. Modulation of Ag-specific T cell differentiation by suppressive ODN in vivo. BALB/c mice were immunized with OVA in IFA and injected i.p. with free ODN 5 min and 2 days later. On day 14, splenocytes were stimulated with OVA (A and B) for 48 h and anti-CD3ε mAb (C and D) for 16 h and monitored for IFN-γ and IL-4 production. Data represent the mean ± SD of four independently analyzed mice per group, and were confirmed in three additional experiments. * p < 0.05 (compared with the PBS treatment group). Note that in the absence of OVA or anti-CD3ε Ab stimulation, no cytokine production was detected. Sup,Suppressive; Cont, control.

Figure 4. Inhibition of IFN-γ production by various stimulations in T cells. Purified CD4+ T cells were cultured with ODN for 30 min and then stimulated with anti-CD3ε mAb, anti-CD28 mAb, or ODN with or without IL-12 for 2 days. The concentration of IFN-γ and IL-4 in culture supernatants was determined by ELISA. Results represent the average ± SD of four animals per group, and the experiment was repeated three times with similar results. In the absence of anti-CD3ε mAb, neither cytokine was produced. * p < 0.05 (compared with the (−) group). Sup, Suppressive; Cont, control.
triggered the production of both IL-4 and IFN-γ (Fig. 3, C and D). Of interest, there was no difference in the amount of cytokine produced by cells from mice treated with control vs suppressive ODN. Thus, administering suppressive ODN during the induction of Ag-specific immunity selectively promoted a Th2-biased Ag-specific response, but did not alter the global Th1:Th2 balance in vivo.

Suppressive ODN inhibit the early differentiation of naive CD4+ T cells into Th1 effectors

Naive CD4+ T cells produce IFN-γ and/or IL-4 in response to TCR stimulation (21). The resultant cytokine milieu influences whether these T cells subsequently differentiate into Th1 or Th2 effectors (23). To examine the effect of suppressive ODN on this very early cytokine production, purified CD4+ lymphocytes were stimulated in vitro with anti-CD3ε plus anti-CD28 mAbs. The cells produced low but detectable levels of IFN-γ, an effect reduced by co-culture with suppressive ODN (p < 0.05, Fig. 4A). Adding IL-12 to these cultures amplified IFN-γ production, but this effect was significantly reduced by inclusion of suppressive ODN. Of interest, suppressive ODN did not increase IL-4 production by naive CD4+ T cells at this early time point (Fig. 4B). These findings suggest that suppressive ODN selectively inhibit the production of IFN-γ but do not alter IL-4 production by naive T cells. Similar results were obtained when naive CD4+ cells were stimulated by other polyclonal activators and when naive CD4+ cells from TLR9-deficient mice were studied (data not shown).

Effect of suppressive ODN on cytokine-induced STAT phosphorylation

The effect of suppressive ODN on IFN-γ-driven T cell differentiation was explored. A critical step in the signaling cascade that culminates in IFN-γ production is IFN-γ-induced STAT1 phosphorylation (24). As seen in Fig. 5A, suppressive ODN block this step. In addition, suppressive ODN inhibit the up-regulation of T-bet, IFN-γ-inducible protein 10, and IFN regulatory factor 1 mRNA expression (Fig. 5B and data not shown). In contrast, suppressive ODN had no effect on IL-2-dependent STAT5 phosphorylation or IL-4-dependent STAT6 phosphorylation (Fig. 5, C and D).

Previous studies established that the IL-12 signaling pathway plays a central role in Th1 differentiation. As seen in Fig. 6A, suppressive ODN inhibited tyrosine phosphorylation of STAT4 and STAT3 in T cells stimulated with IL-12. The magnitude of this inhibition was dose dependent and was mediated by suppressive but not control ODN. To confirm this finding, the IL-12-responsive 2D6 T cell clone was studied. As above, suppressive ODN inhibited IL-12-induced phosphorylation of STAT3 and STAT4 (Fig. 6B). These results suggest that suppressive ODN inhibit T cell differentiation by selectively blocking the phosphorylation of STAT1, STAT3, and STAT4.

Discussion

This study examines the effect of suppressive ODN containing telomeric TTAGGG motifs on naive CD4+ T cells. Results indicate that these suppressive ODN inhibit the differentiation of naive T cells into Th1 effectors, thereby skewing the resultant response to favor type 2 cytokine production (Figs. 1–3). This effect of suppressive ODN is detectable within 2 days of TCR engagement following either Ag-specific or polyclonal T cell activation (Fig. 4). Evidence that suppressive ODN inhibit IFN-γ-induced STAT1 phosphorylation and IL-12-induced STAT3 and STAT4 phosphorylation (Figs. 5 and 6) provides insight into the mechanism underlying this effect.
Available evidence suggests that two distinct “classes” of suppressive ODN may exist. The first class, identified by Krieg and colleagues, is composed of GC-rich DNA and selectively inhibits CpG-induced immune activation (8, 25–28). GC-rich ODN interfere with the binding of CpG DNA to TLR9, thus reducing CpG-dependent activation of NF-κB and AP-1 (28, 29). However, GC-rich ODN do not block other forms of immune activation, nor do they inhibit the IFN-γ or IL-12 signaling cascades (data not shown).

The second class of suppressive ODN contains poly(G) motifs, such as those found in the TTAGGG multimers evaluated in the current work (4). This class of ODN has broader immunosuppressive activity, characterized by the ability to modulate both Ag-specific and polyclonal immune activation (5, 8, 30–32) in a TLR9-independent manner. Consistent with this observation, ODN A151 modulated the Th profile of cells from both WT and TLR9 KO mice (Fig. 2). Although T cells do not bind ODN as effectively as APCs (33, 34), preliminary studies in our laboratory established that FITC-labeled suppressive ODN do bind CD3+ T cells (data not shown). In this context, Halpern and Pisetsky (35) recently demonstrated that poly(G) ODN inhibit IFN-γ production by polyclonally activated T cells.

IFN-γ gene expression during CD4+ T cell activation is mediated by signals transduced through the TCR, IL-12R, and/or IFN-γR (20, 36, 37). Th1 maturation involves IFN-γ-mediated phosphorylation of STAT1, which in turn up-regulates expression of the T-bet transcription factor (38, 39). Current phosphorylation of STAT1, which in turn up-regulates expression of IFN-γ and blocks IL-12-induced STAT3 and STAT4 phosphorylation (40). Our results demonstrate that suppressive ODN block IL-12-dependent STAT3 and STAT4 phosphorylation (Fig. 5). Thus, it appears that broadly suppressive ODN impair the generation of Th1 immunity by blocking both the IFN-γ and IL-12 signaling pathways. Consistent with these findings, Jing et al. (41) recently demonstrated that phosphodiester G-rich ODN inhibit IFN-γ-dependent STAT1 phosphorylation (Fig. 5A) and the concomitant up-regulation of T-bet expression (Fig. 5B). IL-12 also plays a critical role in Th1 differentiation through a signaling cascade that proceeds through STAT3 and STAT4 (40). Our results demonstrate that suppressive ODN block IL-12-dependent STAT3 and STAT4 phosphorylation (Fig. 6). Thus, it appears that broadly suppressive ODN impair the generation of Th1 immunity by blocking both the IFN-γ and IL-12 signaling pathways. Consistent with these findings, Jing et al. (41) recently demonstrated that phosphodiester G-rich ODN inhibit IFN-γ-dependent STAT1 activation and IL-6-dependent STAT3 activation of a cancer cell line. Based on nuclear magnetic resonance studies, those authors suggest that G-rich ODN may interact with the Src homology domain of STAT3. We found the effect of suppressive ODN to be highly specific, since 1) control ODN had no effect on STAT1, STAT3, or STAT4 phosphorylation (Figs. 5 and 6) and 2) suppressive ODN did not inhibit the STAT5 or STAT6 phosphorylation (Fig. 5). Indeed, although suppressive ODN promoted the generation of IL-4-dominated responses, they did not up-regulate STAT6 phosphorylation. Thus, it appears that suppressive ODN, by blocking the production of Th1 cytokines, support the generation of Th2 cells occurring by default.

There is considerable evidence that organ-specific autoimmune diseases are Th1 mediated (9, 42). Thus, the ability of suppressive ODN to selectively inhibit Th1-dependent immune responses is of potential therapeutic importance. In this context, several recent reports indicate that suppressive ODN can prevent/treat autoimmune diseases such as arthritis, systemic lupus erythematosus, and experimental autoimmune encephalomyelitis (5, 8). This is consistent with our hypothesis that TTAGGG motifs released from injured host cells act to down-regulate overexuberant or pathologic immune responses. Ongoing efforts to identify the precise mechanisms(s) through which suppressive ODN limit undesirable immune responses should facilitate the development of agents with greater activity and therapeutic utility.

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**References**


