Published in final edited form as:
Int Immunopharmacol. 2014 December ; 23(2): 516–522. doi:10.1016/j.intimp.2014.09.027.

Suppressive Oligodeoxynucleotides Promote the Generation of Regulatory T Cells by Inhibiting STAT1 Phosphorylation

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Abstract

Suppressive oligodeoxynucleotides (Sup ODN) express repetitive TTAGGG motifs that have proven useful in the treatment/prevention of numerous inflammatory and autoimmune diseases. The mechanism underlying the immunosuppressive activity of Sup ODN is incompletely understood. Regulatory T cells (T_{reg}) play a key role in are generated from controlling a variety of pathologic autoimmune responses. T_{regs} activated $CD4^+$ T cells in a process that involves the phosphorylation of STAT family members. Current studies demonstrate that Sup ODN promote the differentiation of $CD4^+CD25^-$ T cells into functionally active iT_{reg} *in vitro*. When administered *in vivo*, Sup in response to peptide challenge. Central to this ODN promote the generation of iT_{reg} effect is the ability of Sup ODN to block the phosphorylation of STAT1. These findings clarify the mechanism underlying the therapeutic activity of Sup ODN and support their use in T_{reg}-based immunotherapy.

Keywords

suppressive oligonucleotides; regulatory T cells; STAT transcription factors

1 Introduction

DNA has multiple and complex effects on the immune system. Microbial DNA elicits a potent inflammatory response that helps protect the host from infection but can exacerbate autoimmune and inflammatory diseases [1]. Self DNA, in contrast, is anti-inflammatory and can down-regulate pathologic inflammatory responses [2]. The latter effect is mediated, at least in part, by repetitive TTAGGG motifs present at high frequency in mammalian telomeres. Synthetic single-stranded phosphorothioate oligodeoxynucleotides (ODN)

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Conflict of Interest
Members of Dr. Klinman's lab have patents related to the use of suppressive oligonucleotides. All rights to such patents have been assigned to the Federal Government.

expressing TTAGGG motifs mimic the ability of telomeric DNA to prevent/treat a variety of inflammatory and autoimmune diseases [3]. This was established in murine models of lupus, arthritis, encephalomyelitis, iritis and shock [4–7].

The mechanism underlying the anti-inflammatory properties of Sup ODN were initially attributed to their ability to inhibit the maturation of $CD4^+$ T cells into Th1 effectors [7;8]. This was associated with the inhibition of STAT1 and STAT4 phosphorylation and the resultant reduction in IFNg production - a cytokine critical to the generation and maintenance of Th1 immunity. Yet those findings pre-dated the discovery of regulatory T cells (T_{reg}) that are now known to play a key role in restrain the host's suppressing autoimmune and inflammatory diseases [9]. T_{reg} response to self Ags and thus are of enormous importance in the therapy of inflammatory/autoimmune diseases.

There are two broad categories of Tregs. nTreg arise naturally in the thymus while iTreg are induced in the periphery from $CD4^+CD25^-$ T cells [10,11]. Forkhead transcription factor (FoxP) 3 acts as the master transcriptional regulator of Treg development. Deficiency in FoxP3 results in fatal multi-organ autoimmune disease as observed in the scurfy mouse and in patients with immunodysregulation polyendocrinopathy enteropathy X-linked syndrome [10–15]. *In vitro* studies show that $CD4^+CD25^-$ T cells up-regulate FoxP3 and differentiate into functional iTreg when stimulated via their TCR in the presence of TGFß [16;17]. *In vivo*, exposure to Ag generates $FoxP3^+$ iTreg in the periphery. For example, large numbers of $FoxP3^+$ iTreg accumulate in the draining lymph nodes of mice carrying OVA-specific CD4 T cells following challenge with the $_{OVA323-339}$ peptide [18;19].

T_{reg} development is regulated by members of the STAT family. STAT5 binds to the FoxP3 promoter and drives FoxP3 transcription, thereby playing a critical role in T_{reg} generation [20;21]. In contrast, activation of STAT1-dependent pathways prevents FoxP3 expression and blocks T_{reg} development [21,22]. In murine models, unrestrained STAT1 activation results in the breakdown of immunological tolerance and culminates in Th1-mediated pathology [24]. In contrast, deletion of STAT1 promotes the T_{reg} expansion and reduces graft-versus-host disease [25]. These findings suggest that targeting STAT1 represents a promising strategy for therapies designed to improve T_{reg} generation.

Recognizing the critical contribution of T_{reg} to the prevention and treatment of diseases that respond to Sup ODN therapy, we evaluated whether Sup ODN might promote T_{reg} development. Results show that Sup ODN enhance the generation of functional $FoxP3^+$ iT_{reg} from naïve $CD4^+$ precursors *in vitro*. When administered *in vivo*, Sup ODN promote the generation of $FoxP3^+$ iT_{reg} in response to peptide challenge. These effects are linked to the ability of Sup ODN to block STAT1 phosphorylation following the activation of $CD4^+$ T cells. These findings clarify the mechanism underlying the therapeutic activity of Sup ODN in autoimmune disease and provide a rationale for the use of Sup ODN in T_{reg}-based immunotherapy.

2 Materials and Methods

2.1 Mice

Female BALB/c and C57BL/6 mice were bred at the National Cancer Institute (Frederick, MD), FoxP3 eGFP reporter mice were obtained from Dr. Y. Belkaid (National Institute of Allergy and Infectious Diseases, Bethesda, MD), STAT1$^{-/-}$ mice from Dr. D. Levy (NYU School of Medicine, New York, NY) and Rag2$^{-/-}$ DO11.10 mice from Dr. M. Leonardo (National Institute of Allergy and Infectious Diseases, Bethesda, MD)[26;27]. All mice were studied at 6 – 10 wk of age and all experiments were approved by the Institutional Animal Care and Use Committee of the National Cancer Institute at Frederick.

2.2 Oligonucleotides

Phosphorothioate ODN were synthesized at the Core Facility of the Center for Biologics Evaluation and Research facility, Food and Drug Administration (Bethesda, MD). The following ODN were used: suppressive ODN A151 (5′ TTAGGGTTAGGGTTAGGGTTAGGG 3′) and control ODN 1612 (5′ GCTAGAGCTTAGGCT 3′). All ODN were free of detectable protein and endotoxin contamination.

2.3 Isolation of murine CD4$^+$CD25$^-$ T cells

CD4$^+$ T cells were purified from single spleen cell suspensions by negative selection using magnetic beads. These CD4$^+$ cells were then incubated with PE anti-CD25 and anti-PE beads used to isolate CD4$^+$CD25$^-$ T cells. The purity of these T cells typically exceeded 95% and contained fewer than 2% FoxP3$^+$ cells. All reagents were obtained from Miltenyi Biotec, Auburn, CA.

2.4 *In vitro* generation of murine iT$_{reg}$

CD4$^+$CD25$^-$ T cells (10^6/ml) were pre-incubated with 1 uM ODN for 2 h and then transferred to a 96 well plate coated with 3 ug/ml anti-CD3 (2C11; eBioscience, San Diego, CA). Cells were cultured in complete medium (RPMI 1640 supplemented with 10% FCS (both from Lonza, Walkersville, MD), 2 mM glutamine, 100 IU/ml penicillin, 100 ug/ml streptomycin, 25 mM HEPES buffer (all from Invitrogen, Carlsbad, CA), 0.0035% 2 ME (Sigma Aldrich, St. Louis, MO) and stimulated with 2 ug/ml soluble anti-CD28 (37.51; eBioscience) plus 20 ng/ml human TGFb1 (R&D Systems). 20 ng/ml of IL-2 (R&D Systems) was included to support the proliferation of T$_{regs}$ from C57Bl/6 mice. This supplementation was not needed for T cells from BALB/c mice which intrinsically produce sufficient IL-2 when stimulated [28]. In experiments examining whether Sup ODN influenced the differentiation of iT$_{reg}$, only 5 ng of TGFβ1 was added during culture. At the indicated time points, cells were analyzed for FoxP3 expression by flow cytometry or used in functional assays.

2.5 *In vitro* generation of human T$_{reg}$

PBMC were isolated by density gradient centrifugation of buffy coats obtained from normal donors through an IRB approved protocol (NIH, Bethesda, MD). CD4$^+$CD25$^-$ T cells were

isolated by negative selection over MACS using the naive $CD4^+$ T cell isolation kit II (Miltenyi Biotec, Auburn, CA). FACS analysis showed the purity of these cells was >98%. These cells were then stimulated with anti-CD3/28 coated beads plus 2.5 ng/ml TGFβ1 and 500 IU/ml IL-2 (both from eBioscience, San Diego, CA) in the presence or absence of 3 uM suppressive ODN for 5 days. FoxP3 expression was monitored using a Treg detection kit (Miltenyi Biotec (Auburn, CA).

2.6 Flow cytometry

After blocking FcR with 2.4G2 Ab, cells were stained with PerCP–Cy5.5–anti-CD4 (RM4 5), PE–anti-CD25 (PC61), PE anti-DO11.10 TCR (KJ1-26, all from BD Biosciences, San Jose, CA) and/or APC anti-FoxP3 (FJK 16s, eBioscience). Fluorescence was monitored using a LSRFortessa cell analyzer (BD Biosciences). Intracellular staining was performed with the FoxP3 staining buffer kit, according to the manufacturer's protocol (eBioscience). Events were collected and analyzed using FlowJo software (Tree Star, Ashland, OR).

2.7 RNA Isolation and Quantitative Real time PCR

Total RNA was isolated from T cells using the RNeasy Mini Kit (Quiagen, Valencia, CA). cDNA was synthesized with a QuantiTect Reverse Transcription kit according to the manufacturer's instructions (Applied Biosystems, Carlsbad, CA). Gene expression levels (normalized to GAPDH) were analyzed using the StepOnePlus RT PCR system and all reagents were from(Applied Biosystems).

2.8 T_{reg} suppression assay

$CD4^+$ T cells from FoxP3 eGFP reporter mice were isolated using a FACSAria II (BD Biosciences) and cultured in the presence of Sup ODN under T_{reg} polarizing conditions as described above. These FACS sorted $CD4^+CD25^-$ T cells were >97% pure. On day 3, T_{reg} that had matured *in vitro* were isolated by FACS based on their up-regulation of GFP. Simultaneously, naïve $CD4^+CD25^-$ responders (T_{resp}) were isolated from congenic C57BL/6 spleens and stained with 2.5 μM cell trace violet (Invitrogen, Carslbad, CA). 10^5 T_{resp} were stimulated with 0.25 μg/mL soluble anti-CD3 Ab and then mixed with mitomycin C inactivated syngeneic T cell depleted splenocytes (5×10^4) in 96 well round bottom plates for 3 days. T_{reg} generated in the presence of Sup ODN were added at the indicated ratios. Proliferation was measured by monitoring cell trace violet dilution by flow cytometry. The proliferation of stimulated T_{resp} was set to 100% and the percent suppression observed following the addition of T_{reg} calculated.

2.9 *In vivo* generation of iT_{reg}

$CD4^+CD25^-$ T cells were isolated from the spleens of $Rag2^{-/-}$ DO11.10 mice and stained with 5 μM CFSE (Invitrogen, Carslbad, CA) for 5 min at RT. 3×10^6 cells were injected i.v. into BALB/c mice. 24 hr later, these mice were immunized i.v. with 5 μg of $OVA_{323\text{-}339}$ peptide (Gift from Dr. A. Hurwitz, National Cancer Institute, Frederick, MD). Sup ODN (300 ug/mouse) was injected i.p. 3 h before OVA administration. Four days later, cells were isolated from axillary, brachial and inguinal lymph nodes, stained for expression of CD4, FoxP3, and the DO11.10 TCR and analyzed by flow cytometry as described above.

2.10 Flow cytometric analysis of phospho-STAT expression

$CD4^+CD25^-$ T cells were cultured under T_{reg} polarizing conditions ± 1 uM Sup ODN. Cells were fixed with BD Lyse/Fix Buffer for 10 min at 37° C, washed, permeabilized in ice cold BD Perm Buffer III for 30 min and then stained with PE anti-STAT1 (pY701) or PE anti-STAT4 (pY693) Ab (all reagents from BD Biosciences) for 30 min at RT. Flow cytometric analysis was performed on a LSRFortessa cell analyzer.

2.11 Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). Student's t test was used to examine all results. p values <0.05 were considered to be statistically significant.

3. Results

3.1 Suppressive ODN enhance the generation of murine and human T_{reg} *in vitro*

The ability of Sup ODN to reduce the duration and strength of inflammatory and autoimmune diseases [3] led us to examine their effect on the generation of T_{reg}. To enable the detection of either a positive or negative effect of Sup ODN on T_{reg} differentiation, splenic $CD4^+CD25^-$ precursors were incubated *in vitro* under conditions that induced a significant but suboptimal increase in iT_{reg} frequency. These 'suboptimal' conditions involved conventional stimulation with anti-CD3 plus anti-CD28 Abs but lower concentrations of TGFβ (see section 2.8).

As seen in Fig 1, ≈1% of $CD4^+CD25^-$ T cells differentiated into iT_{reg} when incubated for 3 – 5 days in medium alone. The frequency of iT_{reg} increased 5-fold when conditions supporting the suboptimal generation of T_{reg} were used during culture ($p < 0.01$). Of importance, >35% of cultured cells differentiated into $CD4^+CD25^+FoxP3^+$ T cells under the same conditions when supplemented with Sup ODN ($p < 0.001$). This effect was sequence specific, as control ODN lacking suppressive TTAGGG motifs had no significant effect on iT_{reg} generation (Fig 1). Sup ODN increased both the percentage and absolute number of iT_{reg} generated *in vitro* (Fig 1C), a finding confirmed by the increase in FoxP3 mRNA levels detected by RT PCR (Fig 1D; $p < 0.05$). To determine whether Sup ODN had an effect on nTregs, CFSE-labeled $CD4^+CD25^+$ T cells were incubated with IL-2 and the proliferation of $FoxP3^+$ cells monitored by flow cytometry. The inclusion of Sup ODN had no effect on the proliferation of $CD4^+FoxP3^+$ Tregs (Supplemental Fig 1). These data indicate that Sup ODN selectively facilitate the differentiation of naive T cells into iTregs.

To examine whether results from these murine studies were relevant to humans, the effect of Sup ODN on the generation of human iT_{regs} was examined. $CD4^+CD25^-$ T cells were isolated from the peripheral blood of normal healthy volunteers. These were stimulated with IL-2, anti-CD3 and anti-CD28 plus a suboptimal amount of TGFβ1 (identified in preliminary studies). After 5 days, cells were analyzed for FoxP3 expression by FACS. As seen in Fig 2, the inclusion of Sup ODN led to a significant increase in both the percentage and absolute number of human $CD4^+CD25^+FoxP3^+$ T cells generated *in vitro* ($p < 0.05$ and $p < 0.01$ respectively)

3.2 iT_{reg} functional activity

Further study focused on the activity of the murine iT_{regs} generated in the presence of Sup ODN. To eliminate any possibility that mature T_{reg} were contaminating the $CD4^+CD25^-$ T cell pool, FoxP3 GFP knock-in mice were used as cell donors [26]. All T_{reg} from these animals are GFP^+, so FACS isolation of $CD4^+GFP^-$ T cells insures that no T_{reg} are present in the starting population. The cells were then cultured under the T_{reg} polarizing conditions described above. Consistent with results obtained above using cells from WT mice (Fig 1), the addition of Sup ODN resulted in a >4-fold increase the frequency of $CD4^+GFP^+FoxP3^+$ T_{reg} generated (from $3.1 \pm 0.4 \times 10^5$ to $12.8 \pm 1.3 \times 10^5$).

The functional activity of these iTreg was evaluated based their ability to inhibit the proliferation of activated T cells. Consistent with previous reports, T_{reg} suppressed the proliferation of syngeneic T cells in a dose dependent manner (Fig 3 and [29]). The suppressive activity of the T_{reg} generated in the presence of Sup ODN was nearly identical to that of control T_{reg} on a per cell basis (Fig 3). These findings indicate that Sup ODN promote the generation of functionally active iT_{reg}.

3.3 Suppressive ODN promote the generation of T_{reg} *in vivo*

To determine whether Sup ODN support the generation of T_{reg} under physiologically relevant conditions, a well established murine model of peripheral tolerance was used [18]. $CD4^+CD25^-$ T cells were isolated from Rag2 KO donors expressing the DO11.10 TCR (such T cells are specific for OVA). These T cells were labeled with CSFE and transferred into WT recipients. The recipients were then injected with soluble $OVA_{323\text{-}339}$ peptide and the CFSE-labeled $FoxP3^+$ cells monitored. Consistent with previous findings, $CD4^+CD25^-$ T cells from Rag2 KO / DO11.10 donors differentiated into iT_{reg} when exposed to OVA peptide *in vivo* (Fig 4 [19;30]). When the recipient mice were treated with Sup ODN, the number of T_{reg} generated rose by >3-fold (from $4.8 \pm 1.1 \times 10^3$ to $15.7 \pm 3.9 \times 10^3$, $p < 0.05$).

3.4 Role of STAT1 in the generation of iT_{reg} by Sup ODN

Previous studies established that i) Sup ODN can bind to and prevent the phosphorylation of STAT1 and STAT4 in Th1 cells [8] and ii) the phosphorylation of STAT1 and STAT4 can inhibit T_{reg} generation [22]. These observations led us to examine the effect of Sup ODN on STAT phosphorylation in $CD4^+CD25^-$ T cells. In the absence of stimulation, STAT phosphorylation was nearly undetectable (Fig 5A). When $CD4^+CD25^-$ T cells were cultured under suboptimal T_{reg} polarizing conditions, the phosphorylation of STAT1 and STAT4 rose rapidly to 45% and 85%, respectively (Fig 5A, B). The addition of Sup ODN reduced the phosphorylation of STAT1 in these cells by >75% ($p < 0.05$) but had no effect on the phosphorylation of STAT4 (Fig 4A, B). This effect of Sup ODN was sequence specific as control ODN did not significantly alter STAT phosphorylation.

To explore whether the inhibition of STAT1 phosphorylation represented the mechanism by which Sup ODN promoted the generation of iT_{reg}, the response of T cells from WT and STAT1-deficient mice was compared [27]. When $CD4^+CD25^-$ T cells were cultured under identical conditions, $\approx$ 2.5-fold more iT_{reg} were generated from STAT1-deficient vs WT donors (Fig 5C,D; $p < 0.01$). Adding Sup ODN to these cultures increased the frequency of

T_{reg} generated from WT precursors to that observed in the STAT1 KOs (Fig 5C,D; $p < 0.01$). Consistent with the inhibition of STAT1 phosphorylation being the mechanism by which Sup ODN support the generation of T_{reg}, Sup ODN did not further boost the generation of T_{reg} from targets deficient in STAT1.

4. Conclusions

One attractive strategy for treating autoimmune and inflammatory diseases is to capable of down-regulating these pathological immune responses. This provide T_{reg} study examines the effect of synthetic ODN expressing immunosuppressive TTAGGG motifs on iT_{reg} generation. Results indicate that Sup ODN promote the differentiation of both human and murine $CD4^+CD25^-$ T cells into functionally active iT_{reg} under conditions in which either TGFβ or IL-2 is limiting (Figs 1–4). Evidence that Sup ODN inhibits the STAT1 phosphorylation of activated T cells provides insight into the mechanism underlying this effect (Fig 5).

Sup ODN contain multiple TTAGGG motifs identical to those found at high frequency in mammalian telomeres and mimic the ability of telomeric DNA to reduce inflammation and autoimmune reactions [5;6;31–33]. Previous studies found that Sup ODN inhibited the differentiation of naive $CD4^+$ cells into Th1 effectors [7;8]. That effect was postulated to explain their therapeutic utility [8] as the importance of T_{reg} in modulating immune activation and suppressing pathological immune responses had not yet been discovered. Current findings establish that in addition to altering the balance between Th1, Th2 and Th17 [7;8;34] cells that Sup ODN also promote the differentiation of $CD4^+CD25^-$ T cells into iT_{reg}.

Naive $CD4^+$ T cells can differentiate into a diverse array of effector phenotypes under appropriate conditions [35]. For example, CD4 T cells in a pro-inflammatory environment differentiate into Th17 cells when exposed to TGFβ while the same cells exposed to TGFβ in the absence of inflammatory cytokines differentiate into T_{reg} [36]. Our group recently showed that the addition of Sup ODN to CD4 T cells facilitated their maturation into Th17 cells under pro-inflammatory Th17 polarizing conditions [34]. Results from the current work show that adding Sup ODN to the same cells in the absence of pro-inflammatory cytokines induces them to differentiate into T_{reg}. From this we conclude that similar to TGFβ, the effect of Sup ODN on CD4 T cell generation is context-dependent.

Previous work established that Sup ODN were useful in the treatment of various autoimmune diseases [4–7]. To date, efforts to determine whether that effect was mediated by Sup ODN increasing the frequency of T_{reg} have been unrevealing. As previously shown, successful treatment of murine autoimmunity requires that Sup ODN be administered repeatedly over many weeks. This prolonged intervention has many effects on the immune milieu, preventing us from establishing a cause-effect relationship with T_{reg} generation. To circumvent this problem, we used a well established model of *in vivo* tolerance that allowed precise analysis of the effect of a single treatment with Sup ODN on T_{reg} generation (Fig 4).

Sup ODN are fabricated from nuclease-resistant phosphorothioate nucleotides to improve their *in vivo* half life and activity. Bouladoux et al reported that Sup ODN could prevent

CpG ODN from inhibiting T_{reg} generation but did not examine whether Sup ODN directly promoted T_{reg} generation [37]. Kim et al reported that phosophorthioate ODN could stablilize the expression of FoxP3 in a sequence non-specific manner but did not examine whether Sup ODN promoted the generation of T_{reg} from $CD4^+$ precursors [38]. While phosphorothioate ODN can exert sequence non-specific immune effects at the high concentrations used by Kim et al [38;39], the control ODN used in our studies and by other groups uniformly failed to increase T_{reg} development and/or function [37;40–42]. Similarly, studies performed by multiple groups show that suppressive but not control ODN down regulate inflammatory and autoimmune responses *in vivo* [5;6;31–33].

Current findings are consistent with recent reports that T_{reg} generation is inhibited by STAT1 phosphorylation and that the deletion of STAT1 promotes T_{reg} differentiation [21–23,28]. The precise mechanism by which a decrease in STAT1 activation promotes the generation of T_{regs} is unclear. STAT1 may interfere with STAT5 driven T_{reg} production or it may trigger pathways that inhibit T_{reg} development. Specifically, we find that i) $CD4^+$ T cells from STAT1-deficient mice generate significantly more iT_{reg} than those from WT donors, ii) Sup ODN block STAT1 phosphorylation and simultaneously enhance iT_{reg} generation and iii) Sup ODN do not enhance the generation of iT_{reg} from mice deficient in STAT1 (Fig 5). These findings suggest that the mechanism by which Sup ODN promote the generation of iT_{reg} is by inhibiting STAT1 phosphorylation. Consistent with that conclusion, we previously demonstrated that Sup ODN inhibited the differentiation of naive CD4 T cells into Th1 effectors by reducing STAT1 phosphorylation [8]. Other members of the STAT family also play a role in T_{reg} generation. FoxP3 expression and T_{reg} differentiation are enhanced by STAT5 phosphorylation [20;21] but inhibited by activation of the STAT4 and STAT6 signaling pathways [22]. We find that Sup ODN inhibit the phosphorylation of STAT1 but have no effect on STATS 3, 4 or 5 under culture conditions conducive to the generation of iT_{reg} (Fig 5 and data not shown).

The therapeutic benefit of increasing T_{reg} frequencies in patients with autoimmune and inflammatory disorders is being examined clinically [43]. Such therapy currently requires the adoptive transfer of T_{reg}, a strategy that is difficult, time consuming, and expensive [44]. The same outcome might be achieved by administering Sup ODN. Our studies of $CD4^+CD25^-$ T cells from normal human donors shows that Sup ODN induces them to differentiate into iT_{reg}. To study the effect of Sup ODN *in vivo*, the well defined DO11.10 Tg / Rag2 KO mouse model was employed [18]. This model has been used to identify other agents capable of eliciting Ag-specific T_{reg} responses [19;45]. Our results establish that Sup ODN significantly increase the number of $CD4^+CD25^-$ T cells that differentiate into T_{reg} *in vivo* (Fig 4). Other agents designed to increase T_{reg} production caused moderate-severe adverse events in clinical trials [43;46;47]. By comparison, pre-clinical studies found that Sup ODN are safe even when administered repeatedly and at high doses [48]. Taken together, these findings suggest that Sup ODN may be a promising tool to augment the generation of iT_{reg} in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding

This research was supported by the Intramural Research Program of the National Cancer Institute of the National Institutes of Health.

The assertions herein are the private ones of the authors and are not to be construed as official or as reflecting the views of the National Cancer Institute at large.

Abbreviations

ODN	phosphorothiate oligodeoxynucleotide
Sup	suppressive

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Highlights

1. Supressive oligonucleotides (Sup ODN) enhance the generation of functionally active iT_{reg} *in vitro*
2. Sup ODN promote the generation of iT_{regs} in response to peptide challenge *in vivo* 3) The inhibition of STAT1 activation by Sup ODN increases the generation of iT_{reg}

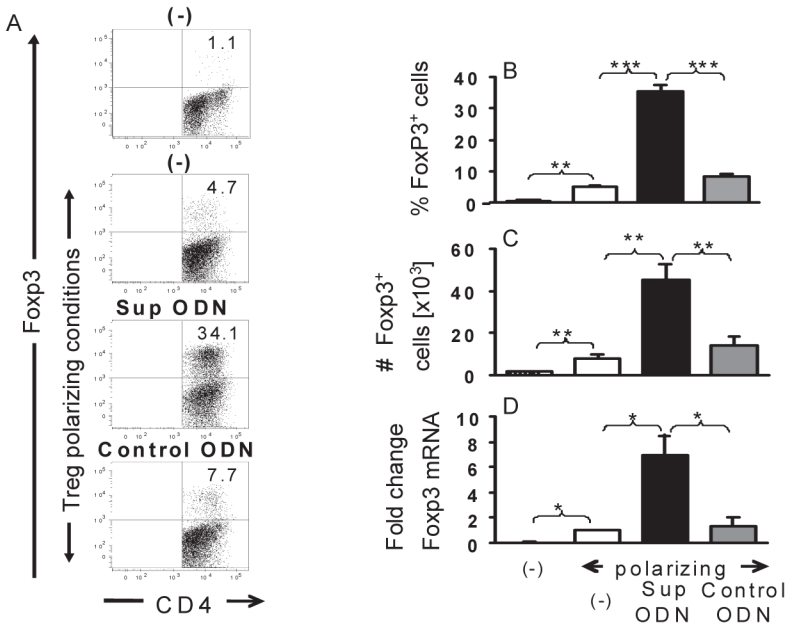

Figure 1. Suppressive ODN promote the generation of iT_{reg} *in vitro*

$CD4^+CD25^-$ T cells were isolated from female BALB/c mice by negative selection. They were incubated for 2 h with 1 uM of suppressive or control ODN and then cultured under T_{reg} polarizing conditions (3 ug/ml of anti-CD3, 2 ug/ml of anti-CD28 and 5 ng/ml of TGFβ) for 5 days. iT_{reg} were identified based on their expression of CD4 and FoxP3 by flow cytometry. B, C) Combined results from 4 independent experiments showing the frequency and absolute number of $CD4^+$ $FoxP3^+$ T_{reg} (mean + SE) generated by each type of treatment over 3–5 days. (D) mRNA was isolated after 5 days of culture and analyzed for FoxP3 gene expression by RT PCR. Relative mRNA levels were calculated by comparison to cells cultured under the same conditions in the absence of ODN. Results from each group were normalized to GAPDH mRNA levels. Each bar represents the mean + SE of 3 independent experiments.

*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$

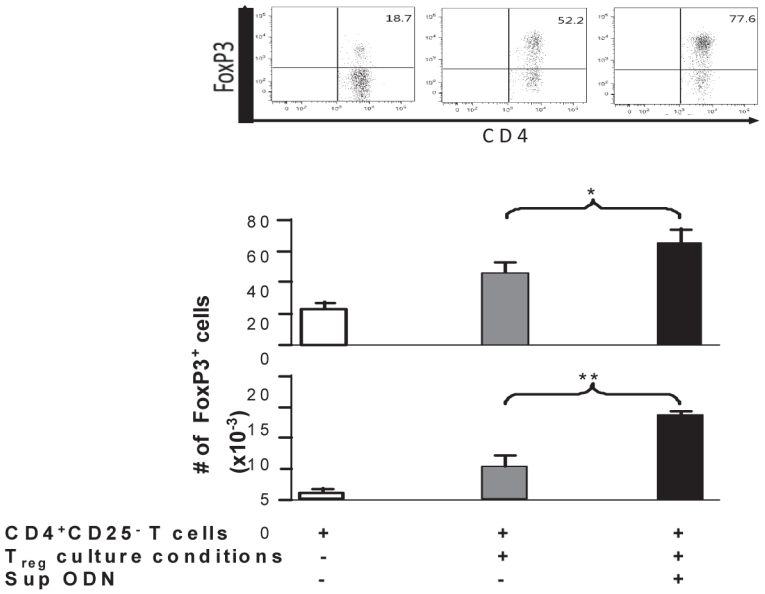

Figure 2. Suppressive ODN promote the generation of human iT$_{reg}$ *in vitro*

$CD4^+CD25^-$ T cells were isolated from normal healthy volunteers (N=3) and incubated for 2 h with 3 uM of suppressive ODN in medium supplemented with IL-2, anti-CD3/CD28 and TGFβ1 as described in the methods section. The cells were stained to detect expression of CD4 and FoxP3 and analyzed by FACS on day 5. Each donor was analyzed independently on a different day and results show the mean + SD of all samples.

*, $p<0.05$; **, $p<0.01$

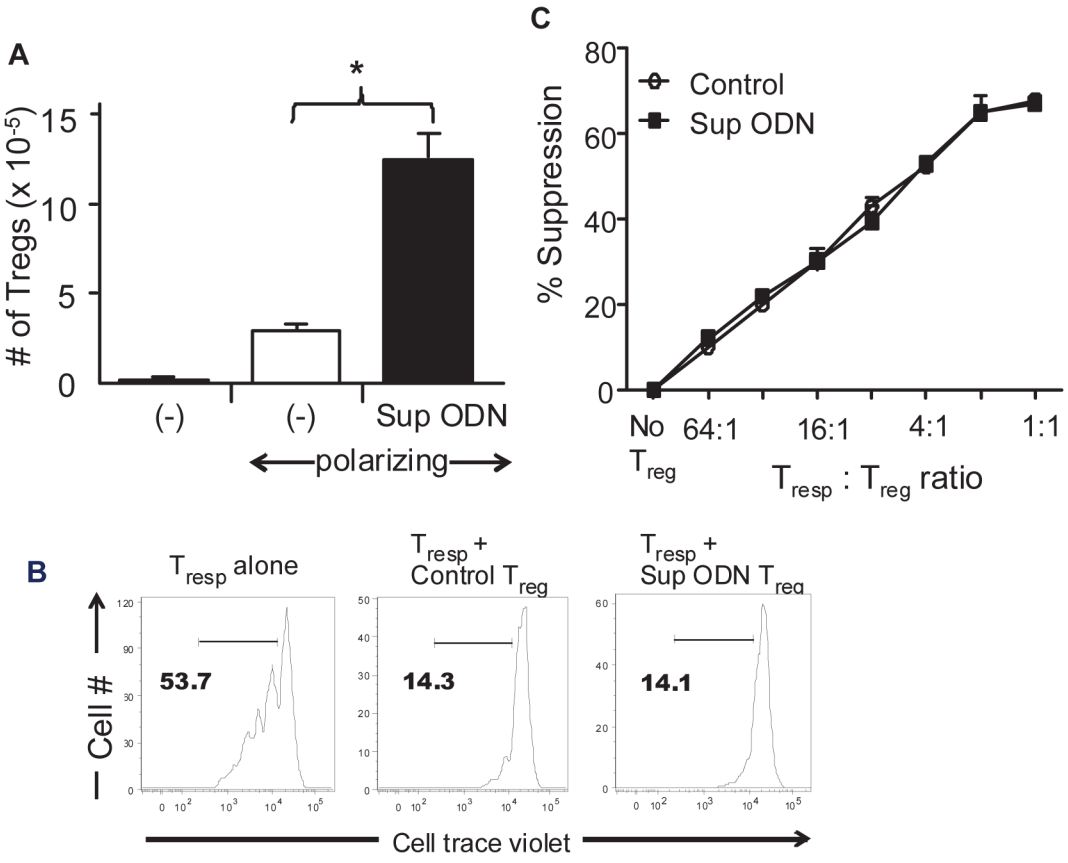

Figure 3. Suppressive ODN enhance the generation of functional iT$_{reg}$

CD4^{+}FoxP3^{-} T cells were FACS isolated from the spleen of FoxP3 GFP knock-in mice and induced to differentiate under T$_{reg}$ polarizing conditions for 3 days as described in Fig 1. The CD4^{+} FoxP3^{+} T$_{reg}$ generated *in vitro* were purified and mixed with CD4^{+}CD25^{-} responders (T$_{resp}$) labeled with cell trace violet and stimulated with anti-CD3 plus mitomycin C treated APCs. A) Absolute number of iT$_{reg}$ generated. B) Effect of T$_{reg}$ on the proliferation of activated T$_{resp}$ at a 1 : 1 ratio. C) Effect of T$_{reg}$ frequency on the proliferation of T$_{resp}$. All data represent the mean of two independent experiments using T$_{reg}$ generated in the presence or absence of Sup ODN. *, $p<0.05$.

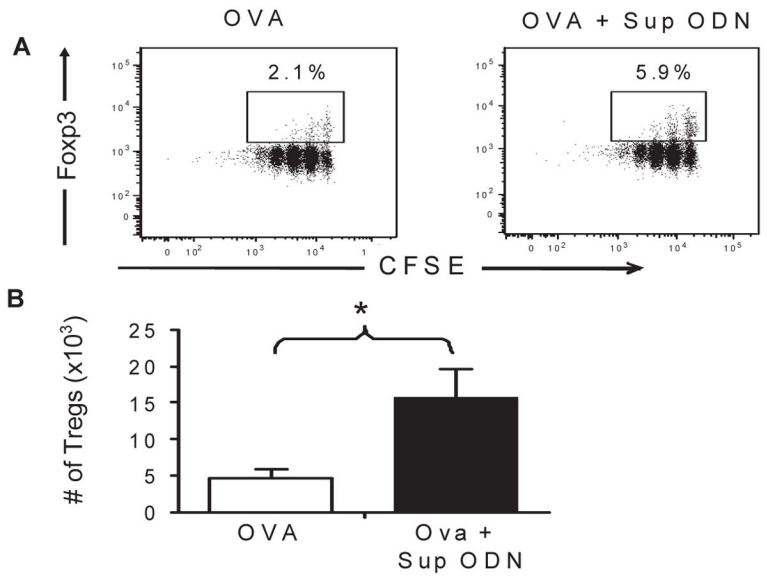

Figure 4. Suppressive ODN increase the number of Ag-specific iT$_{reg}$ generated in vivo

FoxP3^{-} T cells were isolated from Rag2$^{-/-}$ / DO11.10 mice, labeled with CSFE, and injected i.v. into syngeneic BALB/c mice. Recipients were treated on day 1 with 5 ug of OVA$_{323-339}$ peptide ± 300 ug of Sup ODN. On day 5, peripheral lymph nodes were isolated and FACS analyzed for the presence of CFSE^{+} donor cells. A) The percent of transferred cells maturing into FoxP3^{+} T$_{reg}$ *in vivo* and B) the absolute number of T$_{reg}$ (defined as CD4^{+} FoxP3^{+}CFSE^{+}KJ1-26^{+} cells). Data represent the mean + SD of three independent experiments involving 4 independently studied mice/group.

*, $p<0.05$

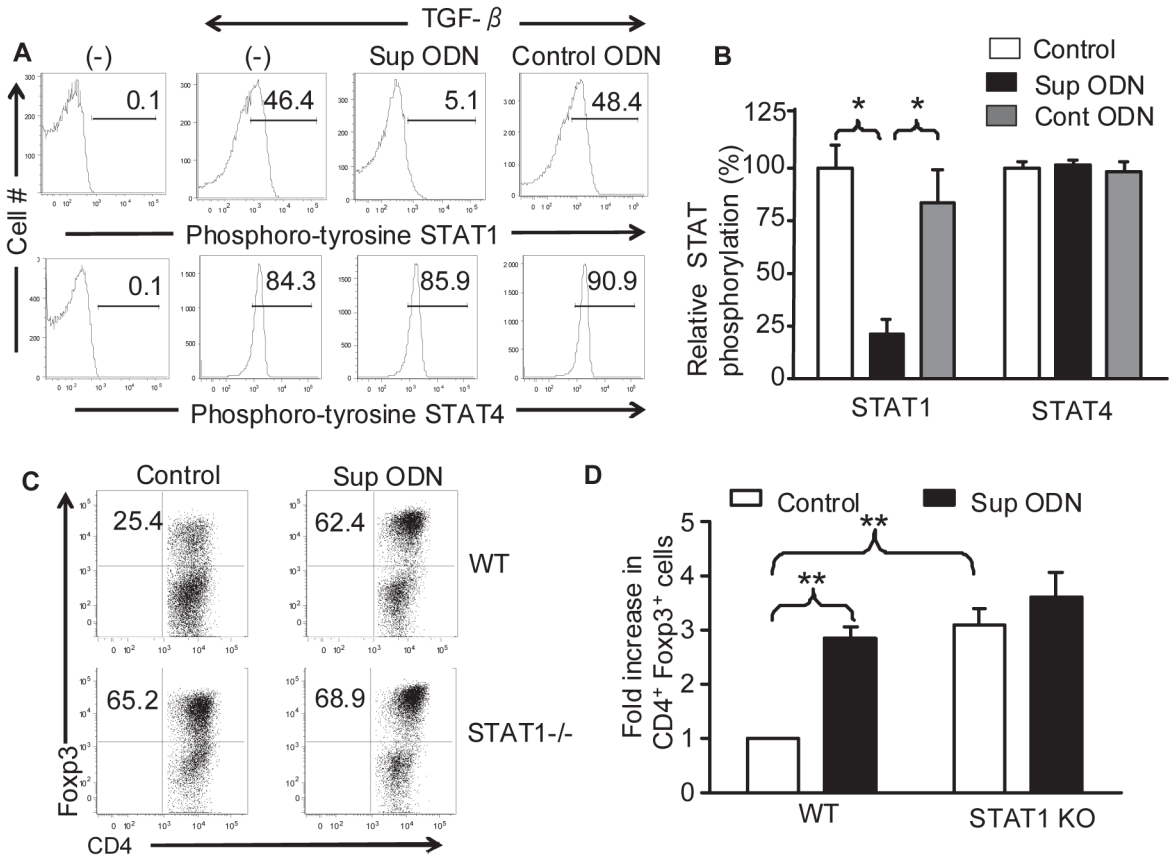

Figure 5. Effect of Suppressive ODN on STAT 1 phosphorylation and iT$_{reg}$ generation

A,B) CD4$^+$CD25$^-$ T cells from BALB/c mice were induced to differentiate under T$_{reg}$ polarizing conditions as described in Fig 1. The level of STAT1 and STAT4 phosphorylation was determined by flow cytometry using phospho-specific Abs at 4 h (A). The mean + SE level of STAT phosphorylation from 4 independent experiments (B). C,D) CD4$^+$CD25$^-$ T cells from C57Bl/6 or STAT1-deficient C57Bl/6 mice were induced to differentiate under T$_{reg}$ polarizing conditions. T$_{reg}$ were identified on day 3 on the basis of CD4 and FoxP3 expression (C). The fold increase in CD4$^+$ FoxP3$^+$ T$_{reg}$ frequency was calculated by comparison to cells from WT mice cultured under optimized conditions but in the absence of Sup ODN (D). Data represent the mean + SE of 3 independent experiments. **, p<0.01