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Development and Partial Characterization of Agonistic OX40 Aptamer anti-CBLB siRNA Conjugates

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DEVELOPMENT AND PARTIAL CHARACTERIZATION OF AGONISTIC OX40 APTAMER αCBLB siRNA CONJUGATES

By

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Tumors that develop in patients with fully functional immune systems evolve different mechanisms of immune suppression that must be counteracted for tumor immunotherapy to be effective. The proposed therapeutic aims to co-stimulate T-cells via OX40, a co-stimulatory receptor expressed on activated T-cells. Signaling via OX40 leads to prolonged survival of activated T-cells, enhanced memory formation, and protection against immunosuppression. In addition to boosting co-stimulation, we aim to enhance T-cell activation by downregulating Cbl-b, an E3 ubiquitin ligase that suppresses T-cell activation. An RNA aptamer that binds OX40 was conjugated to anti-Cbl-b siRNA and was shown by a luciferase reporter assay to have approximately 80% knockdown efficiency. Its co-stimulatory function as a monomer was tested using proliferation assays, but showed no effect on polyclonal activated $CD4^+$ T-cells. When the OX40 aptamer component was dimerized, the OX40-Cbl-b siRNA conjugate was shown to enhance activation of ova-specific $CD4^+$ T-cells when compared to the control OX40-GFP siRNA conjugate, or monoclonal OX40 antibody. After testing for *in vivo* costimulatory effects, the conjugates were not found to be effective. Development of a trimeric OX40 aptamer and conjugate may yield better results *in vivo* as it would emulate the trimeric nature of OX40L.

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LIST OF ABBREVIATIONS

Ab-	Antibody
APC –	Antigen Presenting Cell
Cbl-b –	Casitas B-lineage Lymphoma protein – b
CTL –	Cytotoxic T Lymphocyte
CTLA-4 –	Cytotoxic T Lymphocyte Antigen - 4
IFNγ –	Interferon Gamma
mAb –	Monoclonal antibody
NK –	Natural Killer
OT-II –	Ovalbumin Transgenic MHC class II restricted
Ova –	Ovalbumin
PD-1 –	Programmed Death 1
qPCR –	Quantitative Polymerase Chain Reaction
RING –	Really Interesting New Gene
TCR –	T Cell Receptor
TGFβ –	Transforming Growth Factor beta
TNFSFR –	Tumor Necrosis Factor SuperFamily of Receptors
Treg –	Regulatory T cell

Chapter 1: Introduction

1.1 Anti-tumor immunity: Equally important and opposite approaches that should be combined.

The immune system is very powerful where a delicate balance between activation and suppression must be maintained. Unregulated activation of the immune system can result in autoimmune diseases, while too much suppression and/ or not enough activation, leads to a lack of protection against infectious agents. A developing tumor may co-opt the immune system that is supposed to be responsible for its elimination by inducing suppressive programs that are regularly used during wound healing, or peripheral tolerance[1]. When these programs are engaged, it becomes very difficult for an immune response to be mounted against the tumor. While many strategies appreciate that a strong vaccine initiated immune response may have an effect against tumors, it is often not enough because of these programs that are usually already in place. One approach to clear the way for an effective anti-tumor response would be to first or simultaneously reduce suppressive mechanisms. Many of the suppressive mechanisms used by a tumor are used in peripheral tolerance; ineffective activation (lack of licensing and costimulatory factors), regulatory T cells, surface expression or secretion of suppressive molecules (TGFβ, HLA-G[2], PD-1[3]). It is reasonable to postulate that a therapy that can both enhance activation of an anti-tumoral response, as well as reduce the responders' sensitivity to suppressive mechanisms at the tumor site, would be more effective than either strategy alone.

1.2 Cblb – A key inhibitor of lymphocyte activation

Casitas B lineage Lymphoma protein B is an E3 ubiquitin ligase, an enzyme that modifies proteins by adding a ubiquitin protein (or several) to proteins targeted for proteosomal degradation. It was first discovered as v-Cbl, a truncated form of the Cbl protein lacking a RING domain present in a B lineage lymphoma. Cblb expression in T cells increases when the TCR intracellular signaling cascade is activated. Its expression leads to the ubiquitination and subsequent proteosomal degradation of target proteins Zap70, LAT, PLC-γ and p85 of PI3K, all part of the TCR signaling cascade, effectively shutting it off. Cblb is negatively regulated by phosphorylation by PI3K when CD28 expressed on the surface of the T cell is ligated to B7 expressed on activated antigen presenting cells (APCs). Phosphorylation of Cblb marks it for ubiquitination by another E3 ligase, Itch. This two-step process of TCR ligation to its antigen, followed by CD28 ligation to B7 (licensing), allows for a T cell to become fully activated. Cblb also acts in later stages of shutting down T cell responses. T cells lacking Cblb show reduced sensitivity to TGFβ[4, 5]. CTLA-4 ligation causes upregulation of Cblb expression[6]. It is also postulated that PD1 negatively regulates T cell activation through Cblb. Thus the threshold of tolerance, i.e. the minimum amount of activating stimuli[7] required to activate a T cell, is set by the changes in Cblb levels in a T cell.

T cells lacking Cblb can be fully activated independent of CD28 signaling[8]. When the TCR is engaged, as Cblb is deficient, there is no negative signal stopping the TCR cascade from being turned on, hence there is no need for CD28 signaling to reduce non-existent Cblb levels in the cell. This deficiency in Cblb does not seem to affect the development of Treg cells in the thymus[9]. Peripheral tolerance, however, is severely

affected by a lack of Cblb, with transgenic mice often succumbing to hyperproliferative disease and organ infiltration[10].

There is evidence to support Cblb as a target towards enhancing immunogenic effects. Transgenic mice deficient in Cblb are unable to grow large tumors. Adoptive transfer of Cblb knock out $CD8^+$ T cells to tumor bearing mice leads to reduced tumor burdens and enhanced survival[11]. The aim of this project is to selectively deliver Cblb siRNA to T cells, hoping that their activation can be prolonged, giving time for an effective antitumoral response to be successful.

1.3 – OX40 adjuvants in cancer therapy

OX40 (CD134) is a costimulatory receptor expressed on activated T cells, preferentially on $CD4^+$ T cells, and to a lesser extent on $CD8^+$ T cells, as well as some other immune cells. OX40 is highly expressed on memory T cells as well as on Tregs. It belongs to the tumor necrosis factor superfamily of receptors (TNFSFR4). OX40 ligation on T cells leads to an increase in NFκB, Bcl-2 and hence an increase in survival. It also causes $CD4^+$ T cells to undergo enhanced proliferation, and is necessary for the formation of memory $CD4^+$ and $CD8^+$ T cells[1213], and is a key factor in B-cell class-switching[14]. OX40 costimulation also reduces sensitivity of $CD4^+$ T cells to TGFβ, preventing their induction into Tregs[15], as well as inhibits the inhibitory activity of Tregs[1617]. Another albeit disputed characteristic of OX40 ligation is its ability to turn Treg cells into T effector cells[18]. The implications for this are of vast importance if we were to look at reducing tumor immunity as overcoming peripheral tolerance. If tumor specific iTregs were to be able to be reverted to Teffector cells, then the sheer numbers of Tregs usually

associated with the stroma of a tumor would be co-opted into an anti-tumoral attack. Unfortunately, this also means there is a greater risk for inducing rampant autoimmunity.

1.4 – Aptamers as therapeutic agents

Aptamers are short oligonucleotides made from RNA or DNA that are selected to have a high affinity for its target molecule. In this project, an RNA aptamer of approximately 80 nucleotides was chosen for its high binding affinity to its target, murine OX40. The sequence published[19] called OX40- Sull. 9.8, was generated from a process called SELEX: Systematic Evolution of Ligands by EXponential enrichment[20]. These generated ligands can be used for diagnostic purposes, or can be used in lieu of antibodies in agonistic or antagonistic fashions. Unlike antibodies, aptamers have much smaller sizes, can be easily chemically produced, and are also more easily altered, both in sequence and with functional group additions. Due to their specificity, aptamers, like antibodies, can be used as delivery agents[21]. In this project, the OX40 aptamer is being used to deliver siRNA against Cblb, to activated T cells. The OX40 binding unit, an approx. 60nt structure, when oligomerized (in this case, doubled), can act as an agonistic ligand engaging OX40 on T cells.

1.5 – Rationale

Immunotherapies to cancer are increasingly being considered in the scientific community as ideal agents not only to specifically target tumors, but also, due to principles like epitope-spreading[22, 23], to generate immune memory to help prevent recurrences. While many of these therapies seek to directly enhance activity of cytotoxic

cells like NK and CTLs, the T-helper portion of the adaptive immune response may be capable of strongly synergizing cytotoxic effects of killer cells. As OX40 is highly expressed on $CD4^+$ T cells and plays an important role in their optimal activation, there are increasing numbers of OX40 based immunotherapies that are being translated into clinical trials[24]. The approach of using agonistic aptamers to OX40 has a significant advantage over monoclonal antibodies or OX40L fusion proteins because an antidote aptamer[25] (complementary in sequence) could be administered to quickly neutralize possible autoimmune side-effects. Also being a chemical as opposed to a cell based product, the regulatory process to approve such a drug for use in clinical trials, in theory, would be quicker. Being chemically based also means cheaper production, increasing the cost effectiveness of using other aptamers in combinations as has been proven effective with antibodies[26] (e.g. 4-1BB agonistic aptamer[27] with IL10 blocking aptamer[28], etc). Additionally, its lack of inherent immunogenicity (an immune response to the drug itself) would decrease adverse affects due to repeated administrations (a common issue for other protein based drugs[29]). These attributes would make an agonistic OX40 aptamer an appealing immunotherapy.

Although Cblb is the inhibitory protein being targeted for knock down in this project, there are a number of other possible inhibitory proteins that could be targeted. These include FoxP3, Bim, STAT6, CTLA-4, PD-1 and PTEN. To combine the agonistic function of OX40 aptamer with the ability to deliver siRNA and thus decrease expression of immune inhibitors, is an effective way to maximize the therapeutic benefit of this one drug.

This study therefore aims to

1) Develop an agonistic OX40 aptamer – Cblb siRNA conjugate,

2) Test the ability of the conjugate to enhance costimulation *in vitro*,

3) Test the conjugate for costimulatory and/or antitumoral effects *in vivo* using murine models.

Chapter 2: Materials and Methods

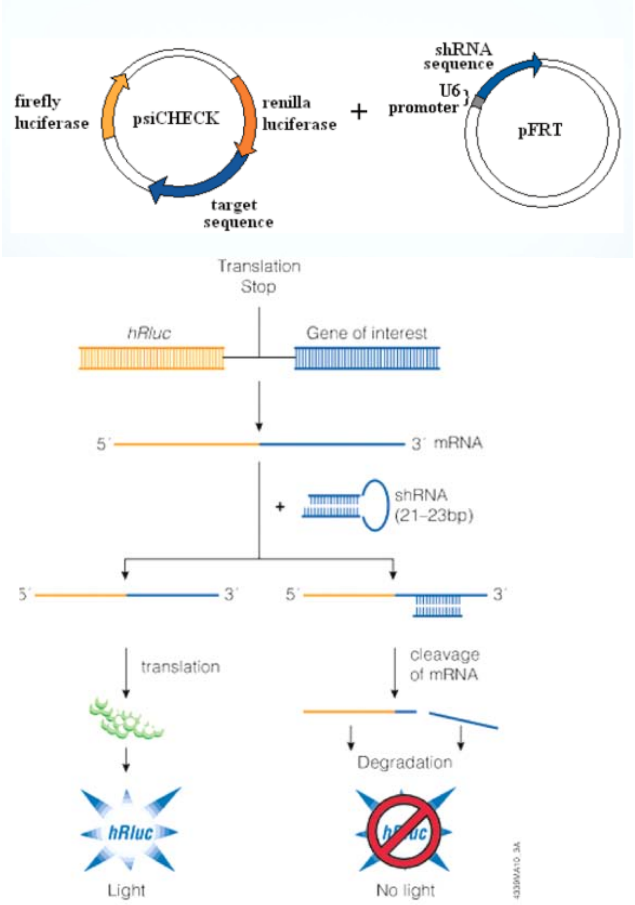
2.1 – Selection of siRNA sequences.

The mRNA sequence for Cbl-b in FASTA format was entered into different siRNA sequence generating algorithms, from Open Biosystems, Dharmacon and HPC Dispatcher (City of Hope). The sequences were ranked according to the lowest difference in free energy ΔG between the 5' and 3' ends, then according to the lowest melting temperatures. Sequences containing nucleotides in repeats of more than 3 were omitted. Six RNA sequences were originally selected; Cblb1: 5' ACUUCCUCCAGAACUCAGC 3', Cblb2: 5' AAAUCAGGGUUAUAGCUCC 3', Cblb3: 5'AAUUCUCGAAGUAUGCUCC 3', Cblb4: 5' UGUGAGAUUCCGUCUGUCC 3', Cblb5: 5' UAUUAACAUAUUUCCAGGC 3', Cblb6: 5' AAAUUAUUUACACUUCCCU 3'.

2.2 - Testing siRNA sequences using psiCHECK2 dual luciferase system.

Oligos were designed to carry the target of each Cblb siRNA sequence selected. These were cloned into the psiCHECK2 vector, which contains a firefly luciferase cassette as a transfection control, and a renilla luciferase cassette, the 3' end of which, the target DNA sequence is cloned. The DNA counterparts to the siRNA sequences were made into oligos containing the sense, loop and antisense. These oligos were cloned into the pFRT vector, which contains a U6 promoter, allowing transcription of shRNA. Both vectors

were cotransfected into HEK 293T cells in 24well plates. 48 hours later, cells in each well were treated using Promega's Dual Glo Luciferase kit (illustrated in Box 1) according to its manual, and the firefly and renilla luciferase activities were measured using a luminometer. The relative knock down activity of each siRNA sequence was calculated by normalizing the renilla luciferase output values to firefly luciferase. This same cotransfection procedure was used to measure knock down activities of free duplex siRNA as well as conjugates containing siRNA sequences.



Box 1. Illustration of Promega's Dual Luciferase system.

2.3 – Production of RNA aptamer and conjugates

The OX40 aptamer sequence 9.8 including the forward and reverse primers was taken from Sullenger et. al. (2008). Oligos were ordered from SIGMA and cloned into pGEMT Easy vector system (Promega). Large batches of template DNA were amplified by PCR to be used in i*n vitro* transcription reactions. T7 transcription kits (Epicentre) were used to make RNA aptamers with 2'Fluoro modified cytosines and uracils. These aptamers were purified on PAGE gels, with bands the appropriate size cut and RNA eluted overnight at 37ºC in Tris-EDTA. Eluates were purified of gel matter and excess nucleotides by repeated centrifugation in 30kD size exclusion columns obtained from Millipore. In the cases of conjugates and dimers, antisense RNA strands or DNA stabilizing oligos were hybridized to aptamers/ dimers by annealing reactions with ratios of 1:3. The annealed aptamers were quantified, aliquotted and stored at -80ºC.

2.4 – Isolation of T cells and functional assays

In vitro functional assays were performed with primary T cells isolated from Balb/c mice, C57B6 mice, or whole splenocyte preparations from OTII transgenic mice. For the polyclonal assays, T cells were isolated by negative selection with magnetic beads using Miltenyi Kits (Cat.# 130-095-248) . These T cells were then incubated with different titrations of CD3 antibody (BD Biosystems Cat. # 553057) in round-bottomed 96 well plates. Proliferation assays were done either by flow cytometry, utilizing CFSE, or by tritiated thymidine assays as described below.

CFSE Assay: Purified T cells were stained with 1uM CFSE in1mL of PBS for 5 minutes in the dark, followed by 2 washes in PBS (5% FBS). 10^5 CFSE labeled T cells would then be plated in wells precoated with CD3 antibody, ranging from 0.05μg/mL – 2μg/mL. After approx. 72hours, cells were collected and stained with CD4-APC and OX40-PE antibodies (BD Biosciences Cat. # 553051 and eBioscience Cat. # 12-1341 respectively)

Tritiated Thymidine Assays: 100K purified T cells were plated in wells precoated with CD3 antibody, and left to proliferate for approx 72hrs. Then, tritiated thymidine would be added to each well (μCi) and incubated at 37ºC for 6 or 18 hours. Then the amount of thymdine incorporated into the proliferating cells was measured using a scintillation counter.

OTII proliferation assays. As OTII mice have $CD4^+$ T cells specific to chicken ovalbumin presented by APCs whole splenocyte preparations were incubated in 96 well plates along with a range of ova II peptide. Proliferation was measured using tritiated thymidine assays.

OX40-Costimulation assays. To test the costimulatory function of the conjugates, a positive control was required. Functional grade OX40 antibody (eBioscience Cat.# 14-1341-81) was used in both the polyclonal and transgenic T cell models at a final concentration of 5ug/mL. Costimulation was measured either by proliferation assays or IFNγ secretion assays. IFNγ secretion was tested by ELISA using kits obtained from BD Biosciences (Cat. # 551866). Supernatants from 96 well plates were harvested after approx. 72hrs after being plated.

2.5 - Quantitative PCR

In order to determine whether the siRNA was successful at reducing mRNA expression of Cbl-b in T cells, qPCR was done on cultures at different points of incubation, using a primer-probe set from Applied Biosystems (Cat.# 4331182). RNA was isolated using RNEasy kits (Invitrogen) and cDNA was made from 1ug of RNA samples using Taqman Reverse Transcription Kits from Applied Biosystems (Cat.# N8080234)

2.6 – *In vivo* models of costimulation

To assess whether conjugates were able to costimulate T cell activity *in vivo*, an adoptive transfer assay involving the transgnic OTII mice was employed. Briefly, $CD45.2^+$ $CD4^+$ T cells were purified from OTII splenocytes, counted, and 10^6 cells transferred into congenic $CD45.1^+$ C57/B6 mice that were given vaccines (2mg ovalbumin + 50ug LPS subcutaneously) 1 day prior to transfer. On days 1 and 2 after the transfer, the mice were treated with OX40 conjugates or OX40 antibody, then blood samples were obtained from tail snips and were stained for flow cytometry in order to determine the percentage of $CD45.2^+$ $CD4^+$ T cells were present in the host's periphery blood. Antibodies used include anti-mouse CD45.1 APC (eBioscience Cat.# 17-0453-81), anti-mouse CD45.2 FITC (eBioscience Cat.# 11-0454-81) CD4 Pacific Blue (Bio Legend Cat.# 100534)

Chapter 3: Results

3.1 – Selection of Cblb siRNA sequences.

Submission of the mRNA sequence to HPC Dispatcher (City of Hope) resulted in an excel file containing hundreds of sequences that were subsequently ranked according to DG and lowest melting temperatures. Six sequences that ranked highly were cloned into shRNA expressing vector pFRT, and tested using a dual luciferase expression vector containing the target of the shRNA sequences cloned to the 3' end of the renilla luciferase cassette. The first 3 sequences were tested both as 21-mers and 27-mer's, to test whether the longer form, as a dicer substrate, would be any advantage to knocking down the target message.

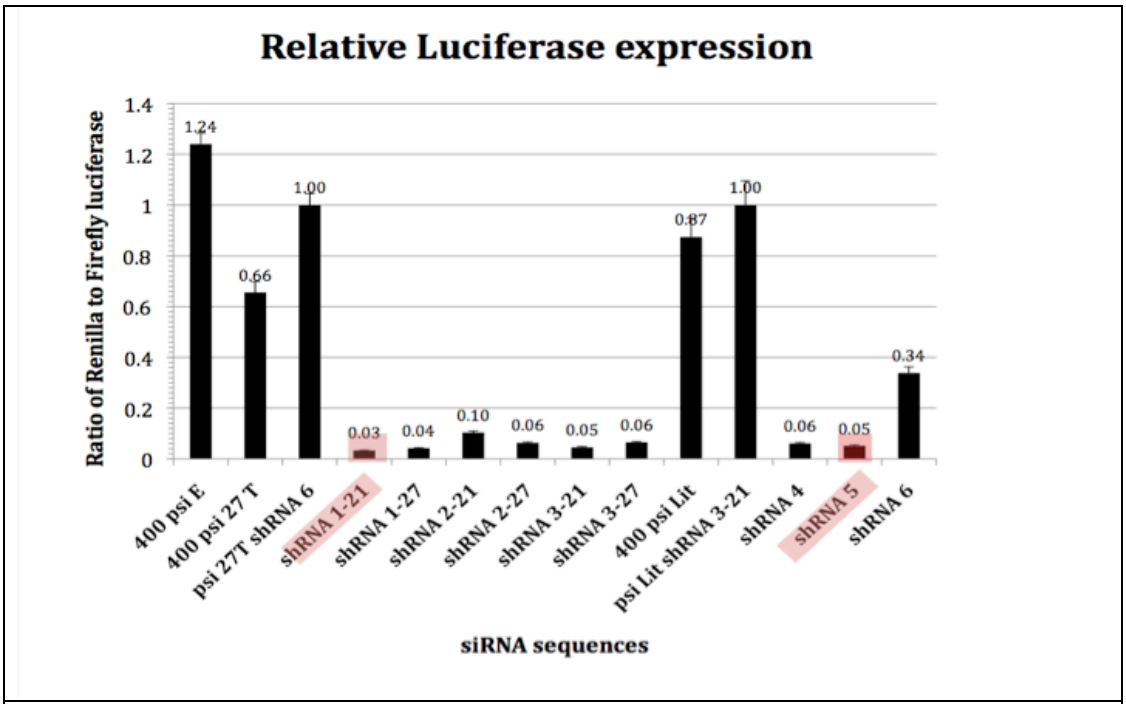


Figure 1. Efficacy of siRNA sequences determined by dual luciferase reporter assay

From the results of these assays, three sequences were selected to be incorporated into aptamer siRNA conjugates, namely, Cblb1, Cblb3 and Cblb5. After these sequences were incorporated into monomeric OX40 aptamer conjugates, the efficacy of the siRNA sequences were tested to ensure knock down of their target after hybridization to the aptamer. Figure 2 shows the effects on target reduction when Cblb3 and Cblb5 were incorporated into conjugates.

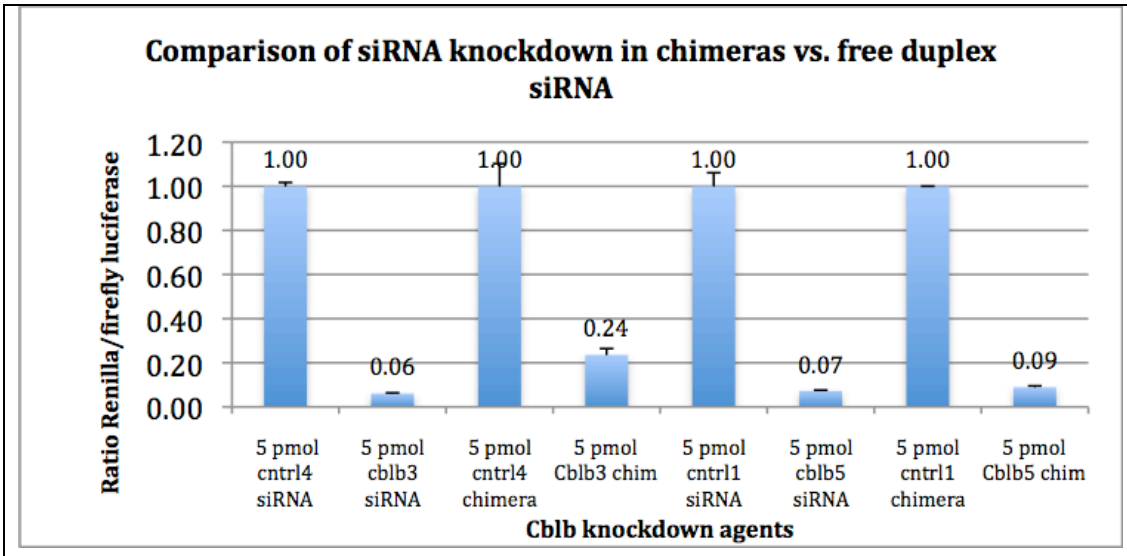


Figure 2. Luciferase assay showing that the Cblb siRNA sequences retain similar activities when incorporated into the OX40 monomer chimeras.

As the Cblb5 sequence was shown to have similar affects when incorporated into the conjugate, the dimer OX40 aptamer conjugate was produced and the activity of the sequence tested (Figure 3).

3.2 – Activity of OX40 Conjugates on activated T cells

Different T cell assays were set up to measure the effects of the conjugates on activated $CD4^+$ T cells. First, levels of Cblb mRNA were measured in proliferating T cell

cultures at 24hr time points to establish a baseline pattern of Cblb expression. Aptamers were added at Day 2 when OX40 expression on activated $CD4^+$ T cells usually peaks. At the indicated time points, cultures were harvested, RNA isolated, cDNA reverse transcribed and Cblb mRNA levels measured using qPCR. Figure 4 shows that while the OX40-Cblb conjugates all seemed to lower the amount of Cblb relative to the OX40-Cntrl conjugate, the levels of Cblb in the proliferating cells that had not received any conjugate was sometimes less than all the other conditions. These results suggested that

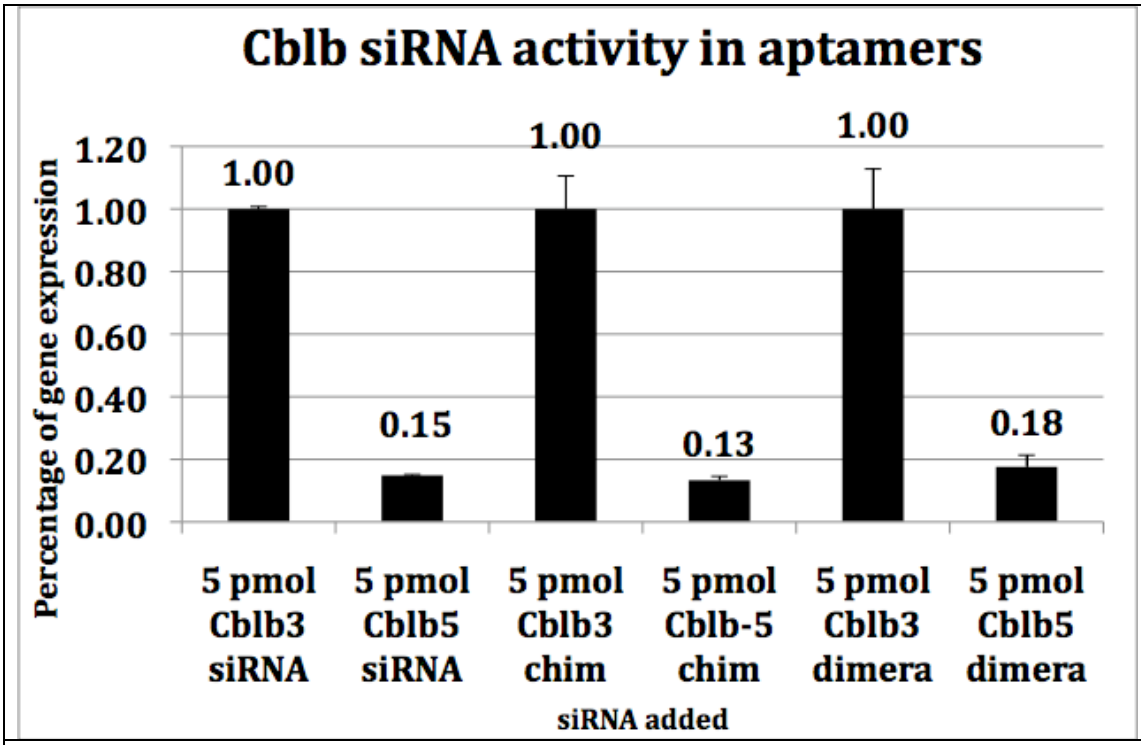


Figure 3. Cbl-b siRNA in monomeric and dimeric OX40 conjugates have similar knockdown activity compared to free duplex siRNA.

the monomer conjugate may not have been optimal for delivering siRNA to T cells, and that the increased recycling rate of receptors that were being activated by an OX40 dimer conjugate may have been a better candidate.

The dimer conjugate was tested using the OTII system. 10^5 splenocytes purified from OTII mice were incubated with ova peptide at specified concentrations. Dimer conjugates were added at the time of plating, and costimulation was assayed using different methods. In figure 5, both OX40 conjugates with Cblb3 and Cblb5 sequences were tested alongside OX86 (mOX40 mAb) in a costimulation assay where proliferation was measured using a thymidine uptake assay. In this and repeated assays, OX40 costimulation seemed to inhibit proliferation.

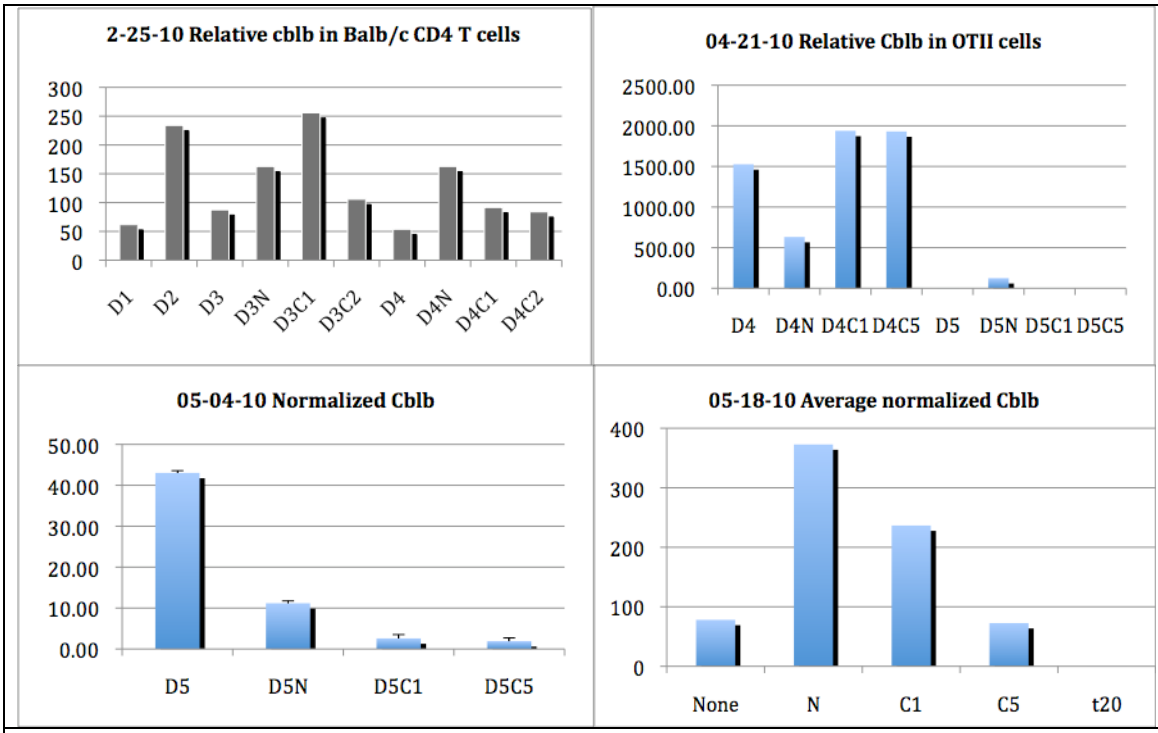


Figure 4. Quantification of Cbl-b levels in activated CD4+ T cells relative to GAPDH expression after treatment with chimeras. Four representative experiments are shown. KEY: D-day; N-coNtrol chimera; C-Cbl-b chimera; C1: Cblbsi#1 chimera; C5=Cblb si#5 chimera

It was later determined that the initial stimulation of the OTII splenocytes may have been too high. The OTII stimulation was re-titered and OX86 costimulation measured via a thymidine uptake proliferation assay. Figure 6 shows that OX86 costimulation resulted in slightly enhanced proliferation with suboptimal stimulation.

Costimulation assays were repeated with three different levels of initial stimulation with ova peptide. Instead of proliferation, costimulation was measured using IFNγ secretion.

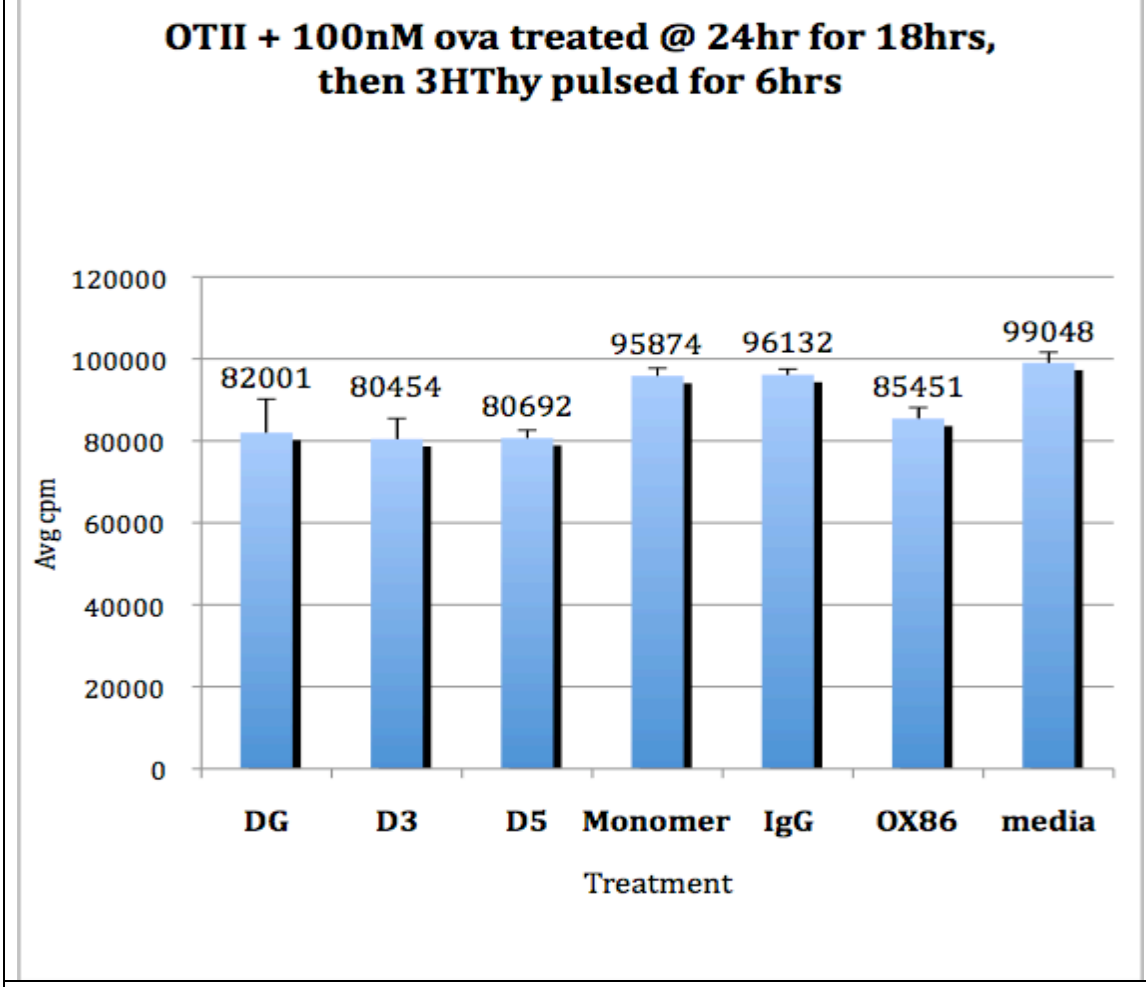


Figure 5. Inhibitory action of OX40 agonists. This proliferation assay shows that OX86, Dimer OX40-GFP and Dimers OX40-Cblb#3 and #5 inhibit proliferation of T cells relative to negative controls.

Figure 7 shows IFNγ secretion of suboptimally stimulated OTII splenocytes that were costimulated with OX86, OX40 dimer GFP control, and OX40 dimer Cblb5 at three different concentrations: 33nM, 66nM and 120nM. The general trend of the results shows costimulation with both OX86 and OX40 aptamers, with the Cblb5 conjugate performing better than the GFP control conjugates.

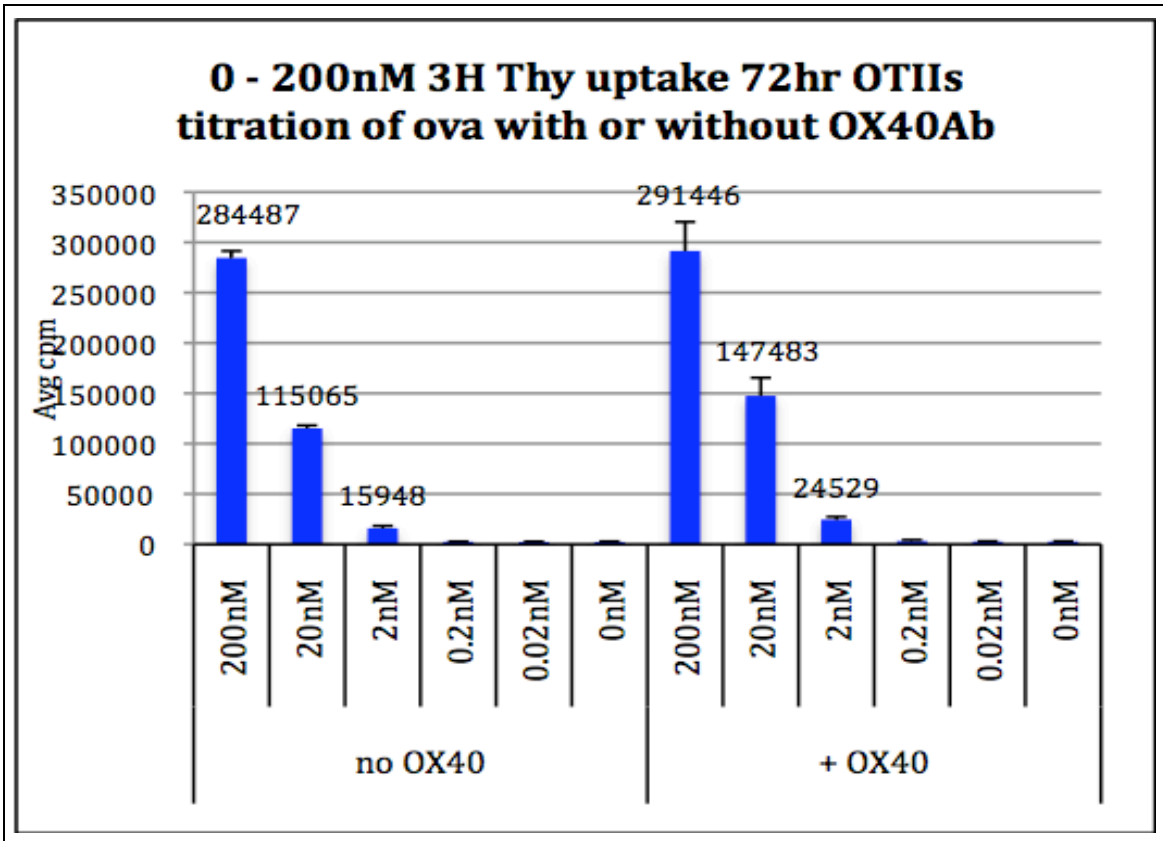


Figure 6. Proliferation of OTII's in presence of OX40Ab with decreasing ova peptide

These IFNγ secretion assays were repeated with similar trends of results. In Figure 8, a pool of random aptamers (library) was used as a negative, non-OX40 specific control. The results showed that while the OX40 dimer with control GFP was

comparable to OX86, the positive control, the OX40 dimer with Cblb5 siRNA outperformed them both. Based on this data, *in vivo* proliferation assays were planned to see if this effect was perpetuated.

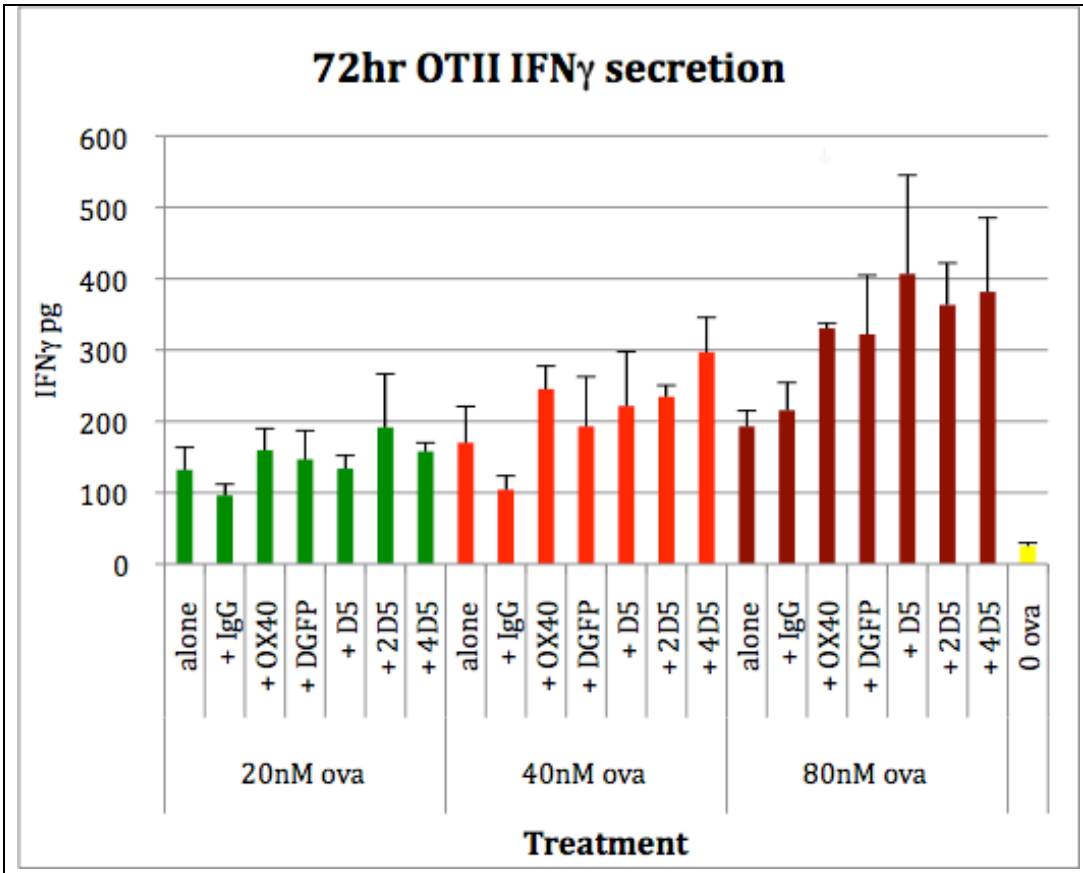


Figure 7. IFNg secretion of OTII cells stimulated with varying ova peptide plus dimer OX40 conjugates or control antibodies. DG- dimer OX40-GFP, D5-dimer OX40 Cblb#5, 2D5 - 2x D5

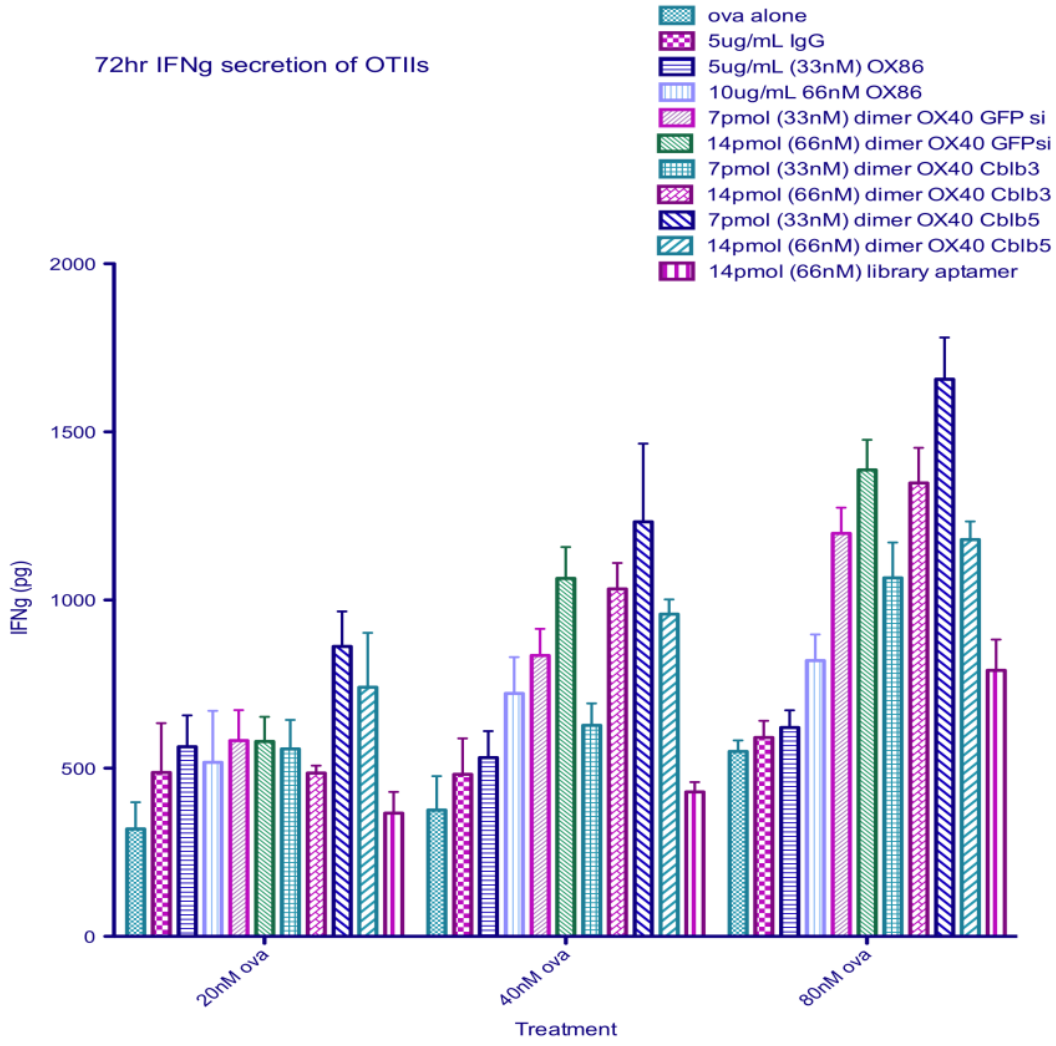


Figure 8. IFNg secretion of ova peptide stimulated OTII's with OX40Ab, OX40 dimer GFP and OX40 dimer Cblb5

3.3 *In vivo* characterization of conjugates

An adoptive transfer model was established to examine costimulatory effects on OTII $CD4^+$ T cells transferred from CD45.2+ OTII transgenic mice, into CD45.1+ congenic mice. The cells were transferred 1 day prior to being administered an ovalbumin vaccine on the inguinal flanks. The OX40 treatment of either OX86 or dimer

conjugates were administered as described in the methods, and the expansion of OTII cells as a proportion of all peripheral $CD4^+$ T cells was measured by flow cytometry from samples obtained by tail bleeds. Setting up a base line expansion and contraction of OTIIs was done, where OX40 costimulation caused the expansion to increase from approx. 12% of all CD4s to approx 25%, at day 3(Figure 9).

When this assay was repeated with two groups receiving the OX40-GFP conjugate, the positive control failed to enhance expansion (Figure 10). This was perhaps due to insufficient antibody being used, as in that specific assay, 50ug of antibody was given instead of the typical 100ug. Upon repeating this a third time, results confirmed that the OX40-Cblb conjugate was not able to enhance expansion of the OTIIs *in vivo* (Figure 11) compared to OX86.

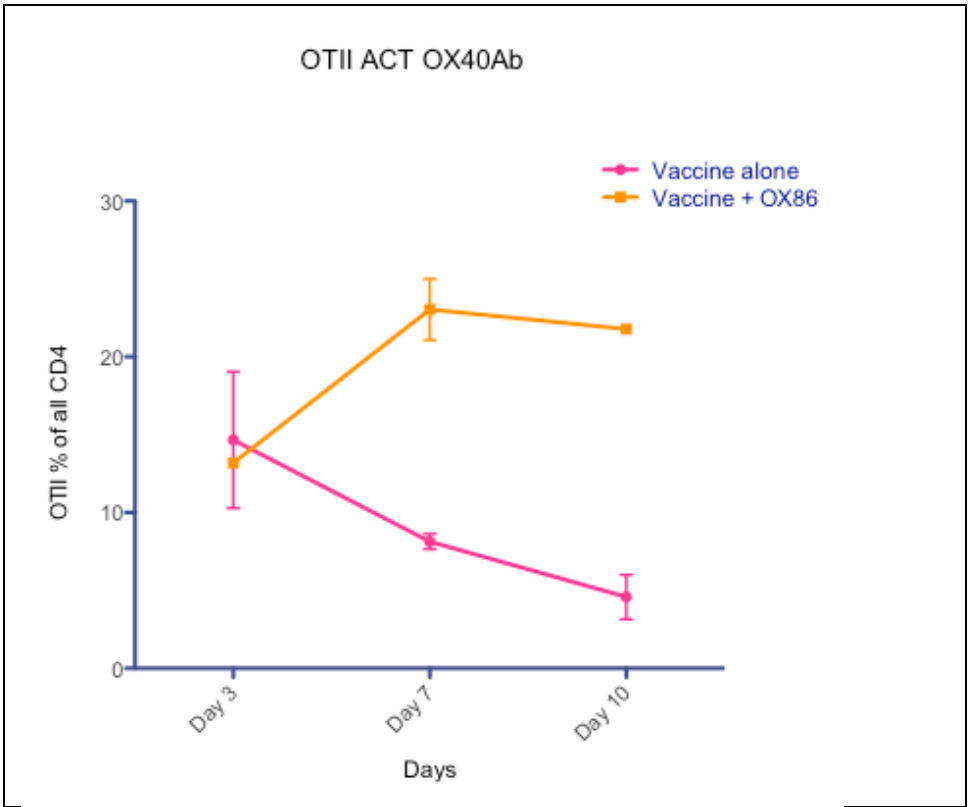


Figure 9. Vaccine induced expansion of OTII T-cells with OX40 mAb therapy

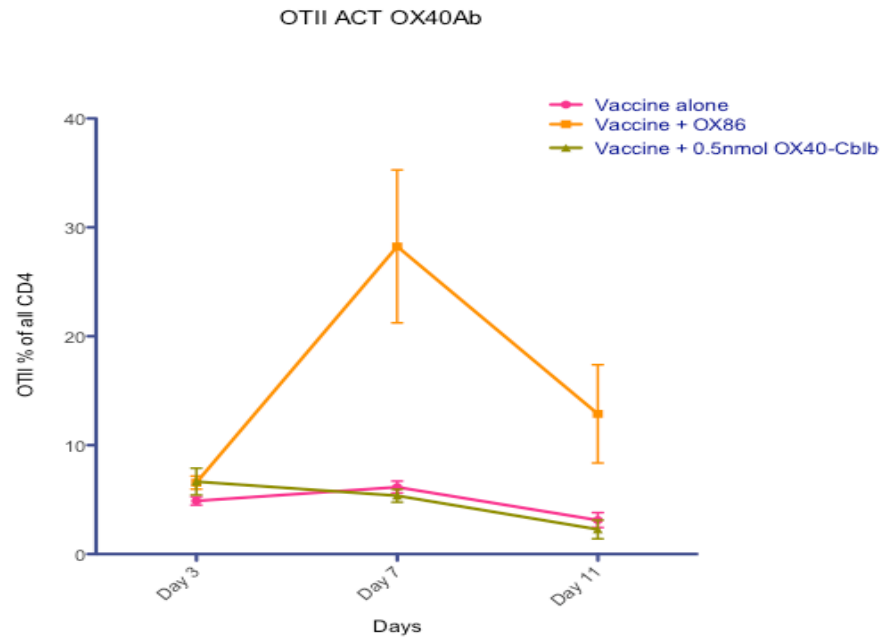


Figure 10. Expansion of OTII's with vaccine and OX40 mAb or OX40-GFP conjugate

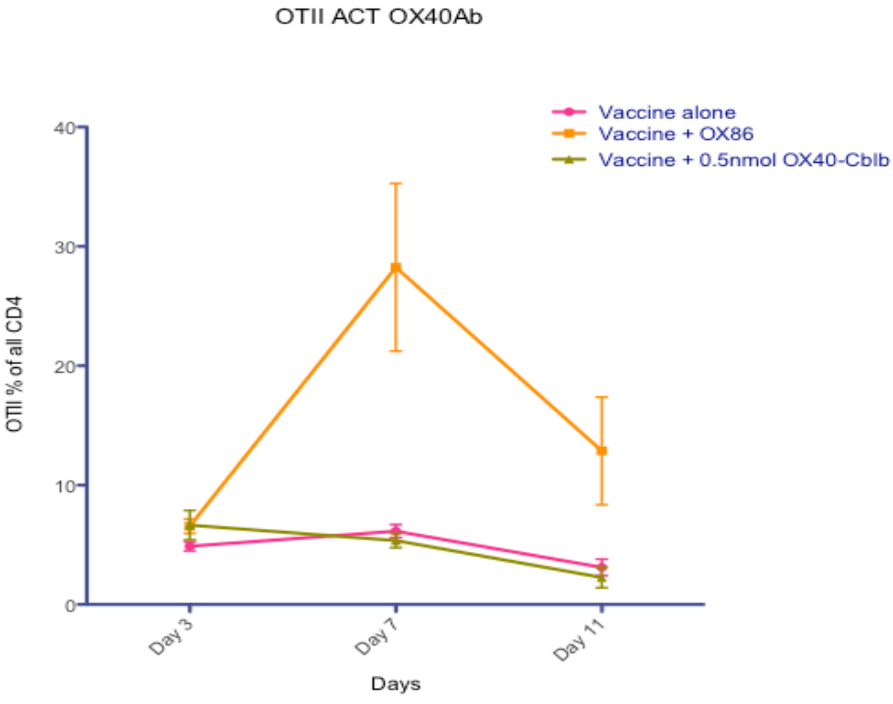


Figure 11. Expansion of OTII's with vaccine and OX40 mAb or OX40-Cblb conjugate

Chapter 4: Conclusions

In summary, siRNA to Cbl-b was designed, sequences were tested using an artificial system, and candidates incorporated into the 3' end of the OX40 aptamer. The conjugate was first tested using the same artifical luciferase system, after which it was tested via qPCR for Cblb knock down in proliferating T cells. There was no consistent knock down observed. The OX40 dimer Cblb conjugate was synthesized and knock down activity tested *in vitro*. Afterwards, its activity in costimulating T cells via OX40 ligation was tested *in vitro*. Although costimulation was achieved *in vitro*, the dimer conjugates were not effective when tested *in vivo* for costimulation of antigen specific $CD4^+$ T cells.

Chapter 5: Discussion

The dimeric OX40-Cblb conjugates that were produced were shown to have a phenotypic effect on isolated T cells. This effect was not seen with non specific RNA aptamers, and was similar to the effects seen with the costimulatory antibody. However, there are quite a few things that remain to be proven about the OX40-Cblb conjugate's *in vitro* and *in vivo* efficacy. *In vitro*, there was no direct evidence that Cblb mRNA expression in T cells was affected by the conjugate. While it was tested as a monomeric conjugate with inconsistent results, it was never tested in its dimeric form.

While binding assays were performed with the original OX40 aptamer, subsequent assays were not performed with the conjugate in its dimeric or monomeric form. Secondary structures of the OX40 conjugates were performed using RNAStructure 5.3 software from the Mathews Lab University of Rochester Medical Center. In theory, all modifications to the original sequence, when hybridized and stabilized, did not lead to significant changes in the secondary structure of the OX40 binding unit. This can be confirmed using 32P filter binding assays or by confocal microscopy.

Another thing not shown was OX40 dependent knock down of Cblb. One way to show this distinctly would be to have two cell lines; the parental, and one that is transfected to express OX40 on its surface. This parental cell line would ideally express enough Cblb to have its knockdown measurable by qPCR. After adding the conjugate to the media of both cell lines, if Cblb expression were reduced in the OX40 expressing cell line and not the parental, that would confirm that the conjugate was being internalized by binding OX40 on the surface, and subsequently knocking down Cblb expression. We examined different cell lines for endogenous Cblb expression, with discouraging results.

The levels of Cblb expressed were very low, being picked up around cycle 30 on qPCR. Cell lines that had slightly higher expression were not easily tranfected. A retroviral construct to transduce OX40 into Hepa1-6 cells was developed by another member of the lab. The resulting cell line Hepa1.6-OX40, could be used in delivery assays.

An interesting phenomenon was observed during the first *in vitro* OX40 costimulation assays; the presence of the OX40 antibody, as well as the dimeric conjugates, inhibited proliferation of $CD4^+$ T cells. The assay was repeated with few alterations, with similar results. Even though it was eventually theorized that lowering the initial stimulation could allow OX40 to costimulate instead of inhibit proliferation, the fact that OX40 could inhibit proliferation / activation in such small, tightly controlled environments as a 96 well plate, implies that in the complex system of a living organism, like a mouse, the effect of adding OX40 could go either way. This was partially shown by the fact that when ½ the dosage of OX40 mAb was administered to mice in order to costimulate proliferating OTII T cells (Fig. 10), their expansion was worse than the group that got ova vaccine (signal 1) alone. The dimeric OX40 conjugate at two different dosages also seemed to inhibit expansion in both that assay, and the following assay when the full OX40 mAb treatment was administered. It can therefore be theorized that, perhaps the OX40 mediated inhibition observed and trouble-shooted *in vitro* could also be corrected *in vivo,* for example, by titering down the ova vaccine.

OX40 usually needs to be trimerized on the cell surface to activate a signaling cascade leading to NFkB nuclear translocation. While OX40 mAb can, depending on the conditions, costimulate OX40 on T cells, for optimal signaling, an artificial ligand should act as its natural ligand, OX40L, and cause trimerization. An aptamer containing three

OX40 binding units would probably show optimal costimulation compared to the dimerized aptamer. This possibility should be investigated before any determination is made as to the feasibility of an activating OX40 aptamer conjugated to Cblb siRNA.

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