

Depleting regulatory T cells with arginine-rich, cell penetrating, peptide-conjugated morpholino oligomer targeting FOXP3 inhibits regulatory T cell function

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ABSTRACT

- Immunotherapy using vaccines to induce effector T cell responses against cancers and viral infections has demonstrated promising clinical activity recently; however, an immunosuppressive tumor microenvironment maintained in part by regulatory T cells remains a challenge. CD4⁺CD25⁺regulatory T cells (Treg) impair antitumor and antiviral immunity, therefore is considerable interest in eliminating them or altering their function as part of cancer immunotherapy strategies. The scurfin transcriptional regulator encoded by the member of the forkhead winged helix protein family (FOXP3) is critical for maintaining function of Treg cells. In the present study, amongst breast cancer patients, for those with more advanced disease (higher stage, positive axillary nodal metastases), a greater percentage of Treg was observed. We hypothesized that targeting FOXP3 expression with a novel arginine rich cell-penetrating peptide-conjugated phosphorodiamidate morpholino (PPMO) based antisense would eliminate Tregs and enhance the induction of effector T cell responses. Morpholino oligomers are the newer generation antisense molecules, wherein the deoxyribose moiety of DNA is replaced with a 6-membered morpholine ring and the charged phosphodiester internucleoside linkage is replaced with phosphorodiamidate linkages which increases stability and specificity. The mechanism of action involves both steric blockade of ribosomal assembly thus preventing translation, and the interference with intron-exon splicing of pre-mRNA preventing appropriate translation of selected mRNA. We observed that the PPMO was taken up by activated T cells in vitro and could downregulate FOXP3 expression which otherwise increases during antigen-specific T cell activation. This is timely and highly relevant as delivery of oligomers in immune cells has been a major issue in manipulation of targets in T cells. Generation of antigen-specific T cells in response to peptide stimulation was enhanced by pre-treatment of peripheral blood mononuclear cells with the FOXP3 targeted PPMO. The successful and widespread use of PMOs for knockdowns in exquisitely sensitive embryonic systems such as zebrafish and *Xenopus* show that they are attractive human therapeutics. Further, specificity and lack of immune response are critical characteristics that allow for antisense PPMOs to be used in humans. In summary, modulation of Treg levels using the FOXP3 PPMO antisense-based genomic strategy has the potential to optimize immunotherapy strategies in breast cancer immunotherapy. DOD W81XWH-07-1-0392 (GRD).

Tregulatory cells

- CD4+ Treg cells have traditionally been defined based on their expression of the IL-2 receptor alpha chain (CD4+CD25+) *Baecher-Allan et al. 2002, J Immunol.*
- Selective depletion of Treg in animal models can increase effector T cell responsiveness to pathogenic and autologous antigens (*McHugh et al. 2002, J Immunol., Hisaeda et al. 2002, J Immunol.*)
- Treg cells are not only important in modulating immune responses, but also may allow cancer cells to escape immune destruction and limit responses to cancer vaccines.
- The forkhead box transcription factor FoxP3 seems to be essential for the development of regulatory T cells *in vivo*. FoxP3 thus appears to be a specific marker for Treg identification and quantification (*Fontenot et al. 2003, Nature Immunol., Yagi et al. 2004, Int Immunol.*)

Figure 1. CD4+CD25^{Hi} T cells are predominantly FOXP3-expressing Tregs.

(A) PBMC were stained with anti-CD25, anti-CD4, and anti-CD3 and gated as shown for cell sorting by FACS (CD4+CD25^{Hi} = ~1-3% of total CD4+ cells in normal donors). (B) Sorted CD4+FoxP3^{Hi}, CD4+FoxP3^{Med} and CD4+FoxP3⁻ cells were analyzed by quantitative real-time PCR with probes and primer specific for FOXP3 and HPRT. FOXP3 expression is presented as relative expression to HPRT (where HPRT=1). Representative data from 3 normal donors. (C) CD4+CD25⁻ and CD4+CD25^{Hi} were cultured alone or together (plus 105 accessory cells) at a 1:1 ratio and stimulated 10 mg/ml anti-CD3 in a proliferation assay. CD4+CD25^{Hi} cells inhibit CD4+CD25⁻ proliferation. The mean cpm +/- SD is presented (*p ≤ 0.02 by Student's t test).

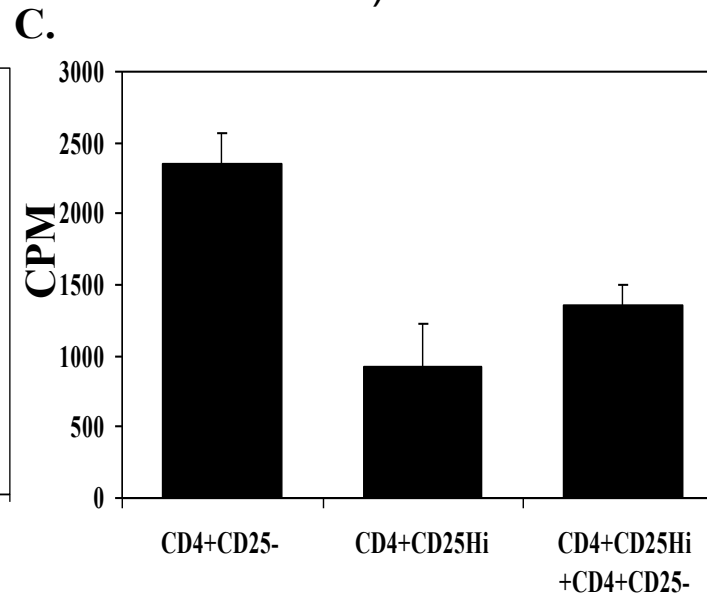
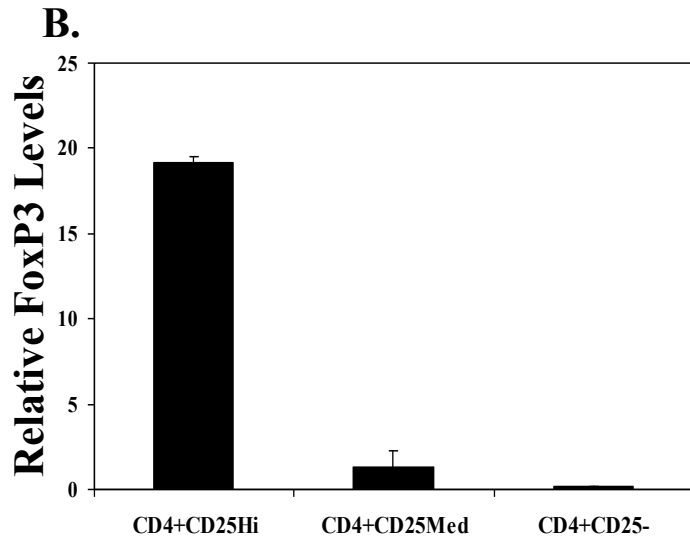
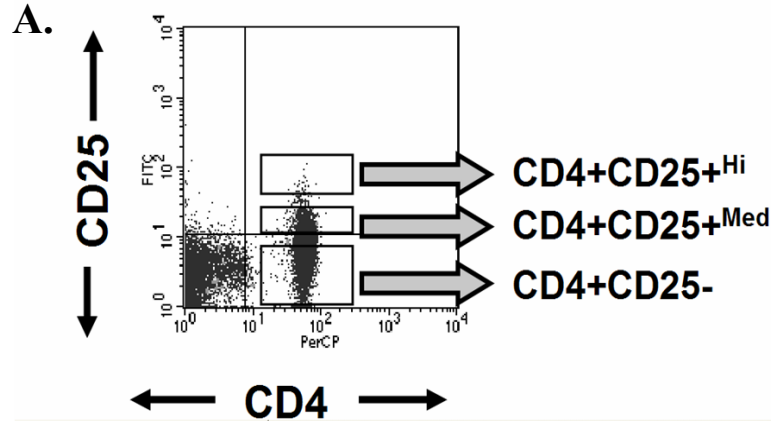
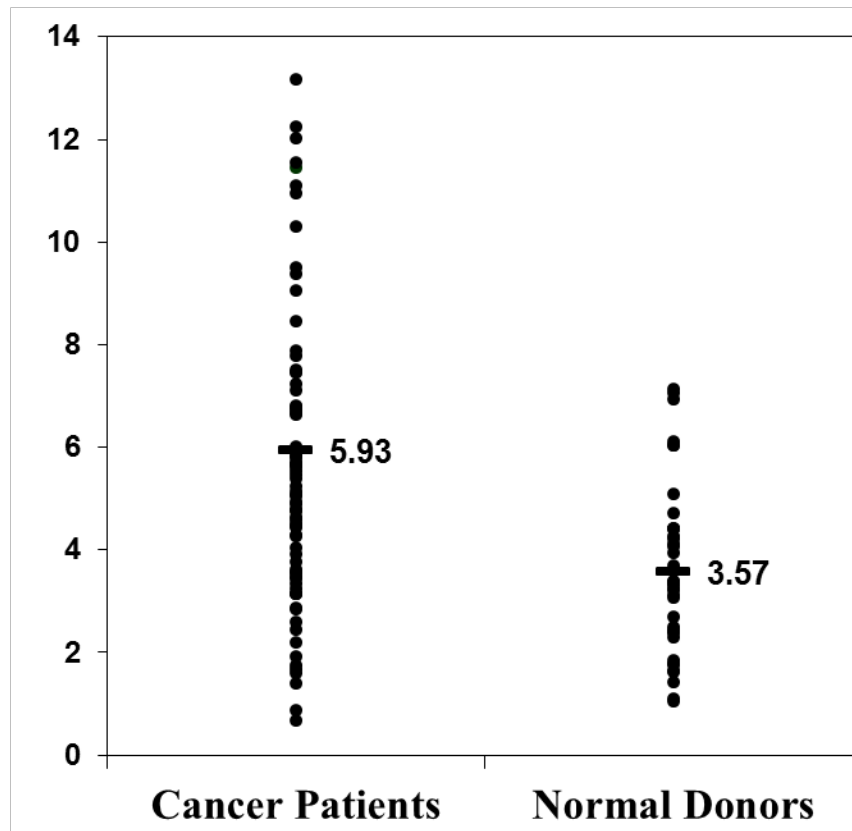


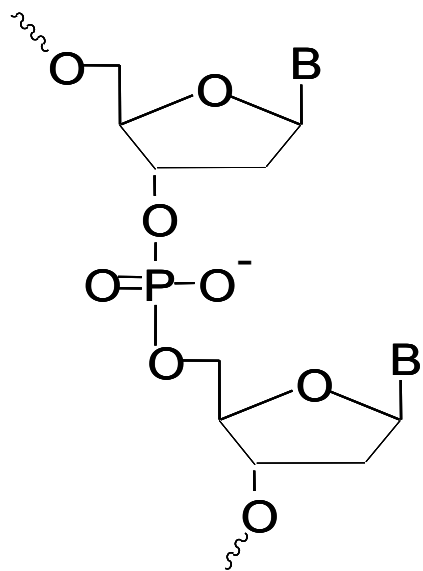
Figure 2. Cancer patients have significantly higher numbers of regulatory T cells.



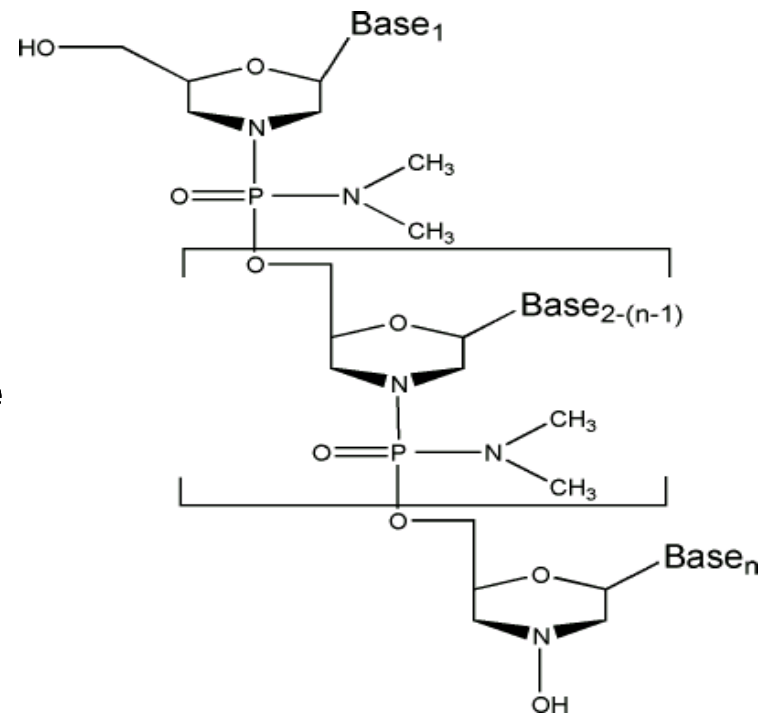
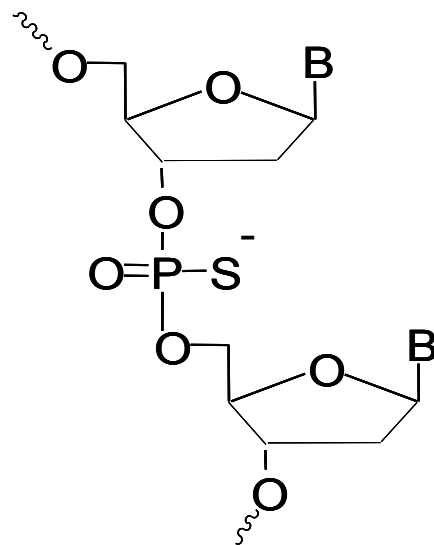
Blood was collected on 41 normal donors and 78 cancer patients and analyzed for the expression of CD4+CD25Hi by flow cytometry. Each solid dot represents an individual donor. One cancer patient was an extreme outlier and not included in the graph. The mean percentage of expression for each group is designated by a solid bar with the corresponding value. T-test $p < 0.0001$.

Phosphorodiamidate Morpholino Oligomer (PMO) Chemistry

Phosphodiester

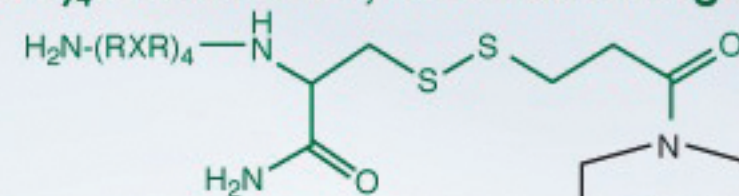


Phosphorothioate

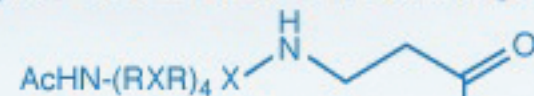


Phosphorodiamidate
Morpholino Oligomer
(PMO)

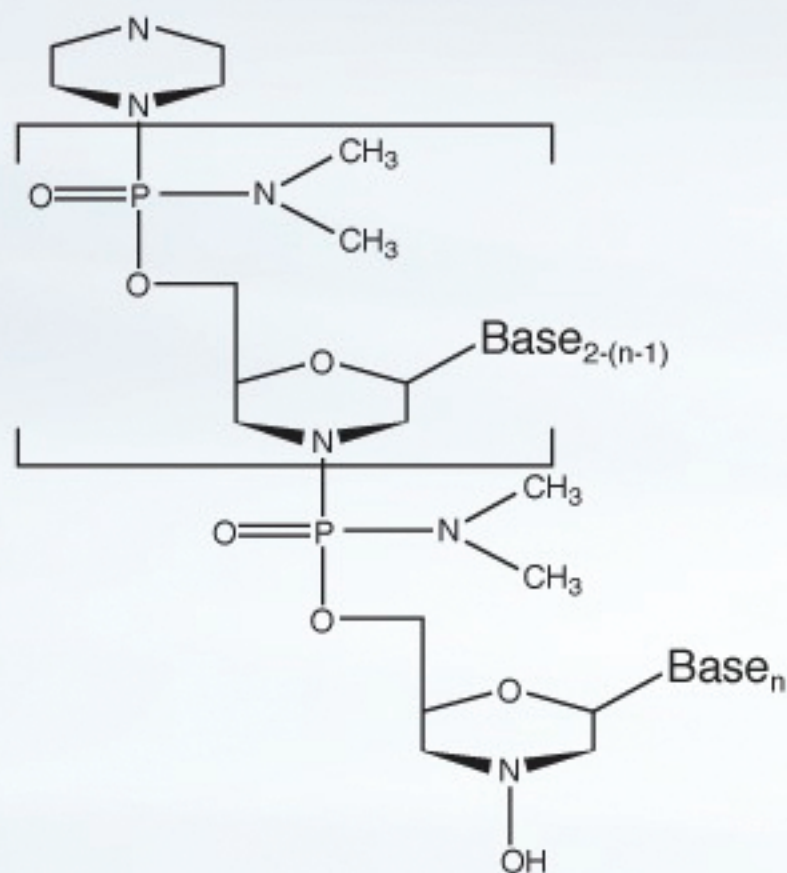
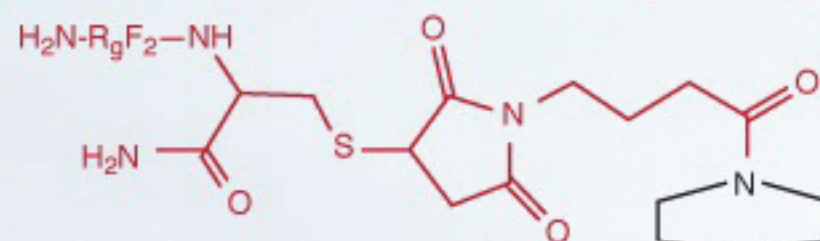
(RXR)₄C-S-S-PMOF; disulfide linkage



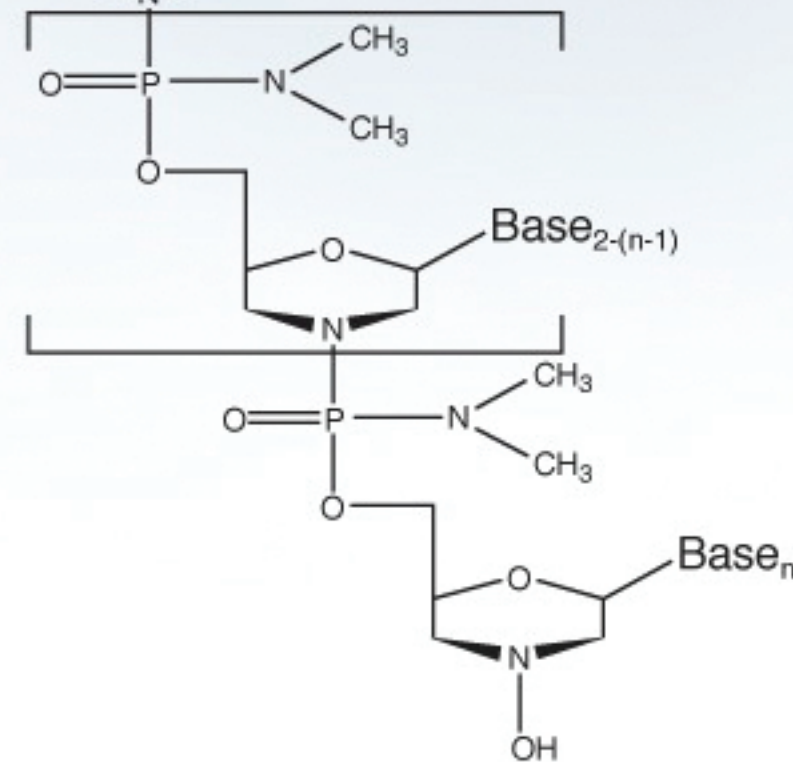
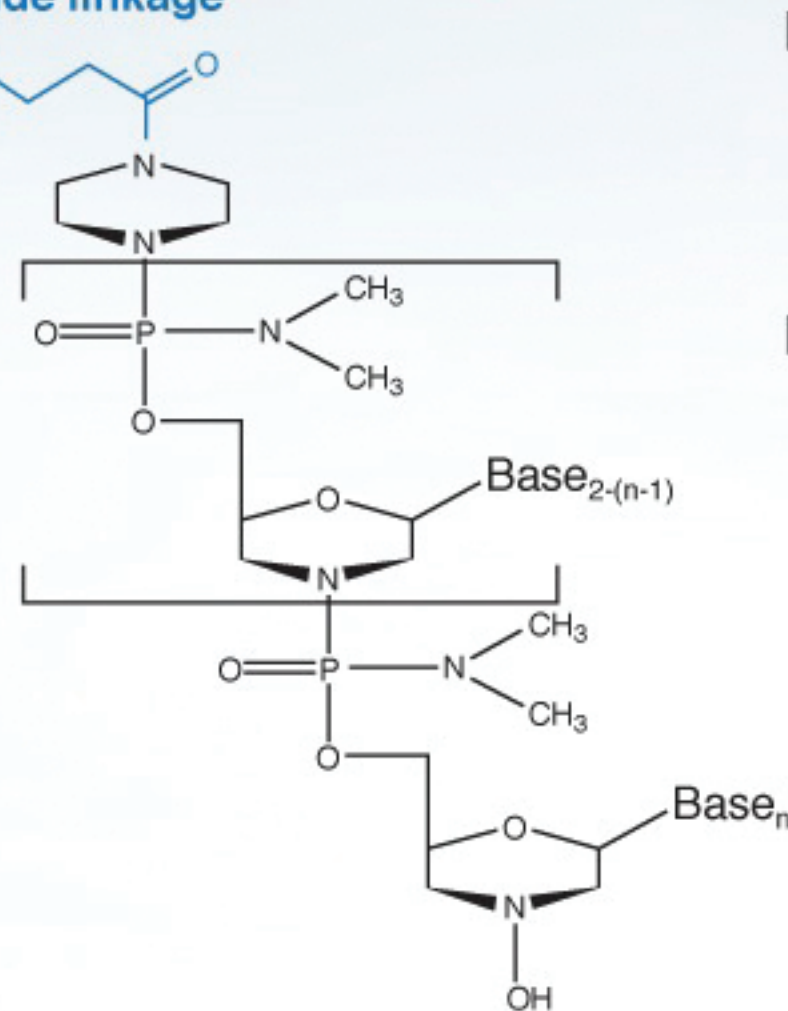
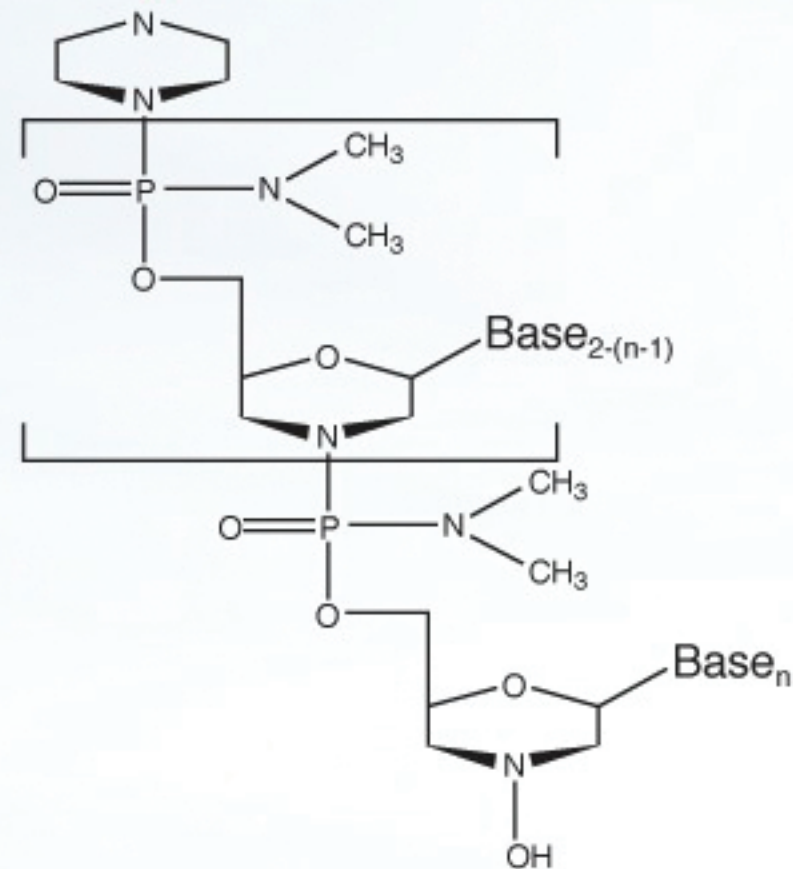
(RXR)₄XB-PMOF; amide linkage



R_gF₂C-PMO; thioether (maleimide) linkage

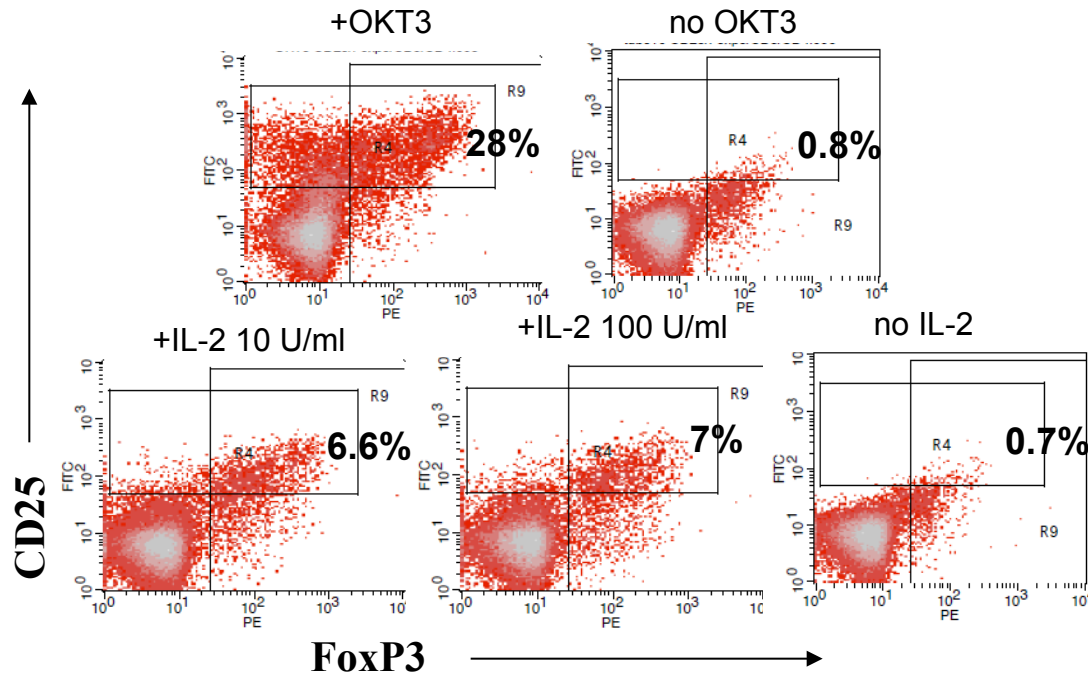


Phosphotodiamidate Morpholino Oligomer (PMO)



Peptide-conjugated Phosphorodiamidate Morpholino Oligomer (PPMO)

Figure 3. Activated T cells exhibit high FoxP3 expression.



Normal donor PBMC activated overnight with OKT3 or IL-2 (10 or 100 U/ml) and non-activated controls were gated on CD4+ cells and then analyzed for CD25 and FoxP3 expression.

The percentage CD25+FoxP3+ is presented for each activation group and controls. Data representative of multiple experiments.

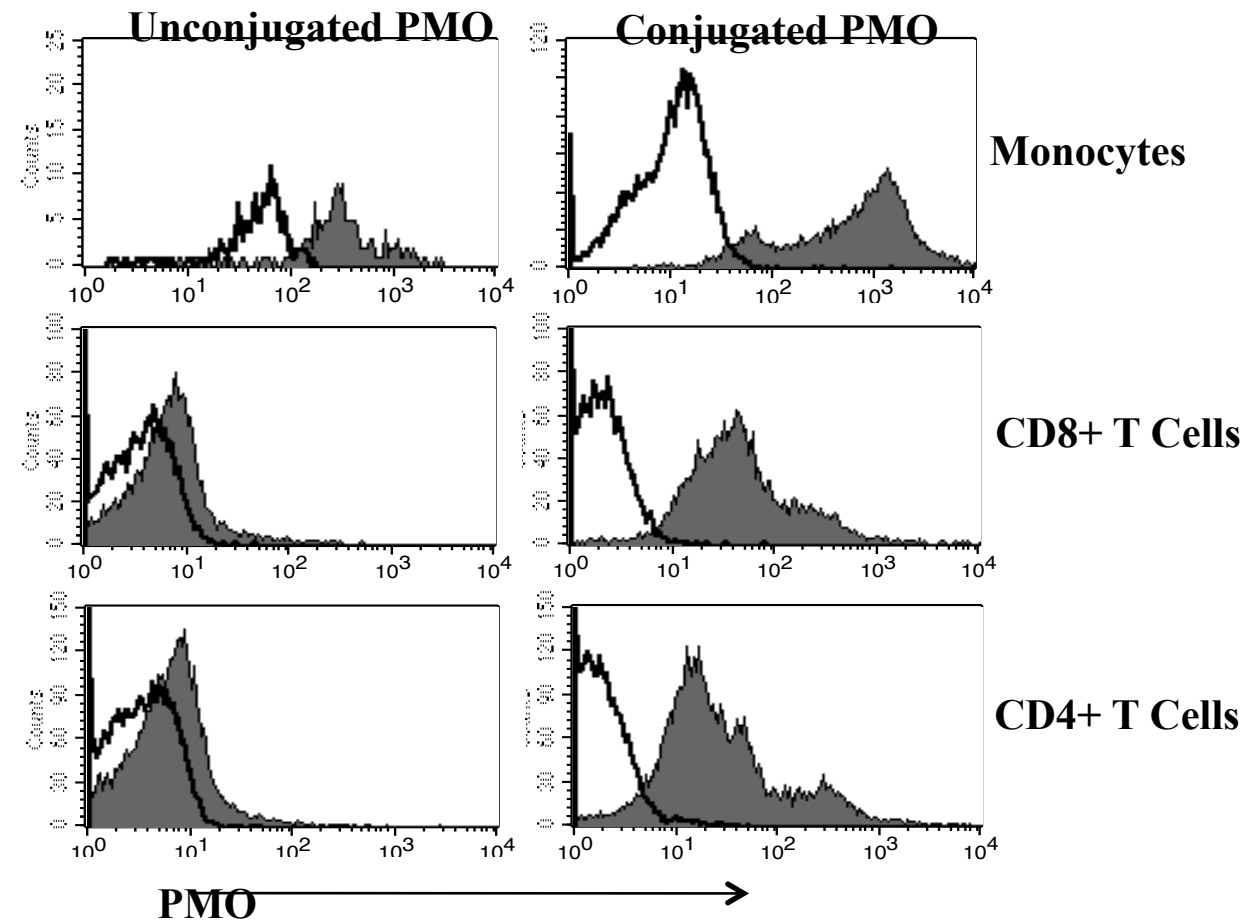
Peptide-conjugated Phosphorodiamidate Morpholino Oligomer (PPMO)

- Phosphorodiamidate morpholino oligomers (PMO) were synthesized at AVI BioPharma (Corvallis, OR) as described earlier.²⁴ Purity was >95% as determined by reverse-phase HPLC and matrix-assisted laser desorption/ionization time-of-flight mass spectrometer. PPMO was produced by attaching the C-terminal cysteine of the peptide (RRRRRRRRRFFC) to the 5'-end of the PMO through a cross-linker *N*-[α -maleimidobutyryloxy]succinimide ester (BGBS).³¹ PPMO was dissolved in sterile H₂O before use in cell cultures.
- The base composition of the oligomers are:
 - *FOXP3 PPMO sequence:* 5'-CTGGGGTTGGGCATCGGGTC -3'.
 - *Scrambled PPMO sequence:* 5'- AGTCTCGACTTGCTACCTCA-3'.
 - *Unrelated Target PPMO sequence:* 5'- CTGTTAAAAGTCATCTTCTC-3'.

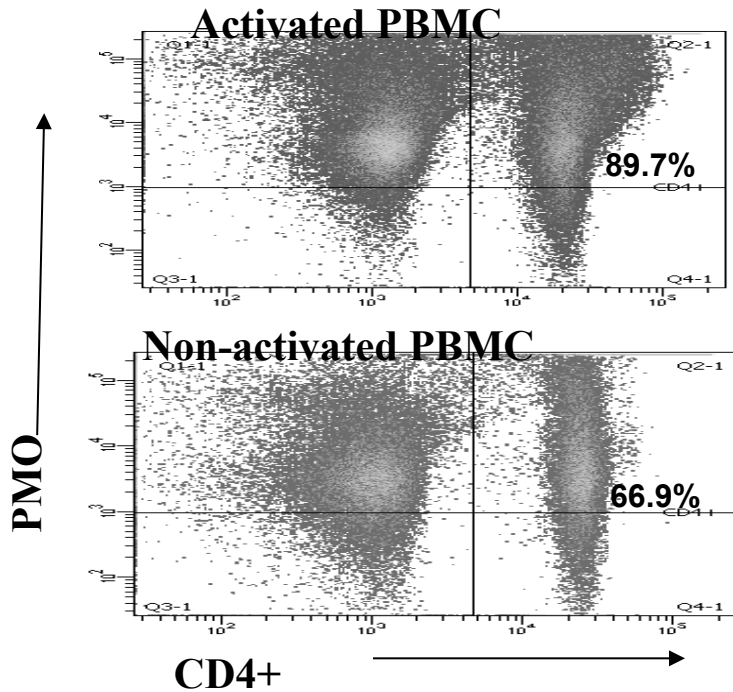
Therapeutic Advantages of Synthetic Oligonucleotides to modulate FOXP3

- This approach exploits a gene specific mechanism of action.
- Employ 15 to 25 bases for therapeutic action.
- No concern for vector/host untoward interactions.
- No metabolism and no chance for accumulation of mutation in therapeutic sequence.
- Pharmacokinetic calculation of dose and duration of action. (Like traditional drugs)

Figure 4. Uptake of FITC-labeled unconjugated PMO and PPMO in human immune cells.

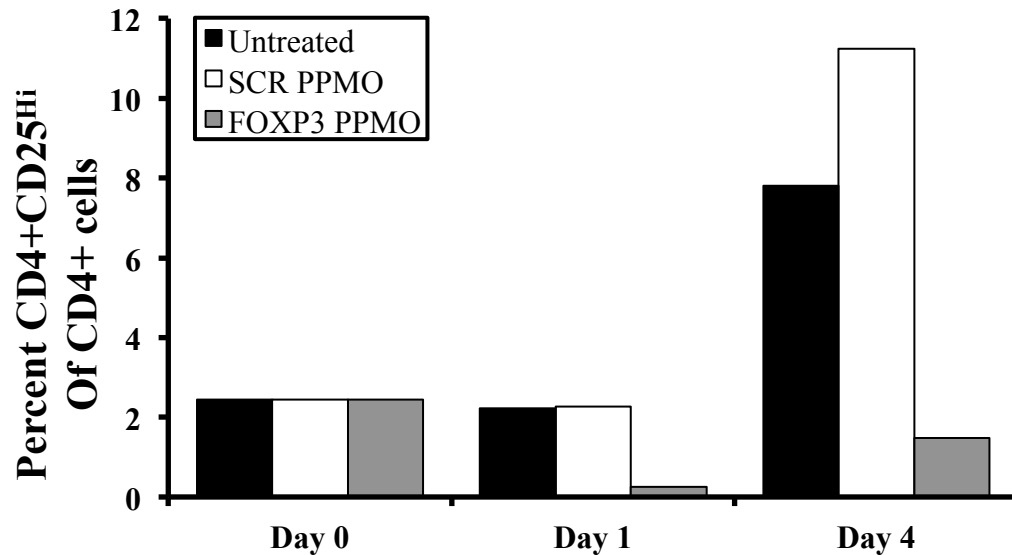


(A) Uptake by monocytes (HLA-DR +CD14+) and CD4+ and CD8+ T cells analyzed by flow cytometry. Unconjugated morpholino oligomer (PMO) or conjugated morpholino oligomer (PPMO) uptake is represented by the grey/solid peak and IgG control by black line/open peak.



(B) PPMO uptake in activated versus non-activated T cells. Normal donor PBMC were activated with IL-2 (10U/ml), incubated with 2.5microM FITC labeled PMO (24 hrs) and analyzed by flow cytometry. The percent CD4+PMO+ cells for activated and non-activated (no IL-2) is indicated for each plot. Data representative of 3 experiments.

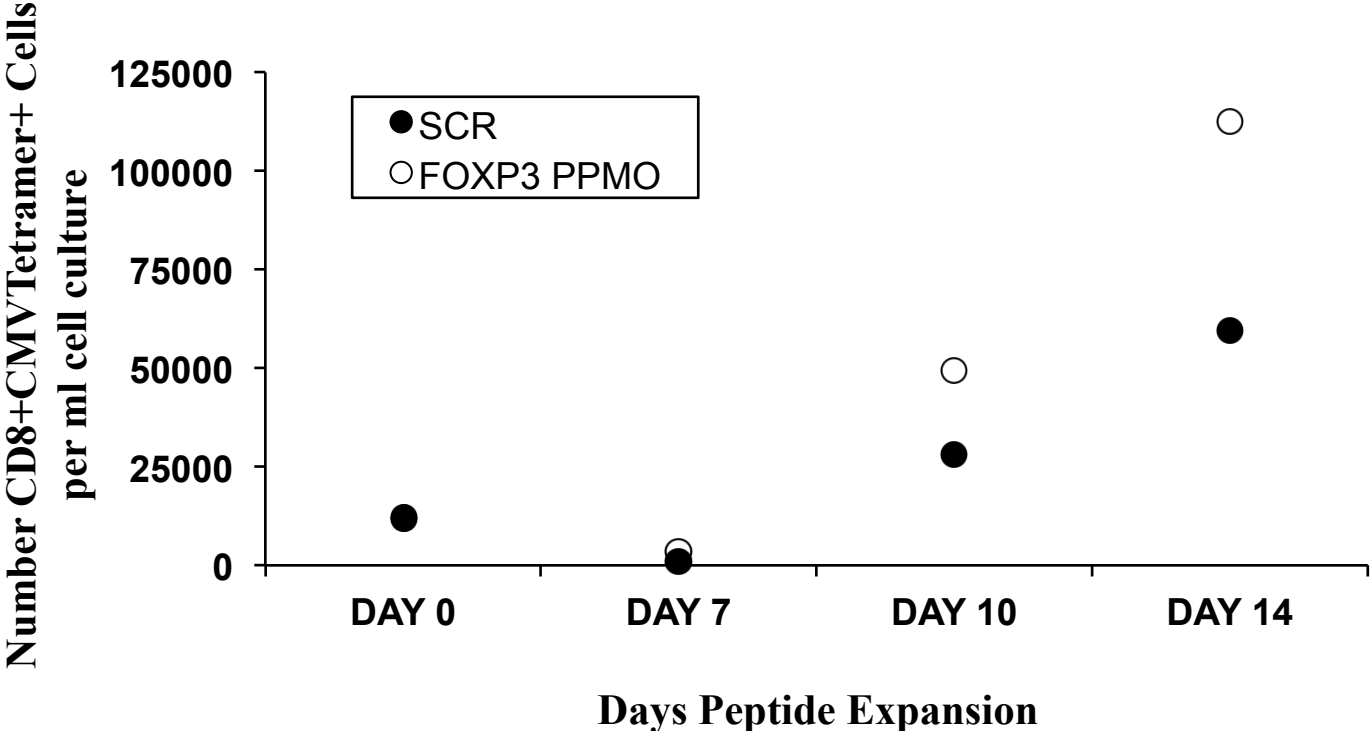
Figure 5. FoxP3-specific PPMO inhibits FoxP3 protein expression in CD4+CD25^{hi} cells.



PBMC were analyzed by flow cytometry for FoxP3 expression on days 0 (i.e., pre-treatment), 1 and 4 days following overnight treatment with 2.5 microM SCR PPMO, FOXP3 PPMO or media alone (untreated).

Frequency of CD4+CD25^{hi} T cells (as a percentage of all CD4+ T cells) for each treatment (Results representative of 5 separate experiments).

Figure 6. Inhibition of FOXP3 expression with FOXP3-specific PPMO leads to enhanced immune responses.



PBMC from a CMV sero-positive normal donor were treated overnight with FOXP3 PPMO or media alone. After 4 days in low dose IL-2 (10 U/ml), cells were stimulated in vitro with 1 mg/ml CMVpp65 peptide plus IL-2 (600 U/ml) for 14 days. T cell cultures were analyzed by flow cytometry for CMVpp65- specific T cells using CMVpp65 peptide-MHC tetramer. The number of CD8+CMVpp65tetramer+ cells per ml of culture is presented for 0, 7, 10, and 14 days following media control and FoxP3 PPMO pre-treatment of PBMC.

Summary

- The present study demonstrates that PPMO antisense-mediated targeting of FOXP3 reduces Treg frequency as suggested by the attenuation in the expansion of CD4+CD25^{bright} T cells otherwise seen during the induction of effector immune responses. Although most CD4+CD25^{bright} T cells are regulatory T cells, there can be transient upregulation of CD25 on activated CD4+ T cells. Interestingly, we also observed a small decrease in the percentage of CD4+CD25+ T cells which expressed FOXP3 after the PPMO antisense application. We speculate that this may be evidence of CD4+ T helper cell activation and expansion during Treg depletion.
- An important consequence of the depletion of Treg was the ability to activate a greater frequency of CMV-specific T cells. This has important implications for use of the PPMO in patients with chronic viral infections and suggests that the PPMO may be used to enhance vaccine induced immune responses as well. Furthermore, because we have shown that other methods of Treg depletion lead to enhanced anti-tumor immune responses to cancer vaccines, we propose that the PPMO may be useful to administer as part of cancer immunotherapy strategies. Indeed, we now propose to study the PPMO in *in vivo* models of tumor immunotherapy.