# Characterizing and modulating the canine T-cell costimulatory molecules CTLA-4 and CD28

James Bertram Jaffe

A thesis
submitted in partial fulfillment of the
requirements for the degree of

Master of Science

University of Washington
2016

Committee:
Roland Strong
Jesse Bloom
Ronald Stenkamp
Alexey Merz
Jennifer Lund

Program Authorized to Offer Degree:
Biochemistry

©Copyright 2016
James Bertram Jaffe

University of Washington

## Abstract

Characterizing and modulating the canine T-cell costimulatory molecules CTLA-4 and CD28

James Bertram Jaffe

Chair of the Supervisory Committee:
Dr. Roland Strong
Biochemistry Department

Current treatments for graft versus host disease, a major side effect of hematopoietic stem cell transplantation, have serious harmful side effects. The complexity of the cellular mechanisms leading to effector T-cell activation and deactivation is a hindrance to attempts to create more specifically acting immunosuppressive drugs. T-cell costimulation receptors CD28 and CTLA-4 and their associated antigen presenting cell ligands CD80 and CD86 have a poorly understood relationship. In particular, the mechanisms by which CTLA-4 expressed on the surface of effector and regulatory T-cells leads to suppression of T-cell activity is still unclear. In this project I attempted to characterize the relationships between these proteins including the canine analogues, which have so far been neglected, despite the importance of the canine model for development of treatments for graft versus host disease. I developed cell based reagents that will be useful tools for addressing these questions. I also attempted to develop and validate a specific CTLA-4 agonist for the purpose of suppressing active T-cells. This reagent is based around a soluble multivalent CD80 or CD86 molecule. I was able to produce milligram amounts of a reagent with desirable and functional binding properties. Further optimization of the expression system is required to produce sufficient product for in vivo testing.

**BACKGROUND**

For the last sixty years, efforts to treat leukemia have been plagued with obstacles. While whole body radiation was successful at suppressing cancer, it also obliterated a patient's immune system. The solution, hematopoietic stem cell transplantation (HCT), allowed an individual's immune system to be reconstituted from donor stem cells. This did not become a feasible procedure until understanding of the human leukocyte antigen (HLA) gene locus in the 1960's allowed doctors to match donors and recipients. Unfortunately, after successful transplantation donor immune cells (or grafted cells) would activate and attack the graft recipient leading rapidly to fatal complications. In the 1970s, cyclosporin was developed as the first immunosuppressive drug and allowed stable long term transplants. The rate at which HCT, or bone marrow transplants, was performed increased rapidly and in 2006, 50,000 HCT were performed[1]. Traditionally, HCT is used after myeloablative radiation treatment that completely eradicates the patient's cancer along with his or her immune system. An increasingly common regimen uses nonmyeloablative radiation treatment to first knock down a patient's immune system. Next, donor immune cells, which are not a perfect HLA match, are transplanted into the recipient[2] where they proceed to attack and eliminate the remainder of the patient's cancer in a "graft vs. leukemia response," leaving a functional chimeric immune system. One advantage of this method is that at no point is the patient without a functioning immune system. Unfortunately, the donated immune cells sometimes continue to target host cells resulting in graft versus host disease (GVHD)[1].

Acute GVHD occurs because of several steps. First, the host's tissue is damaged by radiation therapy. Damage in the gastrointestinal tract leads to the release of inflammatory stimuli, such as

lipopolysaccharide, into circulation. This leads to upregulated activity in antigen presenting cells (APCs), such as dendritic cells. These cells then have increased surface expression of major histocompatibility complex (MHC) and adhesion molecules. Active APCs then activate donated T-cells. Active CD4$^+$ T-cells release cytokines that upregulate activity of many components of the immune system including APC's and CD8$^+$ T-cells. Active CD8$^+$ T-cells begin destroying cells and contribute to further tissue damage. This creates a potentially fatal feedback loop of T-cell activation known as a cytokine storm[1].

Current treatment for GVHD requires heavy suppression of the immune system. This is normally accomplished with calcineurin inhibitors (CNI) such as cyclosporin. Calcineurin is a phosphatase that controls the activity of the nuclear factor of activation (NFAT) which controls IL-2 transcription and T-cell activation[3]. Unfortunately, CNI drugs lead to a wide variety of adverse reactions including serious kidney damage[4]. My goal is to better characterize the receptors involved in T-cell activation and deactivation in order to develop a more specific, less dangerous T-cell suppressing reagent.

T-cell activation and deactivation requires simultaneous engagement of both the T-cell receptor and either CD28 or CTLA-4, respectively[5]. Structurally homologous B-cell surface proteins, CD80 and CD86, bind to both CD28 and CTLA-4 (Figures 1, 2, 3) through a conserved MYPPPY motif. While similar structurally, there appear to be important functional differences between CD80 and CD86. These two ligands have their expression controlled by different cytokine signals. CD86 is expressed constitutively at low levels on dendritic cells while CD80 is not. Also, while expression of both CD80 and CD86 is upregulated on activated B-cells, T-cells,

macrophages, and dendritic cells, CD86 expression peaks much earlier. In the current model, CD86 is responsible for early T-cell activation in lymphoid tissue, while CD80 is responsible for maintaining T-cell activation in the periphery[6, 7].

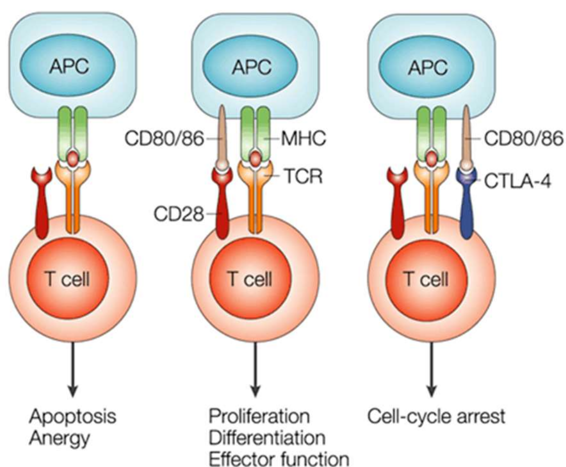



**Figure 1:** T-cells require a costimulatory signal along with the primary T-cell receptor signal in order to become active. Lack of a costimulatory signal leads to anergy (unresponsiveness). CD80/86 binding to CD28 and CTLA-4 leads to T-cell activation and deactivation respectively. Interactions between CD80/86 and CD28/CTLA-4 are in the μM range, though CD28 binding has a $K_d$ approximately 10x greater than CTLA-4. Figure borrowed directly from Alegre, et al.[8]

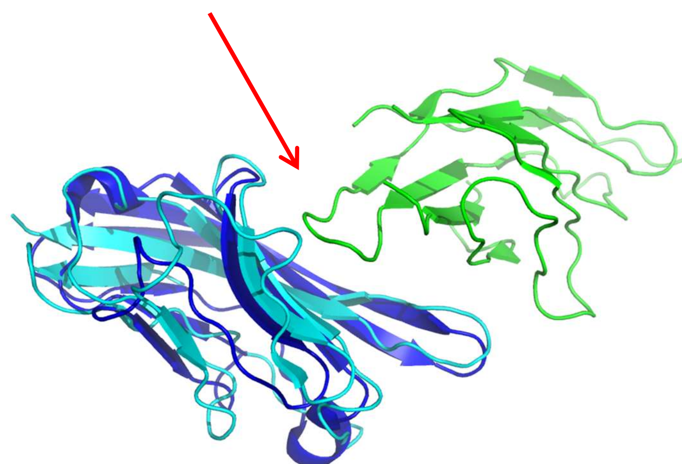


**Figure 2:** Crystal structure of human CD86/CTLA-4 (blue and green respectively) binding with the human CD80 (cyan) structure overlaid[9, 10]. The red arrow indicates the loop containing the MYPPPY motif on CTLA-4 which mediates binding. CD80 and CD86 have very similar binding sites for CTLA-4.

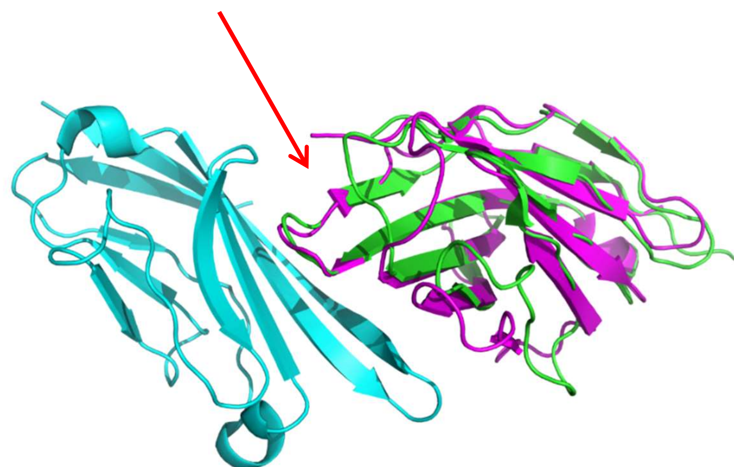


**Figure 3:** Crystal structure of human CD86/CTLA-4 (cyan and green respectively) binding with human CD28 (purple) overlaid[9, 11]. The red arrow indicates the almost identical loops on CD28 and CTLA-4 that contain the MYPPPY motif.

CD28 is a T-cell receptor that is constitutively expressed on 80% of human T-cells and 100% of murine T-cells. After T-cell activation, CD28 is transiently downregulated[12]. CD28 costimulation leads to an increase in transcription and mRNA stability for IL-2 (a key T-cell growth factor) and increased expression of anti-apoptotic protein Bcl-$X_L$[7]. The dependency of T-cells for CD28 costimulation before activation is situationally dependent. T-cells in the presence of tightly binding or large amounts of antigen and some memory T-cells may not need CD28 costimulation to activate. However, most T-cells become anergic when the TCR is engaged in the absence of CD28 costimulation. CD28 stimulation is also required for the activation and maintenance of regulatory T-cells (Tregs)[7, 13]. Active Tregs downregulate effector T-cell (Teff) function and they are necessary to avoid autoimmune responses[14].

In contrast to the well-studied CD28, the mechanism of CTLA-4 function is still uncertain. CTLA-4 expression is expressed on effector T-cells after they are activated, with a peak in expression at 48-96 hours after the activation event. Even activated T-cells express very little CTLA-4 on the surface[12]. Most is stored in intracellular vesicles. In contrast to this, Tregs express high levels of surface CTLA-4 constitutively[15]. While it is clear that the presence of CTLA-4 leads to a decrease in T-cell activity and proliferation, the mechanism of this pathway is currently uncertain[16]. There is some evidence that an intracellular signal is transduced by CTLA-4 leading to arrest of the cell cycle. Studies have shown CTLA-4's intracellular chain binding to several effectors including phosphoinositide 3-kinase and the tyrosine phosphatase SHP-2[17, 18]. However, other studies have failed to observe this interaction[19, 20]. Also, chimeric mice containing both T-cells with and without CTLA-4 are healthy[21]. This observation suggests that

the cells expressing CTLA-4 are affecting other cells rather than just being suppressed themselves.

Other evidence suggests that CTLA-4's sole function is to bind and block CD80 and CD86 to *prevent* a T-cell activation signal through CD28. Increased CTLA-4 on T-cells is also followed by a decrease in CD80 and CD86 levels on the surface of APC's and one group claims to have observed T-cells using CTLA-4 to remove CD80 directly from the surface of an APC via transendocytosis[22]. Also, while it is clear that Tregs require CTLA-4 to fulfill their Teff function, it is unknown how exactly this effect is mediated[15].

Regulatory T-cells were first identified in the 1970s, but detailed understanding of their function has remained elusive. In particular, the role of Tregs in the failure of the immune system to detect and attack tumors has driven a desire to understand these non-effector T-cells[23]. Tregs are the only cells that constitutively express CTLA-4 and so they are critically important for our understanding of the CTLA-4 mechanism. Much of the work on Tregs has been done in the murine system. Development of Tregs is driven by the transcription factor forkhead box P3 (FOXP3). These T-cells are $CD4^+$ and $CD25^+$ and are able to suppress the function of $CDd4^+CD25^-$ T-cells (effector T-cells or Teffs). Isolating human Tregs has proven to be a greater challenge. In humans, several subtypes of Tregs have been identified with different markers and different mechanisms of Teff disruption. Also, in humans Teffs will transiently express FoxP3 and CD25 while active precluding these receptors from being the sole Treg markers. However, evidence suggests that Tregs can be distinguished from Teffs by low CD127 levels. Further markers are being developed to improve isolation of Tregs from Teffs and to isolate Treg

subtypes. Unfortunately, this has led to different labs using different combination of markers to define Tregs throughout the literature. At present, the best way to identify Tregs is functionally, although this can be a laborious process and may not work well for small samples. Tregs suppress Teffs through several mechanisms. They induce Teff apoptosis, interrupt T-cell metabolism and release anti-inflammatory cytokines. They also bind to CD80 and CD86 on APCs via CTLA-4. This leads to a decrease in APC CD80/86 surface expression and decreases the ability of APCs to activate effector T-cells[24].

A large challenge in interpreting the literature concerning this system is the lack of good quality binding data. Many studies measure binding between these species through indirect observations[25] or simply quote previous binding numbers[26]. The most reliable and consistently quoted studies report that CD80 and CD86 both bind to CTLA-4 with a $K_d$ in the low μM range and both bind to CD28 about 10x more weakly[27, 28]. Evidence suggests that CD80 behaves as an obligate dimer, while CD86 behaves as a monomer in solution. Also, CTLA-4 appears to bind ligands divalently while CD28 appears to bind to ligands monovalently[28]. It is important to note that even well designed SPR binding experiments from fifteen years ago are inferior to experiments that can be conducted with current instruments and our ability to analyze data is vastly improved with modern computers that allow global fitting of sophisticated models to SPR data[29, 30].

While only 36 residues long, the intracellular domain of CTLA-4 is the focus of a great deal of attention. Several contradictory claims have been made about its functionality. Different labs have determined that the CTLA-4 intracellular domain binds to both kinases and phosphatases

and other labs have determined that CTLA-4 does *not* bind to these same ligands[17-20]. There is a consensus that the CTLA-4 intracellular domain is involved in regulating CTLA-4 localization, which may be very important for functionality as ~90% of expressed CTLA-4 does not reside on the surface of T-cells[16]. The YVKM motif interacts with AP-2 and allows CTLA-4 to be internalized via clathryn-mediated endocytosis[31]. The YFIPIN motif may also allow weak endocytosis to occur through the same system[32]. The destination of internalized CTLA-4 is not understood well. It may be sent for recycling, but evidence does exist that the lysosome sorts it into vesicular storage. Understanding the signals that control CTLA-4 localization and the amount available at the T-cell surface is fundamental to understanding overall CTLA-4 function.

Many drugs have previously been designed to interact with this system. Most efforts are aimed at activating T-cells in the context of tumor immunotherapy. Recently there has been increasing interest in the mechanisms some tumors develop to avoid immune surveillance. These mechanisms range from increased expression of signaling molecules that downregulate APC activity to the release of chemokines that attract Tregs. The overall effect is a tumor microenvironment with dysfunctional T-cells[14, 33]. Using an anti CTLA-4 antibody to blockade CTLA-4 and prevent T-cell deactivation has proven effective clinically against some melanomas[34]. Abatacept is a CTLA-4-Ig fusion that is used as an immunosuppressant clinically to treat rheumatoid arthritis. By blockading CD80 and CD86, this drug prevents T-cell activation through CD28. Belatacept is a second generation version of this drug that is used as an immunosuppressant for kidney transplants[5, 35, 36]. Attempting to use an anti CD28 monoclonal antibody as an immunosuppressant was a notorious failure in this field. The antibody, TGN1412, was intended to activate Tregs which would suppress other T-cells. Despite all suffering

multiorgan failure, none of the six human recipients of TGN1412 died during the clinical trial[37]. This failure highlights the importance of differences in behavior of these proteins in different systems. Tregs in particular behave quite differently in the human, murine, and other systems[14, 38, 39]. While markers for Tregs are fairly well-established in mice, there is still no defined, consistent marker definition for human Tregs and we are still exploring the variety of ways Tregs can develop in humans. While the discussion about human Treg markers continues, we are left with a functional definition of a Treg as a T-cell that suppresses Teffs[38, 40].

## OBJECTIVES/PROJECT GOALS

Aim 1:

*Completely characterizing the soluble binding behavior of human and canine CTLA-4 and CD28 Fc fusions with CD80 and CD86.*

Well-controlled quantitative data on the binding properties of CTLA-4 and CD28 with their ligands is sparse in the literature. In addition, information on the binding properties of the canine analogues of these proteins is nonexistent, despite the importance of the canine model in the development of T-cell suppression therapeutics. I attempted to use surface plasmon resonance (SPR) experiments to determine the binding kinetics of soluble constructs of these proteins.

Aim 2:

*Reengineering CD80 and/or CD86 to produce a reagent with high avidity and highly specific binding for CTLA-4.*

I attempted to achieve highly avid versions of CD80 and CD86 by fusing them with multimerization domains, such as the trimerization domain from human collagen or the heptamerization domain from human C4b (a participant in the complement system). A large

variety of designs can be achieved by mixing and matching domains from CD80/86, multimerization domains, and other useful domains. I attempted to use these tools to take advantage of slightly different binding properties observed in the canine system in order to create a reagent that binds specifically to CTLA-4 with little or no CD28 binding. Reagents were validated in a variety of systems ranging from soluble binding studies, to human embryonic kidney cell (HEK 293) based binding assays, to T-cell binding assays, to T-cell functionality assays.

## METHODS/TECHNIQUES

Protein production: All proteins are expressed in HEK 293 cells using the Daedalus lentiviral expression system. Daedalus allows the rapid generation of stably transduced mammalian cell lines for the reliable production of large amounts of protein that do not express well in bacteria or other cell lines. Typical constructs include a signal peptide for protein export, the protein of interest, and at least one tag for purification (usually 6xHis). Siderocalin is also commonly included. This protein expresses very strongly and can pull less well-behaved domains through the cell's folding and export systems. Target sequences for proteases such as thrombin and/or TEV sites can be used to remove some or all components other than the protein of interest after purification. Several examples of constructs are included in Figure 4.

Protein purification: Proteins are harvested from the HEK cells' growth media and purified in two steps. The first step depends on a tag or the Fc domain of an Fc fusion. The second step is size exclusion chromatography (SEC). Well folded proteins display single monodisperse peaks on SEC and clean, nonladdered bands on nonreduced and reduced SDS-PAGE gels. Protein

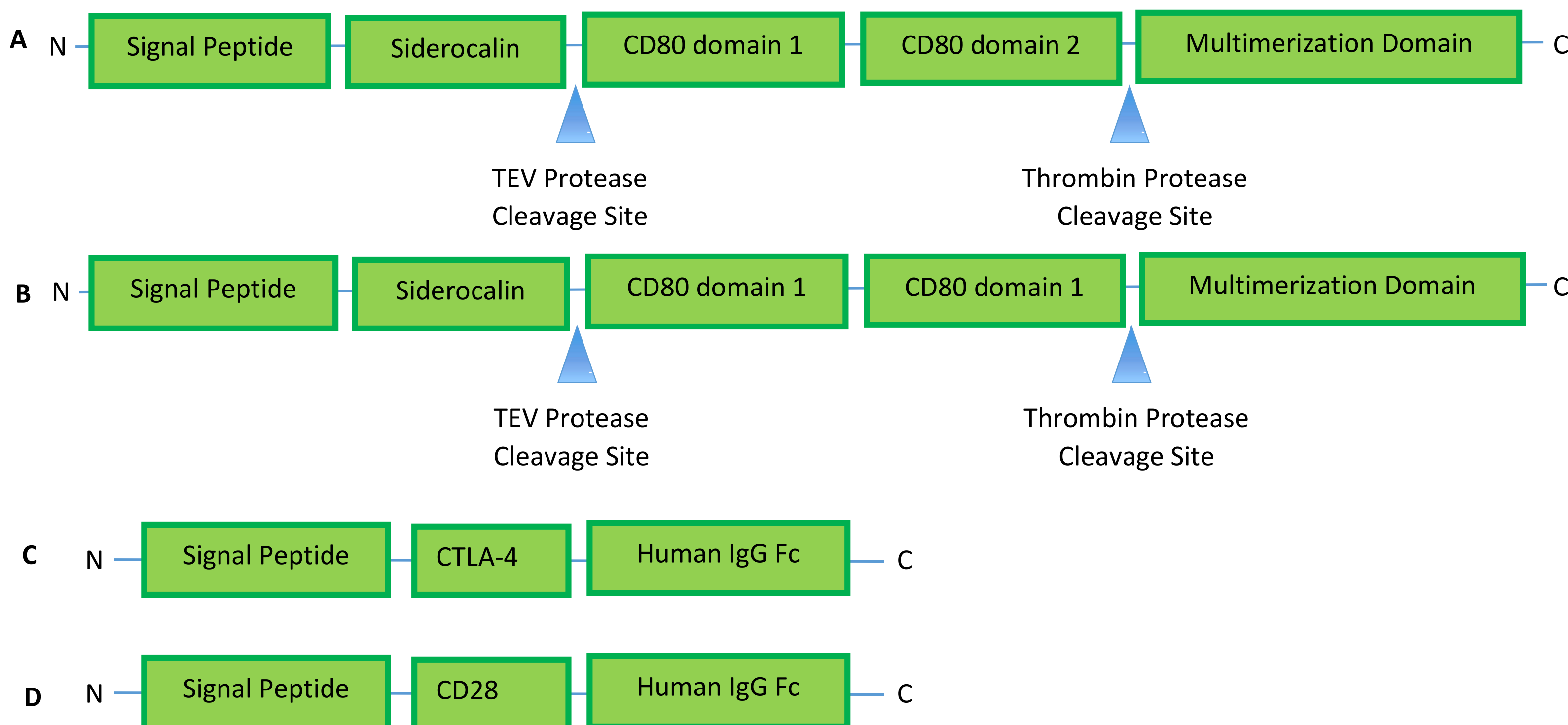



**Figure 4:** Examples of constructs for soluble protein reagents. A: After TEV cleavage this construct produces a heptamerized CD80 containing both the binding and dimerization domain. The signal peptide instructs the HEK 293 cell to process and export the protein. Siderocalin is a removable fusion protein that folds very well and helps pull other proteins through the folding pathways. B: In order to generate more consistent CD80 dimers, we can leave out the dimerization domain and directly link two copies of the CD80 binding domain. C/D – we can create soluble, obligate dimers of CTLA-4 and CD28 by adding an IgG Fc domain.

background in the HEK cell media is limited. Since I am not lysing cells, often only a single purification step is necessary.

Biacore: CD28 and CTLA-4 Fc fusions are anchored on a sensor chip using antibody capture. Different concentrations of analytes, spanning from 10x to 0.1x the $K_d$, are injected in randomized duplicate runs. Sensorgrams are blank-corrected by the double-subtraction method and the final double-referenced data is analyzed with BIAevaluation 4.1 software, globally fitting

data to derive kinetic or equilibrium binding parameters. Commercial monoclonal antibodies against the extracellular domains of human CTLA-4 and CD28 are available for use as controls. 1c6, a monoclonal antibody against canine CD28, is also used as a control.

Cell based assays: HEK 293 cells with CTLA-4 and/or CD28 expressed on the surface are generated with similar constructs to those described above. However, the WT transmembrane domain are included so that, upon export, the receptors end up anchored in the cell membrane. Also, mCherry is added to the C-terminal (intracellular) end of the construct. Fluorescence microscopy is used to visually verify that protein is migrating to the cell surface. These cells are used for binding assays with soluble CD80 or CD86 constructs. Fluorescein isothiocyanate (FITC) is conjugated to the soluble proteins. Qualitative binding can be determined by visually inspecting cells for colocalization of FITC and mCherry. Quantitative binding comparisons can be achieved with flow cytometry. T-cells are obtained from our collaborators in the Storb lab at the FHCRC. Naïve T-cells should express only CD28 while activated T-cells express both CTLA-4 and CD28. Binding of CD80 or CD86 constructs to naïve or activated T-cells can also be accomplished with flow cytometry.

Functional Assay: I can measure the ability of a reagent to suppress T-cell proliferation using the mixed leukocyte reaction (MLR). Blood containing T-cells is exposed to irradiated allogeneic lymphocytes (from another animal of the same species). The living T-cells will rapidly proliferate over a period of several days. This assay tests the reagents' ability to suppress proliferation of activated T-cells.

**CHALLENGES**

Protein Expression: I minimized the risk that the proteins fail to express by working with the mammalian expression system. HEK cells should produce appropriate glycosylation patterns and disulfide bonds. Using HEK cells has a high cost in time, money, and yield when compared to E. coli expression systems. However, the Daedalus system allows me to achieve high yields from human cell lines without subcloning. Most of the constructs made express sufficiently for SPR and cell based binding assays. The CD80 heptamer construct has proven toxic to HEK cells, possibly due to aggregation. Even monomeric CD80 has a tendency to dimerize in solution and the CD80 heptamer may be inappropriately and inconstantly multimerizing. I attempted several different iterations of the CD80 heptamer construct, but I was not able to solve this problem. However, heptameric CD86 shares useful properties with CD80 and it expresses far better in HEK cells.

Difficulties Measuring Binding: Determining binding kinetics using SPR requires running several different concentrations of analyte over a surface with bound CD28 and CTLA-4 Fc fusions. A model is generated to fit this data and generate binding numbers. Binding interactions with first order kinetics can be modeled to obtain values for $K_d$. It is also possible to accurately model a system with a bivalent analyte. Modeling anything more complicated is currently not useful for obtaining accurate binding numbers. Whether the soluble CD80 and CD86 constructs are monomer or dimers in solution, binding numbers can be obtained. However, if they are a mixture, it is not possible to obtain an accurate $K_d$. Accurate quantitative binding kinetics are difficult with my constructs.

No Intracellular Signal: It is still unknown exactly how CTLA-4 leads to T-cell suppression. It is possible that CTLA-4 acts completely extrinsically of the cell. In this case it would be impossible to create a CTLA-4 agonist, since CTLA-4 doesn't actually transduce a signal. Any reagent that binds CTLA-4 would block it from functioning and lead to T-cell activation. While this result would make aim 2 impossible to complete, having a panel of CTLA-4 specific reagents based on CD80 or CD86 with a variety of possible oligomeric states is still a very powerful tool. It can be used to further probe CTLA-4 mechanisms in Tregs and Teffs in the future.

## Results & Discussion

Creating a soluble specific agonist for CTLA-4 requires overcoming two challenges. A reagent that is specific for CTLA-4 without binding to CD28 is required. This has already been produced in the form of CTLA-4 specific antibodies. However, creating a reagent that is an agonist is more challenging. In order to accomplish this I attempted to take CTLA-4's natural ligands, CD80 and CD86, and modify them to have the required properties. By removing the intracellular and transmembrane domains, I produced soluble CD80 and CD86. The wild type versions of these proteins do not bind their ligands very well ($K_d$ in the low μM range) and depending on how the signal is transduced through CTLA-4, a single CTLA-4/CD80 interaction may not have any effect. In order to solve this I fused multimerization domains to the c-terminus of CD80 (Figure 5). These domains are borrowed from other human proteins and when fused to the c-terminus of CD80, they generate dimers, trimers, or heptamers of CD80. As the multimerization order increased, so did the binding strength to CTLA-4 Fc fusions (Figure 6).

Multimeric wild type CD80 was originally produced to serve as a control for other CD80 variants. However, initial data from SPR binding studies showed this construct able to preferentially bind to CTLA-4 over CD28 (Figures 7,8). This result was both surprising and promising as heptameric CD80 might fulfill the requirements. At this point all of the constructs

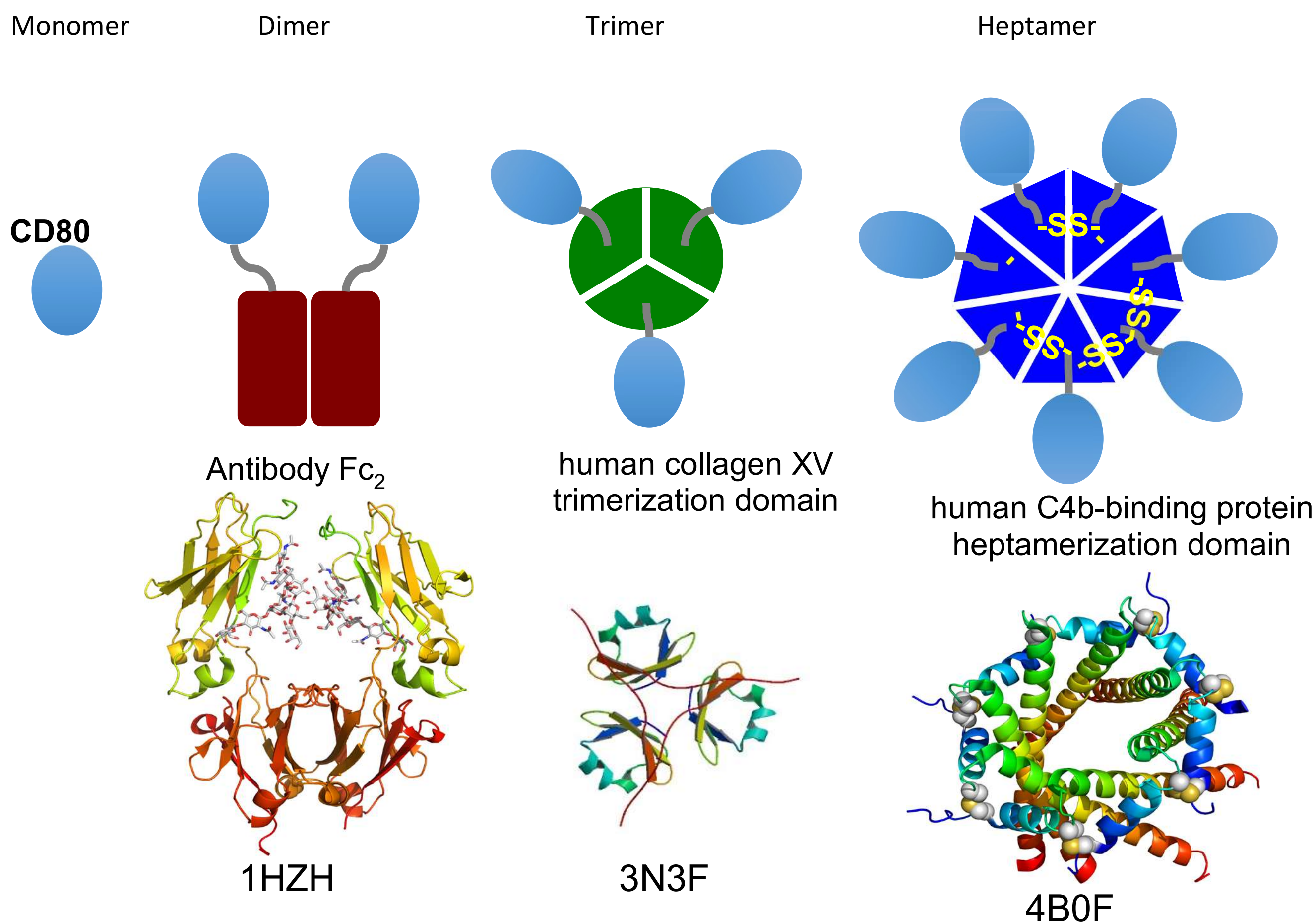
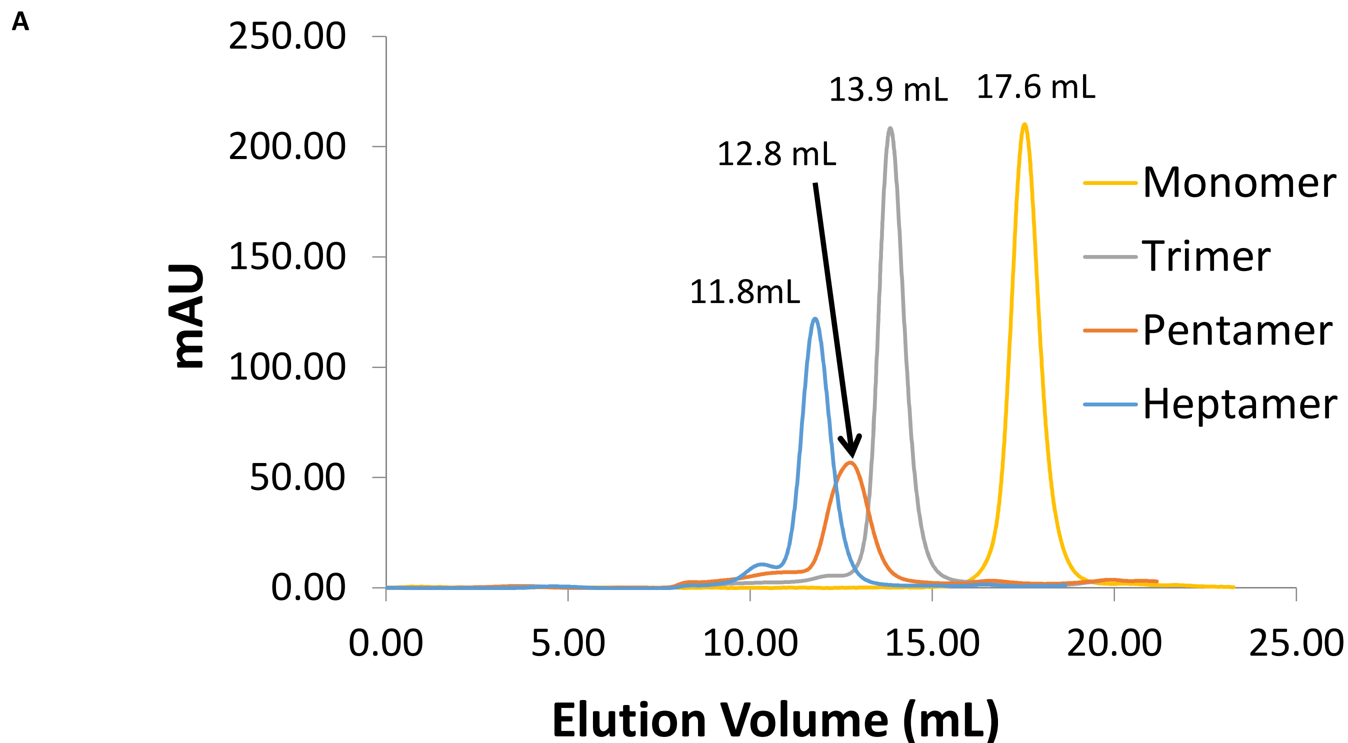



**Figure 5:** By attaching monomeric soluble CD80 (indicated by blue ovals) to multimerization domains we can achieve varying levels of avidity. The top row is a schematic showing how each construct is organized. Below are structures showing the organization of each multimerization domain with the corresponding pdb code. Note that each subunit of the heptamerization domain is disulfide linked to its neighbors producing a highly stable multimer.

A




B

Peak Fractions

CD80 WT

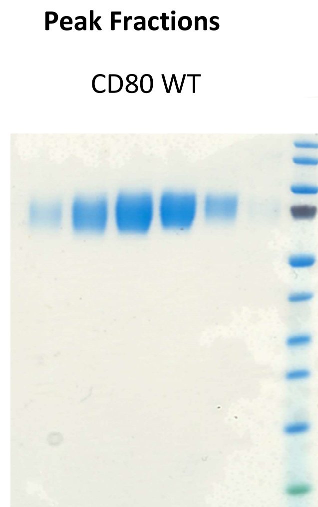


**Figure 6:** A: Overlay of size exclusion column traces for monomeric CD80 and several CD80 multimers. Larger elution volumes correlate with smaller proteins. Single monodisperse peaks indicate that the protein is well folded and sufficiently purified. B: An SDS-PAGE with material from several peak fractions of CD80 monomer. The single band indicates that the sample has been successfully purified. Band fuzziness is due to the inherent randomness of glycosylations generated on the protein surface.

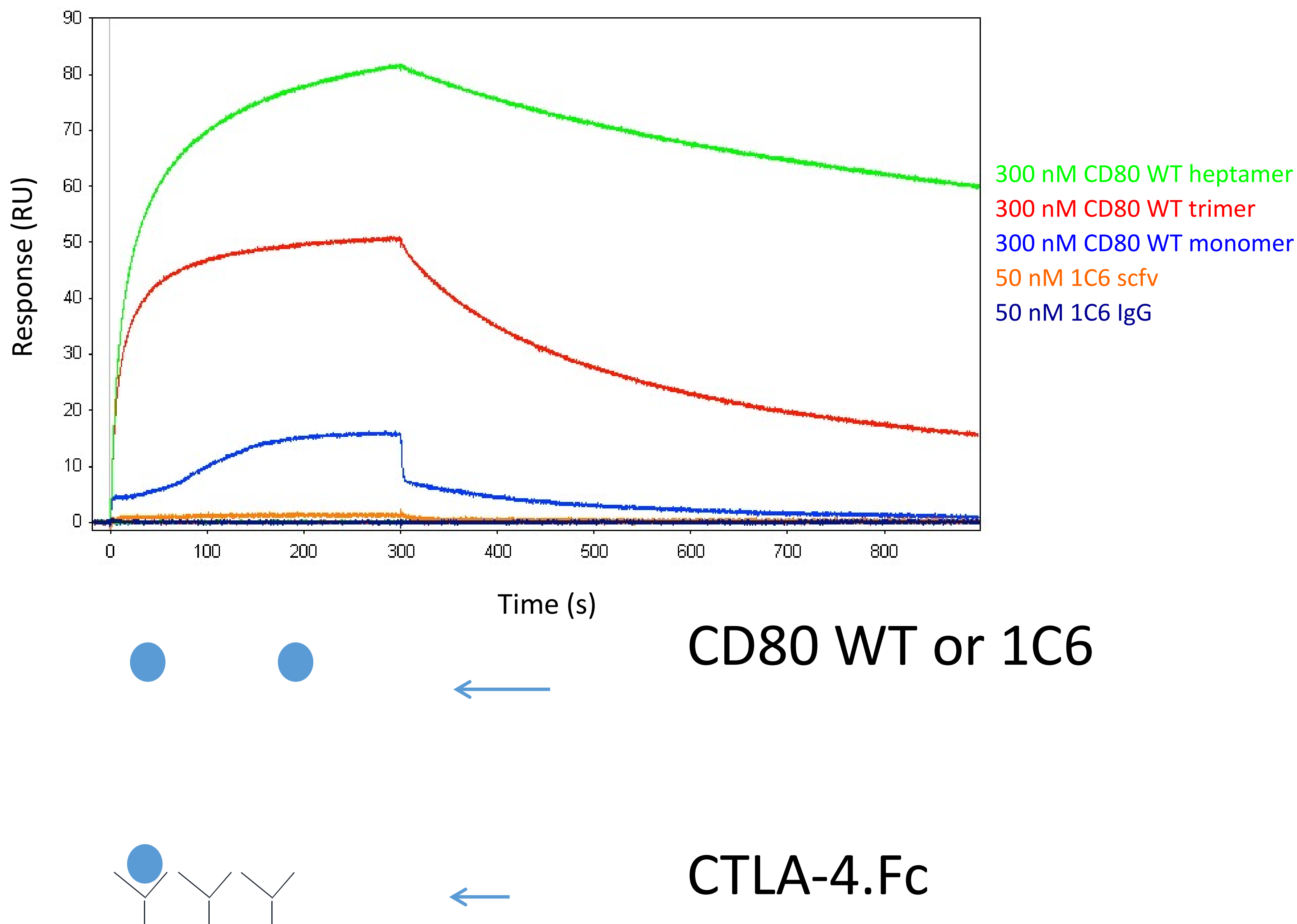



**Figure 7:** A Trace from a surface plasmon resonance experiment. As indicated in the schematic, the CTLA-4 Fc fusion was attached to a surface and CD80 monomers or multimers or 1c6 was flowed over it. The increasing section of the curve is the association phase. The decreasing section is dissociation. Higher responses are correlated with tighter binding. 1c6, an antibody for canine CD28, failed to bind. Increasing oligomerization states of CD80 bound increasingly well to CTLA-4 with the heptamer showing the tightest binding.

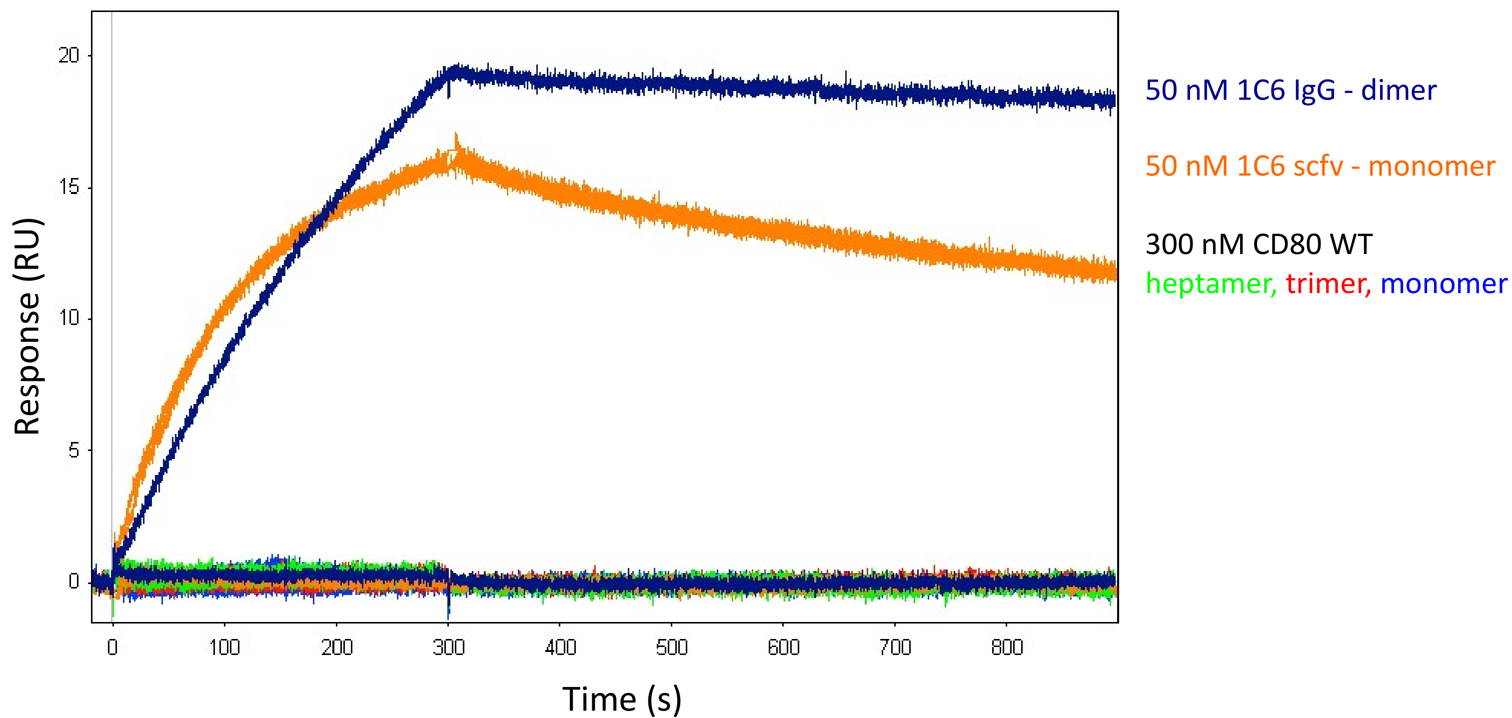





**Figure 8:** A Trace from a surface plasmon resonance experiment. As indicated in the schematic, the CD28 Fc fusion was attached to a surface and CD80 monomers or multimers or 1c6 monomers or dimers were flowed over it. The increasing section of the curve is the association phase. The decreasing section is dissociation. Higher responses are correlated with tighter binding. 1c6, an antibody for canine CD28, successfully bound. No CD80 showed any indication of binding to CD28. Of note, the CD80 heptamer with a very high potential for avidity showed no binding activity.

were made using the canine analogues of CTLA-4, CD28, CD80, and CD86. The intention was to generate canine reagents that could be tested for functional properties in the canine system. Interestingly, canine CD80 heptamers also bind preferentially to human CTLA-4 over human CD28 (Figure 9). However, CD80 heptamer production provided two major hurdles. It was difficult to produce without endotoxin contamination. Endotoxin are mainly bacterial components that are potent activators of the innate immune system. The ultimate goal for these reagents was animal testing, and the limit for endotoxin contamination in the sample was very low in order to maintain animal safety and to avoid masking toxic effects of the reagent itself. Unfortunately, our lab was not set up for endotoxin free production and removing endotoxin from the purified protein product resulted in too much loss of material. After testing many different processing and cleaning steps for the equipment and the sample, I determined that the best solution was using equipment that had never been exposed to bacteria and eventually was able to access this equipment and produce clean protein.

Unfortunately, the other challenge for CD80 multimer production proved insurmountable. In order to collect more than preliminary data as to the efficacy and safety CD80 heptamers in cell and animal assays we required significantly more protein than for SPR experiments. While, HEK 293 cells could produce small amounts of product, CD80 heptamer production is associated with rapid cell death and large scale production has been impossible. Adjustments to the organization of the construct including the order of domains, the type of linkers used, and the signal peptide as well as adjustments to the HEK 293 transduction protocol did not improve the yield.

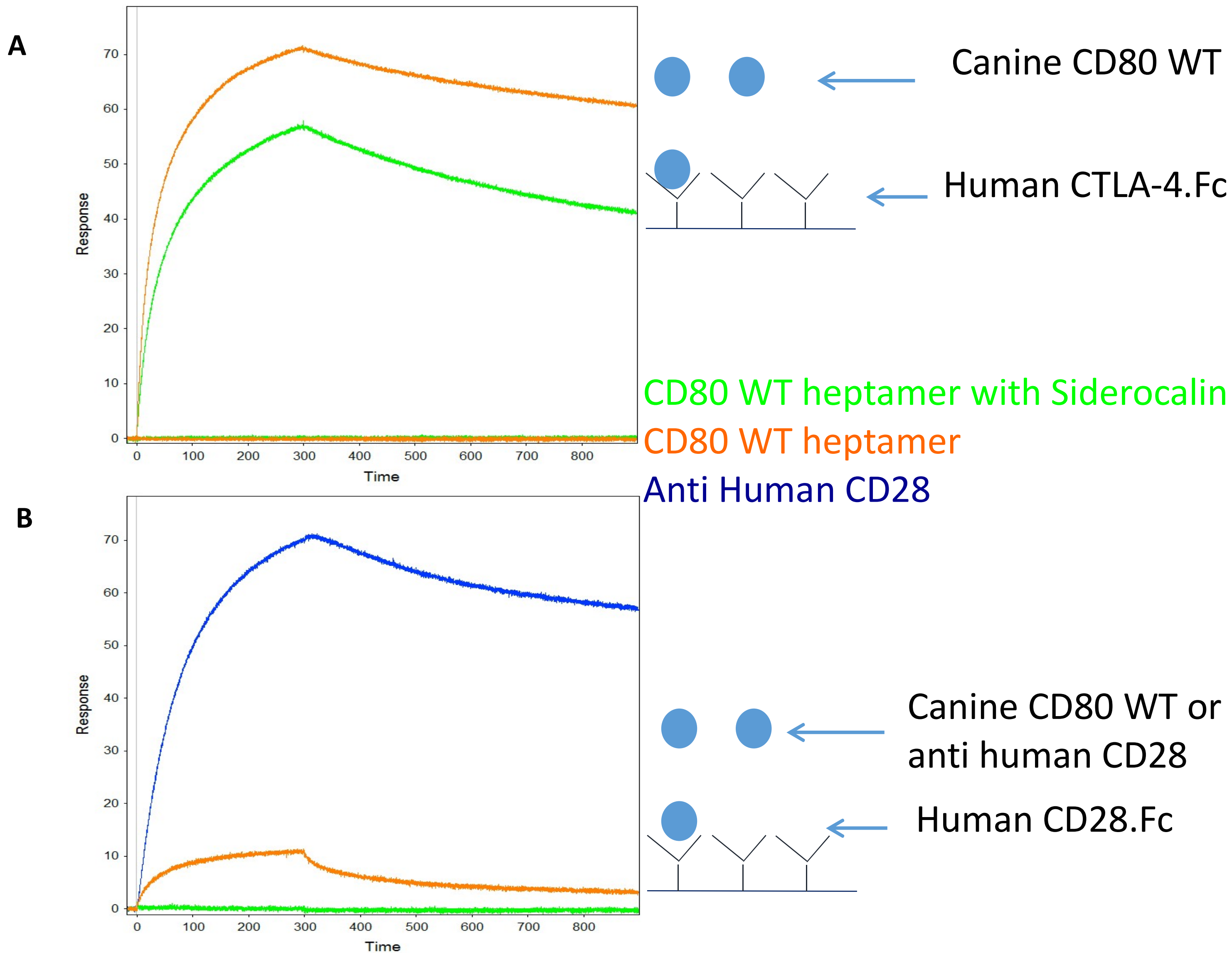



**Figure 9:** Traces from surface plasmon resonance experiments. The increasing section of the curve is the association phase. The decreasing section is dissociation. Higher responses are correlated with tighter binding. A: As indicated in the schematic, the human CTLA-4 Fc fusion was attached to a surface and CD80 heptamers or CD80 heptamers with fused siderocalin were flowed over it. B: As indicated in the schematic, the human CD28 Fc fusion was attached to a surface and CD80 heptamers or CD80 heptamers with fused siderocalin or anti human CD28 mAbs were flowed over it. Canine CD80 heptamers show relatively stronger binding to human CTLA-4 compared to human CD28. This effect is accentuated by fused siderocalin.

Despite these setbacks, the progress of canine CD80 multimers was promising. Preliminary experiments were done to test binding of this reagent to both naive and activated canine T-cells using FACS (Figure 10). FITC was conjugated to CD80 in order to detect which cells CD80 was binding to. Activated T-cells should have both CTLA-4 and CD28 receptors while naive T-cells should have only CD28. The CD80 multimers did preferentially bind to naive T-cells. A mixed leukocyte reaction was also performed in order to determine if CD80 multimers are able to inhibit T-cell proliferation (Figure 11). Mixing T-cells from different animals normally leads to rapid T-cell activation and proliferation. Preliminary results indicated that the CD80 heptamer was able to significantly decrease T-cell proliferation.

In order to do cell based assays to test binding and CTLA-4 trafficking I needed cell based receptor constructs. I produced full length CTLA-4 and CD28 constructs. These include both the intracellular domain and the transmembrane domain along with a C-terminal (intracellular) linked fluorescent protein. CD28 had a linked green fluorescent protein. I used a fluorescence microscope to visualize HEK 293 cells that had been transduced with either CD28 or CTLA-4. CD28 fused to green fluorescent protein (GFP) was observed successfully localizing on the HEK 293 cell surface (Figure 12A). CTLA-4, fused to mCherry (a red fluorescent protein), did not localize to the cell surface. Initially, it appeared that CTLA-4 was localizing to intracellular vesicles (Figure 12B). This is an unsurprising result as approximately 90% of wild type CTLA-4 expressed in T-cells is sequestered intracellularly. However, based on results in the literature the clathryn mediated endocytosis of CTLA-4 can be interrupted by adjustments to the intracellular domain of CTLA-4. I intended to determine the minimum mutation for CTLA-4 that would generate HEK 293 cells with observable amounts of CTLA-4 on the surface for cell based

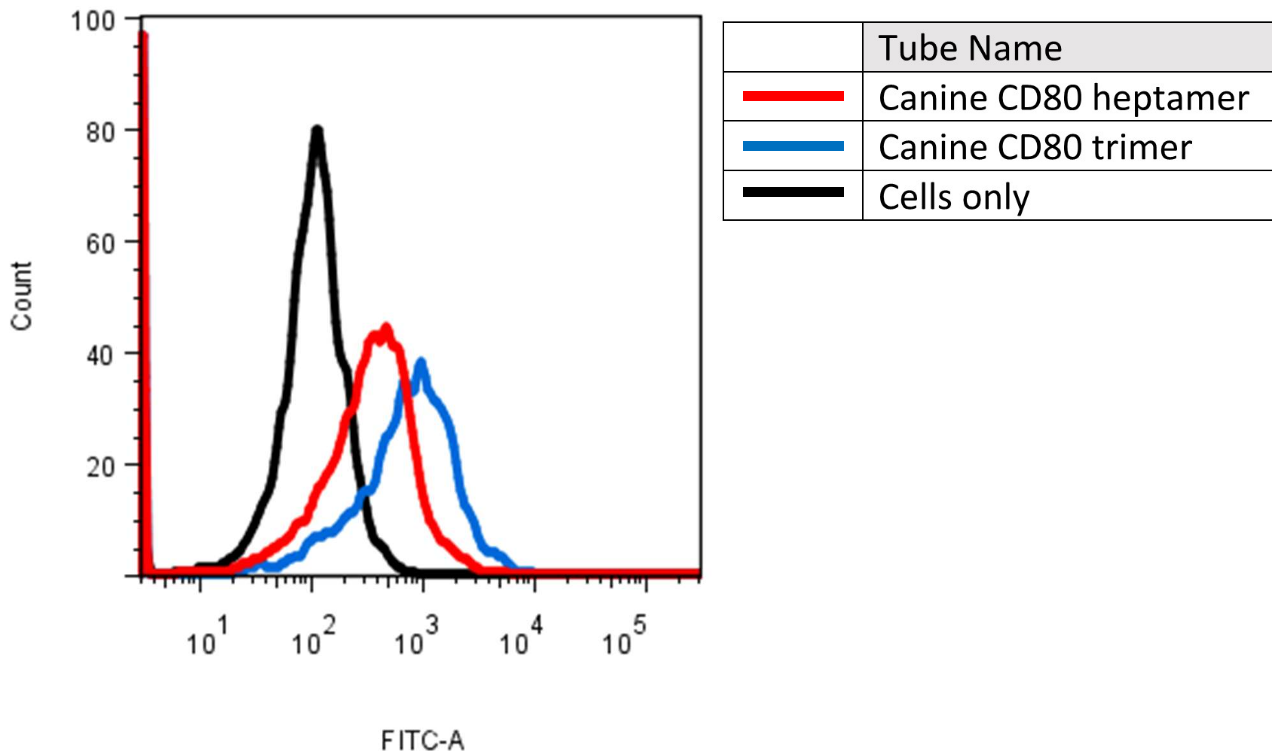



**Figure 10:** Fluorescence activated cell sorting (FACS) traces quantifying the relative amount of canine CD80 heptamer with attached FITC bound to binding to activated T-cells. The vertical axis counts the number of events with signal intensity defined by the horizontal axis. Both CD80 constructs show binding to cells. In a similar experiment with naïve T-cells, no binding was observed.

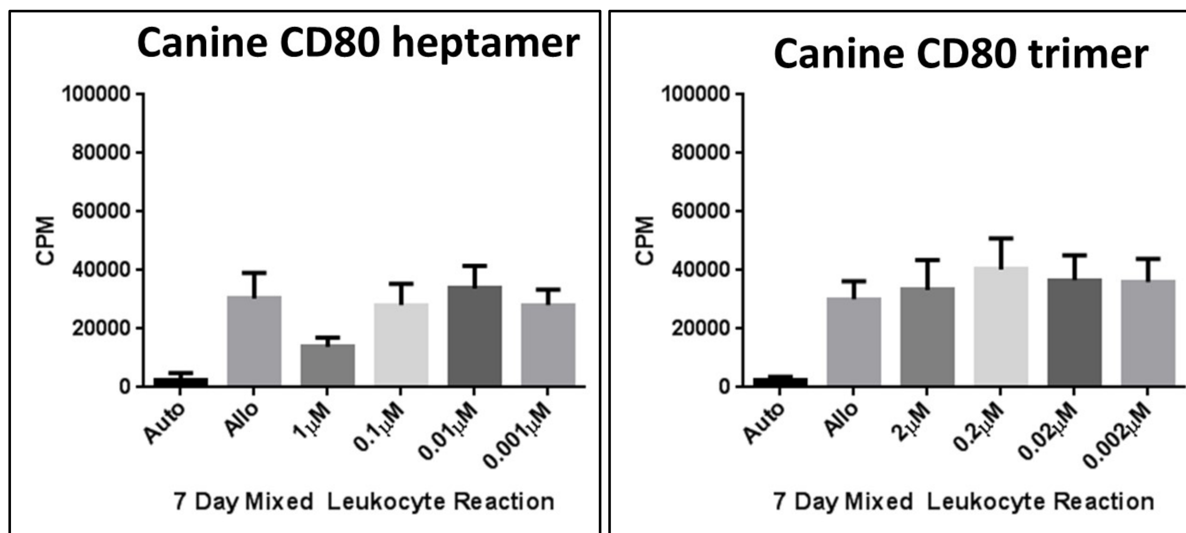



**Figure 11:** Mixed leukocyte reaction showing T-cell proliferation after 7 days. Counts per minute (CPM) on the vertical axis measures the amount of radioactive thymidine cells incorporate during division. T-cells are not exposed to foreign cells in the "Auto" negative control and "Allo" positive control does not include any CD80 reagent. T-cells exposed to 1μM canine CD80 heptamer showed significanlty less proliferation.

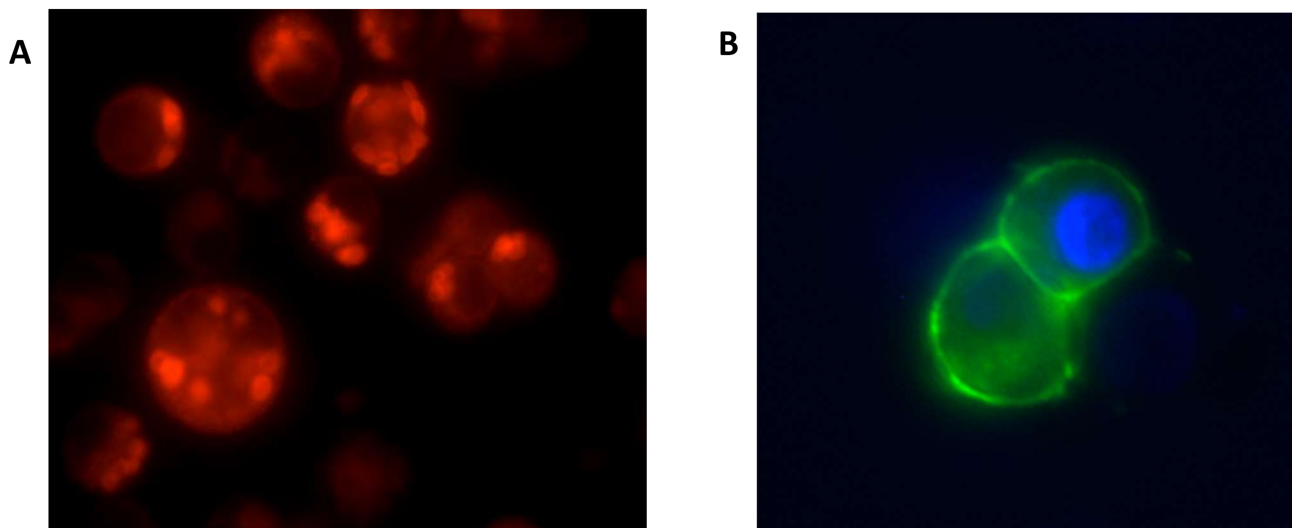



**Figure 12:** Fluorescence microscope photos of HEK 293 cells with virally transduced membrane anchored constructs. A: Full length wild type CTLA-4 with fused mCherry (see construct #1 in figure 13) appears to localize to vesicles. B: Full length wild type CD28 with fused GFP (see cCD28 construct in figure 13) localizes to cell surface. DAPI can be used to stain nucleus blue.

binding studies. The CTLA-4 constructs attempted are summarized in Figure 13. I was unable to generate a CTLA-4 construct that was reliably expressed on the surface. I am also uncertain if the constructs I did create were being rapidly trafficked from the cell surface or if they never made it there in the first place, or if they were being successfully expressed fully and stable.

## CONCLUSION

While trying to create a soluble specific agonist for CTLA-4 I was enticed by heptameric canine CD80 which seemed to possess the properties of interest. However, I did not achieve efficient, practical production of this reagent. In the process I learned a great deal about construct design as well as protein production, purification, and validation. The multimerization domains worked very well. They are fairly stable and allow the design of proteins with varying avidities. The heptamerization domain from C4b binding protein has since been applied successfully to several

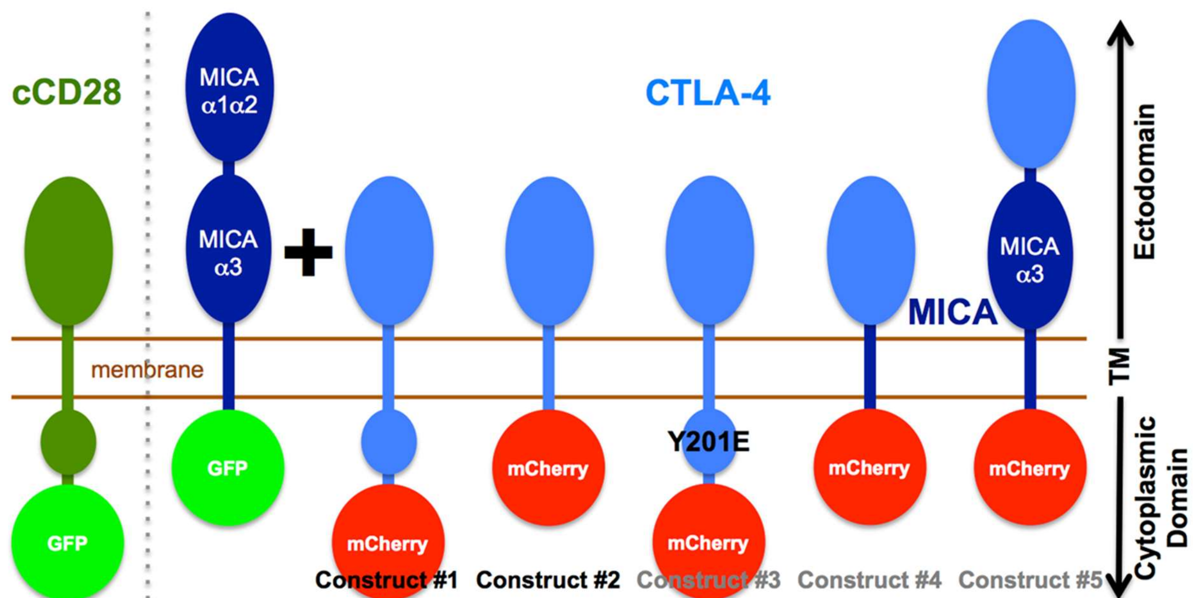



**Figure 13:** Full length cell surface constructs used for fluorescence microscopy. CD28 and MICA have fused GFP and CTLA-4 has fused mCherry for visualization. From L to R: full length canine CD28, extracellular and transmembrane MICA domain, full length CTLA-4, full length CTLA-4 without the intracellular domain, full length CTLA-4 with Y201E mutation in AP-2 binding site, CTLA-4 extracellular domain with fused MICA transmembrane domain, and CTLA-4 extracellular domain with fused MICA extracellular and transmembrane domains.

other projects. In the future, heptameric canine and/or human CD86 could make an excellent alternate reagent.

For the cell surface studies, I produced HEK 293 cells with GFP tagged CD28 reliably anchored to the surface. However, the lack of a counterpart CTLA-4 cell line precluded the cell based binding assays. There is a great deal of potential for the study of cell based CD28 and CTLA-4 binding behavior as well as CTLA-4 trafficking if this model system is completed.

## FUTURE DIRECTIONS

Production of a soluble specific CTLA-4 agonist would still be very useful. There are a great many potential reagents that could have these properties. Heptameric CD80 was a promising yet challenging reagent. In the future, heptameric CD86 may be a superior alternative. CD86 was initially bypassed due to its lower binding strength to CTLA-4 in the literature and the initial success with CD80. Preliminary data from heptameric canine CD86 indicates that it also preferentially binds to CTLA-4 over CD28 (Figure 14). Importantly, heptameric CD86 seems to avoid the expression problems of CD80. Next steps would require larger scale production of the reagent and further validation of its binding properties followed by testing binding to activated and naive T-cells and a test of its ability to prevent T-cell proliferation.

Further reagent improvement can be accomplished using library production.1000s of unique sequences of CD80/86 can be displayed on the surface of HEK293 cells with the addition of the WT transmembrane domain. The differential binding of fluorescently labeled CD28 and CTLA-4 can be used to organize cells using fluorescence activated cell sorting (FACS). Selection of residues for adjustment will be made based on sequence differences between human and canine proteins as well as crystal structures I obtain and previous crystal structures. Residue selection can be adjusted for further rounds of selection.

A great deal is still unknown about the physical properties of CTLA-4 and CD28 as well as the relationship between effector T-cells and regulatory T-cells and the mechanism by which CTLA-4 leads to T-cell deactivation. There are several competing models in the literature and many

pieces of contradictory evidence. Does CTLA-4 act intracellularly, extracellularly or both? How do Tregs use CTLA-4 to suppress Teffs? The question of how CTLA-4 signaling is transduced is

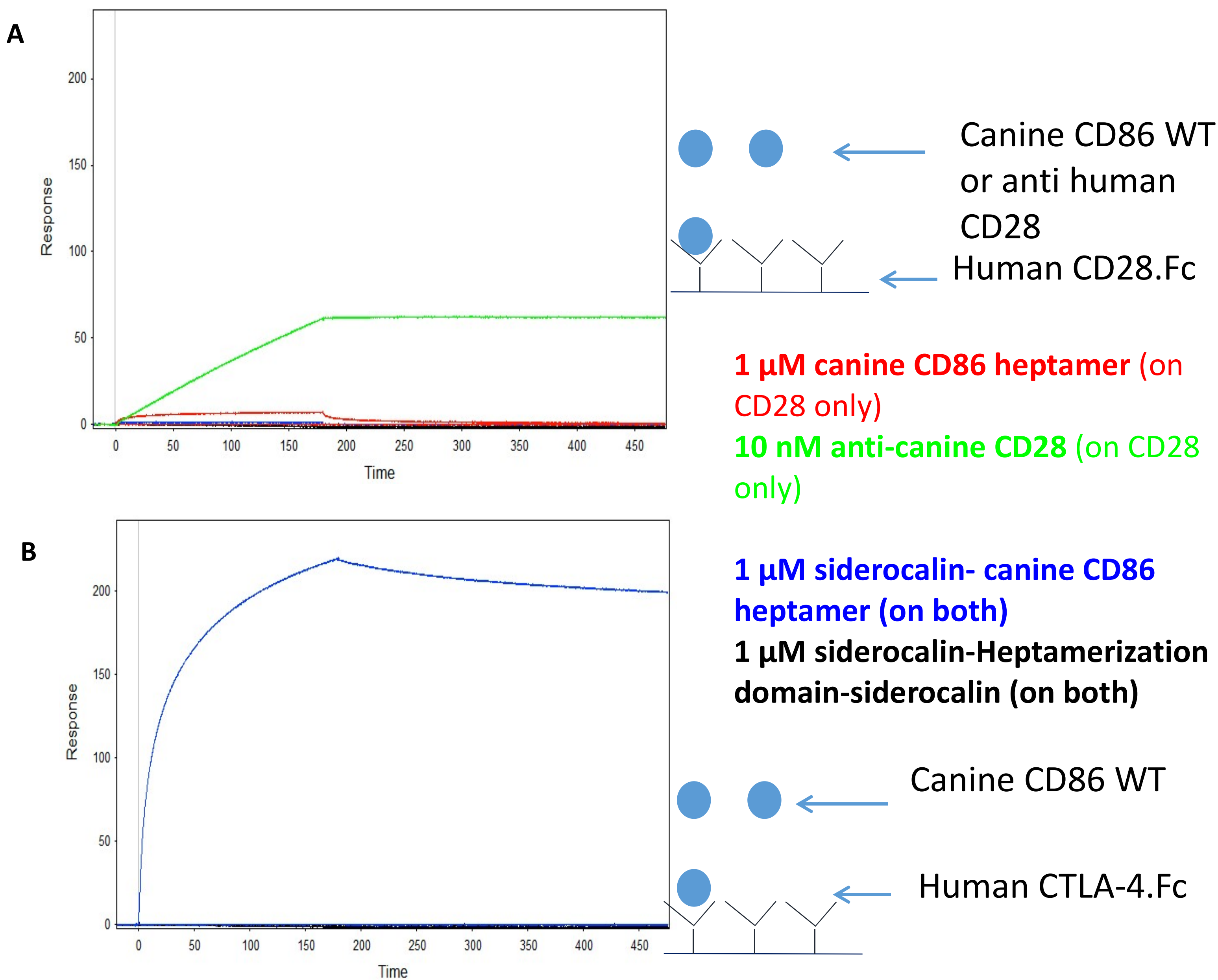



**Figure 14:** Traces from surface plasmon resonance experiments comparing binding of wild type canine CD86 heptamers to either human CD28 or human CTLA-4. CD86 with or without fused siderocalin failed to bind to human CD28. The only reagent to bind canine CD28 was ant-canine CD28 mAbs. In contrast, heptameric canine CD86 did bind to CTLA-4. Heptamerization domain fused to siderocalin (with no CD80 domain) failed to bind to both CD28 and CTLA-4.

very important since several drugs in clinical use have been developed to interact with CTLA-4 directly.

Further development of HEK 293 cell based CTLA-4 and CD28 reagents will be useful in the pursuit of these questions. It is a controllable cell based platform that compliments the use of T-cells for binding studies. A panel of reliable CTLA-4 reagents with different localization properties along with the CD28 reagents currently existing would provide an excellent toolbox for answering these questions.

Several important protein structures are also notably absent from the literature (Table 1). Especially when considered in the context of binding kinetics data, crystal structures provide a great deal of insight into observed protein binding behavior. It would be useful to determine the crystal structures of CD28 bound to CD80/86 as well as the bound and unbound structures of canine CTLA-4, CD28, CD80, and CD86; none of which have yet been reported.

| PDB code | Source Organism | Protein |
|---|---|---|
| 1AH1 | Human | CTLA-4 (NMR structure) |
| 3OSK | Human | CTLA-4 homodimer |
| 1DQT | Murine | CTLA-4 |
| 4KKN | Bovine | CTLA-4 |
| 1I85 | Human | CTLA-4 bound to CD86 |
| 1I8L | Human | CTLA-4 bound to CD80 |
| 1YJD | Human | CD28 bound to antibody |
| 4RWH | Mouse | CD80 |
| 1DR9 | Human | CD80 |
| 1NCN | Human | Receptor binding domain of CD86 |
| 4LCI | Murine | Anti-canine CD28 antibody 1c6 |

**Table 1:** PDB codes of relevant crystal structures.

1. Vogelsang, G.B. and S. Pavletic, *Chronic graft versus host disease: interdisciplinary management*2009, New York: Cambridge University Press.
2. Linhares, Y.P.L., S. Pavletic, and R.P. Gale, *Chronic GVHD: Where are we? Where do we want to be? Will immunomodulatory drugs help?* Bone Marrow Transplantation, 2013. **48**(2): p. 203-209.
3. Macian, F., *NFAT proteins: key regulators of T-cell development and function.* Nat Rev Immunol, 2005. **5**(6): p. 472-484.
4. Naesens, M., D.R.J. Kuypers, and M. Sarwal, *Calcineurin Inhibitor Nephrotoxicity.* Clinical Journal of the American Society of Nephrology, 2009. **4**(2): p. 481-508.
5. Vincenti, F. and M. Luggen, *T cell costimulation: A rational target in the therapeutic armamentarium for autoimmune diseases and transplantation.* Annual Review of Medicine, 2007. **58**: p. 347-358.
6. Lanier, L.L., et al., *CD80 (B7) and CD86 (B70) Provide Similar Costimulatory Signals for T Cell Proliferation, Cytokine Production, and Genertion of CTL.* Journal of Immunology, 1995. **154**(1): p. 97-105.
7. Salomon, B. and J.A. Bluestone, *Complexities of CD28/B7: CTLA-4 costimulatory pathways in autoimmunity and transplantation.* Annual Review of Immunology, 2001. **19**: p. 225-252.
8. Alegre, M.L., K.A. Frauwirth, and C.B. Thompson, *T-cell regulation by CD28 and CTLA-4.* Nature Reviews Immunology, 2001. **1**(3): p. 220-228.
9. Schwartz, J.-C.D., et al., *Structural basis for co-stimulation by the human CTLA-4/B7-2 complex.* Nature, 2001. **410**(6828): p. 604-608.
10. Stamper, C.C., et al., *Crystal structure of the B7-1/CTLA-4 complex that inhibits human immune responses.* Nature, 2001. **410**(6828): p. 608-611.
11. Evans, E.J., et al., *Crystal structure of a soluble CD28-Fab complex.* Nature immunology, 2005. **6**(3): p. 271-9.
12. Lenschow, D.J., T.L. Walunas, and J.A. Bluestone, *CD28/B7 system of T cell costimulation.* Annual Review of Immunology, 1996. **14**: p. 233-258.
13. Salomon, B., et al., *B7/CD28 costimulation is essential for the homeostasis of the CD4(+)CD25(+) immunoregulatory T cells that control autoimmune diabetes.* Immunity, 2000. **12**(4): p. 431-440.
14. Nishikawa, H. and S. Sakaguchi, *Regulatory T cells in cancer immunotherapy.* Curr Opin Immunol, 2014. **27**: p. 1-7.
15. Wing, K., et al., *CTLA-4 control over Foxp3(+) regulatory T cell function.* Science, 2008. **322**(5899): p. 271-275.
16. Walker, L.S. and D.M. Sansom, *Confusing signals: Recent progress in CTLA-4 biology.* Trends Immunol, 2015.
17. Hu, H., C.E. Rudd, and H. Schneider, *Src kinases Fyn and Lck facilitate the accumulation of phosphorylated CTLA-4 and its association with PI-3 kinase in intracellular compartments of T-cells.* Biochemical and biophysical research communications, 2001. **288**(3): p. 573-578.
18. Marengere, L.E.M., et al., *Regulation of T cell receptor signaling by tyrosine phosphatase SYP association with CTLA-4.* Science, 1996. **272**(5265): p. 1170-1173.
19. Stein, P.H., J.D. Fraser, and A. Weiss, *The Cytoplasmic Domain of Cd28 Mediates Costimulation of Interleukin-2 (Il-2) Secretion and Association with Phosphatidylinositol 3' Kinase.* Clinical Research, 1994. **42**(2): p. A206-A206.
20. Schneider, H. and C.E. Rudd, *Tyrosine phosphatase SHP-2 binding to CTLA-4: Absence of direct YVKM/YFIP motif recognition.* Biochemical and Biophysical Research Communications, 2000. **269**(1): p. 279-283.

21. Bachmann, M.F., et al., *Cutting edge: Lymphoproliferative disease in the absence of CTLA-4 is not T cell autonomous.* Journal of Immunology, 1999. **163**(3): p. 1128-1131.
22. Qureshi, O.S., et al., *Trans-Endocytosis of CD80 and CD86: A Molecular Basis for the Cell-Extrinsic Function of CTLA-4.* Science, 2011. **332**(6029): p. 600-603.
23. Grant, C.R., et al., *Regulatory T-cells in autoimmune diseases: Challenges, controversies and-yet-unanswered questions.* Autoimmunity Reviews, 2015. **14**(2): p. 105-116.
24. Grant, C.R., et al., *Regulatory T-cells in autoimmune diseases: challenges, controversies and - yet - unanswered questions (vol 14, pg 105, 2015).* Autoimmunity Reviews, 2015. **14**(9): p. 845-846.
25. Linsley, P.S., et al., *HUMAN B7-1 (CD80) AND B7-2 (CD86) BIND WITH SIMILAR AVIDITIES BUT DISTINCT KINETICS TO CD28 AND CTLA-4 RECEPTORS.* Immunity, 1994. **1**(9): p. 793-801.
26. Chakrabarti, R., et al., *A mutant B7-1/Ig fusion protein that selectively binds to CTLA-4 ameliorates anti-tumor DNA vaccination and counters regulatory T cell activity.* Vaccine, 2005. **23**(37): p. 4553-4564.
27. vanderMerwe, P.A., et al., *CD80 (B7-1) binds both CD28 and CTLA-4 with a low affinity and very fast kinetics.* Journal of Experimental Medicine, 1997. **185**(3): p. 393-403.
28. Collins, A., et al., *Binding affinities within the CD28/CTLA4 and B7-1/B7-2 family.* Immunology, 1999. **98**(suppl. 1): p. 2-2.
29. Sigmundsson, K., et al., *Determination of active concentrations and association and dissociation rate constants of interacting biomolecules: An analytical solution to the theory for kinetic and mass transport limitations in biosensor technology and its experimental verification.* Biochemistry, 2002. **41**(26): p. 8263-8276.
30. Ben Khalifa, M., et al., *BIACORE data processing: An evaluation of the global fitting procedure.* Analytical Biochemistry, 2001. **293**(2): p. 194-203.
31. Mead, K.I., et al., *Exocytosis of CTLA-4 is dependent on phospholipase D and ADP ribosylation factor-1 and stimulated during activation of regulatory T cells.* Journal of Immunology, 2005. **174**(8): p. 4803-4811.
32. Zhang, Y. and J.P. Allison, *Interaction of CTLA-4 with AP50, a clathrin-coated pit adaptor protein.* Proceedings of the National Academy of Sciences of the United States of America, 1997. **94**(17): p. 9273-9278.
33. Tucci, M., et al., *The immune escape in melanoma: role of the impaired dendritic cell function.* Expert Review of Clinical Immunology, 2014. **10**(10): p. 1395-1404.
34. Weber, J., *Ipilimumab: controversies in its development, utility and autoimmune adverse events.* Cancer Immunology Immunotherapy, 2009. **58**(5): p. 823-830.
35. Martin, S.T., E.M. Tichy, and S. Gabardi, *Belatacept: A Novel Biologic for Maintenance Immunosuppression After Renal Transplantation.* Pharmacotherapy, 2011. **31**(4): p. 394-407.
36. Latek, R., et al., *Assessment of Belatacept-Mediated Costimulation Blockade Through Evaluation of CD80/86-Receptor Saturation.* Transplantation, 2009. **87**(6): p. 926-933.
37. Suntharalingam, G., et al., *Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412.* New England Journal of Medicine, 2006. **355**(10): p. 1018-1028.
38. Santana, C.A.G., J.W. Tung, and S. Gulnik, *Human Treg Cells Are Characterized by Low/Negative CD6 Expression.* Cytometry Part A, 2014. **85A**(10): p. 901-908.
39. Qi, L., *Tipping the Balance in Metabolic Regulation: Regulating Regulatory T Cells by Costimulation.* Diabetes, 2014. **63**(4): p. 1179-1181.
40. Abbas, A.K., et al., *Regulatory T cells: recommendations to simplify the nomenclature.* Nat Immunol, 2013. **14**(4): p. 307-8.