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Generation, Identification and Characterization of Novel Monoclonal Antibodies Against CTLA-4, PD-1 and BTLA for the Treatment of Cancer

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ABSTRACT

Members of the CD28 co-inhibitory receptor family, Cytotoxic T-lymphocyte- associated antigen 4 (CTLA-4), Program death-1 (PD-1) and B- and T-lymphocyte attenuator (BTLA) are type I transmembrane proteins expressed on a variety of immune cells. Co- inhibitory receptors deliver "off" signals that play an important role in down regulating immune cell activation. Manipulation of inhibitory signals have shown to be a powerful strategy in the treatment of autoimmune diseases, infectious diseases and various forms of cancer. In fact, the FDA (Food and Drug Administration) has approved the use of monoclonal antibodies against CTLA-4 (Ipilimumab) for the treatment of metastatic melanoma, against PD-1 (Nivolumab, Pembrolizumab and Durvalumab) for the treatment of melanoma, non-small cell lung cancer among other cancers. However, little is known about the therapeutic value of monoclonal antibodies against BTLA and consequently of a combination of these co- inhibitory receptor inhibitors sequentially. To explore the therapeutic potential of targeting these receptors for the treatment of cancer and to further elucidate the effects of BTLA blockade CTLA-4, PD-1 and BTLA novel monoclonal antibodies (mAbs) against unique sites within the extracellular domains (ECD) of human CTLA-4, PD-1 and BTLA were developed. To evaluate the specificity of these novel mAbs, dot blot, western blotting, FACS analysis and cell Immunohistochemistry (IHC) were employed to confirm the *in vitro* ability of these antibodies to recognize their target protein. To evaluate their effect on tumor inhibition *in vivo*, a Balb/c breast cancer model was tested to investigate the effects of new monotherapies and combinational-therapies. This work has identified novel mAbs against three checkpoint inhibitors and were able to inhibit tumor growth in a murine model of human breast cancer. This work suggests that administration of unique checkpoint inhibitors that deploy BTLA harbor therapeutic value.

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CHAPTER I

INTRODUCTION

1.1 Immune system

The human body is protected from foreign antigens such as microbes, viruses, and toxins as well as cancerous cells by the immune system which is categorized by two arms of defense, innate and adaptive immunity. Innate immunity is our body's first line of defense and represents an antigen independent defense mechanism which lacks immunological memory (1). Non-specific defensive barriers such as skin, mucus membranes, fever and low pH are physiological defenses of innate immunity to protect us from disease (1). In contrast, adaptive immunity is antigen dependent, highly specific response that results in our ability to mount a rapid and efficient immune defensive mechanism when exposed to the same antigen a second time known as immunological memory (1). Adaptive immunity is controlled by cells which originate from the bone marrow and migrate to the thymus where they become mature T lymphocytes (T cells) (1). T cells express an antigen binding receptor on their membrane known as the T cell receptor (TCR) and are activated when TCR proteins interact with antigen bound major histocompatibility complex (MHC) molecules displayed on antigen presenting cells (APC). This interaction relies on the recognition of antigenic peptides which initiate an intracellular signaling cascade driven by serine, threonine and tyrosine kinases and specially the Immunoreceptor Tyrosine Activation Motifs (ITAM) to promote tissue glands. (2). Following antigen presentation, T cells differentiate into T helper (Th) cells which are characterized by expression of CD4 co-stimulatory proteins or cytotoxic T cells (Tc), which express CD8 proteins on the cell surface (2). $CD4^+$ cells provide protection by producing cytokines that stimulate B cells, immune cells which mature in the bone marrow and produce antibodies against foreign antigens. Cytotoxic T lymphocytes, $CD8^+$ T cells, are important as they exert their toxic effects on infected cells through the use of perforin or granzyme which cause cells to erupt (lysis) or granulysin which induces cell apoptosis (2). A third type of T cell, T

regulatory (Treg) cells, are important in maintaining immune homeostasis by suppressing or "turning off" the immune system (3).

1.2 T-cell activation

Successful T cell activation requires that three signals be delivered through multiple receptors on the surface of T cells: Signal 1 through the T- cell receptor (TCR), Signal 2 through co-stimulatory receptors (CD28 molecules) and Signal 3 through cytokine receptors (Figure 1.1). Key interactions necessary for delivering Signal 1 and 2 occur between the T cell and antigen presenting cells (APCs) such as macrophages, dendritic cells (DCs) and B cells. Engagement of the TCR and MHC molecules, Signal 1 results in the phosphorylation of ITAM domains within the various chains of the TCR which is necessary for the recruitment of intracellular signaling proteins (Figure 1.1). Co-stimulatory signals are delivered through CD28 proteins, Signal 2, on the surface of T-cells engaging B7-1 (CD80) or B7-2 (CD86) receptors on the surface of APCs resulting in amplified Ras/Raf/Erk and Ca^{2+} mediated signaling (Figure 1.1). The signaling cascades initiated by these first two signals result in transcription of genes important in cell survival and proliferation. The third signal is delivered through cytokine receptors including those for the common gamma chain family, Interleukin-2 (IL-2), IL-4, IL-7, IL-9, IL-15 and IL-21 (Figure 1.1). Similar to Signal 1, cytokine receptors rely on multiple kinases to initiate intracellular signaling and transcription of genes necessary for cell survival and proliferation. Following clonal expansion, activated T cells migrate to sites where the antigenic peptides were initially found to begin cell mediated cytotoxicity and elimination of any foreign, malignant or damaged cells (3). T cell activation is tightly regulated by co-inhibitory signals mediated by receptors of the CD28 family maintaining immune balance (4).

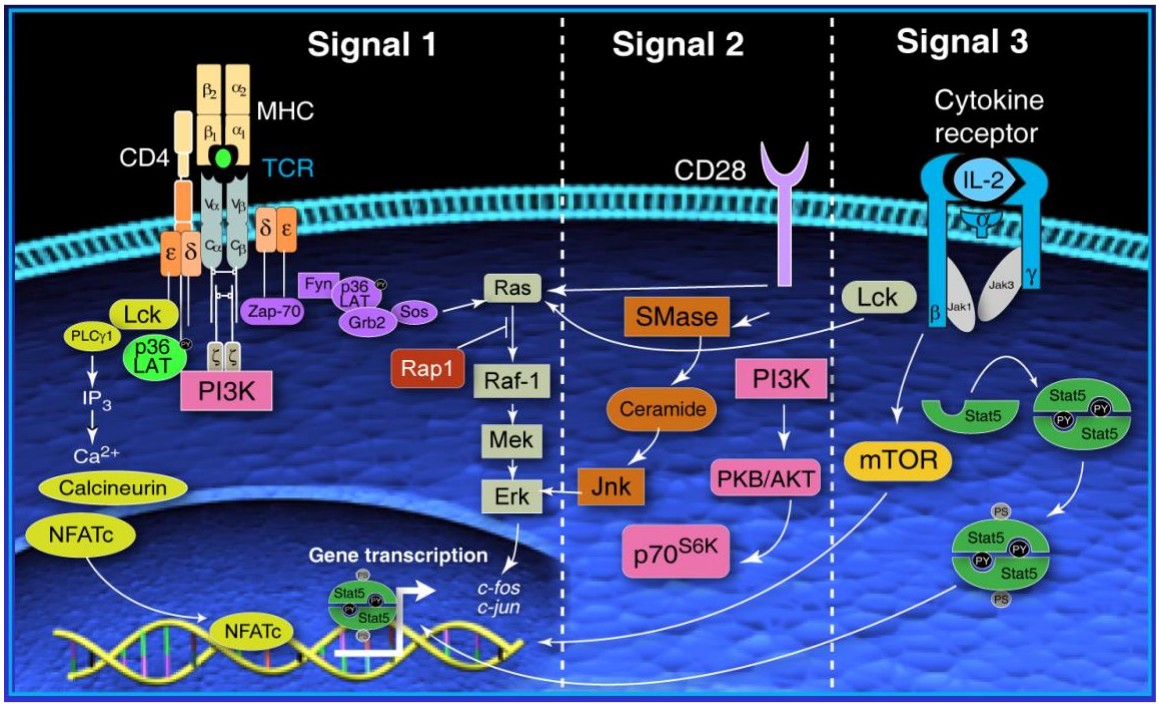


Figure 1.1. Schematic representation of the three signals required for full T-cell activation. Signal 1 is mediate by the T-cell receptor (TCR); Signal 2 is mediated by CD28 and Signal number 3 mediated by the cytokine receptor composed of the β and γ chains (Ross, 2007).

1.3 Co-inhibitory signals

Co-inhibitory signals are necessary for the modulation of the immune response in order to prevent autoimmune diseases (2). Co-inhibitory receptors are essential in this process which main goal is to maintain balance by tightly regulating the extent and duration of the immune response. One of the main cells participating in co-inhibitory processes are T regs, which mainly express $CD4^+$ co-receptors but may sometimes express $CD8^+$ instead. T regs are able to exert their functions assisted by immunosuppressive cytokines such as TGF-β, IL-10, IL-35 and Foxp3 (3). Foxp3 is mainly associated with the inhibition of IL-2 transcription which in turn results in the up-regulation of molecules associated with T reg such as CD25 and cytotoxic T-lymphocyte antigen

4 (CTLA-4). T reg-associated molecules such as CTLA4 are crucial for the direct cell contact necessary for inhibitory effects of T regs (5). CTLA-4, PD-1 and BTLA co-inhibitory receptors are widely known for their ability to attenuate TCR-mediated signals via their ligands; CD80/CD86, PDL1/PD-L2 and HVEM (Herpesvirus Entry Mediator) resulting in decreased cell proliferation, cytokine production and cell cycle progression (Figure 1.2). Over the past decade, these pathways have gained special attention for the development of novel therapeutics aimed at blocking inhibitory signals as a way of treating immune diseases as well as stopping progression of such malignancies. The negative signals delivered by these receptors makes them of great interest due to their role in regulating T cell activation and ensuring peripheral tolerance.

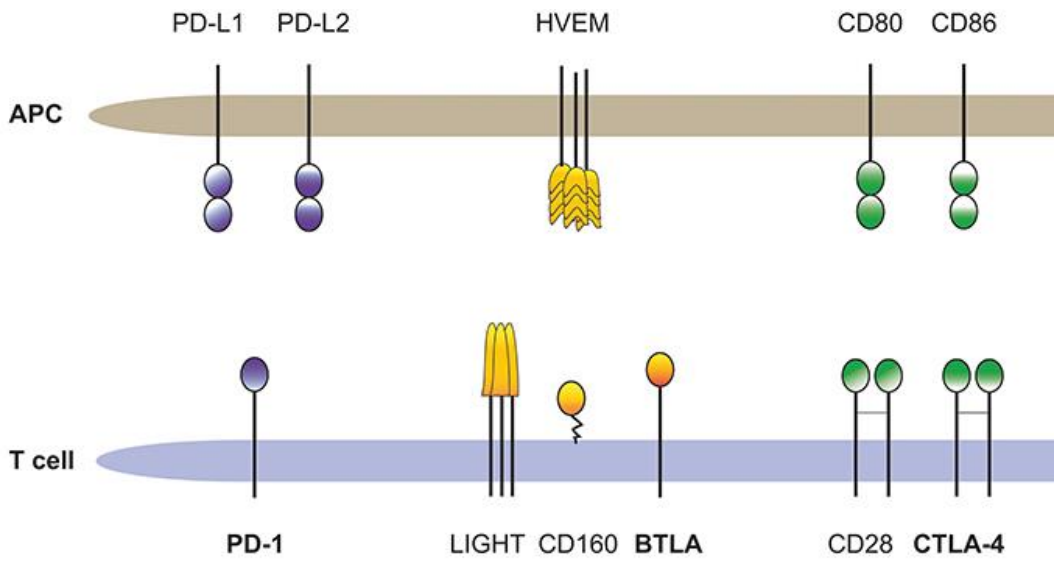


Figure 1.2. Schematic representation of co-inhibitory signals via immune checkpoints and their ligands. T cells express PD-1, BTLA and CTLA-4. Antigen presenting cells (APC) express the ligands, PD-L1/PD-L2, HVEM or CD80/CD86, thus activating inhibitory signaling cascades which inhibit activation signals. (De Sousa Linhares, 2018)

1.4 Immuno-editing

Through a process known as immunosurveillance, the immune system is capable of recognizing and rejecting tumors (7). Understanding the mechanisms by which the immune system is able to detect tumors and stop their proliferation is one of the most recent fields in immunology and cancer referred to as immuno-oncology. This field explores the process tumors cells use to evade the immune response which leads to cancer progression. The goal of immuno-oncology is to study the immune system's interactions with tumor cells instead of looking exclusively at cancer cell behavior and design therapeutics focused on enhancing and directing the patient's immune response. This aim is supported by the idea that the tumors have developed a way to evade the immune response. The immune system is able to suppress the tumor if this limitation is removed. Activating the patient's immune system should result in the system's capacity to recognize tumors and disrupt their immunosuppressive interactions with immune cells (7). The concepts explored in this field, rely on the use of what is called a cancer immunoediting model. This model describes the process by which cancer cells evade the immune response. These phases are referred to as elimination, equilibrium and escape (Figure 1.3). The elimination stage is the one on which the human body relies on to remove cancer cells. It is characterized by the detection of tumor antigens found on the cell surface which trigger an immune response and recruitment of immune cells such as NK cells, dendritic cells, macrophages, $CD8^+$ and $CD4^+$ cells leading to tumor suppression and elimination. During the equilibrium stage, the immune system seeks to sustain a balance between cancer cell death and cell proliferation maintaining the tumor in a quiescent state. Is it during this stage that tumors stop expressing tumor specific antigens on the cell surface which makes them undetectable by the immune system. This gives rise to a different subset of cells with minimal immunogenicity. This phase leads to escape, the stage in which the tumor modifies the immune

response and evades both innate and adaptive immune systems and their attempts at suppressing and eliminating the tumor. The immune evasion described in the escape stage is characterized by the immune system failing at recognizing the tumor cell, the tumor not being susceptible to effector cells leading to immune suppression and tumor progression (7).

One of the ways in which cancer cells modify the immune response which leads to the evasion of immune mechanisms is by surface ligands such as B7.1, B7.2, PD-L1 and HVEM (CTLA-4, PD-1 and BTLA ligands). These ligands interact with co-inhibitory receptors on the surface of T cells suppressing the activity of these immune cells and with-it anti-tumor immunity leading to tumor growth (7). The uncoupling of co-inhibitory receptors and their ligands expressed on cancer cells may be hindered by the use of highly active mAbs specific to the binding site of these interactions. Specific mAbs prevent ligation by occupying the binding region which might defeat tumor growth and progression.

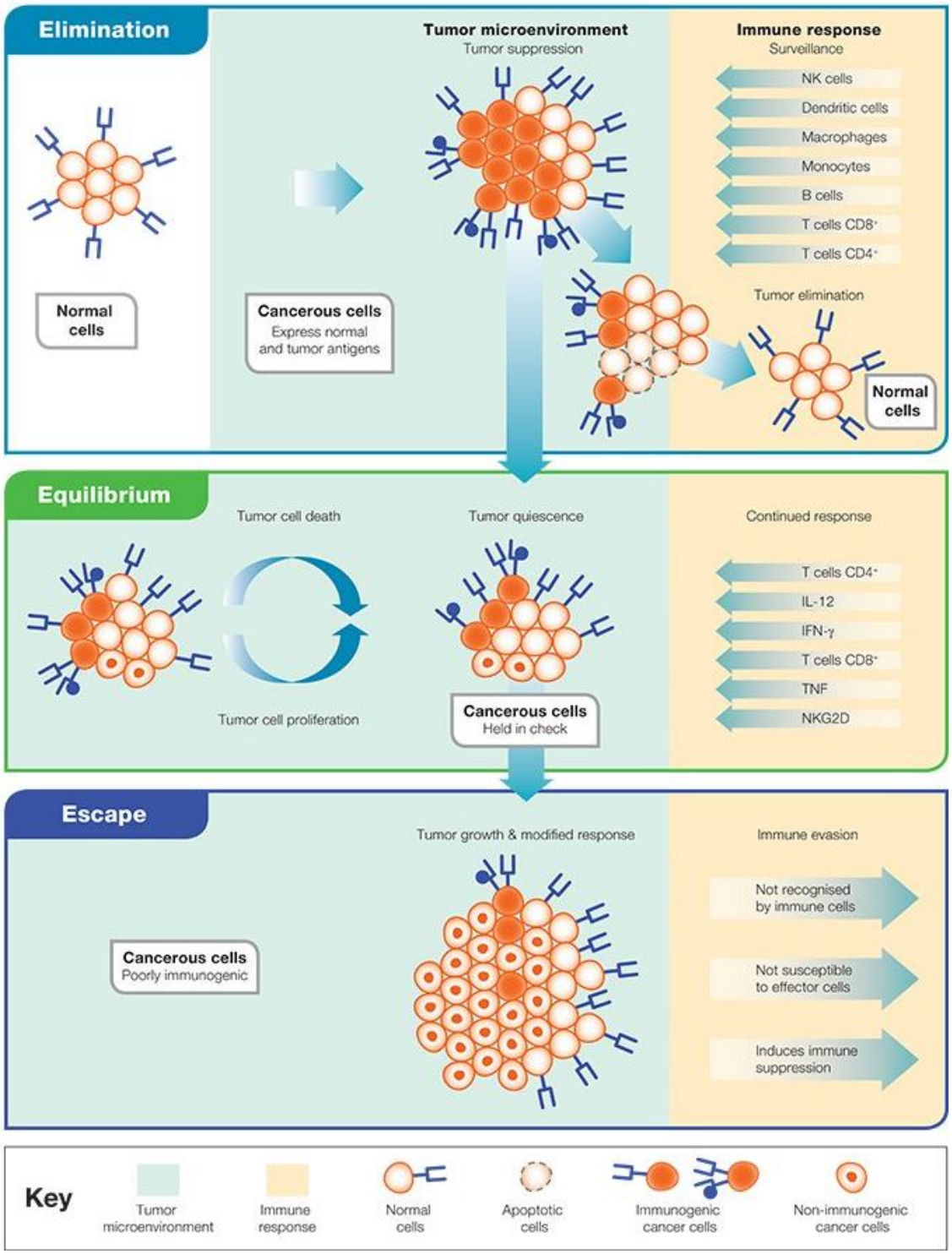


Figure 1.3. Schematic representation of immune-editing model. The stages of immuno-editing; elimination, equilibrium and escape. The mechanisms tumor cells utilize to evade immune response and the cells and events that take place on each stage (Biorad).

1.5 Co-inhibitory receptors

In order to maintain immune homeostasis, immune cells express co-inhibitory receptors, which deliver necessary "off" signals upon culmination of successful antigen clearance from the body. Co-inhibitory receptors, also known as check point inhibitors, include members of the CD28 receptor family: Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), Program death-1 (PD-1) and B- and T-lymphocyte attenuator (BTLA) are transmembrane proteins expressed on T cells, B cells, natural killer (NK) natural killer T (NKT), activated monocytes and dendritic cells (DC).

CTLA-4 is a type I transmembrane protein expressed in T cells upon activation and in T regs cells in a constitutive fashion. This receptor is found inside the cell in vesicles that are rapidly expressed on the surface of the cell upon activation. One of the unique features of CLTA-4 is the fact that it possesses both, extrinsic and intrinsic effects. That is, that the receptor has inhibitory properties that are independent of intracellular activation (8). This receptor can be regulated extracellularly through its interaction with ligands such as CD80 and CD86. Interestingly, CTLA-4 shares the same ligands (CD80 and CD84) with the co-stimulatory receptor CD28 to which CTLA-4 shows a higher affinity (6). The action of CTLA-4 has been reported to block Signal 2 of the T cell activation signaling pathway (9).

PD-1 is a type I transmembrane protein expressed on T cells, T regs, B cells, activated monocytes, DCs, NK cells and NKT cells (10). PD-L1 and PD-L2 are the ligands for the PD-1 receptor expressed on hematopoietic cells, specifically activated T cells, and on dendritic cells (DC's) and macrophages, respectively. It has been reported that the inhibitory properties of PD-1 rely strictly on the ligation of PD-L or PD-L1 (6). While these ligands are expressed on a variety of hematopoietic cells, they are also found on non-hematopoietic cells including tumors of the bladder, gastric, pancreatic, liver, kidney, ovarian and breast cancer (6, 12). PD-1 receptors have

been reported to be of great importance in maintaining a balanced immune response since its absence results in autoimmune diseases. Blocking PD-1/PD-L pathway is a promising approach to achieve immunopotentiation in tumor therapy as it activates pathways responsible for suppressing the immune response against tumors.

Similarly, BTLA is a type I glycosylated transmembrane protein expressed on naïve T cells, Th1 cells, T follicular cells, NK cells, NKT cells, B cells, DCs and macrophages (10). HVEM (Herpes virus entry mediator) is the ligand for BTLA and its deficiency results in hyper-reactive B and T cells (6). BTLA is the most recently recognized member of the CD28 family and has been shown to play an important role in maintenance of immune tolerance and prevention of autoimmune diseases (10). Lastly, a unique feature of BTLA is that of the three members of the CD28 family this is the only one that is present on naïve T cells and its downregulated once the T cell is activated (6, 11). Interestingly, BTLA has been reported to have both inhibitory properties like CTLA-4 and PD-1 and stimulatory properties like the CD28 proteins (12). Even though BTLA is recognized as a member of co-inhibitory receptor family, its mechanism of action and anti tumor potential remains to be elucidated.

1.6 Significance and hypothesis

The advancements in immunotherapies over the past twenty years have proved to be of great importance in the treatment of various malignancies, many questions remain unanswered. CTLA-4 was the first member of the CD28 family to be studied as a target for cancer therapy followed by PD-1 and most recently BTLA. In 2011 the FDA (US Food and Drug Administration) approved Ipilimumab, a fully humanized mAb against CTLA-4 for the treatment of advanced melanoma (13). The use of Ipilimumab for the treatment of metastatic melanoma was the first antibody

against this receptor that was found to improve overall survival and, in combinational therapies with gp100, a melanoma specific peptide vaccine, it significantly increased survival of patients with advanced melanoma by 3.5 months (9, 10). In 2014, Nivolumab, an ani-PD1 monoclonal antibody was approved by the FDA and since then it has been used in combination with Ipilimumab resulting in a higher survival response rate leading many to believe that mAb combination therapies can be more efficient than anti CTLA-4 or PD-1 alone (9). The effect of the CTLA-4 and PD-1 combined antitumor immunity has been reported to be efficient due to their complementary and non-redundant pathways which work synergistically thereby providing a more favorable checkpoint blockade in early clinical trials (13). Levels of certain cytokines involved in proliferation, differentiation and immunosuppression can serve as markers for prognosis, thus monitoring treatment effectiveness and contributing to a better understanding of what is occurring in the tumor microenvironment and the drug response. The characterization of monoclonal antibodies capable of aiding T cell immune surveillance, to fight cancer more efficiently, would be a great strategy for patients where currently available alternatives treatment are no longer effective or tumor resistant. Significant work has been performed to determine the potential of anti CTLA-4 and anti-PD-1 against cancers. However, little has been done to characterize the anti-tumor potential of monoclonal antibodies against BTLA alone, or in combination with other immune checkpoint inhibitors.

To address this deficiency, our group has developed novel antibodies against the full extracellular domains (ECD) of CTLA-4, PD-1 and BTLA. These antibodies might be used to block the activation of signaling pathways that "turn off" the immune response thus releasing the "breaks" of T-cells against cancer. This project is aimed at fully charactering the *in vitro* abilities of our novel monoclonal antibodies to recognize human and mouse CTLA-4, PD-1 and BTLA.

Additionally, we seek to test the hypothesis that our novel mAb are able to effectively reduce tumor burden, *in vivo*, using an animal model system for human breast cancer. **We hypothesize that our novel monoclonal antibodies against CTLA-4, PD-1 and BTLA can have more potent anti-tumor activity.**

CHAPTER II

DEVELOPMENT OF MONOCLONAL ANTIBODIES AGAINST CD28 CO-INHIBITORY RECEPTOR FAMILY MEMBERS

2.1 Introduction

The negative signals delivered by CTLA-4, PD-1 and BTLA make them of great therapeutic interest due to their role in regulating T cell activation and ensuring peripheral tolerance. Blockade of these inhibitory checkpoints can be manipulated through the use of small peptides or monoclonal antibodies as strategies to suppress tumor cell evasion. Over the past decade, the FDA has approved the use of monoclonal antibodies (mAbs) against CTLA-4 (Ipilimumab) for the treatment of metastatic melanoma (14) and against PD-1 (Nivolumab) for the treatment of melanoma, renal, head and neck carcinoma, cervical, uterine and breast cancer, as well as some lymphomas (15). However, less is known about BTLA blockade and a combinational approach potentially using these three immune checkpoints to attack tumors.

To test our hypothesis, we developed novel mAbs against the extracellular domains of CTLA-4, PD-1 and BTLA. The protein sequences of human and mouse were aligned to show a 79% homology for CTLA-4; 59% homology for PD-1 and for BTLA a 48% homology. Conservation of human and mouse sequences are of importance for the development of a mouse model system to study human cancer (Figure 2.1-2.3).

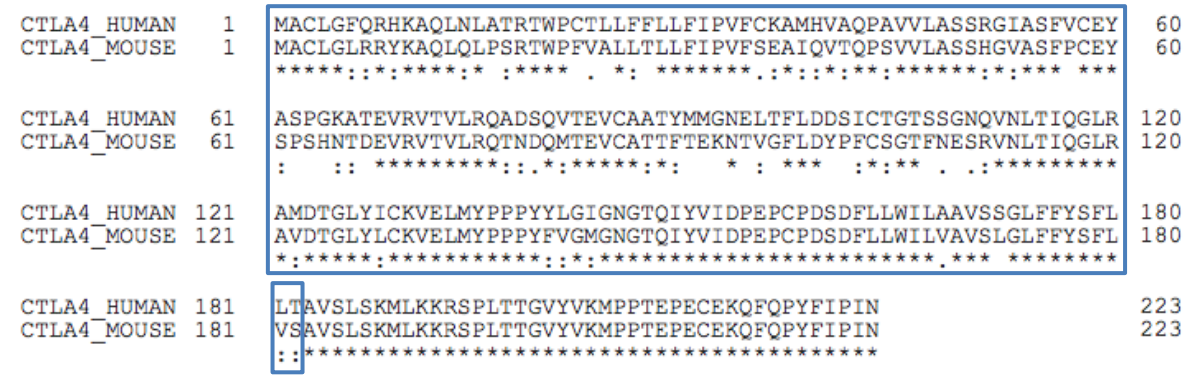


Figure 2.1. Sequence alignment of human and mouse CTLA-4. Asterisk (*) denote conserved residues; colon marks (:) denote residues with similar properties. These sequences share a 74% homology. The region of the sequence corresponding to the extra cellular domain (ECD) is denoted by the boxed area.

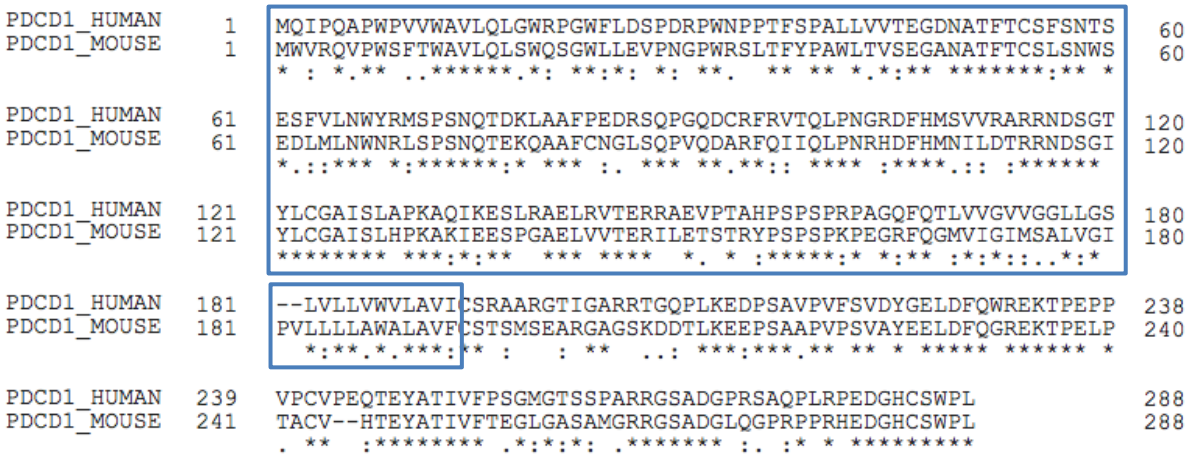


Figure 2.2. Sequence alignment of human and mouse PD-1. Asterisk (*) denote conserved residues; colon marks (:) denote residues with similar properties. These sequences share 59% homology. The region of the sequence corresponding to the extra cellular domain (ECD) is denoted by the boxed area.

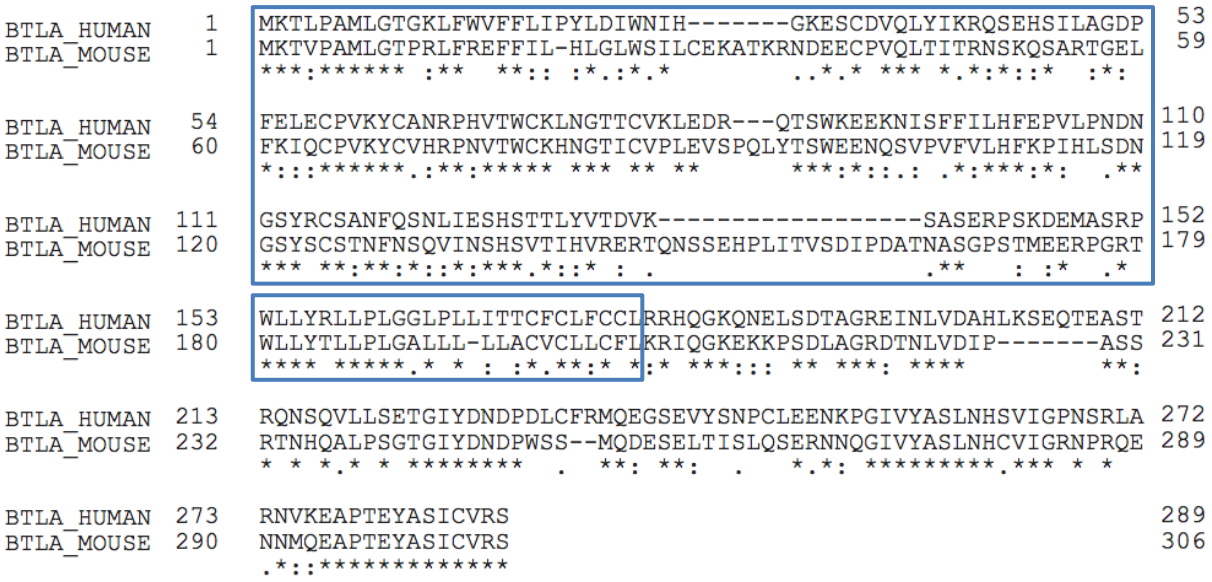


Figure 2.3. Sequence alignment of human and mouse BTLA. Asterisk (*) denote conserved residues; colon marks (:) denote residues with similar properties. These sequences share 48% homology. The region of the sequence corresponding to the extra cellular domain (ECD) is denoted by the boxed area.

2.2 Materials and methods

2.2.1 Hybridoma development: Balb/c mice were injected with the extracellular domain (ECD) of human CTLA-4, PD-1 or BTLA. Hybridoma clones were created by fusion of Balb/c splenocytes with SP2/0 mouse myeloma cells (Genscript).

2.2.2 Cell culture: Hybridoma clones were maintained in 20 % FBS, 4 % L-Glutamine DMEM media at 37 °C in 10cm plates and 6-well plates. Large scale cultures were grown in CELLine 1000 (INTEGRA BIOSCIENCE) bioreactor flasks. Flasks were seeded using hybridoma cells which were pre-acclimated to 100% serum free media (SFM). Antibody rich media from cell compartment was collected every 4 days, stored at -20 °C and replace with 100% serum free media

(SFM). DMEM media with 20 % FBS from nutrient compartment was changed every 8 days according to the protocol described by Treback et al (16).

2.2.3 Monoclonal antibody production and purification: Hybridoma supernatant was purified using Protein G Sepharose beads by column chromatography according to Sigma-Aldrich Protein Purification protocol (17).

2.2.4 SDS-PAGE and Coomassie Staining: Collected antibody rich media and elusions from Protein G Sapharose purification were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and stained using Coomassie Brilliant Blue solution for 1 hr. prior to de-staining with 10 % acetic acid, 40% methanol, 50% water solution.

2.3 Results

The full ECD of each protein was synthesized and used to immunize Balb/c mice (Figure 2.1-2.3). Hybridoma clones capable of producing monoclonal antibodies against human CTLA-4, PD-1 and BTLA were produced by fusing Balb/c splenocytes with SP2/0 mouse myeloma cells. Spleen cells with myeloma cells in the presence of 8-azaguanine are grown in HAT selection media by Genscript Inc. Using this method, our lab obtained seven novel anti-CTLA-4, five anti-PD-1 and five anti-BTLA producing hybridoma cell lines. Hybridoma cells were individually cultured and the antibody-rich supernatant collected. A small scale hybridoma cell culture method in which we used 10 cm plates was first utilized. Then a larger cell culture method was used which consisted of growing hybridomas in T150 flasks followed by a large-scale method using a two-chamber flask. Once the hybridoma cells were cultured using the different methods, the antibodies were

purified from the antibody rich media from the cell culture by column chromatography using Protein G Sepharose beads. Elution's were collected, quantified by nano drop and bicinchoninic acid (BCA) assay technology and tested for purity by separating on a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and staining with Coomassie Brilliant Blue stain. Purified antibodies against human CTLA-4, PD-1 and BTLA were produced and without impurities. Small scale production and purification of antibodies resulted in elusions collected for the different clones; 5C411.0, 5D3H5.3 5D3H5.4, 3E12H2.3, 3E12H2.4,3F12H1.3 and 3F12H1.4 corresponding to CTLA-4; 3H2F4, DB3D5, 2A6E6, 2H11D3 and 10C7D3 corresponding to PD-1 and 4G4H3, 6F1B2, 7F2G5 corresponding to BTLA. CTLA-4 and PD-1 clones were found more abundantly produced and without impurities while BTLA antibodies were detectable but found to be less concentrated (Figures 2.4-2.6). Large scale cell culture of hybridomas using a two chamber CELLine 1000 flask (INTEGRA BIOSCIENCE) showed larger amounts of mAb (Figure 2.7).

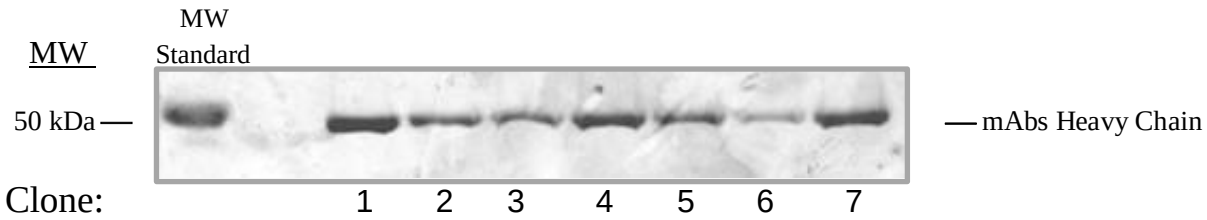


Figure 2.4. Detection of anti-CTLA-4 mAbs following their purification. Purified CTLA-4 antibodies were separated by SDS-PAGE gel and stained with Coomassie Brilliant Blue. Anti-CTLA-4 hybridoma clone 1) 5C411.0, 2) 5D3H5.3, 3) 5D3H5.4, 4) 3E12H2.3, 5) 3E12H2.4, 6)3F12H1.3 and 7) 3F12H1.4.

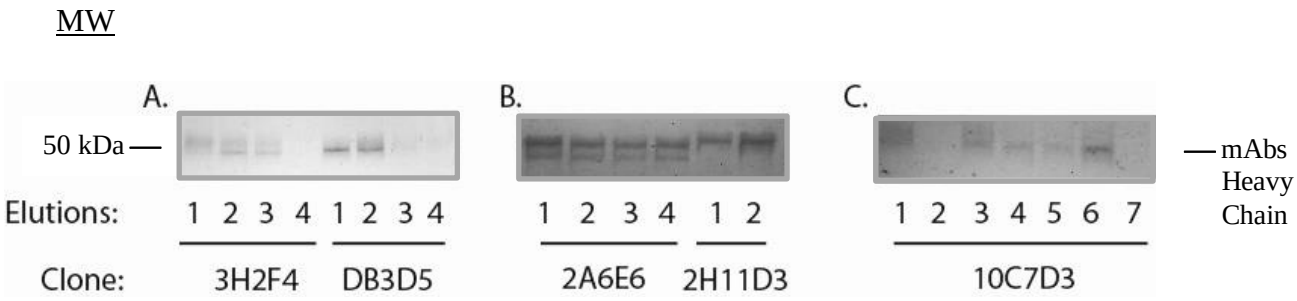


Figure 2.5. Detection of anti-PD1 mAbs following purification. Purified PD-1 antibodies were separated by SDS-PAGE gel and stained with Coomassie Brilliant Blue. Panel A) Anti-PD1 hybridoma clone 3H2F4, elutions 1-4 and clone 5B3D5 elutions 1-4, Panel B) Anti-PD1 hybridoma clone 2A6E6 elutions 1-4 and clone 2H11D3 elution 1-2, Panel C) Anti-PD1 hybridoma clone 10C7D3 elutions 1-7.

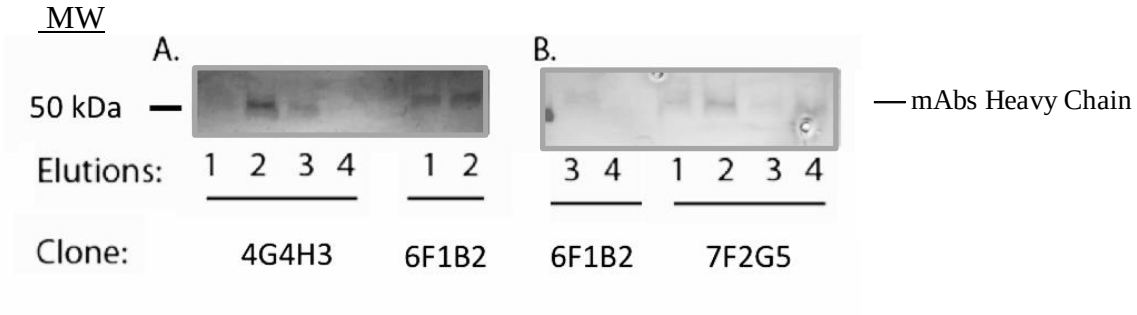


Figure 2.6. Detection of anti-BTLA mAbs following purification. Purified BTLA antibodies were separated by SDS-PAGE gel and stained with Coomassie Brilliant Blue. Panel A) BTLA antibody hybridoma clone 4G4H3, elutions 1-4, and clone 6F1B2 elutions 1-2, Panel B) Anti-BTLA hybridoma clone 6F1B2, elutions 3-4, and clone 7F2G5 elutions 1-4.

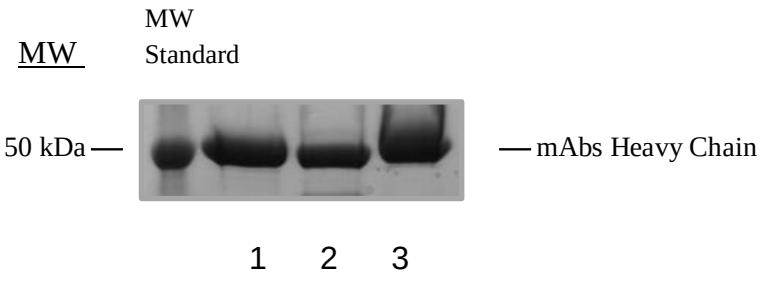


Figure 2.7. Detection of mAbs following Purification of large scale hybridoma cell culture. Purified antibodies were separated by SDS-PAGE gel and stained with Coomassie Brilliant Blue. Lane 1) CTLA antibody clone 5D3H5; lane 2) PD-1 antibody clone 5B3D5; lane 3) BTLA antibody clone 1H3H6.

2.4 Discussion

Monoclonal antibodies are extensively used for the treatment of many alignments including autoimmune diseases, transplant tolerance as well as cancer. Using small molecules or antibodies for the manipulation of activation and co-inhibitory signals for the treatment of cancer is an area of ongoing research. In an effort to provide further insight on the use of mAbs to reduce tumor burden and to shine light on a poorly characterized immune checkpoint, BTLA, seventeen hybridoma clones against CTLA-4, PD-1 and BTLA were developed. These hybridoma clones were developed using the corresponding protein's sequence of interest, more specifically the extracellular domain (ECD) of each protein. Making use of the ECD of each protein ensures for the specificity of each mAbs to recognize and bind to their target.

The development of hybridoma cell lines capable of producing antibodies allowed for the development of a small-scale culture and purification method which was used to generate mAb in house (Figure 2.4-6). Small scale production resulted in 2-4 mg of pure antibody per clone which

allowed for the initial characterization in vitro. However, large amounts of antibodies are needed in order to accomplish further characterization. To meet this need we used of a two-chamber flask as a large-scale culture method to amplify our novel mAb. This approach was successful and resulted in the production of 12-18 mg of pure monoclonal antibody per clone (Figure 2.7). These mAbs were used to characterize their specificity *in vitro* and their effects *in vivo*.

CHAPTER III

IN VITRO CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST CTLA-4, PD-1 and BTLA.

3.1 Introduction

Co-inhibitory receptors have been studied for the past few decades; however, our approach was to use the full-length extracellular domain of each protein in order to facilitate recognition of secondary and tertiary structure, in contrast to previously developed antibodies against these receptors. Additionally, the increasing ongoing research on these and other co-inhibitory receptors has brought light on the mechanism of action of each and facilitated the strategic combination of targeted therapies (6).

In order to determine the specificity of our mAbs against their targets, purified antibodies produced and purified from six CTLA-4 hybridoma clones, five PD-1 clones and five BTLA clones were tested by dot blot, Western blot and flow cytometry for their ability to recognize human and mouse protein selectively and on the surface of living cells.

3.2 Materials and methods

3.2.1 Dot Blot Analysis: Decreasing concentrations (0.1 µg, 0.01 µg, 0.001 µg and PBS diluent (0 µg)), of human extracellular domain (ECD) peptide for CTLA-4, PD-1 and BTLA as well as a non-coinhibitory receptor control protein (CD22 or Jak3) were spotted onto a PVDF membrane and allowed to dry. Membranes were Western blotted as previously described (18) using the designated purified mAbs developed in this study.

3.2.2 Cell culture: Human YT, HH and Jurkat cell lines were maintained in RPMI-1640 medium containing 10 % fetal bovine serum (FBS, Atlanta Biologicals), 2 mM L-glutamine, and penicillin-streptomycin (50 mg/ml). Mouse CTLL-2 cell line (ATCC) was maintained in RPMI-1640 medium containing 10% FBS, 2 mM L-glutamine; additional 1mM sodium pyruvate; 10% T-

STIM with Con A (Becton Dickinson), penicillin-streptomycin (50 IU/ml and 50 μg/ml, respectively), and 25 U/ml of recombinant human IL-2 (25 U/ml). Mouse Baf3 cell lines were maintained in RPMI-1640 medium containing 10 % fetal bovine serum (FBS, Atlanta Biologicals), 2 mM L-glutamine, and penicillin-streptomycin (50 mg/ml) and 10 U/ml of IL-3. Murine EMT-6 cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 2 mm l-glutamine. All cell lines were maintained in a humidified incubator at 37 °C and 5% CO_2.

3.2.3 SDS-PAGE and Western blot Cell pellets were lysed and subjected to SDS-PAGE as previously described (18). Briefly, samples were separated by 10 % SDS-PAGE and transferred to PVDF membrane prior to undergoing Western blot analysis as previously described (18). Antibodies used for Western blot include our novel antibodies against CTLA-4 (clone 5D3D5), anti PD-1 (clone 5B3H5) and anti BTLA (clone 1H3H6) and commercially available antibodies against CTLA-4 clone 9H10 (Millipore 04963), PD-1 (abcam 52587) and BTLA (abcam 212089).

3.2.4 Immunostaining and Flow Cytometry: Actively growing YT, HH, Jurkat, CTLL-2, Baf3 and EMT-6 cells were harvested and immunostained, according to eBiosciences' Staining Cell Surface Antigens for Flow Cytometry protocol (19), using monoclonal anti-CTLA-4, anti-PD1 and anti-BTLA antibodies developed in this study and anti-CTLA-4 clone 9H10 (ebioscience 16-1521-85) and PD-1 (abcam 52587). Fluorescein labeled goat anti-mouse IgG, goat anti-rabbit and goat anti-hamster (H+L) antibody (Pierce) were used to detect anti-CTLA-4, anti-PD1 ad anti-BTLA by flow cytometry analysis. Flow cytometry results were obtained using a Beckman Coulter Gallios flow cytometer using 488 nm laser to detect green fluorescence (500-550 nm).

3.3 Results

Dot blot analysis was performed by spotting decreasing concentrations of human or mouse ECD peptide (0.1 µg, 0.01 µg, 0.001 µg and PBS diluent (0µg)) for CTLA-4, PD-1 and BTLA as well as a non-coinhibitory receptor control protein onto a polyvinylidene difluoride (PVDF) membrane. The resulting membrane was Western blotted using our unique anti-CTLA-4, anti-PD1 or anti-BTLA purified mAbs from each clone. Dot blot analysis showed mAbs developed in this study to recognize each target selectively, as they did not recognize a control peptide. Five of the six anti-CTLA-4 clones and five anti-PD1 clones were found to be sensitive enough to recognize as little as 0.001 µg of peptide. In comparison, all five anti-BTLA clones required 0.1 µg of peptide in order to detect BTLA (Figure 3.1-3.3).

Also, full cell lysate was collected from human cell lines: YT, HH and Jurkat cells and from mouse cell lines: CTLL-2, Baf3 and EMT-6 cells. Whole cell lysate was separated by SDS-PAGE electrophoresis and Western blotted using our novel antibodies against CTLA-4 clone 5D3D5, anti PD-1 clone 5B3H5 and anti BTLA clone 1H3H6 and compared with commercially available antibodies against CTLA-4 clone 9H10 (Millipore 04963), PD-1 (abcam 52587) and BTLA (abcam 212089). Western blot data showed YT, HH, Jurkat, CTLL-2, Baf3 and EMT-6 cell lines express CTLA-4, PD-1 and BTLA (Figure 3.4-3.6). Baf3 cells showed the higher expression of CTLA-4 followed by YT, HH, Jurkat and EMT-6 cells. CTLL-2 cells showed the lower expression of CTLA-4 compared to the other cell lines. Novel mAb against CTLA-4 clone 5D3H5 showed to western blot with a higher specificity than the commercial antibody (Millipore) (Figure 3.4). Similarly, YT, HH, Jurkat and Baf3 cells showed higher expression of PD-1 followed by EMT-6 and CTLL-2 cells which showed lower expression of PD-1. Novel mAb against PD-1 clone 5B3D5 and commercial antibody (abcam) recognized the protein similarly (Figure 3.5).

Lastly, YT, HH, Jurkat, CTLL-2, Baf3 and EMT-6 cells all show expression of BTLA. However, novel mAb against BLTA clone 1H3H6 showed higher specificity as it recognized BTLA on all cell lines while the commercially available antibody (abcam) only recognized the protein on HH and Jurkat cells (Figure 3.6).

Additionally, to test the ability of these mAbs to recognize their target on the cell surface, human cell lines YT, HH and Jurkat and mouse cell lines Baf3 and EMT-6 cells were immunostained using monoclonal anti-CTLA-4, anti-PD1 and anti-BTLA antibodies developed in this study and anti-CTLA-4 clone 9H10 (ebioscience 16-1521-85) and PD-1 (abcam 52587) as controls. Flow cytometry results were obtained using a Beckman Coulter Gallios flow cytometer using 488 nm laser to detect green fluorescence (500-550 nm) (Figure 3.7-3.12). YT cells were used to confirm the expression of PD-1 and the ability of novel mAbs to recognize and bind to their target on the cell surface. (Figure 3.7). YT cells were immunostained with novel mAbs against PD-1 clones 2A6E6, 2H11D3, 3H3F4, 5B3D5 and 10C7D3. Clone 5B3D5 stained 54% of the cells while the other clones stained 40% or below. (Figure 3.8). HH cells were stained with novel mAb against CTLA-4 clone 5D3H5, PD-1 clone 5B3D5 and BTLA clone 1H3H6 and staining was showed to be 53%, 16% and 72% respectively. Antibody against CTLA and BTLA showed the higher staining of HH cells (Figure 3.9). Similarly, Jurkat cells were stained with three noel mAbs and their ability to recognize their target on the cell surface was as between 1.2 and 4.8% (figure 3.10). Baf3 cells were stained with novel mAb against CLA-4 clone 5D3H5, PD-1 clone 5B3D5 and BTLA clone 1H3H6 as well as controls. Novel mAb against BTLA clone 1H3H6 showed the best staining of 21% of the cells (Figure 3.11). Lastly, EMT-6 cells were stained with mAb against CLTA-4 clone 5D3H5 which recognized 60% of the cells (Figure 3.12).

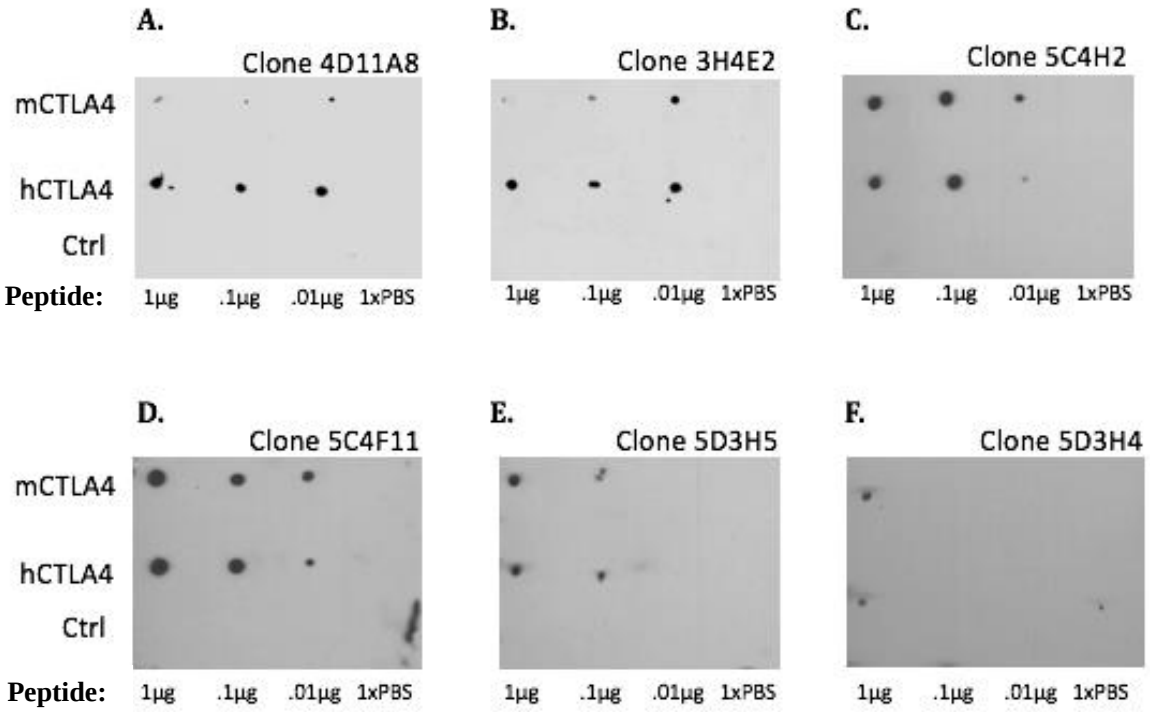


Figure 3.1. Anti-CLTA-4 Dot blot analysis. Decreasing concentrations (shown) of CTLA-4 or control peptide were spotted onto PVDF membrane, allowed to dry and then Western blotted (WB) using CTLA-4 hybridoma clone mAbs. Panel A) WB with clone 4D11A8, Panel B) WB with clone 3H4E2, Panel C) WB with clone 5C4H2, Panel D) WB with clone 5C4F11, Panel E) WB with clone 5D3H5, Panel F) WB with clone 5D3H4.

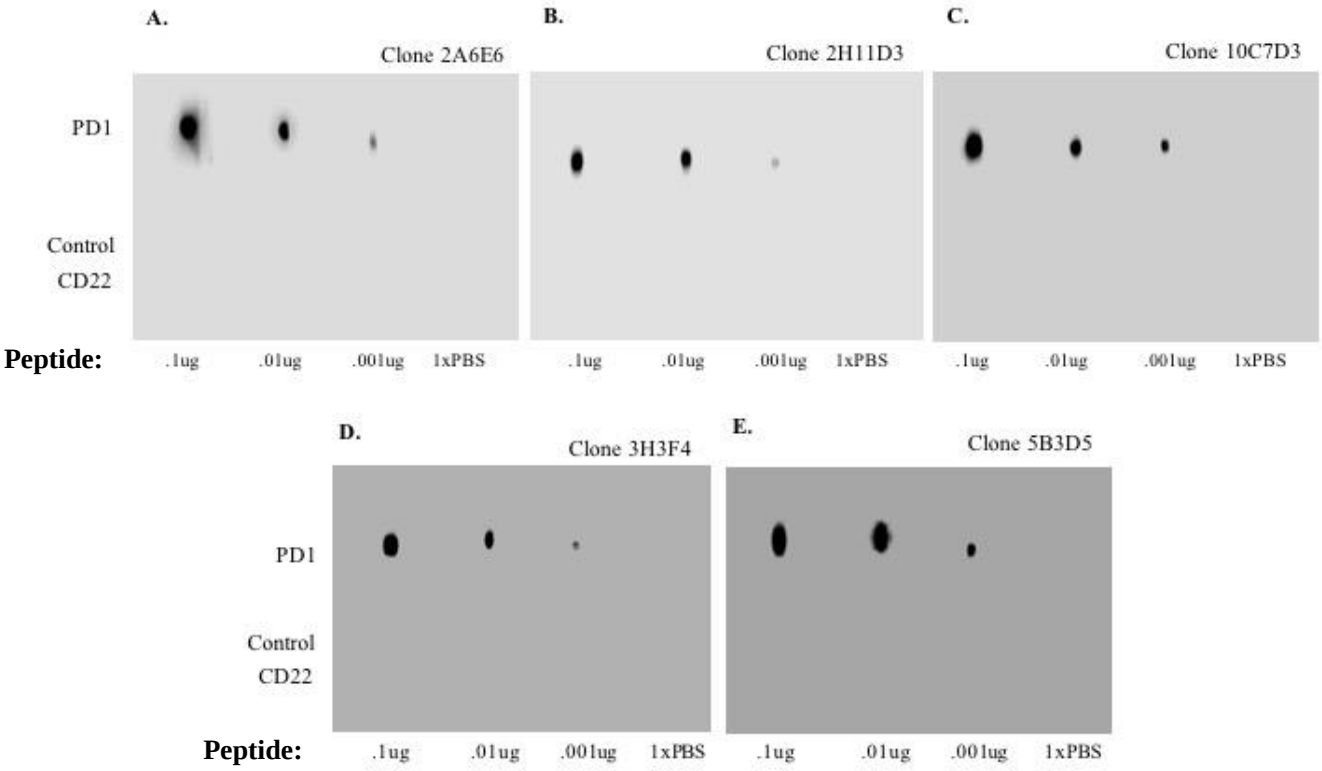


Figure 3.2. Anti-PD-1 Dot blot Analysis. Decreasing concentrations (shown) of PD-1 or control peptide, CD22 were spotted onto PVDF membrane, allowed to dry and then Western blotted (WB) using PD-1 hybridoma clone mAbs. Panel A) WB with clone 2A6E6, Panel B) WB with clone 2H11D3, Panel C) WB with clone 10C7D3, Panel D) WB with clone 3H3F4, Panel E) WB with clone 5B3D5.

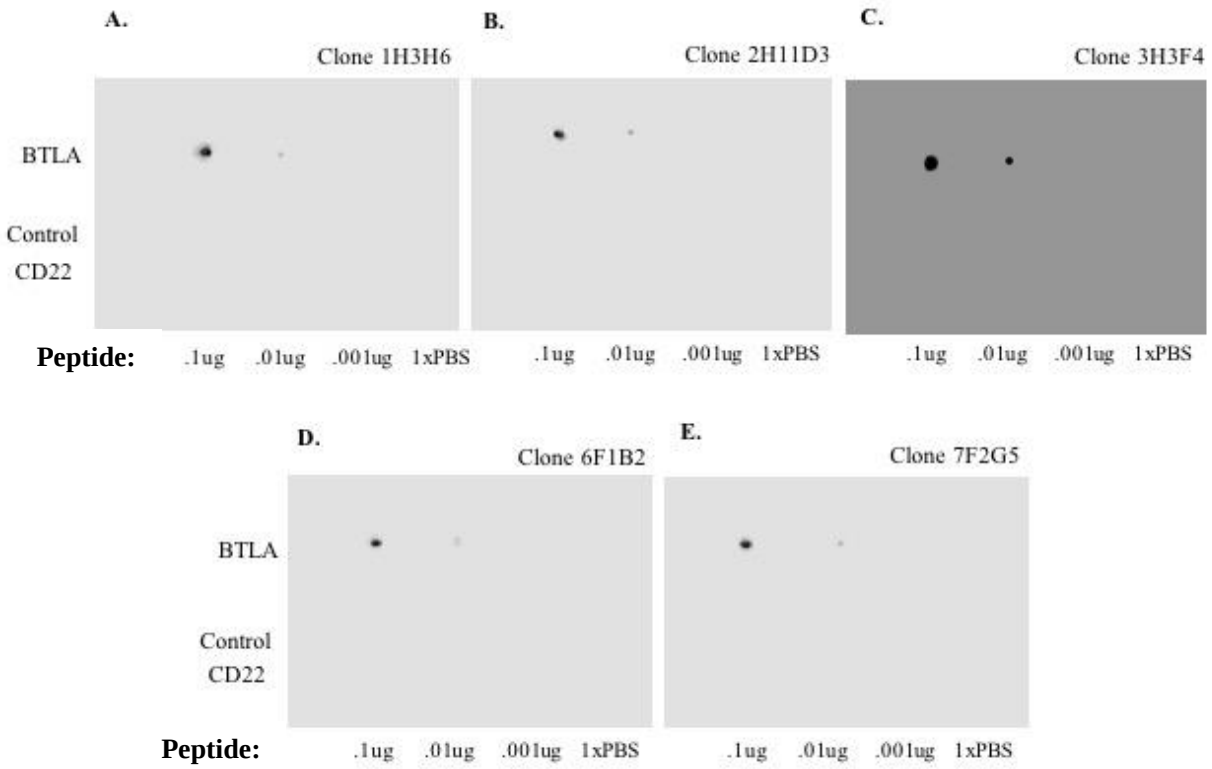


Figure 3.3. Anti-BTLA Dot blot Analysis. Decreasing concentrations (shown) of BTLA or control peptide, CD22 were spotted onto PVDF membrane, allowed to dry and then Western blotted (WB) using BTLA hybridoma clone mAbs. A) WB with clone 2A6E6; B) WB with clone 2H11D3; C) WB with clone 10C7D3; D) WB with clone 3H3F4; E) WB with clone 5B3D5.

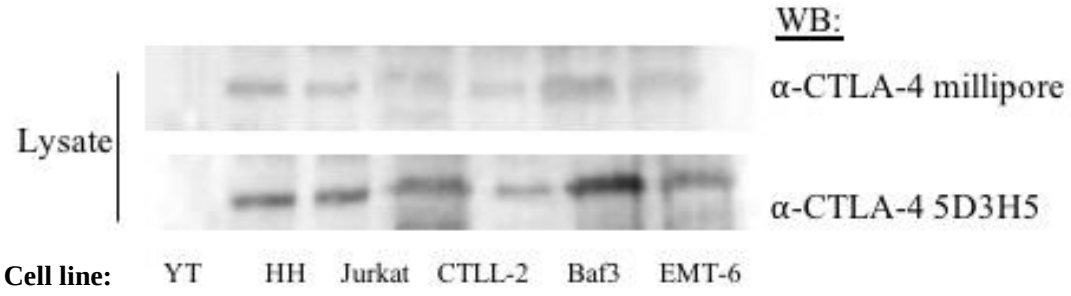


Figure 3.4. Expression of CTLA-4 on human and mouse cell lines. Full cell lysate of YT, HH, Jurkat, CTLL-2, Baf3 and EMT-6 cell lines separated on SDS-PAGE and Western blotted with anti-CTLA-4 antibodies. Top panel shows WB with anti-CTLA-4 (Millipore 04963); bottom panel shows WB with anti-CTLA-4 clone 5D3H5.

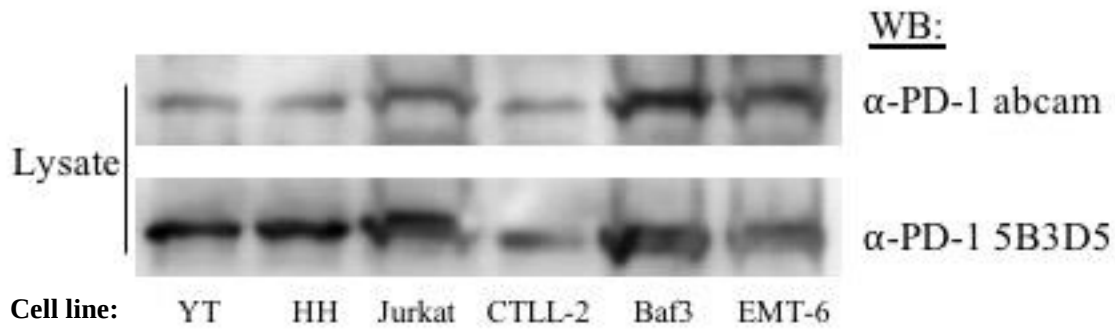


Figure 3.5. Expression of PD-1 on human and mouse cell lines. Full cell lysate of YT, HH, Jurkat, CTLL-2, Baf3 and EMT-6 cell lines separated on SDS-PAGE and Western blotted with anti-PD-1 antibodies. Top panel shows WB with anti-PD-1 (abcam 52587); bottom panel shows WB with anti-PD-1 clone 5B3D5.

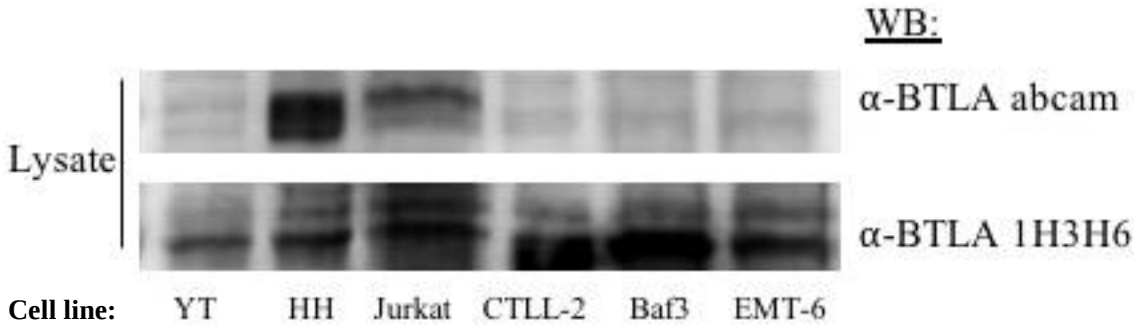


Figure 3.6. Expression of BTLA on human and mouse cell lines. Full cell lysate of YT, HH, Jurkat, CTLL-2, Baf3 and EMT-6 cell lines separated on SDS-PAGE and Western blotted with anti-BTLA antibodies. Top panel shows WB with anti-BTLA (abcam 212089); bottom panel shows WB with anti-BTLA clone 1H3H6.

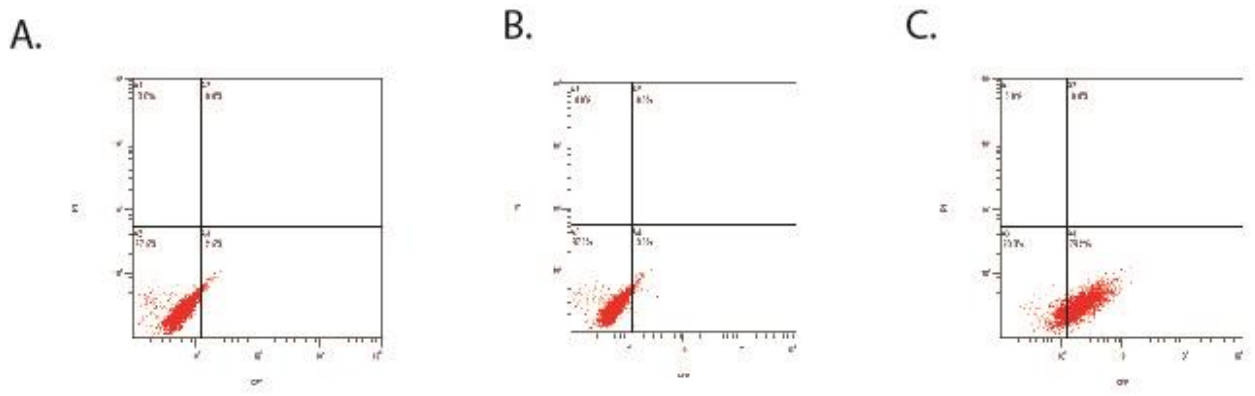


Figure 3.7. Detection of PD-1 receptor on YT cells by Flow Cytometry analysis. A) Unstained YT cells alone; B) YT cells stained with secondary antibody alone; C) YT cells stained with commercially available anti PD-1 (Origene).

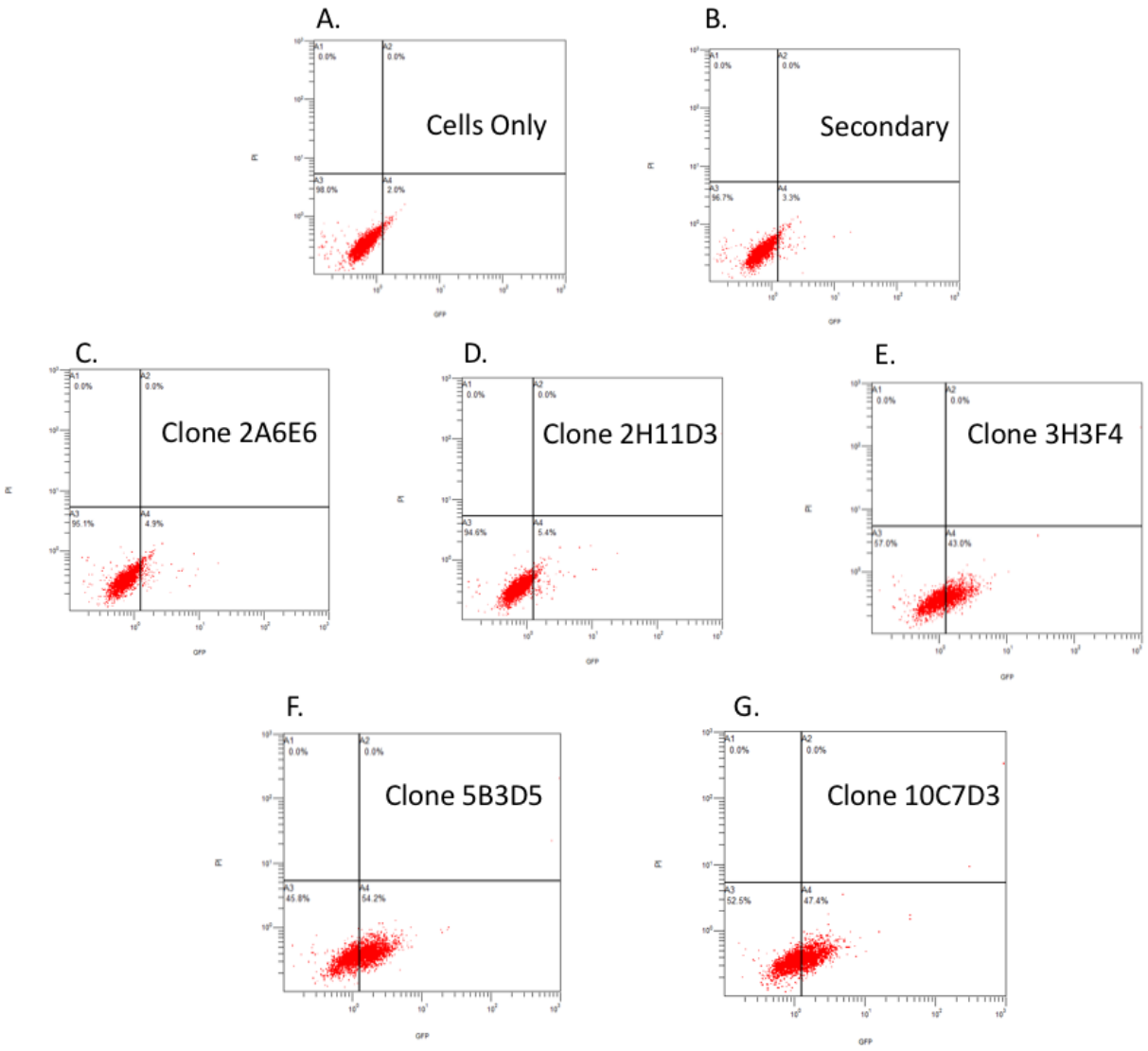


Figure 3.8. Detection of PD-1 receptor on YT cells by flow cytometry analysis using novel mAbs. A) Unstained YT cells alone; B) YT cells stained with secondary antibody alone; C) YT cells stained with anti PD-1 clones 2A6E6; D) anti PD-1 clone 2H11D3; E) anti PD-1 clone 2H3F4; F) anti PD-1 clone 5B3D5 and G) anti PD-1 clone 10C7D3

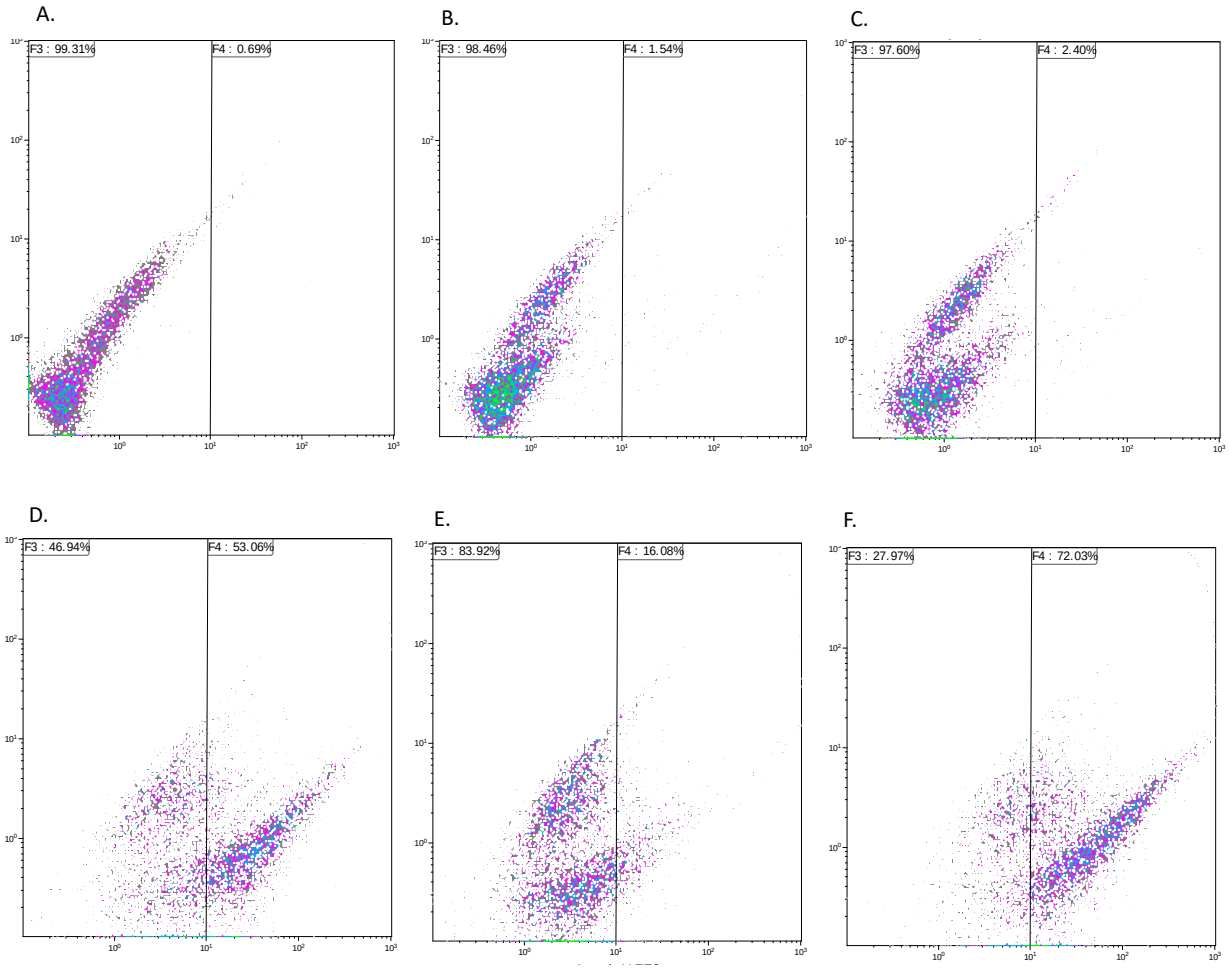


Figure 3.9. Detection of CTLA-4, PD-1 and BTLA receptors on HH cells by flow cytometry analysis using novel mAbs. A) cells only; B) anti-mouse; C) anti PD-1 (abcam); D) anti CTLA-4 clone 5D3D5; E) anti PD-1 clone 5B3H5; F) anti BLTA clone 1H3H6.

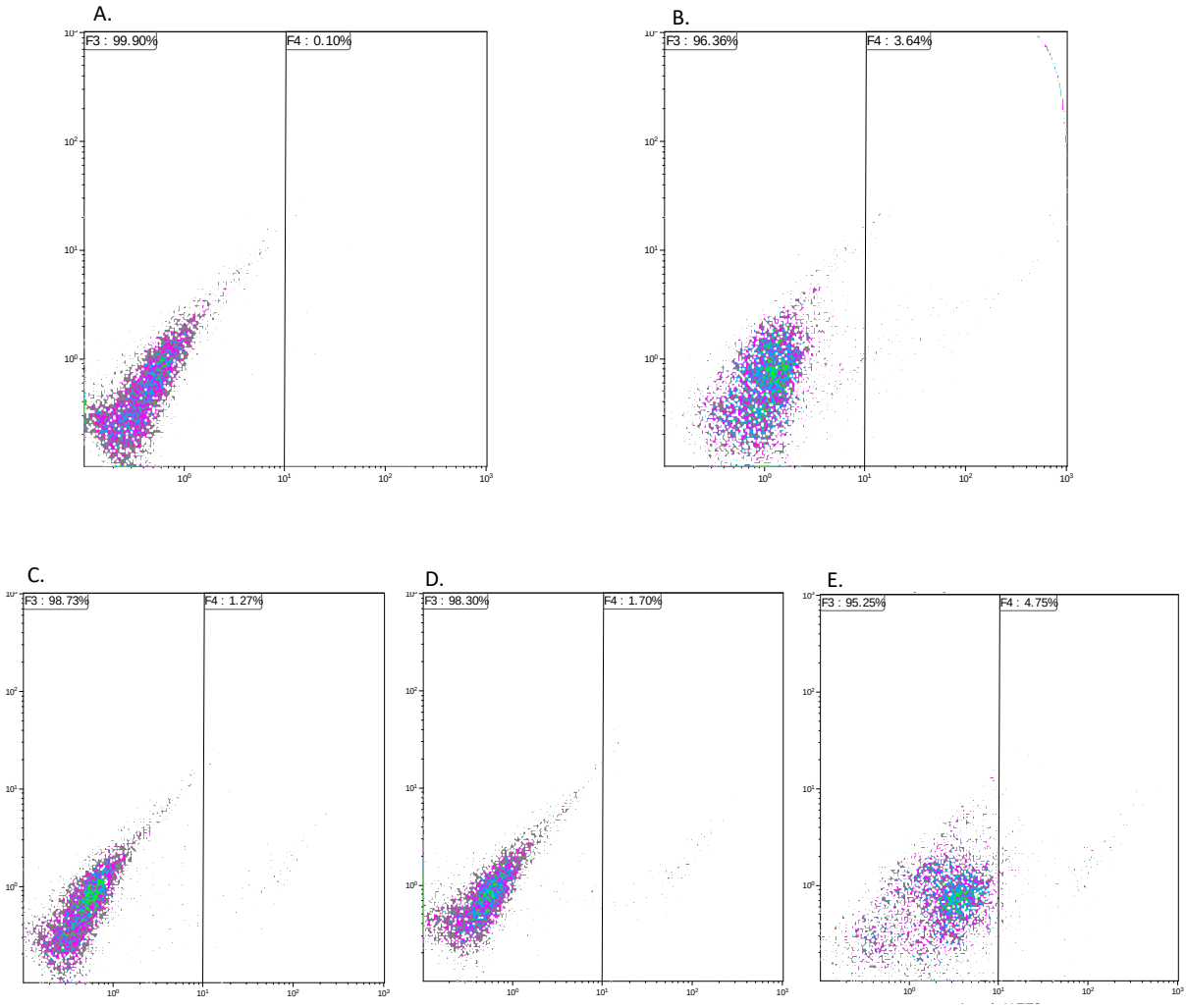
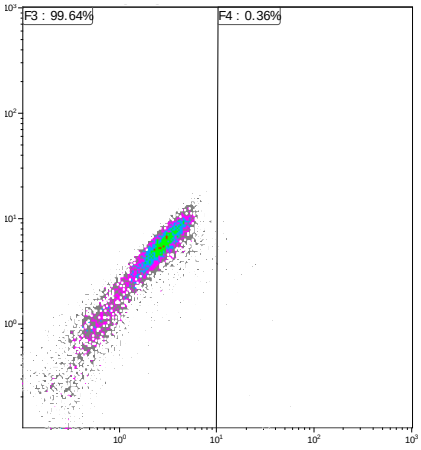


Figure 3.10. Detection of CTLA-4, PD-1 and BTLA receptors on Jurkat cells by flow cytometry analysis using novel mAbs. A) cells only; B) anti-mouse; C) anti CTLA-4 clone 5D3D5; D) anti PD-1 clone 5B3H5; E) anti BLTA clone 1H3H6.

A.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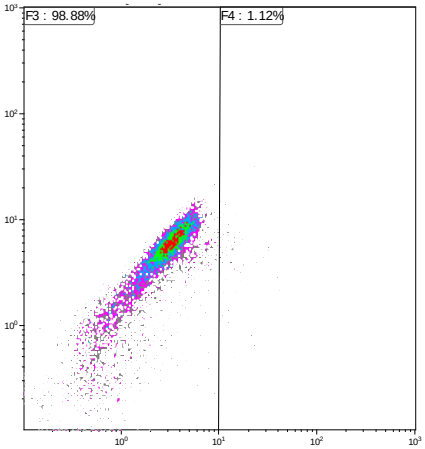
F4 : 0.36%

B.


F3 : 98.88%
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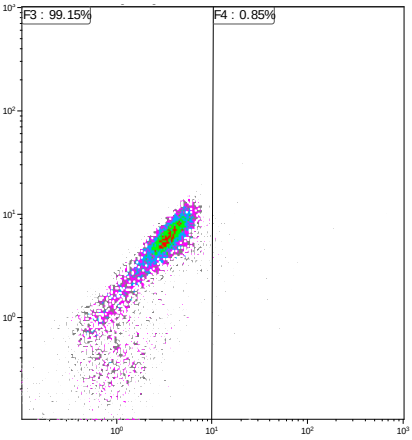
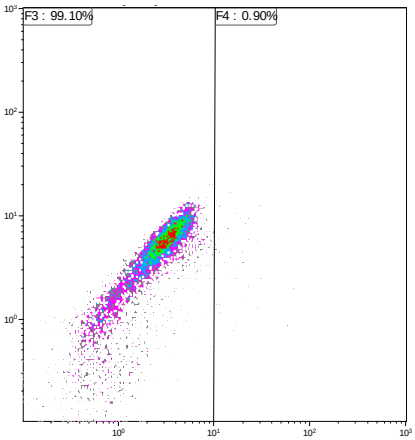
C.


F3 : 99.15%
F4 : 0.85%

D.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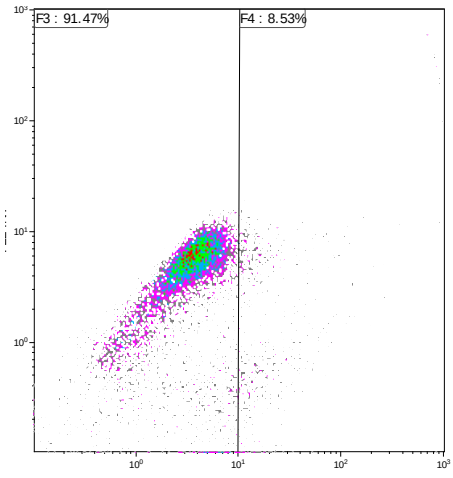
F4 : 0.90%

E.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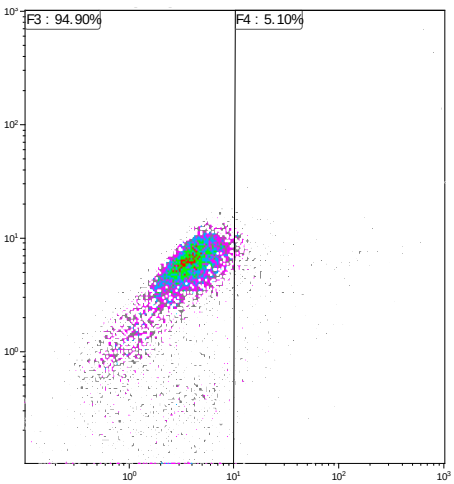
F4 : 8.53%

F.


F3 : 94.90%
F4 : 5.10%

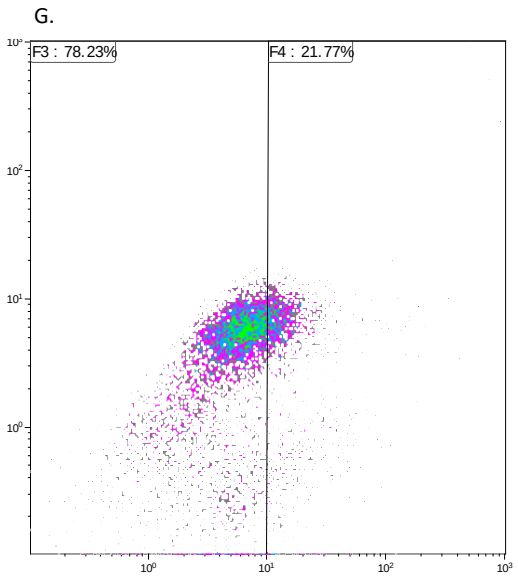


Figure 3.11. Detection of CTLA-4, PD-1 and BTLA receptors on Baf3 cells by flow cytometry analysis using novel mAbs. A) cells only; B) anti-mouse; C) anti CTLA-4 (ebioscience); D) anti PD-1 (abcam); E) anti CTLA-4 clone 5D3D5; F) anti PD-1 clone 5B3H5; G) anti BLTA clone 1H3H6.

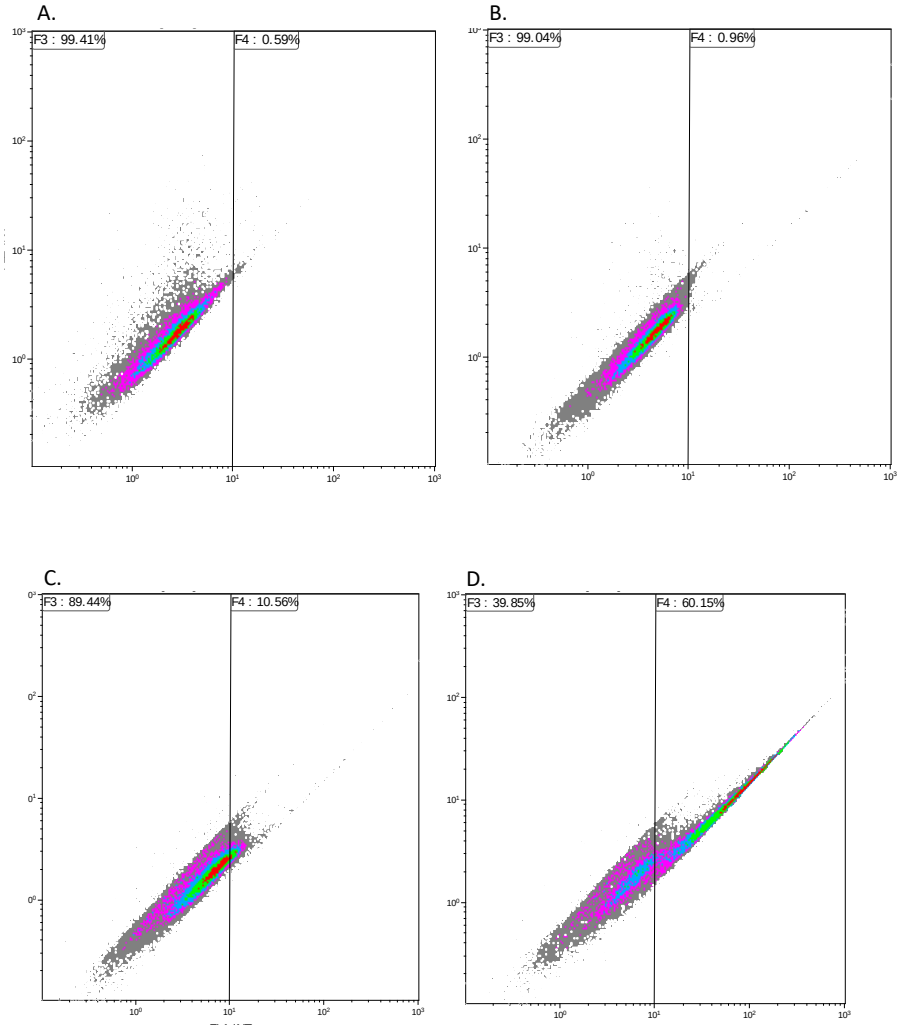


Figure 3.12. Detection of CTLA-4 receptors on EMT-6 cells by flow cytometry analysis using novel mAbs. A) cells only; B) anti-mouse; C) anti CTLA-4 (ebioscience); D) anti CTLA-4 clone 5D3D5.

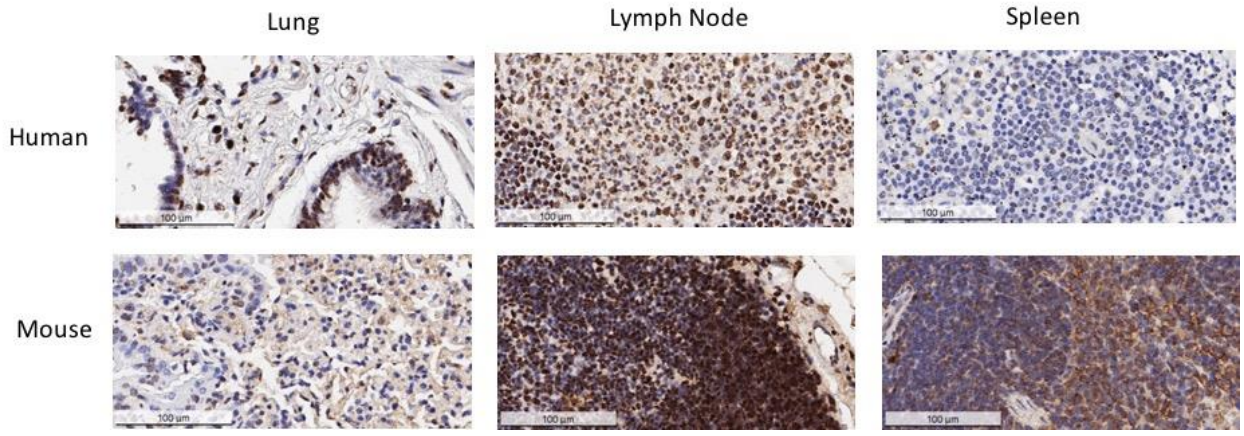


Figure 3.13. Detection of CTLA-4 receptors on human and mouse tissues. Human and mouse lung, lymph node and spleen tissues were stained using novel mAbs against CTLA-4 clone 5D3D5.

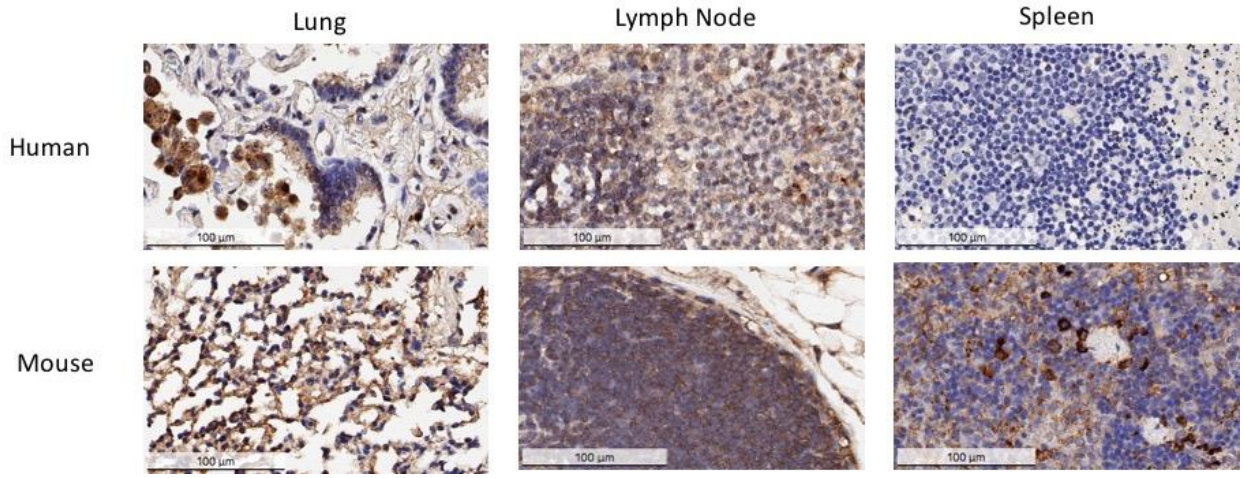


Figure 3.14. Detection of PD-1 receptors on human and mouse tissues. Human and mouse lung, lymph node and spleen tissues were stained using novel mAbs against PD-1 lone 5B3H5.

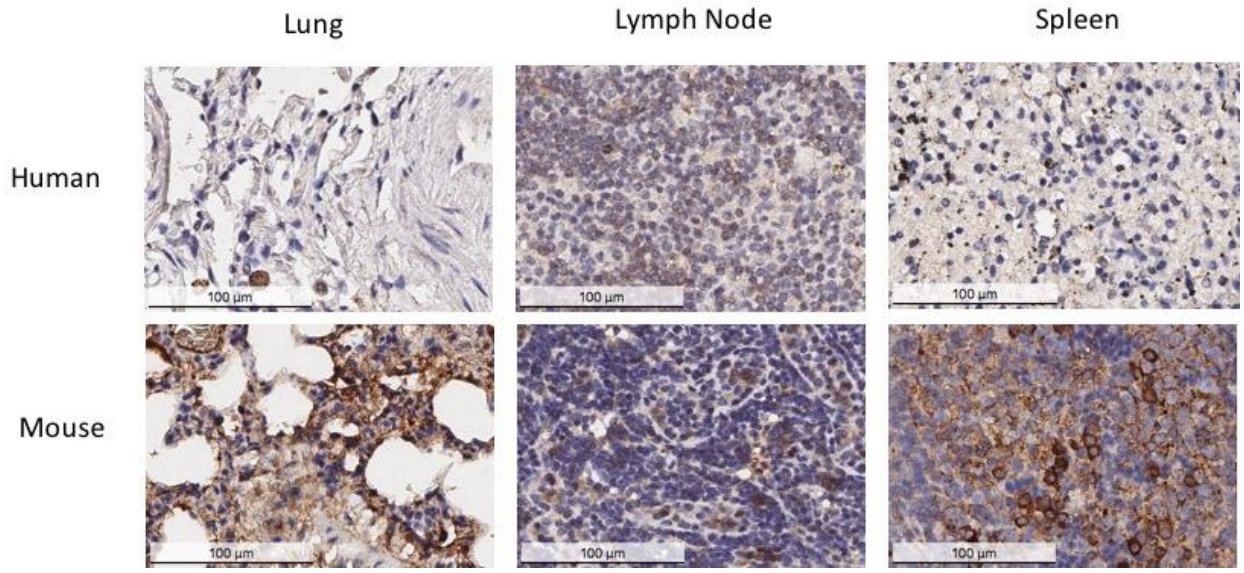


Figure 3.15. Detection of BTLA receptors on human and mouse tissues. Human and mouse lung, lymph node and spleen tissues were stained using novel mAbs against BTLA lone 1H3H6.

3.4 Discussion

The potential of monoclonal antibodies as therapeutic agents relies on their ability to bind and recognize their target. Using the full sequence of the ECD of the proteins of interest hybridoma cells were developed and mAbs produced. The specificity of the novel mAbs to their target was confirmed using full ECD protein as well as human and mouse cell lines (Figure 3.1-3.12). The ECD of the human and mouse proteins were used as the human sequence was used to immunize mice for the development of the different hybridoma clones. Additionally, the mouse ECD was also used in order to observe the recognition patterns of the mAbs developed against the human protein (Figure 3.1-3.6). Similarly, human and mouse cell lines were used in order to determine if the novel mAbs cross react with both human and mouse cell surface receptors. Interestingly, most of the antibodies developed in this study recognized native form exposed on human and mouse

sequence either in dot blot analysis or on the surface of B and T cells as show on the FACS analysis. Moreover, EMT-6 cells are often used to establish tumors in animal models to study the effect of chemotherapeutic agents as well as other mAbs on tumor burden. (20). The data shown above of Western blots using whole cell lysate of several human and mouse cell lines to include EMT-6 cells and on the FACS analysis where EMT-6 cells were stained using novel mAbs suggest that our mAbs could successfully be used on *in vivo* studies using these mouse tumor cell line (figure 3.12). The ability of the novel mAbs to recognize their protein targets in several forms and in a variety of cell lines suggest that these mAbs could successfully bind and recognize their immune checkpoint receptor in an *in vivo* model.

CHAPTER IV

IN VIVO CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST CTLA-4, PD-1 AND BTLA

4.1 Introduction

Ipilimumab (anti-CTLA-4) and Nivolumab (anti-PD-1) are both currently used in the clinic, alone and in combination with each other and in combination with chemotherapeutic agents for the treatment of cancer. Even though the use of these drugs has been reported to be a great advance in the treatment of cancers, such as melanoma, its efficacy it is still limited to certain cancers, for some patients for a determined period of time (21, 22). These patients do not experience the expected outcome and exhibit many side effects, ultimately only to become drug resistant (23, 24).

The objective of *in vivo* characterization of our novel mAbs was to determine their possible effectiveness on a breast cancer tumor model when used alone and in combination. The goal was to first identify the most effective clones as well as the most successful combinational therapies for their ability to reduce tumor volume within a mouse model. The goal is to identify the most effective clones at reducing tumor burden used alone or in different combinations to determine their potential to reduce *in vivo* tumor growth and possible metastasis.

Previously, *in vivo* testing of mAbs against CTLA-4 using a Balb/c mouse model and EMT-6 cells for the establishment of tumors has been successfully performed (20). This model was an appropriate approach to characterize the *in vivo* effects of these novel mAbs against CTLA-4, PD-1 and BTLA based on their ability to recognize their target protein on the surface of EMT-6 cells (Figure 3.12). Using this tumor model mAbs developed as described earlier, were used to test their ability to inhibit tumor growth, *in vivo.* Balb/c mice were injected with EMT-6 breast cancer cells and treated with the different clones of our novel anti-CTLA4, anti-PD1 or anti-BTLA. In the first animal trial, the treatments consisted of two doses were given on the first at day one (35ug) and

the second on day 6 (400 and 800ug). For the combinational trial, the doses were of 35ug and 100ug on day one and day six, respectively.

Additionally, Cytokines are a valuable tool in gaining insight on tumor micro environment (TMI) thus elucidating the immunological events that take place at the molecular level during cancer. To establish the cytokine profile of TMI of *in vivo* testing of mAbs serum collected from mice were submitted to a screening using Luminex technology in which over 15 cytokines are measured. Some cytokines of interest are IL-1, IL-12 and TNF-α which are known to be proinflammatory cytokines and IL-1β found to aid in the recruitment of myeloid cells to lung, breast and pancreatic tumors in a murine model (25). The lack of expression of IL-15 has also been found to be associated with the reduction of natural killer (NK) cells available to be recruited to tumors (25). Furthermore, low levels of IL-2 is related to loss of T cell effector function (13). Lastly, high expression of IL-10, an immunosuppressive cytokine, has been associated with a poor response in the treatment of breast cancer (26, 27).

4.2 Materials and methods

Tumor cell culture: Murine EMT-6/P mammary carcinoma cells were grown in RPMI supplemented with 10% fetal bovine serum and 2 mm l-glutamine. Cells were maintained in a humidified incubator at 37 °C and 5% CO_2. (20)

Antibody preparation**:** Anti-CTLA4, Anti-PD1, Anti-BTLA, Anti-mouse CD152 (CTLA-4), FG purified clone 9H10, purchased from Ebioscience (San Diego, CA, USA), were diluted in PBS

immediately before i.p. injection. Mice were administered 100 μg of the antibody on day 1 of treatment followed by a 35 μg injection on day 6. (20)

Tumor growth assessment**:** Six-week-old female Balb/c mice were purchased from Envigo RMS, Inc. (Indianapolis, IN, USA). Mice were allowed to acclimatize for 2 weeks before implantation of tumor cells. To prepare cells for injection, sub-confluent plates were harvested with 1% trypsin-EDTA, and cells then washed and resuspended in RPMI at 2 million cells per ml. Two hundred thousand EMT-6 cells were injected subcutaneously into the flank of the mice (for CT-26 cells, 1 million cells per mouse were implanted). Mice were monitored twice weekly for fluctuations in body weight and for tumor growth, which was measured by Vernier calipers, and tumor volume was calculated by the formula (length × width^2)/2. Institutional guidelines were followed to determine when the experimental end points were reached. Results were plotted as event-free survival (Kaplan–Meier analysis) over time, where duration of event-free survival is defined as time to primary tumor progression beyond 1200 mm^3 or >15% weight loss, as per previous study (20). All *in vivo* procedures and experiments were performed with the approval of the UTEP IACUC (IACUC reference #: A-201201-1) (20).

Statistical Analysis: The analysis of variance among groups (ANOVA), followed by the Student-Newman–Keuls test, were used to assess the statistical differences of data *in vivo* and of cytokine profile using multiplex assay screening. Tumor therapy results are reported as mean ± s.d. survival curves were plotted by the method of Kaplan and Meier and was tested for survival differences using the log-rank test. The level of significance was set at $P<0.05$. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA) (20).

Multiplex assay for cytokine screening: Serum from Balb/c mice was collected at the end of the study and screened for the presence of mouse cytokines and chemokines. Cytokines and chemokines were measured using the Milliplex MAP Mouse Cytokine/Chemokine Magnetic Bead Panel – Premixed 32 Plex-Immunology Multiplex Assay (Millipore) according to manufacturer's protocol. This kit measures the following cytokines/chemokines: G-CSF, Eotaxin, GM-CSF, IFN-γ, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IP-10, KC-like, LIF, LIX, M-CSF, MCP-1, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNF-α, and VEGF (Millipore).

4.3 Results

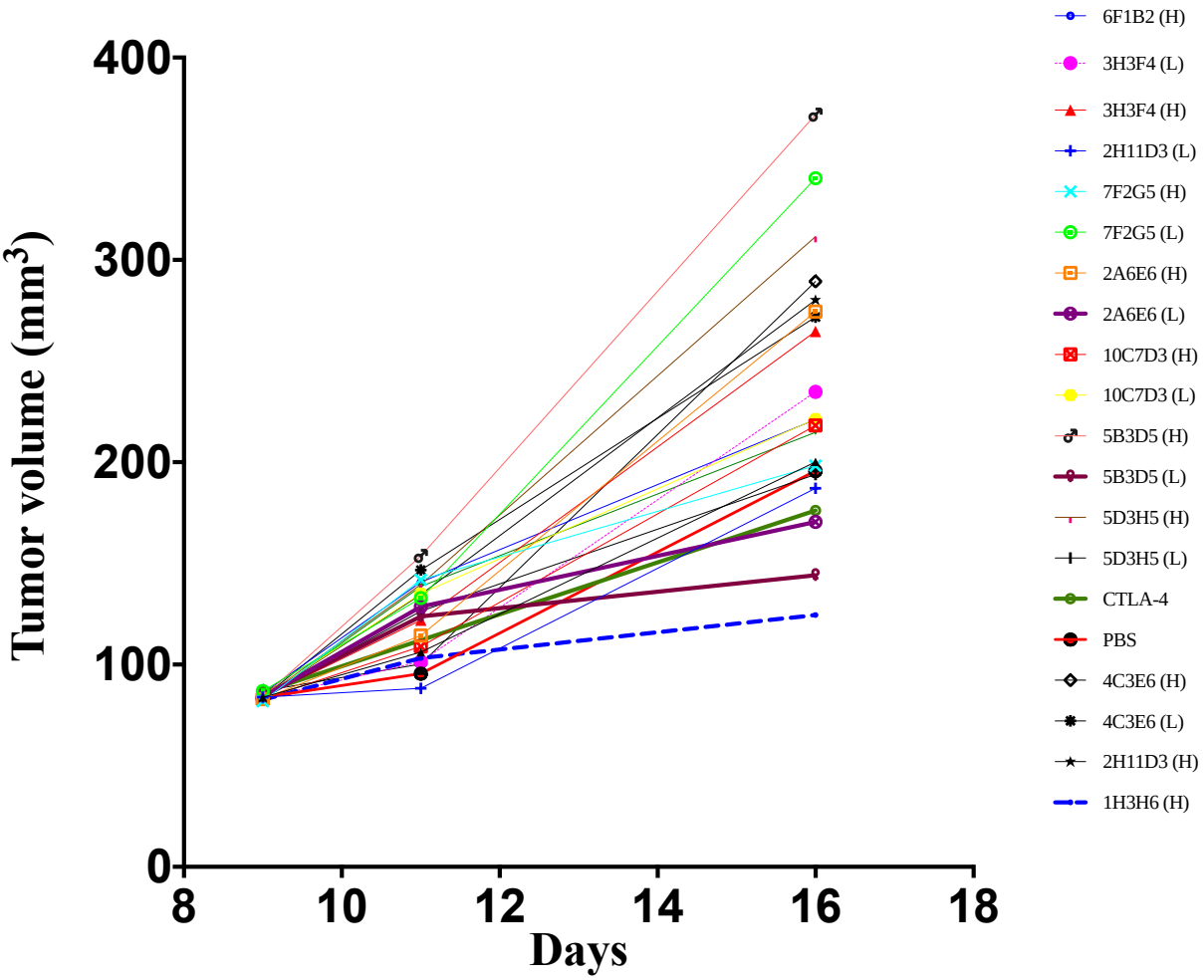


Figure 4.1. Tumor growth curves of in vivo testing of mAb against CTLA-4, PD-1 and BTLA. Novel mAbs against CTLA-4 clone 5D3H5, PD-1 clones 2H11D3, 2A6E6, 3H3F4, 5B3D5 and 10C7D3 and against BTLA clones 6F1B2, 1H3H6, 4C3E6 and 7F2G5 were used to treat Balb/c mice. A high (H) and a low (L) dose were used (H, 800ug; L, 400ug). Shown is the average tumor volume for each treatment group at the time of measurement.

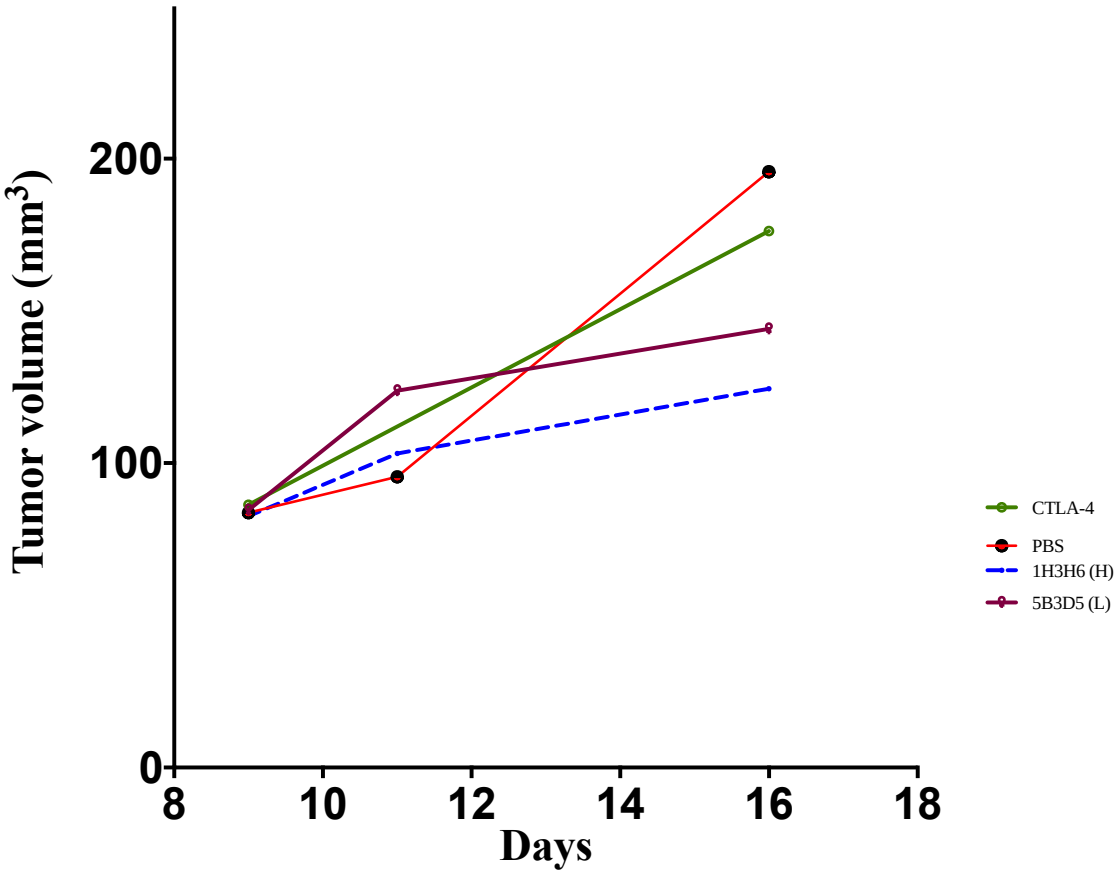


Figure 4.2. Tumor growth curves of in vivo testing of mAb against PD-1 and BTLA. Novel mAbs against PD-1 clone 5B3D5 and against BTLA clone 1H3H6 show reduction of tumor burden on Balb/c mice. A high (H) and a low (L) dose were used (H, 800ug; L, 400ug). Shown is the average tumor volume for each treatment group at the time of measurement.

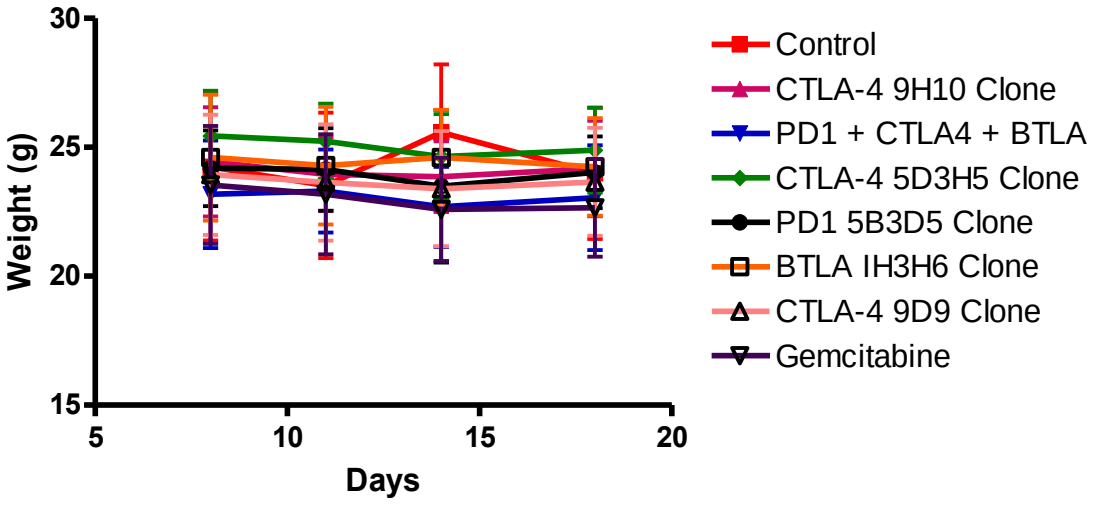


Figure 4.3. Effects of mAb on animal weight as a measure of toxicity. Novel mAbs against CTLA-4 clone 5D3H5, PD-1 clone 5B3D5 and against BTLA clones 1H3H6 were used to treat Balb/c mice and their weight was recorded as a measure of toxicity of treatment. Shown is the average animal weight for each treatment group at the time of measurement.

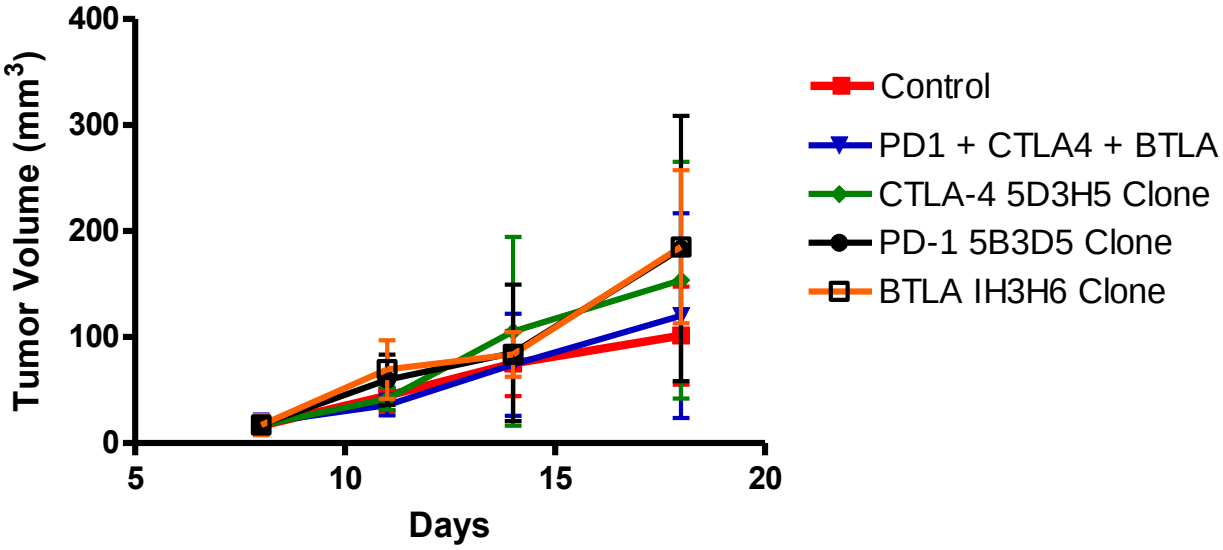


Figure 4.4. Tumor growth curves of in vivo testing of mAb against CTLA-4, PD-1 and BTLA. Novel mAbs against CTLA-4 clone 5D3H5, PD-1 clone 5B3D5 and against BTLA clone 1H3H6 were used to treat Balb/c mice. Shown is the average tumor volume for each treatment group at the time of measurement.

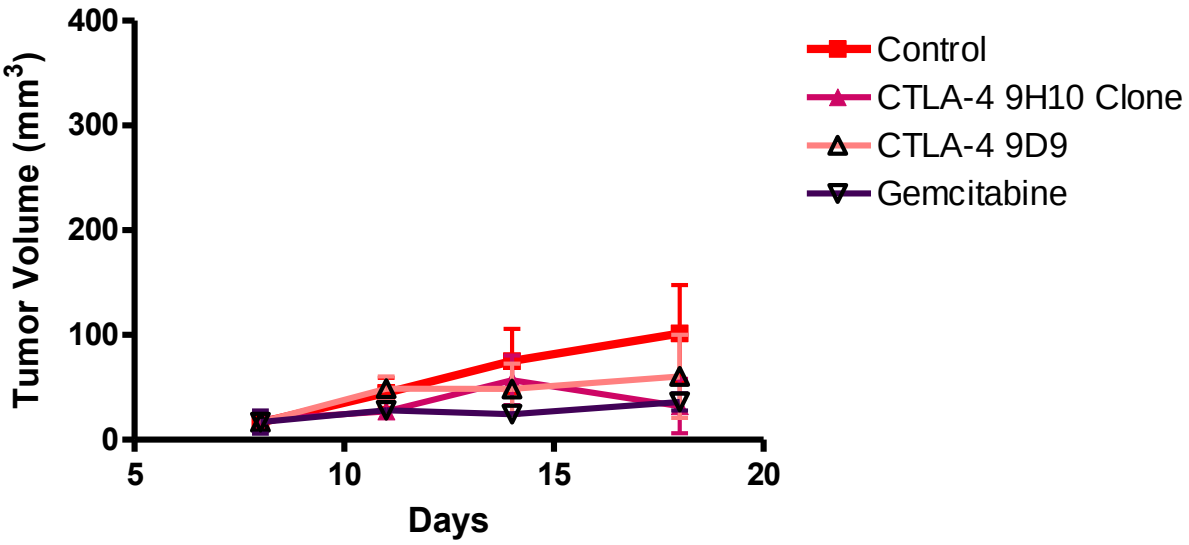
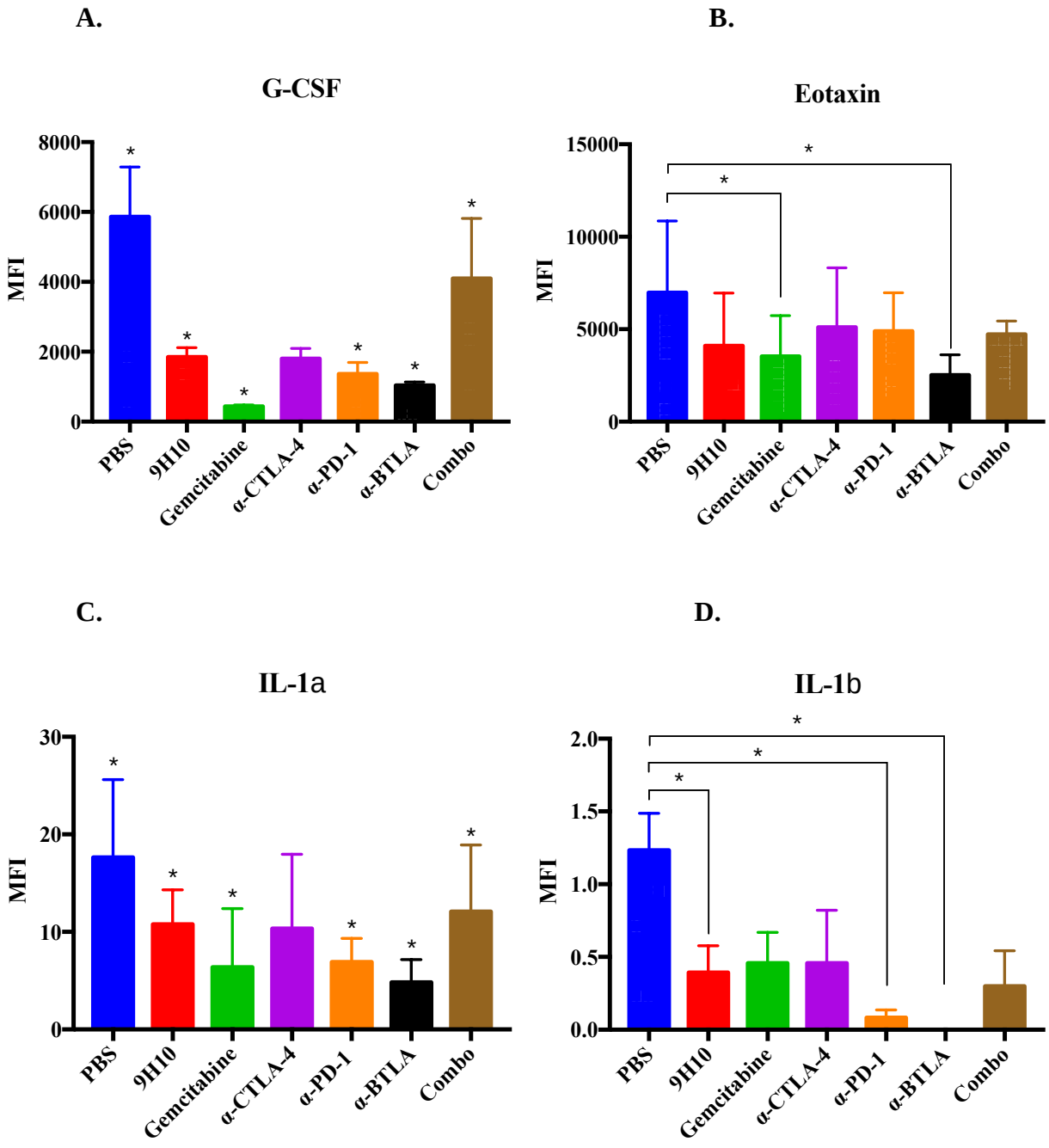
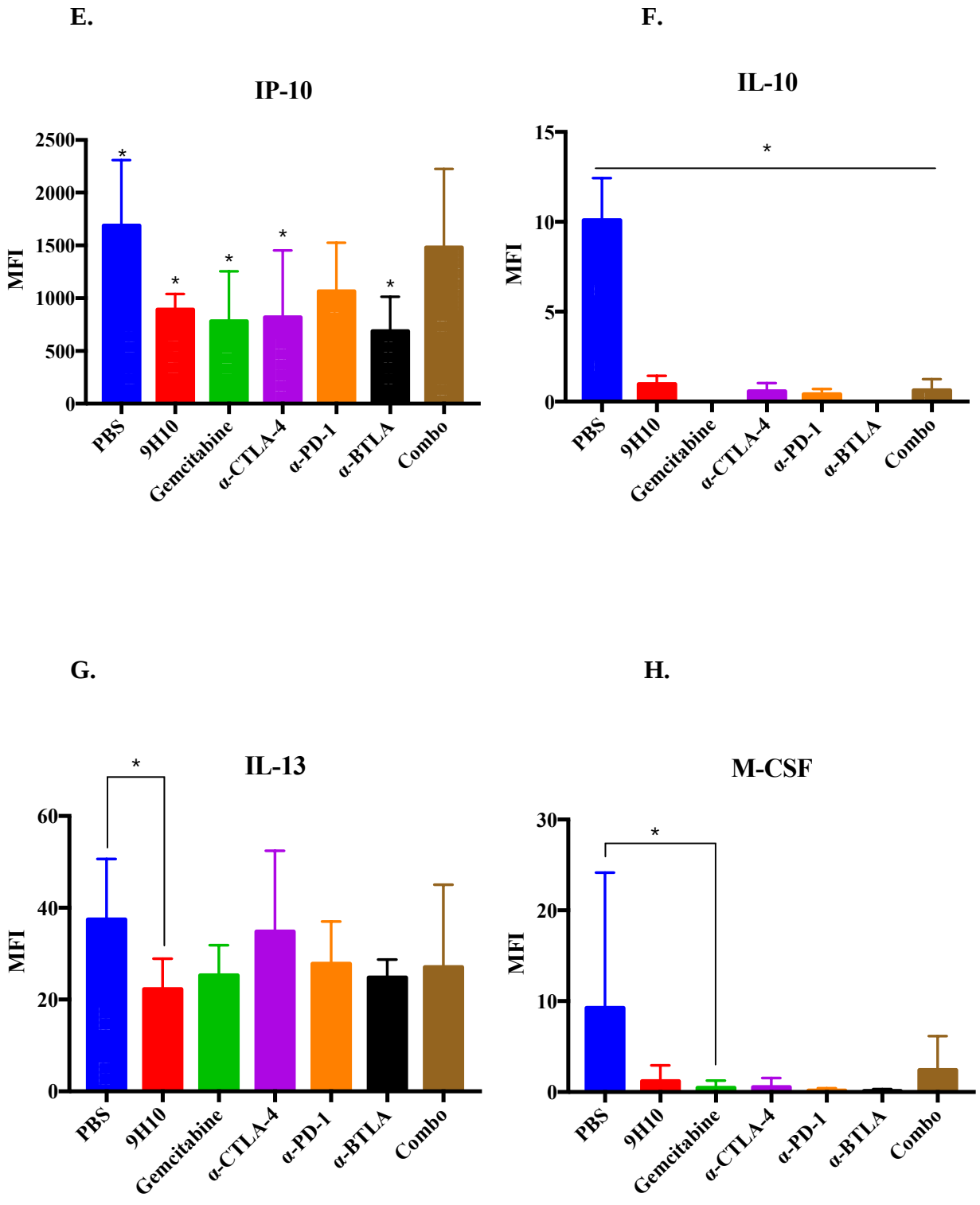
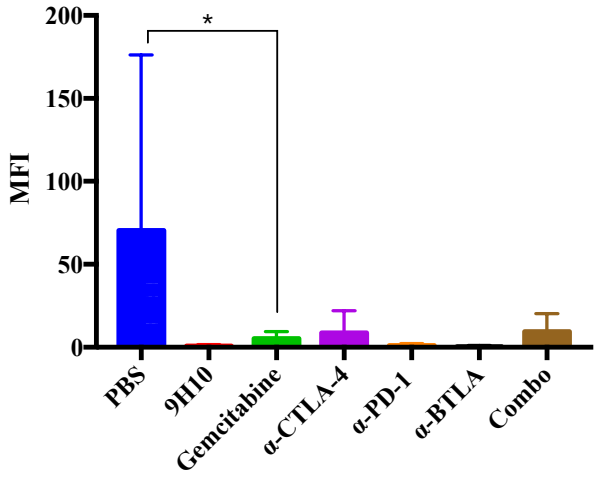


Figure 4.5. Tumor growth curves of in vivo testing of mAb against CTLA-4, PD-1 and BTLA. mAbs against CTLA-4 clone 9H10 and 9D9 and gemcitabine were used to treat Balb/c mice. Shown is the average tumor volume for each treatment group at the time of measurement.

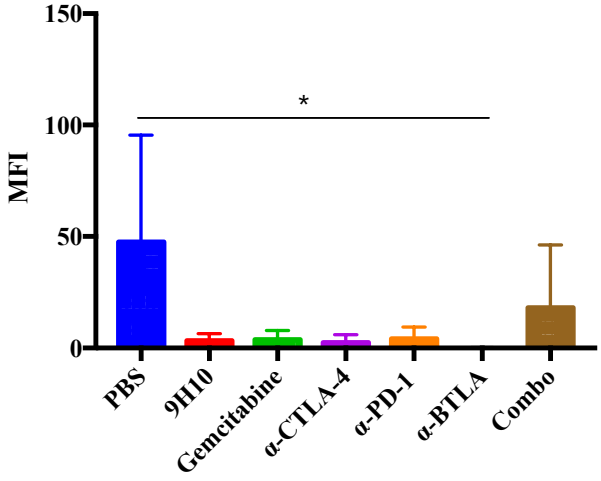

A.
G-CSF
MFI
8000
6000
4000
2000
0
PBS
9H10
Gemcitabine
α-CTLA-4
α-PD-1
α-BTLA
Combo
B.
Eotaxin
MFI
15000
10000
5000
0
C.
IL-1a
MFI
30
20
10
0
D.
IL-1b
MFI
2.0
1.5
1.0
0.5
0.0


E.
IP-10
MFI
2500
2000
1500
1000
500
0
PBS
9H10
Gemcitabine
α-CTLA-4
α-PD-1
α-BTLA
Combo
F.
IL-10
MFI
15
10
5
0
G.
IL-13
MFI
60
40
20
0
H.
M-CSF
MFI
30
20
10
0

I.


MCP-1
MFI
200
150
100
50
0
*
PBS
9H10
Gemcitabine
α-CTLA-4
α-PD-1
α-BTLA
Combo

J.


MIP-1b
MFI
150
100
50
0
*
PBS
9H10
Gemcitabine
α-CTLA-4
α-PD-1
α-BTLA
Combo

K.

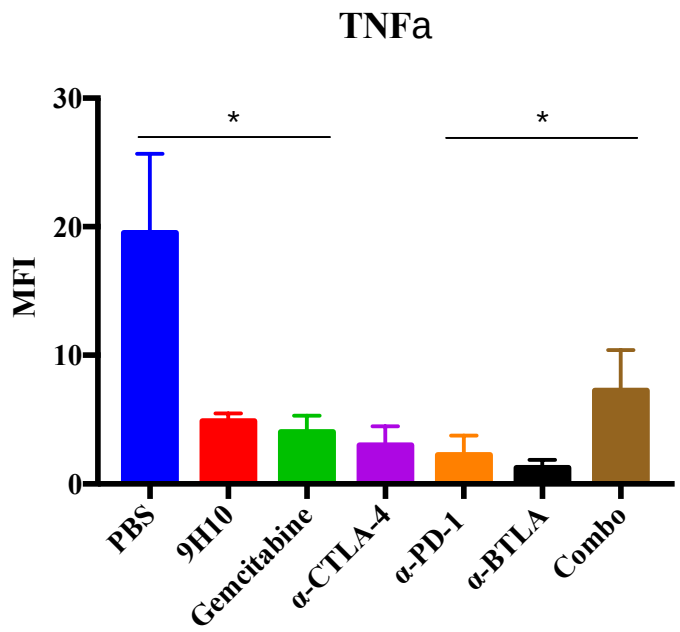

TNFa
MFI
30
20
10
0
*
*
PBS
9H10
Gemcitabine
α-CTLA-4
α-PD-1
α-BTLA
Combo

Figure 4.6. Cytokine profile of *in vivo* testing of mAbs. Cytokines G-CSF, Eotaxin, GM-CSF, IFN-γ, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IP-10, KC-like, LIF, LIX, M-CSF, MCP-1, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNF-α, and VEGF were screened using Milliplex Assay (Millipore). Cytokines that showed statistical significance are G-CSF, Eotaxin, IL-1α, IL-1β, IP-10, IL-10, IL-13, M-CSF, MCP-1, MIP-1β, and TNF-α as shown on panels A-K. Statistical difference was observe between PBS control and an experimental group denoted by the asterisks (*). *The level of significance was set at $P<0.05$.

4.4 Discussion

Hybridoma clones with the ability to produce mAbs against the immune checkpoints of interests were tested *in vivo* for their ability to reduce tumor burden. A total of seventeen clones were generated of which one clone against each immune receptor was isolated for their positive effects on tumor volume reduction (Figure 4.1). Clones 5D3H5, 5B3D5 and 1H3H6 for CTLA-4, PD-1 and BTLA respectively showed to reduce tumor burden on *in vivo* testing. This was followed by the large-scale production of these three clones to be used on additional animal trial where the goal was to explore their combinational effect. It was anticipated to observe a potent protection against tumor progression and growth in the combinational experimental groups of two or more of the novel mAbs developed in this work. However, the additional *in vitro* studies using the three mAbs that had previously shown tumor volume reduction did not show the protective effect expected when used alone or in a triple combination (Figure 4.4). These mAb did not have a protective effect against tumor growth or reduced tumor size once established. In an effort to understand the effect the different treatment groups, control and experiment, had on the molecular mechanism, mouse serum was screened using Milliplex chemokine/cytokine kit.

The cytokine profile established using mouse serum and a multi-plex cytokine screening assay showed cytokine such as G- CSF, Eotaxin, IL-1α, IL-1β, IL-10, IL13, IP-10, MCP-1, MCP-1, MIP-1β, M-CSF, and TNFγ to be different between treated and control groups when compared with non-treated animals. Granulocyte colony-stimulating factor (G-CSF) is a growth factor associated in proliferation and differentiation of hematopoietic neutrophils (29). G-CSF is also known to induce secretion of TNF-α which was also shown to be low in both treated and untreated groups when compared with the vehicle control (PBS). Similarly, TNF-α (tumor necrosis factor) is involved in inflammation and its produced by macrophages and monocytes. It is also associated with necrosis and apoptosis signaling pathways (30). Two other cytokines shown to be lower on experimental groups that on vehicle control were Eotaxin and IL-13. Eotaxin is a cytokine that acts as a chemoattractant and has been reported to play an important role in migration of eosinophils during allergic inflammation of tissues such as during asthma episodes (31). Likewise, IL-13 is a cytokine associated in allergic inflammation and immunoregulation of B cells and monocytes in humans and macrophages in mice (32). The levels of these two cytokines were not increased on animals with established tumors and treated with novel mAbs. This is understandable since both cytokines are known to be active during allergic reactions such as those related to asthma and Eotaxin is not found on the periphery but reside in tissue (33). Moreover, IL-1α, IL-1β, IL-10 and MIP-1β are all proinflammatory cytokines that have immunoregulatory effects of hematopoietic cells (25, 33). IL-1 promotes development of helper T cells (Th17) while IL-10 reduces helper T cell (Th1) cytokines as well as CD80/CD86 which are important costimulatory molecules. Another interesting aspect of IL-10 is that it aids in the survival, growth and antibody production of B cells (33). In the cytokine profile established using Luminex technology and animal serum, IL-10 showed to be considerably lower in all groups when compared with the

vehicle control. This is of interest given the function on IL-10 specially as it relates to its effects on the reduction of CD80/86 costimulatory molecules which are the ligands for CTLA-4. Additionally, IL-10 has been reported to be a characteristic of the immunosuppressive phenotype of myeloid cells in the tumor micro environment (25). In this study, the levels of IL-10 were observed to be low on treated animals which suggests the tumor microenvironment was not immunosuppressed as it relates to IL-10. (Figure 4.6). However, IL-1 has been reported to be important for the recruitment of myeloid cells and based on the cytokine screening its levels were lower in a few of the groups and not all. This suggest that recruitment of immune cells through IL-1 was present but possible affected in some groups such anti-PD-1 and anti-BTLA treated groups (Figure 4.6).

While it was anticipated to observe differences in main pro-inflammatory cytokines such as IL-2, IL-7, IL-9 and IL-15 between treated and untreated groups and on responsive and non-responsive groups, no significant difference was seen. While this data allowed us to observe the cytokines present on animals used in this trial, further analysis of additional studies is needed to draw more concrete cytokine profile.

CHAPTER V

OVERVIEW AND FUTURE DIRECTIONS

5.1 Overview

The work done over the past few decades on immune checkpoint inhibitors has brought light on their structure, function and potential therapeutic use. With the FDA approval of six different monoclonal antibodies against checkpoint inhibitors and associated molecules to include Ipilimumab (anti-CTLA-4), Nivolumab and Pembrolizumab (anti-PD-1) and Atezolizumab (anti-PD-L1) these have become a common second-line of treatment for cancer patients (34). In spite of the advances in the treatment of cancer using these inhibitors, their effect has shown a wide variety of treatment outcome based on individual patients. Due to the limitation of the current applications of approved antibodies against these checkpoints, additional therapies are needed.

The first objective of this study was to generate mAbs against the full ECD domain of CTLA-4, PD-1 and BTLA. This was accomplished by immunizing mice and with the fusion of their spleen cells with myeloid cells generate hybridoma cells. **Through this process, hybridoma cell lines capable of producing antibodies against CTLA-4, PD-1 and BTLA were generated.** To determine their ability to produce mAb and the specificity of these to recognize their target *in vitro* dot blot and Western blot analysis and flow cytometry were used as well as Immunohistochemistry (IHC). **Using these techniques, mAbs were shown to be specific to their full target proteins as well as these proteins found on the surface of mouse and human cell lines and to be specific to their targets on lung, lymph node and spleen tissues . This suggests that our mAbs cross react with both human and mouse proteins.** However, certain clones showed to recognize protein strongly than others such as CTLA-4 clones 5C4H2 and 5C4F11; PD-1 clones 10C7D3 and 5B3D5 and BTLA clone 3H3F4 (Figure 3.1-3.3).

The next objective of this work was to determine the effects of the novel mAbs generated to reduce tumor burden on an animal model and establish the cytokine profile of the treated

animals. This was accomplished by utilizing a Balb/c mouse model and EMT-6 cells in order to establish tumors which were treated with novel mAbs alone and in combination with each other. **Using this model, three of the clones generated where identified to have a protective effect against tumors on a Balb/c EMT-6 mouse model; CTLA-4 clone 5D3H5, PD-1 clone5B3D5 and BTLA clone 1H3H6 (Figure 4.2).** However, these clones did not show a protective effect when used at a dose of less than 100ug bud did show a reduction of tumor burden when used at 400ug and 800ug per dose (Figure 4.4). The serum of these animals was screened with the aim of establishing a cytokines profile of responding and non-responding groups (Figure 4.6). While the cytokine analysis provided some inside on those molecules showing statistical significance more information could be gathered by analyzing the serum of an additional animal study where tumors show a positive respond when treated with our novel mAbs. One cytokine of interest, IL-10 showed to be significantly lower in all groups compared to the non-treated group. This is of relevance due to the fact that IL-10 has been reported to have prognosis value for certain cancer such as prostate and breast cancer (25). Moreover, increased levels of IL-10 are associated with a lack of response after chemotherapy in breast cancer treatments (27). These findings suggest that the immune response with the use of mAbs was complete but a protective effect against tumor progression was not achieved with the low dosage used.

Considering the data gathered in this study, it can be concluded the mAbs developed were able to recognize and binding to their targets *in vitro*. Additionally, *in vivo* studies indicated that the protective effect observed with the mAbs is dosage dependent. Our novel mAbs against CLTA-4, PD-1 and BTLA show a protective effect when used at a concentration of at least 400ug.

5.2 Future Directions

Some of the proposed future directions for this project are to determine the toxicity and cytokine profiles of the 400ug and 800ug treatment strategies. Additionally, to utilize combinational strategies using a metronomic approach and chemotherapeutic agents such as cyclophosphamide. Moreover, the results of this study suggest that or mAbs against BTLA could have a more potent effect so characterizing the additional clones generated in this study will be pursued. Lastly, we propose to look at the binding patters of the novel mAbs developed in this study and compare the data obtained with the binding sequences of the monoclonal antibodies using in the clinical setting.

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VITA

Ms. Rosabril Acuna was born in El Paso, TX and raised in Mexico. Following completion of her high school education, she moved to El Paso, where she continued her education at the local Community College. Due to financial difficulties Ms. Acuna deferred school for a few years to focus on a technical career which she pursuit for eight years culminating in her return to college in 2012. She first enrolled at the local Community College where she was again exposed to science and research through the Research Initiative for Scientific Enhancement (RISE) program. As a RISE scholar, she participated in different research projects, presented her findings at local and national meetings and received many awards and honors including best poster presentation at national meetings and was awarded the STEM scholarship for two years in a row. Thereafter, Ms. Acuna transferred to The University of Texas (UTEP) to pursue a bachelor's degree where she received several scholarships including the Wolslager Foundation scholarship, the UTEP Promise scholarship and the MARC scholarship which funded her participation in scientific research during her undergraduate education. During her second she participated in the SPUR-LABS summer program at The University of California Los Angeles where she learned biochemistry techniques. As an undergraduate student at UTEP, Ms. Acuna participated in Dr. Robert A. Kirken's lab on the characterization of antibodies against PD-1 and BTLA, as a novel treatment strategy for cancer. Upon completion of her Bachelor of Science degree in December of 2017, Ms. Acuna joined the graduate program to pursue a Master's degree in the department of Biological Sciences at UTEP in the area of Immuno-Oncology in the lab of Dr. Robert A. Kirken where she continued working in the development of potential drugs for the treatment of cancer. At the conclusion of her graduate education Ms. Acuna hopes to obtain a research position at the National Cancer Institute (NCI) in the Maryland/DC area. Ms. Acuna can be contacted via e-mail at ro.acu@hotmail.com