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# ACTIVATING THE 4-1BB PATHWAY FOR THE EXPANSION OF TUMOR-INFILTRATING LYMPHOCYTES FOR ADOPTIVE T-CELL THERAPY FOR METASTATIC MELANOMA PATIENTS

Jessica Ann Chacon

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**ACTIVATING THE 4-1BB PATHWAY FOR THE EXPANSION OF TUMOR-  
INFILTRATING LYMPHOCYTES FOR ADOPTIVE T-CELL THERAPY  
FOR METASTATIC MELANOMA PATIENTS**

by

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FOR METASTATIC MELANOMA PATIENTS**

A

DISSERTATION

Presented to the Faculty of

The University of Texas

Health Science Center at Houston

and

The University of Texas

M. D. Anderson Cancer Center

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

By

Jessica Ann Chacon, M.S.

Houston, Texas

May, 2014

## **DEDICATION**

To my husband, thank you for believing in me, for your patience, and for letting me fly.

To my parents, thank you for encouraging me to pursue and follow my dreams, even though it meant being physically apart from each other. Your words, thoughts and prayers kept me motivated and determined to do my best.

To my sister and brother, thank you for providing me with great leadership.

To my grandparents, thank you for all your words of encouragement.

To OC, AC and my nephews, I look forward to reading your thesis/dissertation one day. Thank you for your patience.

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**Jessica Ann Chacon, M.S.**

**Supervisory Professor: Laszlo Radvanyi, PhD**

This dissertation project focused on improving the quality of the tumor-infiltrating lymphocytes (TIL) used in Adoptive T-cell therapy by understanding the role of 4-1BB/CD137 co-stimulation during the expansion of the tumor-infiltrating lymphocytes (TIL). Adoptive T-cell therapy using TIL is a promising therapy for late stage melanoma patients, resulting in a 50% response rate and durable long-term survival in over 20% of patients. Current research is aiming at improving the quality of the expanded cells and their persistence *in vivo* after adoptive transfer to further boost response rates. The specific focus of this dissertation project is the testing of agonistic anti-4-1BB antibodies at different stages of TIL expansion from tumors for its effects on modulating the phenotype and anti-tumor activity of the cells. The expansion of TIL from the melanoma tumor occurs in 2 stages: The first stage involves the initial expansion of the TIL from small cut 3-5 mm<sup>2</sup> fragments of viable tumor (12-24 fragments/tumor) with Interleukin-2 (IL-2) over a 4-5 week period; TIL isolated after this stage are referred to as 'pre-REP' TIL. The second stage involves the pre-REP TIL undergoing a secondary expansion referred to as the rapid expansion protocol (REP) for a period of 2 weeks in which the TIL are activated through the T-cell receptor (TCR) and also provided IL-2 to trigger rapid cell division. The TIL are then referred to as 'post-REP' TIL after this expansion. We have found that 4-1BB is

expressed on freshly isolated T cells that are within melanoma tumor fragments, as well as expressed on pre- and post- REP TIL. This observation prompted us to investigate what the role of 4-1BB ligation would play during the expansion of the TIL. We demonstrated that providing co-stimulation to the TIL during the initial expansion stage and during the secondary expansion using an agonist anti-4-1BB antibody facilitated an increased expansion of CD8<sup>+</sup> TIL with increased cytolytic function and a phenotype of memory T cells with enhanced cell survival gene expression. TIL receiving 4-1BB co-stimulation during expansion also exhibited longer persistence and increased anti-tumor activity in an *in vivo* human TIL adoptive transfer model using NOD-SCID-gamma chain<sup>-/-</sup> (NSG) mice xenografted with HLA-A-matched melanoma cells. The post-REP TIL also exhibited improved responses to antigenic re-stimulation when the anti-4-1BB antibody was added during expansion.

We also investigated the role of 4-1BB ligation in the tumor microenvironment *ex vivo* in the tumor fragments used as the source of the expanded TIL. We found that in addition to 4-1BB being expressed on T cells in these fragments 4-1BB was also expressed on dendritic cells within the melanoma tumor fragments. Addition of agonist anti-4-1BB increased activation and NFκB (a key marker of 4-1BB signaling) in these dendritic cells and T cells, that was associated with the increased proliferation and activation state of the CD8<sup>+</sup> T cells growing out of these fragments. Moreover, 4-1BB co-stimulation in these early tumor fragment cultures also significantly enriched the tumor specificity of the TIL, as found by an increase in the frequency of tumor-specific CD8<sup>+</sup> T cells in single cell and bulk anti-tumor reactivity assays.

In conclusion, our results demonstrate that enhancing 4-1BB co-stimulation at different stages of melanoma TIL expansion *ex vivo* increases the CD8<sup>+</sup> TIL yield, greatly increases tumor specificity, and enhances the effector-memory phenotype of the cells conducive to improved persistence and anti-tumor activity *in vivo* during adoptive cell therapy. Our results indicate that addition of an anti-4-1BB antibody during the initial and/or secondary expansion of the TIL in the clinic will result in a significantly enhanced TIL product than with currently used expansion protocols that will boost clinical response rates and durable long-term survival in treated patients.



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## **ABBREVIATIONS**

**aAPC:** Artificial Antigen Presenting Cell

**ACT:** Adoptive T-cell Therapy

**ADCC:** Antibody-dependent cell-mediated cytotoxicity

**AICD:** Activation Induced Cell Death

**BMS:** Bristol Myers Squibb

**CAR:** Chimeric Antigen Receptor

**CD:** Cluster of Differentiation

**CFSE:** Carboxylfluorescein Succinimidyl Ester

**CTL:** Cytotoxic T lymphocyte

**DC:** Dendritic Cell

**EOMES:** Eomesodermin

**FDA:** Food and Drug Administration

**GB:** Granzyme B

**Gp:** Glycoprotein

**HLA:** Human Leukocyte Antigen

**ICS:** Intracellular Cytokine Staining

**IFN:** Interferon

**IL-2:** Interleukin-2

**IL-6:** Interleukin-6

**IL-7:** Interleukin-7

**IL-15:** Interleukin-15

**ILA:** Induced by Lymphocyte Activation

**I.P.:** Intraperitoneal

**I.V.:** Intravenous

**JNK:** c-Jun N-terminal kinase

**KLRG-1:** Killer cell lectin-like receptor subfamily G member 1

**mAb:** Monoclonal Antibody

**MART:** Melanoma Antigen Recognized by T cells

**MAGE:** Melanoma-associated antigen

**MDSC:** Myeloid Derived Suppressor Cell

**MFI:** Mean Fluorescence Intensity

**MHC:** Major Histocompatibility Complex

**MSD:** Meso Scale Discovery

**NK:** Natural Killer

**NSG:** NOD Scid IL2 receptor gamma chain knockout mice

**Perf:** Perforin

**PBMC:** Peripheral Blood Mononuclear Cell

**REP:** Rapid Expansion Protocol

**SCID:** Severe Combined Immunodeficiency

**TCM:** Central Memory T-cell

**TCR:** T-cell Receptor

**TEFF:** Effector T-cell

**TEM:** Effector Memory T-cell

**TIL:** Tumor-Infiltrating Lymphocyte

**TNF:** Tumor Necrosis Factor

**TNFR:** Tumor Necrosis Factor Receptor

**Treg cell:** T regulatory cell

## **CHAPTER 1**

### **General Introduction**

## 1.1 Melanoma

There are 3 different types of skin cancer; basal cell carcinoma, squamous cell carcinoma, and melanoma (*1*). Melanoma is the deadliest type of skin cancer due to its metastatic behavior. The origin of melanoma starts in the melanocytes. Cancerous cells develop as a result of mutations that develop in the DNA of the melanocytes (*1*). Mutations can occur due to DNA damage (often caused by ultraviolet radiation) that has not been repaired (*1*). The four types of melanoma are superficial spreading melanoma, lentigo maligna, acral lentiginous, and nodula melanoma (*1*). Superficial spreading melanoma is the most common form of melanoma and accounts for 70% of all cases (*1*). Lentigo maligna is the most common form of melanoma in Hawaii. Acral lentiginous usually appears on the soles of the feet, palms of hands and under the nails, and is most commonly seen in the African Americans, Asian and Hispanic populations. Unlike the other types, which first appear on the surface of the skin and then later become invasive, nodular melanoma is invasive at first diagnosis and is the most aggressive form of melanoma (*1*).

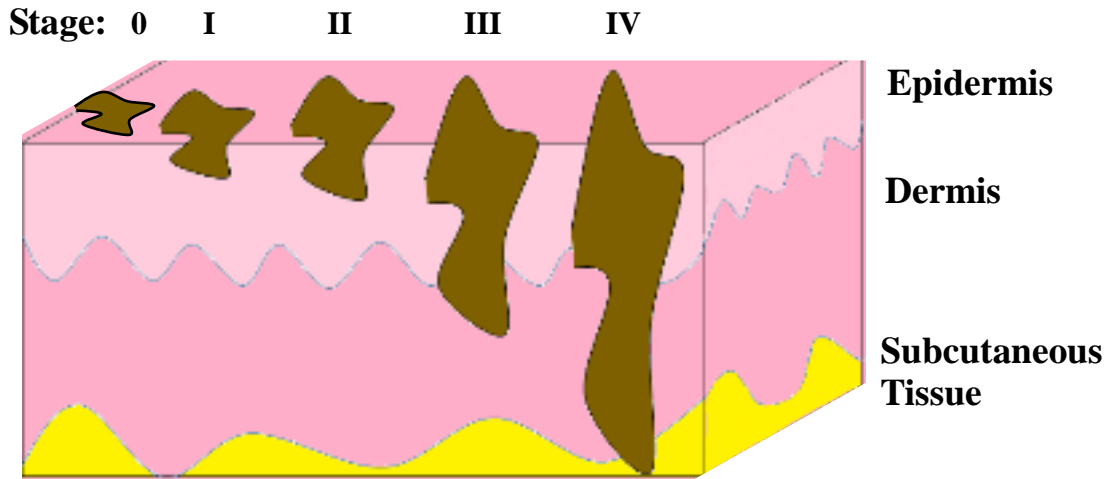
Melanoma has different stages (**Figure 1.1**), the earlier the stage of detection, the better survival rate. Stage 0 melanoma tumors are confined to the epidermis of the skin and can be treated through surgically removing the tumor. Stage I (A and B) is defined by the tumor being less than 1 millimeter and treated through surgery. In stage IA, the tumor is not ulcerated, and stage IB, the tumor is ulcerated (*1*). Stage II (A B, C) is when the tumor is 1-4 millimeters thick and treated using surgery (stage IIA, the tumor is between 1.01mm and 2.0mm and ulcerated or if the tumor is between 2.01 and 4.0mm and not ulcerated (*1*). In stage IIB, the tumor is between 2-

4mm thick with ulceration, or more than 4mm thick with no ulceration. In stage IIC, the tumor is more than 4 millimeters thick and ulcerated, surgery, as well as additional treatment may be necessary (1). In stage III (A-C) melanoma, the tumor is more independent of thickness and more dependent on the ability to spread to nearby tissue and/or lymph nodes. In stage IIIA, the tumor is any thickness, not ulcerated, and spread to 1-3 nearby lymph nodes (1). Stage IIIB, the tumor is any thickness, has spread to nearby lymph nodes, and the lymph nodes are enlarged (1). In stage IIIC, the melanoma has spread to 4 or more lymph nodes, is ulcerated, and lymph nodes are enlarged. Treatment is surgery, as well as targeted therapy or immunotherapy. Stage IV (metastatic melanoma) is the most serious stage because the cancer has spread beyond the lymph nodes, as well as metastasized to other organs of the body (lung, liver, brain, distant areas of skin or distant lymph nodes) (1). The treatment involves surgery, targeted therapy, and immunotherapy.

**Figure 1.1. Characteristics of the different stages of melanoma.** The different stages of melanoma (0-IV) differ in their size, location within the skin, patient survival rate, treatment, and the ability to metastasize. The later the stage of melanoma, the harder the disease is to treat. Surgery is the primary treatment, but in later stages of melanoma, surgery, as well as other forms of therapy, such as chemotherapy, radiation, and even immunotherapy may be needed.



**Figure 1.1. Characteristics of the different stages of melanoma.**



STAGE	0	I	II	III	IV
<b>Characteristics</b>	Confined to epidermis	<1mm <b>1A:</b> Not ulcerated <b>1B:</b> Ulcerated	1-4mm <b>2A:</b> 1<x<2mm (ulcerated) <b>2B:</b> 2<x<4 (non ulcerated) <b>2C:</b> x>4 (ulcerated)	Spread to nearby tissue/ lymph nodes <b>3A:</b> not ulcerated, spread to 1-3 nearby LN <b>3B:</b> Spread to nearby LN and lymph nodes are enlarged <b>3C:</b> Spread to 4 or more lymph and ulcerated	Metastasized
<b>Treatment</b>	Surgery	Surgery	Surgery	Surgery, Immunotherapy, Targeted Therapy	Surgery, Immunotherapy, Targeted Therapy
<b>5 year survival rate</b>		<b>1A:</b> 97% <b>1B:</b> 92%	<b>2A:</b> 81% <b>2B:</b> 70% <b>2C:</b> 53%	<b>3A:</b> 78% <b>3B:</b> 59% <b>3C:</b> 40%	15-20%
<b>10 year survival rate</b>		<b>1A:</b> 95% <b>1B:</b> 86%	<b>2A:</b> 67% <b>2B:</b> 57% <b>2C:</b> 40%	<b>3A:</b> 68% <b>3B:</b> 43% <b>3C:</b> 24%	10-15%

It is estimated, according to the American Cancer Society, that in 2014, there will be 43, 890 new cases of skin melanoma in men and 32, 210 new cases of skin melanoma in women, and 9,710 people will die from this disease. Melanoma is a growing epidemic in the U.S. as one in fifty people has a risk of getting diagnosed with melanoma in their lifetime, someone will be diagnosed with melanoma every eight minutes and melanoma will kill someone every hour of every single day.

Therefore, it is especially crucial that innovative treatments be developed and current treatments become more effective in treating melanoma. Dacarbazine has been the traditional chemotherapy treatment for metastatic melanoma. However, due to the vastly transient nature of tumor relapses occurring, treating melanoma using dacarbazine does not have major impacts on patient overall survival. Cancer cells are highly resistant to cytostatic agents and radiation. One of the newer hallmarks of cancer is avoiding immune destruction, therefore, novel therapeutics that utilize the immune system, such as immunotherapeutic agents and techniques have been developed. These will be described in the next section.

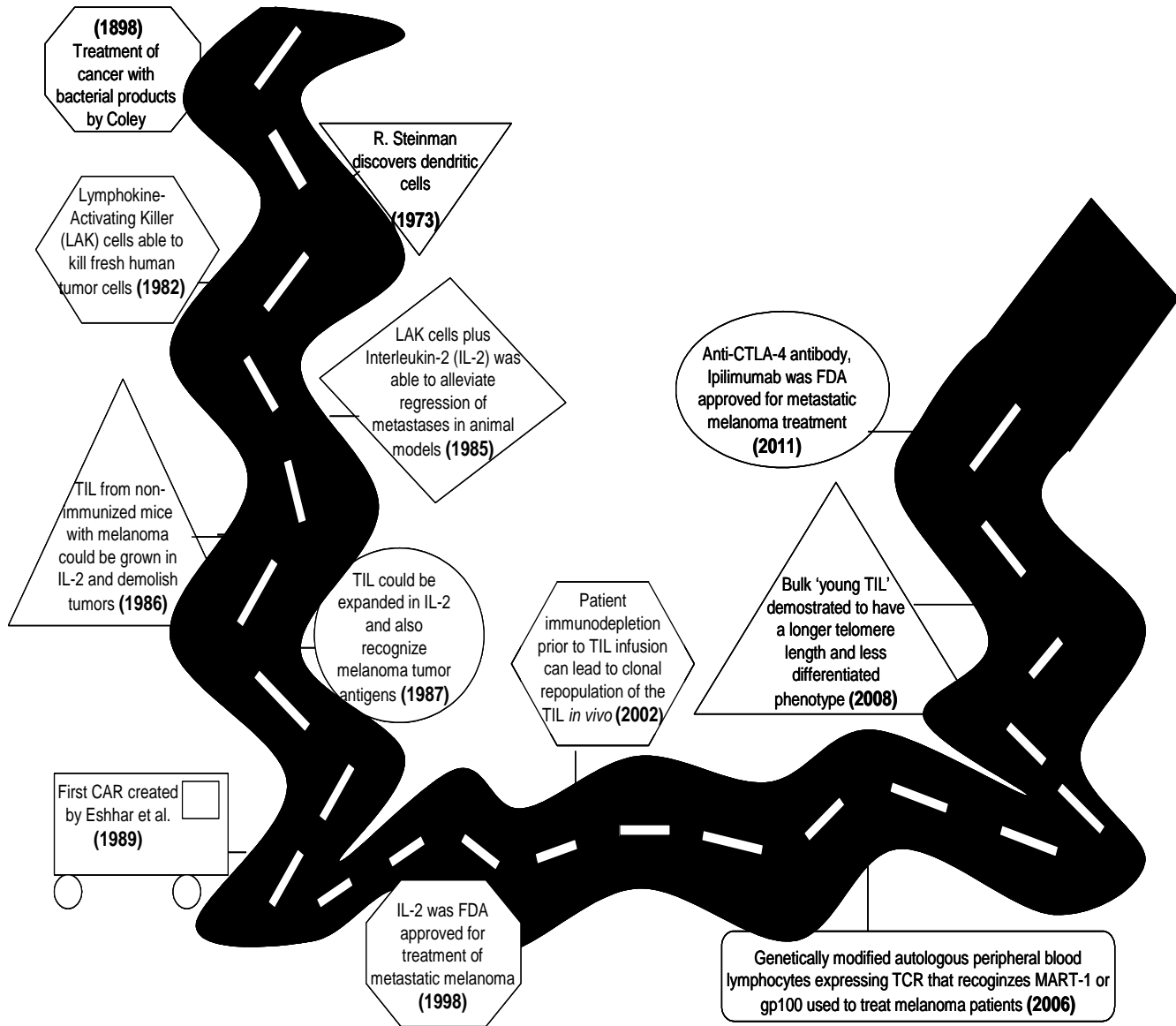
## **1.2 Immunotherapy**

Attempts to augment the immune system to treat cancer began over a century ago. In the 19<sup>th</sup> and 20<sup>th</sup> century, bacteria were injected into tumor-bearing individuals in studies mainly conducted by Coley (2-6). This work was later extended to include treatment of tumor-bearing individuals with injected killed bacteria into the tumor and eradication of the tumor was a result of Tumor Necrosis Factor (TNF) production in response to the bacterial endotoxins (2-6).

In 1926, J.B. Murphy proposed that lymphoid cells played a key role in eradicating solid tumors that had previously been transplanted in animal models (7). In 1958, Sir Peter Medawar coined the term “*immunological competent cell*” to describe a cell that is “*fully qualified to undertake an immunological response* (7, 8).” In the mid-1960s, Alexander and associates treated mice that had sarcomas using lymphocytes from immunized syngeneic animals (8, 9). Alex Fefer and colleagues demonstrated in 1969 that mice containing lymphomas that were virally induced could be treated using chemotherapy and infusion of lymphocytes (10, 11). In the mid-1970s, Eberlein and colleagues demonstrated that immune cells could be expanded using the growth factor Interleukin-2 (IL-2), be injected intravenously into a tumor-bearing mice and eradicate the implanted tumors (11-15). In the early 1990s, Boon and colleagues discovered (from MAGE-3) the first tumor-associated Human Leukocyte Antigen (HLA)-restricted T-cell epitope (16). A timeline of some of these major Immunotherapy breakthroughs is demonstrated in **Figure 1.2**.

**Figure 1.2. The long and winding road of Immunotherapy.** Immunotherapy began over a century ago. Experiments to eradicate tumors using the immune system lead researchers to develop innovative approaches to modify the immune system to treat cancer.

**Figure 1.2. The long and winding road of Immunotherapy.**



The immune system is our first line of defense to help protect us from foreign pathogens. Knowledge regarding the immune system has greatly increased over the years, paving the way for immunotherapy to become a novel way to treat diseases, including cancer. Many cancer cells become resistant to conventional therapy, such as chemotherapy and radiation, resulting in many patients relapsing, therefore immunotherapies have been developed to overcome this resistance (11, 17-19). Passive and active immunotherapies have been developed over the years (**Table 1**). Passive immunotherapies consist of antibodies, cytokines or other elements of the immune system that are constructed in a laboratory and subsequently administered to patients to provide immunity against a disease. On the other hand, active immunotherapy involves the direct stimulation of the host immune system, usually through the use of vaccines. **Table 1** lists different types of immunotherapies that are branched into either passive or active forms.

**TABLE 1. Types of Immunotherapy**

<b>TYPES OF IMMUNOTHERAPY</b>	
<b>PASSIVE</b>	<b>ACTIVE</b>
<p><b>Humanized monoclonal antibodies:</b></p> <ul style="list-style-type: none"> <li>• Anti-CD19 (Rituximab)</li> <li>• Anti-HER2/neu (Herceptin)</li> <li>• Anti-CTLA4</li> </ul> <p><b>Adoptive T-cell Therapy:</b></p> <ul style="list-style-type: none"> <li>• TILs</li> <li>• TCR-transduced</li> <li>• CAR transduced</li> </ul> <p><b>Cytokines and immune modulators:</b></p> <ul style="list-style-type: none"> <li>• IL-2</li> <li>• IFN-alpha</li> <li>• TLR ligands (CpG, Poly I:C)</li> <li>• Anti-CTLA-4</li> <li>• PD-1/PDL-1</li> </ul>	<p><b>Antigen-specific cancer vaccines:</b></p> <ul style="list-style-type: none"> <li>• Peptide vaccine</li> <li>• Viral vector-based vaccines (whole or parts of tumor antigens)</li> <li>• DC vaccines (peptide-pulsed or tumor lysate pulsed)</li> </ul> <p><b>Antigen non-specific vaccines:</b></p> <ul style="list-style-type: none"> <li>• Allovectin (viral induced allo MHC expression in tumors)</li> </ul>

### 1.3. Adoptive T-cell Therapy

Interleukin-2 (IL-2) was FDA approved for the treatment of late-stage melanoma in 1998 (20, 21). The treatment of late-stage melanoma patients consisted of intravenously (I.V.) giving patients high dose infusion (600,000-720,000 IU/kg) IL-2 every 8 hours, with several rounds of these high dose-IL-2 infusions given 3-5 weeks apart (20, 21). Using high-dose IL-2 to treat melanoma patients has resulted in varied response rates. In patients containing one or more visceral lesions, this high-dose IL-2 resulted in clinical response rates of only 10-15%, while patients that contained cutaneous melanoma response rates reached 50% objective response rates (20, 21). In patients with visceral metastasis, treatment with high-dose IL-2 resulted in 5-6% of patients undergoing complete remission that has lasted over 10 years (20, 21).

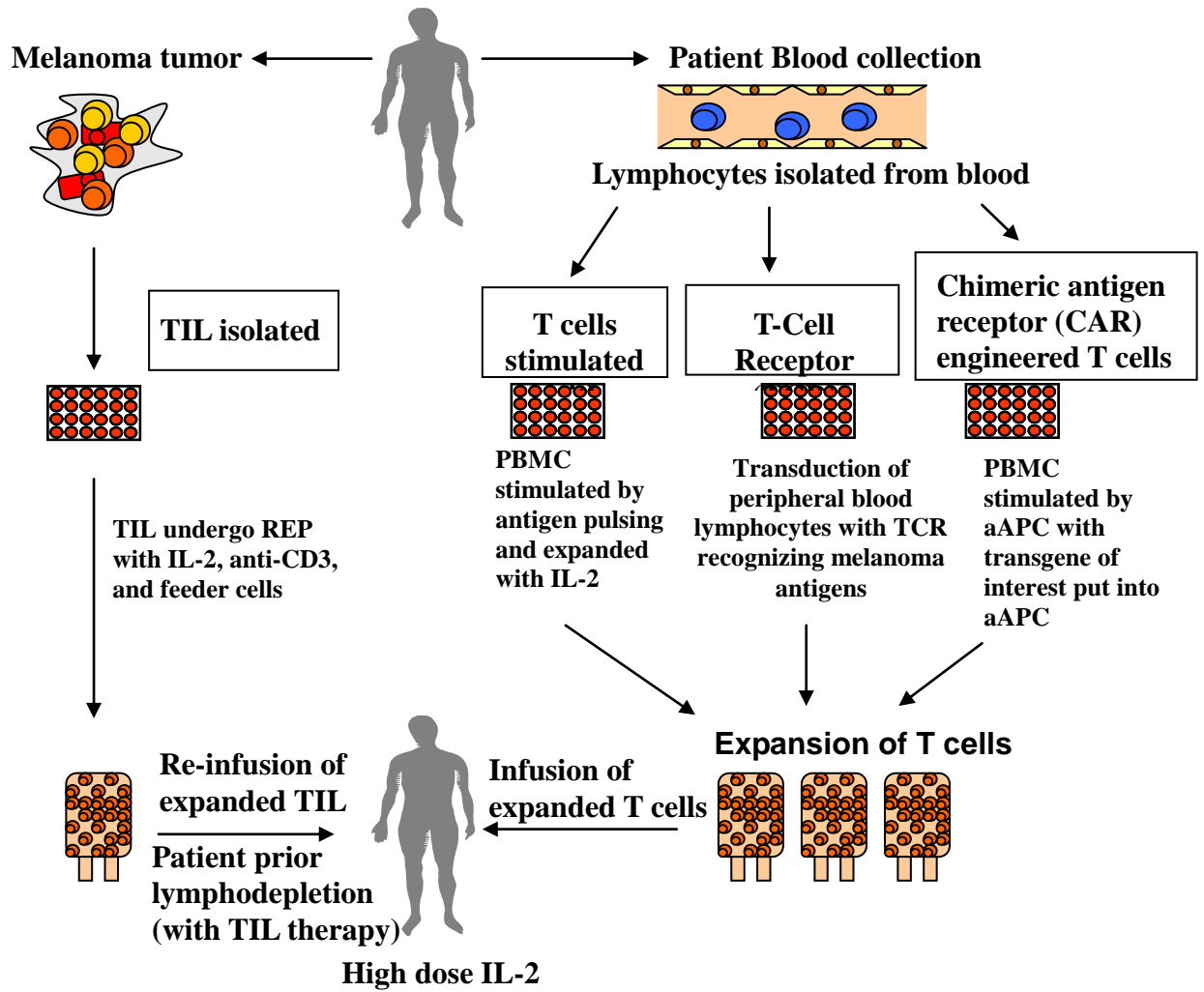
Results from using high-dose IL-2 to treat melanoma patients prompted researchers at the National Cancer Institute (NCI), Bethesda, Maryland to investigate the role of combining high-dose IL-2 with adoptive T-cell therapy (ACT) (12, 22). ACT is a type of passive immunotherapy that acquires either cancer patient peripheral blood or lymphocytes obtained within a tumor and expands these cells into large numbers using growth factors (such as IL-2) in a laboratory (17, 18, 23). These cells are then re-infused into the patient. In the following section, I will discuss the different types of adoptive T-cell therapy, which vary in the site where the lymphocytes are collected and the methods used to expand the cells (**Figure 1.3**). A major advantage of using ACT to treat cancer patients is the opportunity for researchers to select or engineer *in vitro* the desired or optimal T-cell phenotype,

function, anti-tumor response, antigen specificity, and expand this selected population prior to infusing the cells back into the patients.



**Figure 1.3. Different forms of adoptive T-cell therapy.** A schematic representation of the process of large expansion of TILs derived from tumor fragments or circulating antigen-specific T cells from peripheral blood for adoptive T cell therapy.

Figure 1.3. Different forms of adoptive T-cell therapy.



### ***1.3 a) Tumor-Infiltrating Lymphocytes***

When ACT was first combined with high-dose IL-2 at the NCI by Grimm and colleagues in 1982, they developed a novel cytolytic cell system using lymphokine activated killer (LAK) cells (22, 24, 25). LAK cells were generated from cancer patient peripheral blood lymphocytes and normal donors, using high-dose IL-2 to grow the cells (22, 24, 25). Using animal models, infusion of LAK cells and high-dose IL-2 was able to eradicate metastatic tumors (22, 24-27). In 1985, Rosenberg and colleagues demonstrated in a clinical trial that LAK cells infused with high-dose IL-2 had an impact on metastatic melanoma patients resulting in a 21% response rate (26-28). In a follow-up study conducted in 1987, it was demonstrated that combining high-dose IL-2 with LAK cells was more effective in treating cancer patients than high-dose IL-2 alone (26-28). However, in the clinical trial conducted by Rosenberg and colleagues, the majority of the responses were partial responses and this initiated the investigators to inquire about other cells that may induce anti-tumor responses.

It was demonstrated in 1986, using mice models, that tumor-infiltrating lymphocytes (TIL) obtained from melanoma tumors from the mouse could be expanded *in vitro* in IL-2 and subsequently eradicate the tumors when adoptively transferred (11, 29, 30). In a phase II trial, when metastatic melanoma patients were treated with TIL and high-dose IL-2, the response rate was 39% (11, 29). However, in a groundbreaking Phase II clinical trial in 2002, conducted at the NCI, Dudley and colleagues demonstrated that combining cyclophosphamide and fludarabine (a lymphodepleting chemotherapy regimen) prior to infusing the TIL with high-dose IL-2 resulted in 50% response rates in metastatic melanoma patients (17, 31, 32).

Lymphodepletion allowed the transferred TIL to persist better in the patient and rid any cells that may be in competition with the infused TIL for homeostatic cytokines, such as Interleukin-7 (IL-7) and Interleukin-15 (IL-15) (17, 31-34). In addition, lymphodepletion also eliminates endogenous suppressor cells such as T regulatory cells (Tregs) that may inhibit the infused T cells functions (31-36). However, the re-appearance of Tregs and other suppressor cells and factors that may inhibit T-cell function is a concern even after lymphodepletion (31-34) (35, 36). Therefore, new approaches using total body irradiation (TBI) of 2Gy or 12Gy, in addition to the traditional cyclophosphamide and fludarabine lymphodepletion have been developed at the NCI (17, 37). Using the 12Gy TBI plus chemotherapy, the NCI found an impressive objective clinical response rate of 72% and a complete response rate of 40% (17, 37).

The standard protocol for ACT using TIL for the treatment of metastatic melanoma patients is as follows (**Figure 1.4**). The melanoma tumor is surgically resected from the patient. The tumor is then subsequently cut up into multiple 3-5mm<sup>2</sup> fragments or enzymatically digested using collagenase and hyaluronidase and Ficoll to isolate the TIL and placed in a 24-well plate with TIL-media and high-dose IL-2 (6,000IU/ml) for a period of 4-5 weeks (17, 23, 38). After this period, the TIL are referred as 'pre-Rapid Expansion' TIL or pre-REP TIL. The pre-REP TIL are then subjected to undergo the REP. The REP is a 2 week expansion period in which the TIL are placed in flasks with TIL-media, high-dose IL-2, anti-CD3, and irradiated peripheral blood mononuclear cells (PBMCs) that we refer to as feeder cells (18, 23, 39). After the 2 week expansion period, the TIL are referred to as 'post-REP' TIL.

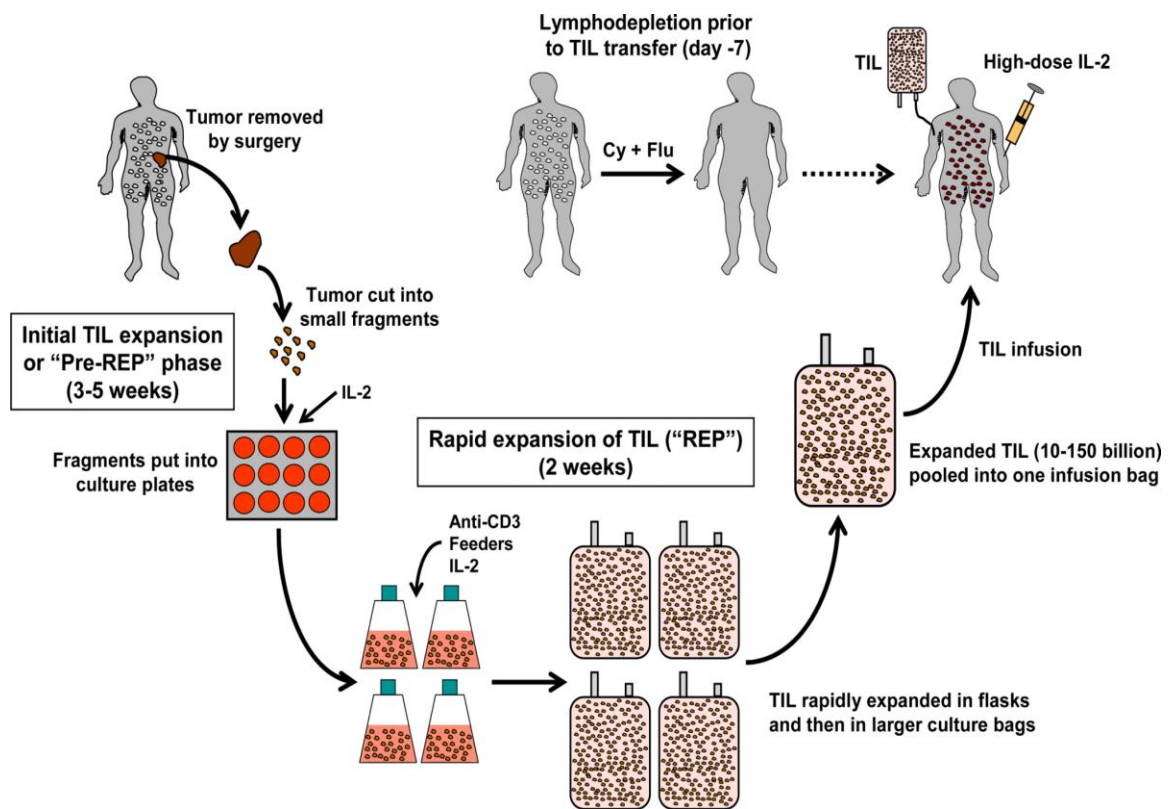
During the expansion period, the TIL numerically expand to billions of cells (18, 23, 39). The billions of post-REP TIL are then infused into a lymphodepleted patient and high-dose IL-2 is subsequently given in order to help the transferred TIL persist (18, 23, 39). Using this TIL to treat metastatic melanoma patients has resulted in clinical response rates of 50% in Phase II clinical trials at NCI, as well as other centers around the world, including at MD Anderson Cancer Center (18, 38, 40). Although TIL therapy was pioneered and has been mainly studied in melanoma, TIL therapy has also been developed to treat patients with breast cancer, renal cancer, ovarian cancer, lung cancer, cervical cancer, gastric cancer, and head and neck cancers.

An advantage of using TIL therapy to treat metastatic melanoma patients is that TIL have a polyclonal nature that recognize multiple tumor-associated antigens (TAA), including melanocyte/melanoma differentiation antigens as well as a wide range of unknown antigens. However, alternative approaches have been developed to expand antigen specific T cells from patient-derived PBMCs.

**Figure 1.4. Schematic representation of the process of TIL expansion and TIL therapy for metastatic melanoma starting from tumor fragments.** Suitable tumors from eligible stage IIIc-IV patients are resected and taken to the lab under sterile conditions where they are cut up into small 3–5 mm<sup>2</sup> fragments and placed in culture plates or small culture flasks with growth medium and high-dose (HD) IL-2. The TIL are initially expanded for 3–5 weeks during this “pre-rapid expansion protocol” (pre-REP) phase to at least  $50 \times 10^6$  cells. The cells are then subjected to a rapid expansion protocol (REP) over two weeks by stimulating the T cells using anti-CD3 in the presence of PBMC feeders cells and IL-2. The expanded TIL (now billions of cells) are washed, pooled, and infused into the patient followed by one or two cycles of HD IL-2 therapy. Before TIL transfer, the patient is treated with a preparative regimen using cyclophosphamide (Cy) and fludarabine (Flu) that transiently depletes host lymphocytes “making room” for the infused TIL and removing cytokine sinks and regulatory T cells in order to facilitate TIL persistence.

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**Figure 1.4. Schematic representation of the process of TIL expansion and TIL therapy for metastatic melanoma starting from tumor fragments.**



### ***1.3 b) Chimeric Antigen Receptor***

Another exciting form of ACT utilizes chimeric antigen receptors (CAR). The first generation of the CAR contained a link between light chain and heavy chain of a monoclonal antibody with variable domains (41, 42). This was associated with transmembrane and the T-Cell Receptor (TCR) complex cytoplasmic tail of the zeta ( $\zeta$ ) chain (41, 42). Engineering T cells using this approach was developed to circumvent Major Histocompatibility Complex (MHC) restriction due to the fact that tumor cells evade recognition by down-regulating the MHC. However, this first generation of the CAR led to poor proliferation of the engineered T cells and it was noted that stimulation of the TCR via the cytoplasmic tail of the zeta ( $\zeta$ ) chain was insufficient to help the T cells persist (41, 42). Therefore, the second generation of the CAR was generated to provide improved co-stimulation to the T cells by adding the CD28 endo-domain, in addition to the zeta ( $\zeta$ ) chain that was already on the first generation CAR in order to mimic the two-signals needed for T-cell activation (43, 44). Other endo-domains from other co-stimulatory molecules have been added to the CAR constructs, such as the endo-domains from CD137 (4-1BB and CD134 (OX40) to generate a third generation of CAR consisting of the zeta ( $\zeta$ ) chain, and the endo-domains of CD28 and CD137 (45).

Adoptive transfer of autologous CAR transduced T cells induced substantial tumor regressions in leukemia and B-cell lymphomas (46, 47). While treating leukemia and B-cell lymphomas, CAR constructs contained a CD19-specific Immunoglobulin G (IgG) single chain variable fusion (scFv) fused to the TCR  $\zeta$  chain and the endo-domains of CD137 (46, 47). Treatment of leukemias and lymphomas



using CAR transduced T cells have shown promising results in regards to anti-tumor activity and persistence (46, 47). In regards to melanoma treatment, targets of CAR transduced T cells include overexpressed genes, such as gangliosides GD2, and GD3, which are over expressed in 50-80% of metastatic melanomas (48, 49). Using IL-2 administration and a second generation CAR that targeted GD2 with endo-domains of CD28 and CD134 significantly improved the survival of mice following transfer (50). In addition, researchers found that administering IL-2 and using a TCR transduced car that targeted GD3 and contained CD28 endo-domain resulted in complete response rate in 50% of melanoma tumor-burdened mice (49).

### ***1.3 c) TCR-transgenic T cells***

Another type of ACT involves TCR-transduced T cells. These T cells are transduced using retroviral or lentiviral vectors containing TCR genes that encode variable regions for different melanoma-associated antigens, such as glycoprotein-100 (gp100) and melanoma antigen recognized by T cells-1 (MART-1) (51, 52).

In a pilot trial, T cells were transduced with a gp-100 specific TCR and infused with IL-2 into melanoma patients that had been previously lymphodepleted (53, 54). The TCR-transduced T cells persisted and no toxicity was detected in the patients, however, the T cells demonstrated minimal effector function due to the low surface levels of the gp-100 TCR (53, 54). However, when high-affinity MART-1 transduced TCR T cells were expanded and infused into melanoma patients, this resulted in a 30% clinical response rate (55, 56). CD4<sup>+</sup> T cells have also been transduced to target another melanoma antigen, NY-ESO-1, and have demonstrated objective response rates in 5 out of 11 patients, with 2 complete responses (57).

### ***1.3 d) Antigen-specific CD8<sup>+</sup> and CD4<sup>+</sup> T cells.***

Using autologous patient PBMCs, tumor-associated antigen (TAA)-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell clones have been expanded using multiple *in vitro* antigen stimulations. CD8<sup>+</sup> T cells are believed to be the optimal population for ACT due to their specialized ability to recognize and kill tumors via the TCR binding to the peptide:Major Histocompatibility Complex (MHC) class I (58). Using ACT, various groups have attempted to use antigen-specific CD8<sup>+</sup> cells to treat metastatic melanoma patients (58, 59). A key approach isolates lymphocytes from peripheral blood of patients. CD8<sup>+</sup> T cells that recognize melanoma antigens gp100, MART-1 or tyrosinase are expanded using autologous mature dendritic cells that have been pulsed with peptide (59-62). In a phase I clinical trial, CD8<sup>+</sup> T-cell clones that recognized MART-1 and gp100 that were expanded and infused into metastatic melanoma patients were able to respond to IL-2, persist *in vivo*, traffic to the tumor sites and induce tumor regression (59, 63, 64). Using this approach resulted in clinical responses of about 30%, with patients experiencing complete responses, partial responses, or stabilization of the disease for up to 11 months (59, 63, 64). In another clinical trial conducted by Mackensen and colleagues, a 30% clinical response was demonstrated when melanoma patients received MART-1-specific T cells that were able to migrate to the tumor sites (59, 62). Butler et al. conducted studies using K562 artificial antigen presenting cells (aAPCs) to expand MART-1 specific CD8<sup>+</sup> T cells (65). The aAPCs were transduced to express MART-1, HLA-A2, CD86 and 4-1BBL and the MART-1 specific CD8<sup>+</sup> cells were obtained from melanoma patient peripheral blood (65).

Currently, the specific role of antigen-specific CD4<sup>+</sup> used to treat metastatic melanoma patients is being investigated (66). Studies have demonstrated that adoptively transferring tumor reactive CD4<sup>+</sup> T helper (Th)17 cells into C57Bl/6 mice resulted in tumor eradication (67, 68). Although the use of antigen-specific CD8<sup>+</sup> T cells have been largely studied, many groups believe that polyclonal CD4<sup>+</sup> and CD8<sup>+</sup> population used for ACT for melanoma patient treatment may be necessary to induce better clinical responses (61, 69-71). In addition to the HLA class I-restricted melanoma antigens mentioned earlier, there are also HLA class II-restricted melanoma antigens, such as tyrosinase, NY-ESO-1 and Melanoma Associate antigen-1 (MAGE-1) (60). A clinical trial using Th1 CD4<sup>+</sup> T cells clones that were specific for tyrosinase or NY-ESO-1 antigens to treat melanoma patients demonstrated a persistence of these cells for up to 2 months (60). In addition, four patients showed a partial response or stabilization of the disease and one patient exhibited complete durable response of more than 3 years (60).

#### **1.4) Drawbacks of ACT using TIL**

##### ***1.4 a) T-cell differentiation***

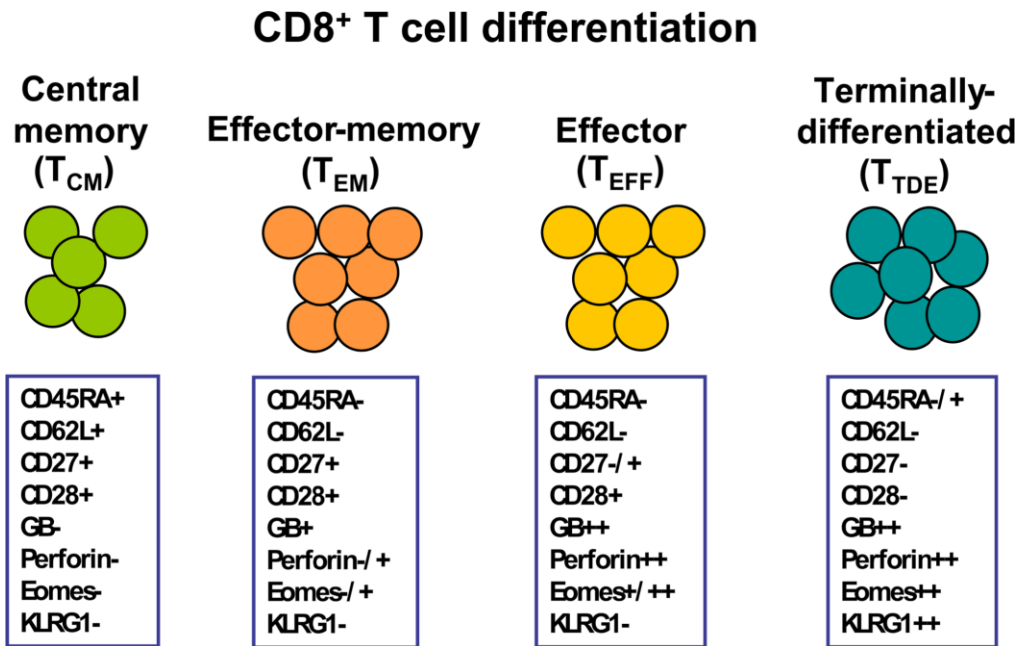
The differentiation stage of the T cells that are infused and subsequently associated with clinical responses has been investigated. The stages of differentiation for majority of CD8<sup>+</sup> TIL used in ACT for metastatic melanoma treatment are T effector-memory, (TEM), effector (TEFF), and terminally differentiated effector cells (TTDE) (72, 73). The more differentiated the cells, the more cytolytic they will become, but they will have a lower proliferative capacity (72, 73). Also, more differentiated cells tend to lose critical co-stimulatory molecules such as CD27 and

CD28 (72-75). The markers for the different stages of differentiation are demonstrated in (**Figure 1.5**). A small percentage of TIL have been found to be central memory T cells (TCM). When TIL are expanded with high-dose IL-2, this can induce differentiation of the T cells (76). TCM are able to traffic to the lymph nodes *in vivo* where they may encounter antigen-presenting cells that present TAA (77, 78). Studies have demonstrated using mice models that antigen-specific TCM persisted long-term *in vivo*, while inducing an anti-tumor response (73, 77, 78). However, some of these findings were observed when a cancer vaccine with a specific TAA was administered following adoptive transfer of the TCM, and it has not been determined whether TCM are the optimal population for ACT in the absence of a cancer vaccine (73, 77, 78).

**Figure 1.5. Stages of CD8<sup>+</sup> T-cell differentiation found in melanoma CD8<sup>+</sup> T-cell in TIL and phenotypic markers assessed by flow cytometry used to delineate these stages. CD8<sup>+</sup> T cells in TIL can be found having markers of central memory (TCM), effector memory (TEM), effector (TEFF) or terminally-differentiated effector (TTDE) stages.** These stages and markers are based on previous work on anti-viral CD8<sup>+</sup> T-cell response and the states of CTL differentiation associated with the control of both acute and chronic viral infections in humans. These markers are only a guide to identify these functional memory or effector stages and may not always fit into these defined categories. In addition, the expression of these subset markers may be transiently down-modulated, permanently lost, or re-expressed under different cytokine or in different tissue niches in vivo. Nevertheless, understanding the changes in these effector and memory markers in TIL is very useful in biomarker studies to identify clinically active T-cell subsets. Melanoma TIL expanded for therapy are a mixture of mostly TEM, TEFF, TTDE (that have lost CD28 expression). Relatively very few TCM are found (<5%). Initially, NK cells are found in initial TIL expansions (pre-REP), but these are lost after the REP which selectively activates the T cells.

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#### ***1.4 b) Long process of Isolating TIL for Adoptive T-cell Therapy***

Although ACT using TIL has resulted in a 50% clinical response rate across various institutes (17, 23, 38, 40), one of the caveats for this therapy is the actual expansion of the TIL. The process of isolating and expanding TIL from melanoma tumors can be time-consuming and labor intensive. In addition, not all TIL from all patients are able to be isolated and expand (17, 23, 38, 40). In order for the TIL to be eligible for the secondary expansion (REP), the pre-REP TIL must reach a minimum of  $50 \times 10^6$  cells following the initial isolation and expansion period (23, 38). However, only 65% of patients pre-REP TIL reach this threshold (23, 38). Although the expansion period induces the growth of billions of TIL, this process may also induce differentiation (23, 38). An approach to shorten the time expanding the TIL without inducing the differentiation of the TIL is referred to as the 'young' TIL method. This approach uses enzymatic digestion, not the traditional fragment set-up to isolate the TIL from the tumor (40, 79-82). The TIL are then expanded with high-dose IL-2 over a period of 3-5 weeks. Using the enzymatic digest method to expand the TIL has shown success in generating the minimum number of pre-REP TIL needed to undergo the secondary expansion in as little as 3 weeks, increasing the number of patients eligible for secondary from 50-60% to 80% (79-82). In addition the 'young' TIL protocol does not test for anti-tumor reactivity in the TIL, therefore all patient TIL undergo the secondary expansion despite their anti-tumor reactivity and are infused into lymphodepleted patients, along with high dose IL-2. Besser and colleagues reported the results of their first clinical trial that utilized the 'young' TIL protocol (79, 83). They reported a clinical response of 50%, which was comparable to the response rates seen with using the conventional method to isolate and expand the TIL

(79, 83). Although the 'young' TIL method did not increase the clinical response rates, it can be alluded that the 'young' TIL are less differentiated and may persist *in vivo* longer, and overall, this method reduces the initial isolation expansion period.

### **1.5) Co-stimulation**

When a T-cell becomes activated, it requires 2 initial signals. Signal 1 occurs when the T-cell receptor (TCR) binds to the MHC: peptide complex on an antigen presenting cell (APC) (84, 85). The first signal initiates the activation of the T-cell. The second signal occurs when CD28 on the T-cell interacts with CD80/86 on an APC (84, 85). The second signal involves co-stimulation and helps the T-cell persist. If T cells receive these 2 signals they will undergo proliferation, differentiation, and then acquire effector functions (84, 85). A third signal involves cytokines released from the APC that may influence the differentiation and proliferation of the T-cell. However, after these initial activation events occur, additional co-stimulatory receptors/ligands are up-regulated on both T cells and APCs. These additional co-stimulatory molecules are members of the Tumor Necrosis Factor Receptor (TNFR) super family, 4-1BB (CD137) and/or OX40 (CD134) (84, 85). In conjunction with the TCR-CD3 complex and CD28 signaling, the ligation of 4-1BB on T cells using an agonistic anti-4-1BB monoclonal antibody (mAb) can present an additional signal (86-88).

These co-stimulatory molecules are important to initiate T-cell survival and proliferation. Co-stimulatory signals may perform several functions, such as augmenting production of IL-2, inducing cytokine production, suppressing cell death by altering bcl-2 and caspase protein function and enhancing memory T cell



development (87-92). In the absence of co-stimulation, a T-cell can become susceptible to become anergic. Co-stimulatory molecules play crucial roles in all phases of T-cell response, not only in the activation phase but also in the effector phase, expansion phase, and memory phase (87-92).

### ***1.5 a) 4-1BB discovery and its signaling pathways***

4-1BB was discovered using a screening method to identify new genes in mice that were induced after T-cell activation (93-95). In humans, 4-1BB was initially termed 'induced by lymphocyte activation (ILA) (93-95) and is 60% homologous to the mouse 4-1BB (93-96). 4-1BB is an activation dependent gene that is a 30-kDa monomer and 55-kDa homodimer (91, 93-96). The mouse 4-1BB gene consists of 10 exons, 8 exons in the coding region and 2 in the 5' untranslated region (91, 93-96). The human 4-1BB gene encompasses 255 amino acids and was initially cloned from the activated human T-cell leukemia virus type-1 (HTLV-1) transformed human T cell library (91, 93-96).

The ligand for 4-1BB is 4-1BB ligand (4-1BBL) (91, 96-98). In 1994, Alderson and colleagues were the first to isolate the human 4-1BBL gene using a fusion protein that contained the extracellular region of 4-1BB couple to IgG1 Fc region (98). 4-1BBL is a Type II transmembrane protein and is primarily expressed on antigen presenting cells (APCs) (96, 98).

4-1BB encodes Type-I surface glycoproteins that consist of extracellular domain, transmembrane region, and intracellular region (91, 99). The intracellular regions of 4-1BB associate with TNFR-associated factor-2 (TRAF 2) and p56<sup>lck</sup> (91, 99). Upon 4-1BB receptor binding to its ligand, 4-1BBL, the cytoplasmic tail of 4-

1BB contains TRAF-binding regions that then recruit TRAFs (91, 99). The TRAF molecules associated with 4-1BB signaling are TRAF1, TRAF2, and TRAF3 (in humans) (**Figure 1.6**) (85, 91, 99). The TRAF trimers (1, 2, and 3) are recruited to TRAF-binding motifs in cytoplasmic tail of 4-1BB upon activation (85, 91, 99). These TRAFs form heterotrimers upon receptor-ligand association (85, 91, 99). After T-cell activation, TRAF1 is up-regulated. Since TRAF1 lacks the RING finger domain needed to induce NF $\kappa$ B activation, TRAF1 mainly acts as a modulator for TRAF2, protecting TRAF2 from undergoing degradation after 4-1BB ligation and signaling (85, 91, 99). TRAF2 employ E3 ubiquitin ligase activity and link 4-1BB to the downstream signaling pathways (85, 87, 100, 101). If T cells are defective or lack TRAF2, cytokine production and p38 activation will be negatively affected in response to 4-1BB activation (85, 87, 100, 101).

The 4-1BB 5' promoter region contains binding sites for NF $\kappa$ B and AP-1, which are involved in TCR activation-dependent transcriptional regulation of 4-1BB promoter (85, 87, 100-102). The expression of 4-1BB is up-regulated through MEK and C-Jun-N-terminal kinase-1 (JNK) activity (85, 87, 100, 101). 4-1BB signaling through the NF $\kappa$ B, phosphoinositide 3-kinase (PI3K) pathways regulate cytokine production, cell proliferation, memory development, and anti-apoptosis molecules, such as bcl-2 and bcl-xL (**Figure 1.6**).

### ***1.5 b) The role of 4-1BB in Immunotherapy***

4-1BB expression is up-regulated after activation. 4-1BB is expressed on T cells, B cells, Natural Killer (NK) cells, neutrophils, monocytes, and dendritic cells (DCs) (85, 89, 103). The expression of surface 4-1BB reaches maximal levels

between 24-48 hours (85, 89, 103). Ligation of 4-1BB receptor with 4-1BBL induces anti-apoptotic signals, proliferation and cytokine production via NFκB, p38 and PI3K pathways (85, 89, 103). 4-1BB activation also prompts the proliferation and expansion of CD8<sup>+</sup> T cells, resulting in their production of IL-2, IFN-gamma, and Tumor Necrosis Factor (TNF)-alpha that initiates an anti-tumor response (85, 89, 103). In antigen specific T cells, the expression of 4-1BB is rapid and diverse in CD4<sup>+</sup> versus CD8<sup>+</sup> T cells (85, 89, 103). An increase in 4-1BB expression was not seen in T cells until 12 hours after activation, where CD8<sup>+</sup> T cells expressed higher levels of 4-1BB than CD4<sup>+</sup> T cells (85, 89, 103). 4-1BB expression in CD8<sup>+</sup> and CD4<sup>+</sup> T cells was decreased to baseline by 48 hours (104, 105). Many researchers speculate that CD4<sup>+</sup> T cells may be less responsive to 4-1BB ligation than CD8<sup>+</sup> T cells and this could explain the preferential expansion and effect on CD8<sup>+</sup> T cells upon 4-1BB activation (104, 105). 4-1BB ligation induces a profound effect on CD8<sup>+</sup> T cells (104, 105). Researchers have found that 4-1BB activation can inactivate CD4<sup>+</sup> T-cell effector functions or even kill CD4<sup>+</sup> cells (104, 105).

As mentioned earlier, 4-1BB activation leads to increased CD8<sup>+</sup> proliferation and effector functions. Investigating the anti-viral effects in a 4-1BB<sup>-/-</sup> mice demonstrated that the 4-1BB<sup>-/-</sup> mice have normal primary CD8<sup>+</sup> responses, but exhibit a decrease in secondary responses (106). These findings suggest that 4-1BB activation may be critical for late anti-viral responses (85, 106).

When the anti-tumor effect was compared in a 4-1BB<sup>+/+</sup> and 4-1BB<sup>-/-</sup> mice using the B16.F10 melanoma cell line to challenge the mice, there was increased mortality in the 4-1BB<sup>-/-</sup> mice compared to the 4-1BB<sup>+/+</sup> mice (96, 97). This anti-

tumor effect was due to the increased survival of the CD8<sup>+</sup> T cells, and increased IFN-gamma production in the 4-1BB<sup>+/+</sup> mice (96, 97).

4-1BB ligation by cell surface 4-1BBL or using agonistic anti-4-1BB antibodies initiates a co-stimulatory signal to cells and enhances T-cell survival via increased anti-apoptotic molecules (96, 97). Melero and colleagues were the first to demonstrate that treating tumor-bearing mice using an agonistic anti-4-1BB antibody eradicated the tumors in the mice (107). However, when Sabel et al. co-injected tumor cells with PBMCs in a severe combined immunodeficiency (SCID) mouse and administered an anti-human anti-4-1BB antibody, there was no lymphocyte-mediated tumor eradication or suppression, and the tumors continued to grow (108). The authors speculated that this may have been a result of the adoptively transferred PBMCs being killed by endogenous NK cells in an antibody-dependent cellular cytotoxicity (ADCC) manner (108). Bertram et al. illustrated that in 4-1BBL<sup>-/-</sup> mice, there was decreased antigen specific (Db/NP366-374) CD8<sup>+</sup> T cells responses (85, 109).

Miller and colleagues demonstrated that treating tumor challenged mice with an anti-4-1BB antibody was only effective once the tumor was already established (92). Their study indicated that the anti-4-1BB antibody acted on T cells that were already antigen-experienced (92). The effect of an anti-4-1BB antibody was also determined in a lung metastases model. When T cells and anti-4-1BB antibody were administered to mice bearing lung metastases, the mice exhibited tumor regression, compared to when T cells alone were administered (88).

### ***1.5 c) 4-1BB signaling outside of the T-cell realm***

4-1BB is expressed on a variety of cells (T cells, B cells, NK cells, and DCs), thus the effects of an anti-4-1BB antibody can mediate effects in these cells as well. Activation of monocytes using an agonistic anti-4-1BB antibody exhibited an increase in the production of IL-8 and TNF-alpha, but a decreased production of IL-10 (110). Zhang et al. demonstrated that when PBMCs were activated using pokeweed mitogen, human B cells expressed 4-1BB (110, 111). The expression of 4-1BB on B cells was also up-regulated using an anti-CD40/anti-Ig in combination with IFN-gamma (110, 111). In addition, when B cells were incubated with the P815 mastocytoma cell line that had been transfected with 4-1BBL, the B cells' survival and proliferation was enhanced (110, 111). In addition, the B cells increased TNF-alpha and TNF-beta, indicating that the expression of 4-1BB on B cells was functional (110, 111). 4-1BB has been demonstrated to be expressed on activated NK cells, but not on resting NK cells (110). 4-1BB activation using an agonistic anti-4-1BB antibody did not affect the NK cell cytolytic function or anti-tumor function during tumor progression (110).

4-1BB is expressed on DCs and signaling via CD40 can down-regulate the 4-1BB expression (110, 112-114). Wilcox demonstrated that *in vivo* administration of an agonistic anti-4-1BB antibody to naïve mice increased the ability of DCs to activate T cell and induce their proliferation (110, 112-114).

#### ***1.5 d) 4-1BB monoclonal antibodies in the clinic***

4-1BB is transiently expressed on CD4<sup>+</sup> and CD8<sup>+</sup> T cells, although it is preferentially expressed more on CD8<sup>+</sup> T cells. 4-1BB expression is increased after TCR activation and its expression can be seen for up to 4-5 days after TCR activation

(105). Agonists to 4-1BB and/or 4-1BBL have been found to induce an increase in cytolytic T lymphocyte (CTL) activation, Interferon-gamma (IFN-gamma) secretion and initiate an anti-tumor and anti-viral response (86, 91, 96, 99). In addition, 4-1BB has recently been indicated as a potential biomarker for tumor-specific T cells (115). Due to the numerous studies demonstrating 4-1BB as a powerful co-stimulatory molecule for the expansion and effector function of CD8<sup>+</sup> T cells (86, 90, 104), agonists to 4-1BB have been developed to use in the clinic.

Bristol Myers Squibb (BMS) developed a fully-human agonistic anti-4-1BB antibody (BMS-663513 or Urelumumab) that demonstrated anti-tumor effects in a Phase I clinical trial in patients with melanoma renal cell carcinoma, and ovarian cancer (89, 116). There were tolerable side effects in the phase I trial, so this led to the development of phase II trials in melanoma patients, in which the anti-4-1BB antibody was used alone for treatment or in combination with chemotherapy or radiation (89, 116). However, when higher doses of the anti-4-1BB antibody were used, there was a considerable amount of liver toxicity observed (89, 116). Although the reason behind the liver toxicity was not determined, it is speculated that NK cells present in the liver, were non-specifically activated (89, 116).

In a mouse study, it was observed that when high concentrations or repeated doses of anti-4-1BB agonistic antibody were injected into naïve or irradiated mice, the mice exhibited adverse side effects, such as induction of lymphopenia and decreased lymphokine trafficking (87, 89, 99). The antibody also induced an intense accumulation of T cells in the liver (87, 89, 99). This detrimental finding may be explained by a number of other reasons. The surface expression of 4-1BB, as well as

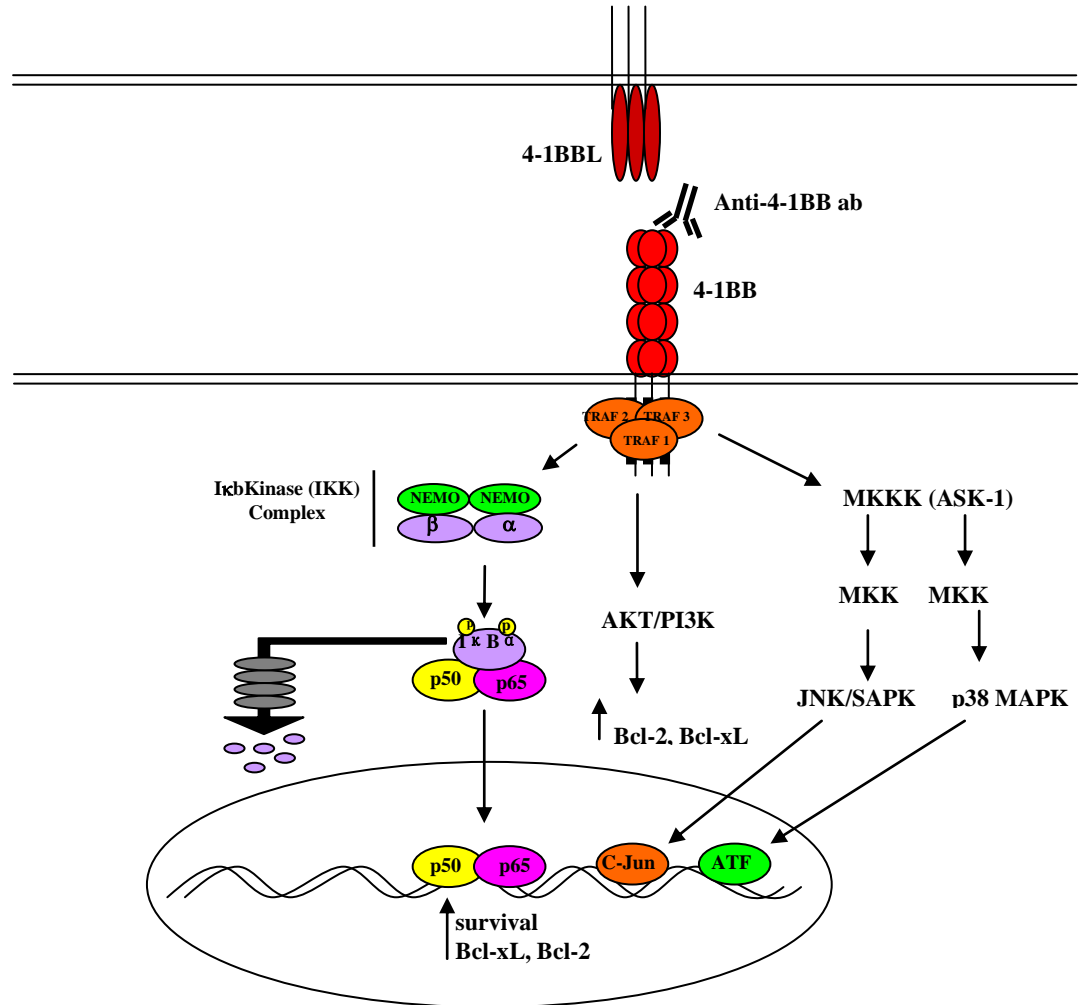
the recruitment of the TRAF members, depends on the activation state of the cells (87, 89, 99). This needs to be taken into consideration when administering the anti-4-1BB antibody systemically.

Pfizer developed an agonistic antibody against 4-1BB (PF-05082566, GTC Biotherapeutics) and has started clinical testing and is currently being tested in synergy with rituximab for the treatment of lymphoma patients (117).

**Figure 1.6. 4-1BB signaling pathways.** When 4-1BB is activated by 4-1BBL or agonistic antibodies, it triggers the TRAF heterodimers to form and initiates downstream signaling. 4-1BB signaling induces the proliferation and survival of cells via PI3K, NF $\kappa$ B, and up-regulation of bcl-2 and bcl-xL.



Figure 1.6. 4-1BB signaling pathways.



## 1.6 Main theoretical questions posed in this dissertation

Although activating the 4-1BB pathway using agonistic anti-4-1BB antibodies exhibit an increase in cytotoxic T lymphocyte (CTL) activity in tumor and viral models, there was liver toxicity observed in melanoma patients in phase II clinical trials. Therefore, we have investigated ways to utilize the anti-4-1BB antibody in improving adoptive T-cell therapy used to treat melanoma, without the possibility of inducing adverse side effects, such as non-specific activation of other cells. We have previously found that post-REP TIL are susceptible to activation-induced cell death (AICD). However, we observed that using agonistic anti-4-1BB antibodies protected the post-REP TIL from AICD. This initial observation prompted us to further investigate the role of activating the 4-1BB during the expansion of the TIL. In our study, our overall goal was to try to improve the overall quality of the TIL used for Adoptive T-cell therapy. Although ACT using TIL has resulted in 50% response rates, we believe there are innovative ways to try and improve this therapy during the different stages of generating the tumor-infiltrating lymphocytes. In this study, we activate the 4-1BB pathway by utilizing an anti-4-1BB antibody during the expansion of the TIL. We wanted to investigate whether activating the 4-1BB pathway would induce an optimal T-cell product that would generate anti-tumor functions and persist *in vivo*. In addition, if providing co-stimulation to the TIL during the initial expansion period using the anti-4-1BB antibody, we wanted to further investigate the possible mechanism by which this could be occurring.

## **1.7 Overall hypothesis and Specific Aims**

Our overall hypothesis is that providing additional co-stimulation to the TIL through the 4-1BB signaling pathway can improve the TIL phenotype, effector function, and *in vivo* persistence, providing an overall better TIL product for melanoma adoptive T-cell therapy. In this dissertation, we tested our hypothesis under the following specific Aims:

**AIM I:** Determine whether activation of the 4-1BB pathway during the initial TIL isolation and expansion affects melanoma TIL proliferation and phenotype.

**AIM II:** Determine whether 4-1BB ligation augments the signaling pathways in the T cells during the initial TIL isolation.

**AIM III:** Determine whether activation of the 4-1BB pathway affects melanoma TIL phenotype, cytolytic activity and persistence during secondary expansion.

## **CHAPTER 2**

**Early activation of the TNFR super family member 4-1BB pathway  
for the expansion of tumor-infiltrating lymphocytes for Adoptive T-  
cell Therapy for metastatic melanoma patients**

## 2.1 Rationale and Hypothesis

My first Aim seeks to extend the number of metastatic melanoma patients who can benefit from Adoptive T-cell therapy (ACT) and improve the efficacy of the treatment. ACT using tumor-infiltrating lymphocytes (TIL) has emerged as a powerful therapy for patients that have failed first and second line therapies. T-cell-based therapies have emerged to be powerful mediators of anti-tumor responses in both hematologic and solid tumors (11, 17). Over the past 3 decades, accumulating evidence has demonstrated the potential of treating metastatic melanoma patients with their own tumor-infiltrating lymphocytes (TILs) as a form of personalized therapy (11, 17, 38). TIL therapy for melanoma is predicated on the enriched tumor antigen-specificity of T cells infiltrating tumors which can be expanded to high numbers and re-infused. The protocol used in most centers involves the initial outgrowth of TILs from 3-5 mm<sup>2</sup> cut fragments from metastatic melanoma surgical resections or biopsies using IL-2 as growth factor (11, 17, 38). These initial tumor fragment cultures usually take about 3-5 weeks to yield enough TIL for secondary expansion in larger scale to yield the final infusion product. Although earlier clinical trials were not that encouraging, the introduction of a non-myeloablative transient lymphodepleting chemotherapy regimen before adoptive transfer of autologous TILs expanded *ex vivo* has significantly boosted clinical response rates to around 45-50% (29, 30). Phase II clinical trials in a number of TIL therapy centers around the world using this general approach of tumor fragment-derived TIL has reproduced these results and long-term follow-up of patients treated with their own TIL is now also showing a survival benefit over other conventional therapies, especially in patients that have progressed

even after other immunotherapies such as IL-2, anti-CTLA-4, and anti-PD-1(11, 17, 38, 79).

CD8<sup>+</sup> TILs enriched in tumor specificity have emerged to be critical in mediating tumor regression in a number of Phase II TIL trials, and current efforts are now aimed to not only increase the expansion of CD8<sup>+</sup> T cells from tumor tissue, but also increase the anti-tumor activity and effector-memory phenotype of the final TIL infusion product to improve persistence after adoptive transfer (17, 18, 38, 58, 79, 118). In addition, accelerating the rate of TIL expansion from the initial tumor fragment cultures has also become a priority to minimize the time the cells are in culture and shorten the time from surgery to treatment and prevent patient protocol withdrawal, especially when contemplating out-scaling TIL manufacturing to larger cohorts of patients (79, 80, 82, 119). Metastatic melanomas contain a population of CD8<sup>+</sup> T cells expressing activation markers, such as PD-1 and 4-1BB/CD137, indicating a recent history of antigenic stimulation in the tumor microenvironment *in vivo* (115). Recent studies have found that CD8<sup>+</sup> TIL expressing 4-1BB especially represent the most highly enriched tumor-specific sub-population of T cells in melanoma (115). Protocols are now being developed to purify 4-1BB<sup>+</sup> CD8<sup>+</sup> T cells from melanoma tissues and expand these selected cells for infusion. Although this approach is promising, it has a number of caveats, including the need to prepare single cell suspensions from tumor tissues, the variable and sometimes low frequency of 4-1BB<sup>+</sup> T cells in tumors that can make positive selection methods difficult, the small sizes of tumor tissue available in many cases from biopsies of surgical resections that yield few cells after enzymatic digestion or mechanical disaggregation,

and the possibility that not all tumor-specific CD8<sup>+</sup> T cells may be in an activated (4-1BB<sup>+</sup>) state at the time the tumor was taken out and processed for TIL expansion.

An alternative approach that may overcome these issues, and also opens up more translational possibilities, is to directly manipulate co-stimulatory pathways within the initial melanoma tumor fragment cultures themselves to capitalize on the *de novo* expression of co-stimulatory molecules due to previous antigenic stimulation on resident CD8<sup>+</sup> T cells which can accelerate the rate of TIL expansion out of the tumor fragments and may also enrich the tumor-specific T cells at the same time. In addition, additional antigenic stimulation may occur during the early tumor fragment culture from antigen presentation that may induce further T-cell co-stimulatory molecule expression that can be further used to further enhance T-cell outgrowth. Tumor fragments have been used for years to expand TIL by simply adding exogenous IL-2, but whether other immunomodulators can be added in these tumor fragment cultures to affect TIL expansion and phenotype has basically been ignored.

In this study, we surmised that the 4-1BB co-stimulatory pathway could be exploited in these initial melanoma tumor fragment cultures in an active rather than a passive way and **hypothesized** that activating 4-1BB signaling using agonistic anti-4-1BB antibodies would enhance the output of CD8<sup>+</sup> T cells, their tumor reactivity, and memory properties. We found that 4-1BB expression is commonly seen on resident CD8<sup>+</sup> T cells in melanoma metastases and is maintained during the culture of tumor fragments for at least a week in a significant frequency of CD8<sup>+</sup> T cells in these early cultures *ex vivo*. We tested the effects of an agonistic anti-4-1BB antibody added during the initiation of individual tumor fragment cultures and found that this

increased the rate of CD8<sup>+</sup> TIL expansion as well as the tumor reactivity of the expanded product.



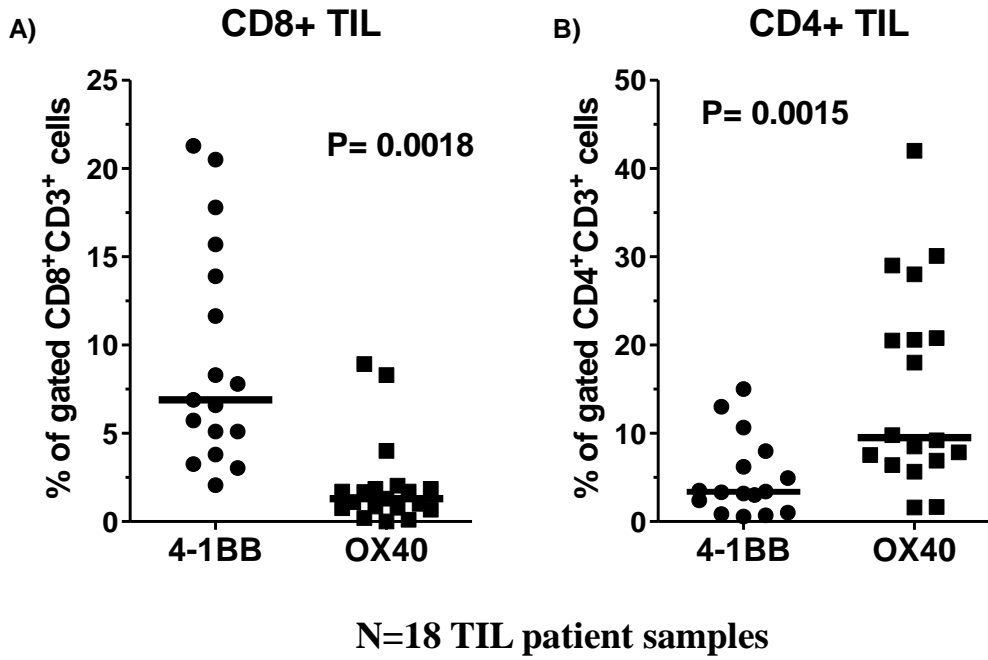
## 2.2 Results

### **4-1BB is expressed on freshly isolated melanoma tumor-infiltrating lymphocytes.**

We wanted to investigate what co-stimulatory molecules were expressed on freshly isolated melanoma TIL. We wanted to determine whether 4-1BB and OX40 co-stimulatory molecules were expressed on freshly isolated TIL from melanoma tumors. Melanoma tumors were surgically resected from metastatic melanoma patients and single cell suspensions were prepared and stained for CD4, CD8, 4-1BB and OX40. In 18 independent TIL samples, we found that 4-1BB was mainly expressed on CD8<sup>+</sup> TIL (**Figure 2.1A**), whereas OX40 was mainly expressed on CD4<sup>+</sup> TIL (**Figure 2.1B**).

**Figure 2.1. Detection of 4-1BB and OX40 on freshly-isolated CD8<sup>+</sup> TIL.** Using flow cytometry, we measured the expression of 4-1BB and OX40 on CD8<sup>+</sup> and CD4<sup>+</sup> TIL single cell suspensions from 18 freshly-isolated melanoma metastasis (A). 4-1BB and OX40 was expressed on CD8<sup>+</sup>, but OX40, was expressed at lower levels on CD8<sup>+</sup> TIL (A and B).

Figure 2.1. Detection of 4-1BB and OX40 on freshly-isolated CD8<sup>+</sup> TIL.



**Addition of agonistic anti-4-1BB antibody increases TIL expansion *in vitro*.** Since we found that 4-1BB was expressed on our freshly isolated CD8<sup>+</sup> TIL, we wanted to determine whether adding an agonistic anti-4-1BB antibody to our initial isolation of the TIL would affect the TIL expansion. In our phase II clinical trial at UT MD Anderson Cancer Center, we are currently only able to successfully grow TIL from 65% of metastatic melanoma patients. In addition, the initial TIL isolation process can take up to 5 weeks. Since we have previously had success in expanding TIL CD8<sup>+</sup> subsets using this anti-4-1BB antibody (90), we wanted to determine if activating the 4-1BB pathway using the anti-4-1BB antibody would be able to increase the number of melanoma patients from which we are able to grow TIL and decrease the initial TIL isolation time.

Melanoma tumors were surgically resected from patients and cut into 3-5mm<sup>2</sup> fragments. The fragments were placed in a 24 well plate over a 3 week period. We conducted antibody dose titration experiments to test what the optimal concentration of antibody was (**Figure 2.1**). Each TIL sample was set up with IL-2 and different concentrations of anti-4-1BB antibody or IL-2 alone (**Figure 2.2A**). After 3 weeks, viable cell counts were done using a hemocytometer after Trypan Blue staining for each condition (**Figure 2.2A**). In our clinical trials at UT MD Anderson Cancer Center (38), we have found that a minimum of 50x10<sup>6</sup> pre-REP TIL is needed in order to expand during the secondary expansion (REP) to a sufficient number. We found that 10µg/ml anti-4-1BB antibody was the optimal dose to expand the pre-REP TIL to 50x10<sup>6</sup> after just 3 weeks in culture. Normally, it takes TIL 3-5 weeks to reach the minimum number (50x10<sup>6</sup>) needed for secondary expansion. We also observed

that only 65% of metastatic melanoma patients' TIL grow from the melanoma fragments. We found that the 10 $\mu$ g/ml anti-4-1BB antibody allowed TIL growth from some patients whose TIL would not have typically reached the 50x10<sup>6</sup> threshold (**Figure 2.2B**). Since we found our optimal concentration was 10 $\mu$ g/ml anti-4-1BB antibody, we subsequently added this dose each time the TIL were fed with fresh IL-2 and media and used this concentration for the remainder of our experiments.

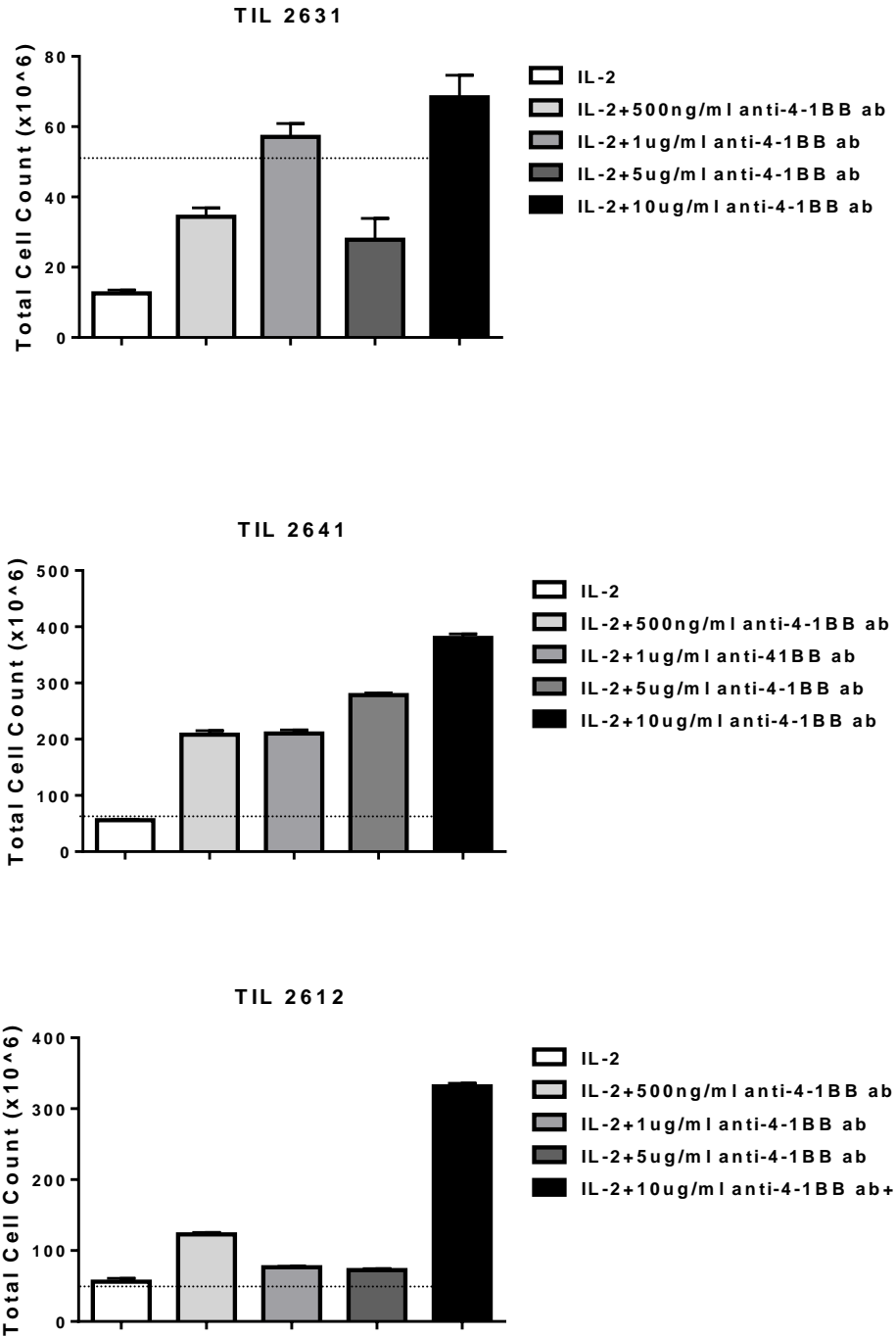
Once we found the optimal dose of the anti-4-1BB antibody, we wanted to determine how the anti-4-1BB antibody would compare to another antibody currently being used in research and clinical trials at expanding the TIL. We added different antibodies (anti-4-1BB agonistic antibody, anti-CTLA-4 blocking antibody (Yervoy), or IL-2 alone control) to the initial TIL cultures and determined cell counts after 3 weeks using a hemocytometer after Trypan Blue staining for each condition (**Figure 2.3A**). We found that in comparison to anti-CTLA-4, the anti-4-1BB antibody was superior in expanding the TIL, specifically the CD8<sup>+</sup> TIL (**Figure 2.3A**).

We usually observe the TIL to begin to grow out from the melanoma fragment after 7 days in culture. We wanted to determine whether the addition of the anti-4-1BB antibody was inducing more proliferation of the cells that grew out from the tumor fragment. After 1 week in culture with or without the anti-4-1BB antibody, we collected the supernatant and cells from the conditions and prepared the samples for cytospin. We cytospun the cells onto glass slides and stained for the proliferation marker Ki67 using Immunocytochemistry (ICC) (**Figure 2.4A**). Interestingly, we found that as early as 7 days after the initial fragment set-up, the anti-4-1BB antibody was initiating the TIL to proliferate more than the traditional culture set-up (IL-2)

(**Figure 2.4A**). We then went on to investigate the total TIL expansion after a 3 week period. When we looked at viable cell counts using Trypan Blue and a hemocytometer, we found that out of 12 independent TIL lines, when the TIL were grown with IL-2 alone, only 4 out of 12 TIL lines were able to reach the  $50 \times 10^6$  TIL needed for secondary expansion **Figure 2.4B**. When the TIL were expanded with IL-2 and anti-4-1BB antibody, 9 out of 12 independent TIL lines were able to reach the  $50 \times 10^6$  TIL threshold needed for secondary expansion **Figure 2.4B**.

**Figure 2.2. Optimal concentration for pre-REP TIL expansion.** A dose titration experiment was conducted to determine the optimal dose for the anti-4-1BB antibody for initial TIL expansion. Cells counts were done after 3 weeks of culture for the different conditions.  $50 \times 10^6$  TIL were needed to initiate a successful REP expansion (dotted, black line). 10ug/ml anti-4-1BB antibody was found to expand the TIL to over  $50 \times 10^6$  and was subsequently the concentration used for the remainder of the experiments.

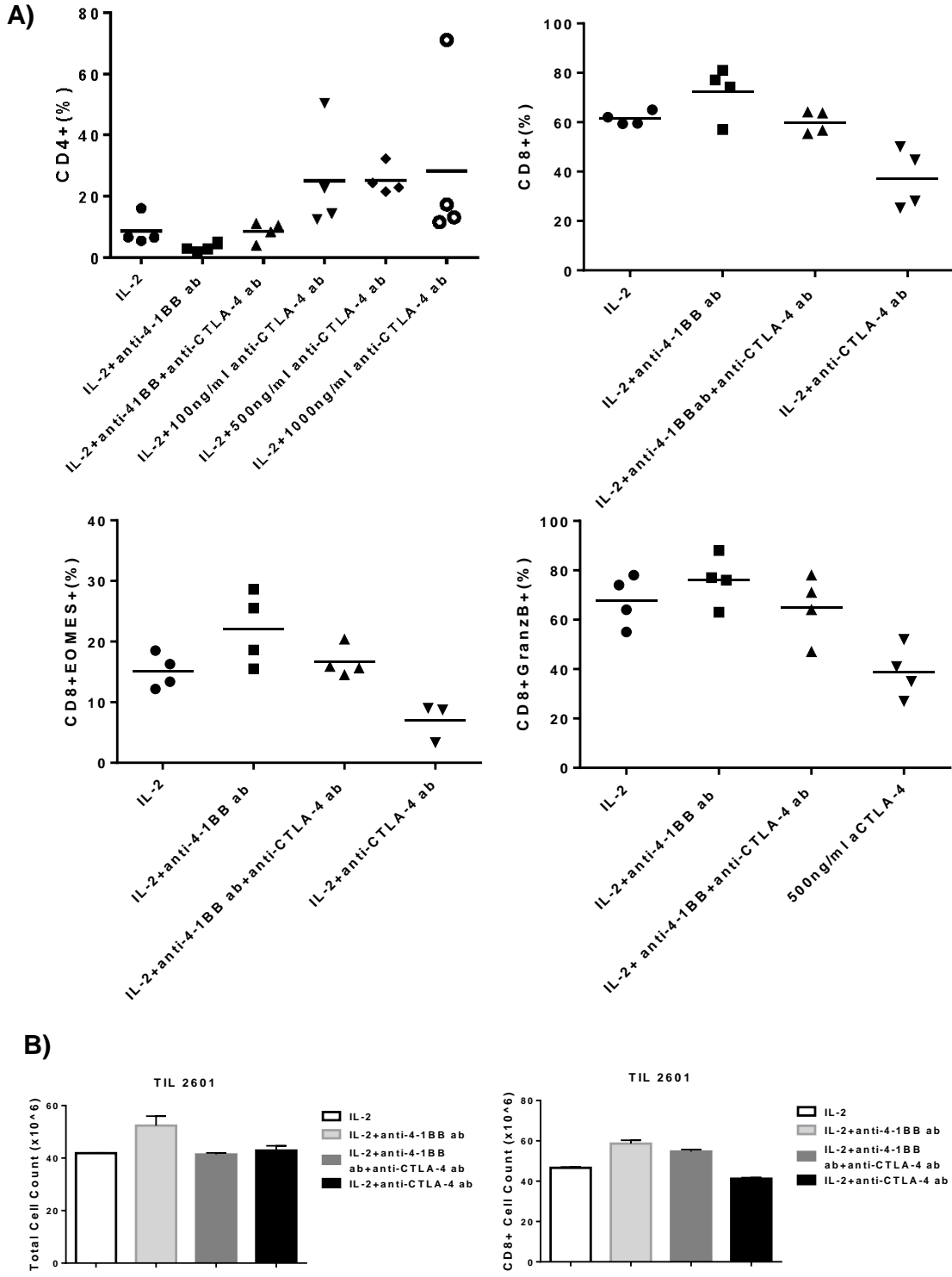
**Figure 2.2. Optimal concentration for pre-REP TIL expansion.**





**Figure 2.3. Addition of anti-CTLA-4 antibody to the fragments increases the percentage and expansion of CD4<sup>+</sup> TIL.** Melanoma tumors were surgically resected and cut up into multiple fragments and placed in a 24 well plate with IL-2. The addition of anti-4-1BB antibody, anti-CTLA-4 antibody or the combination of both was given to the cells each time the cells were fed. After 3 weeks, the TIL were counted and stained with CD3, CD4, CD8, and a viability dye. The cells were then gated on the viable, CD3<sup>+</sup> population (**A**). We found that the TIL grown with the anti-CTLA-4 antibody (Yervoy) preferentially expanded the CD4<sup>+</sup> population, compared with the TIL expanded with the anti-4-1BB antibody or IL-2 alone (**B**).

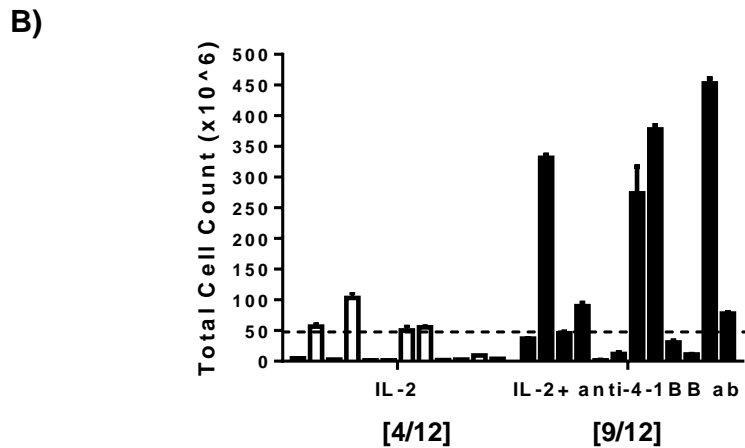
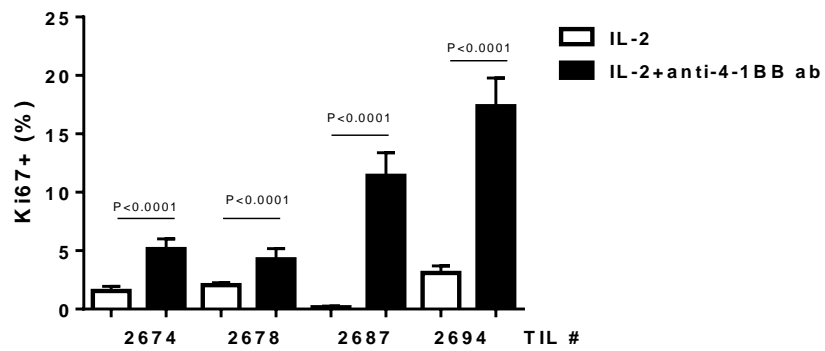
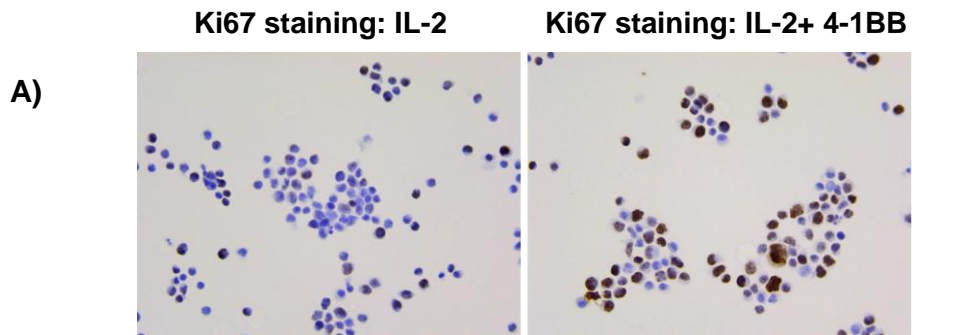
**Figure 2.3. Addition of anti-CTLA-4 antibody to the fragments increases the percentage and expansion of CD4<sup>+</sup> TIL.**



**Figure 2.4. Anti-4-1BB agonistic antibody increases TIL expansion *in vitro*.**

When determining the proliferation of the TIL at early time points (1 week in culture), we found an increased expression of Ki67 proliferation marker in the 4-1BB activated TIL as compared to the control (A). We then went on to determine the total TIL expansion after a 3 week period. In order to be eligible for secondary expansion (REP), the pre-TIL need to reach a minimum of  $\sim 50 \times 10^6$  TIL. When we determined the expansion of the total pre-REP TIL for the different conditions (IL-2 and IL-2+anti-4-1BB antibody), we found that 4 out of 12 TIL were able to reach the  $50 \times 10^6$  threshold needed to be eligible for secondary expansion when expanded with the anti-4-1BB antibody, compared to IL-2 alone.

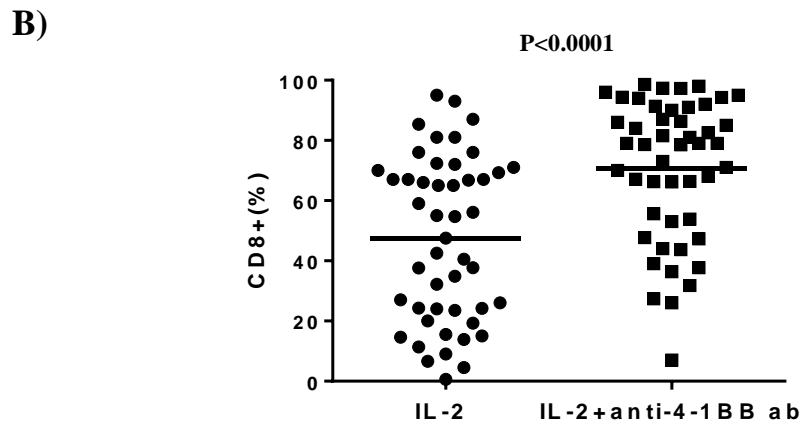
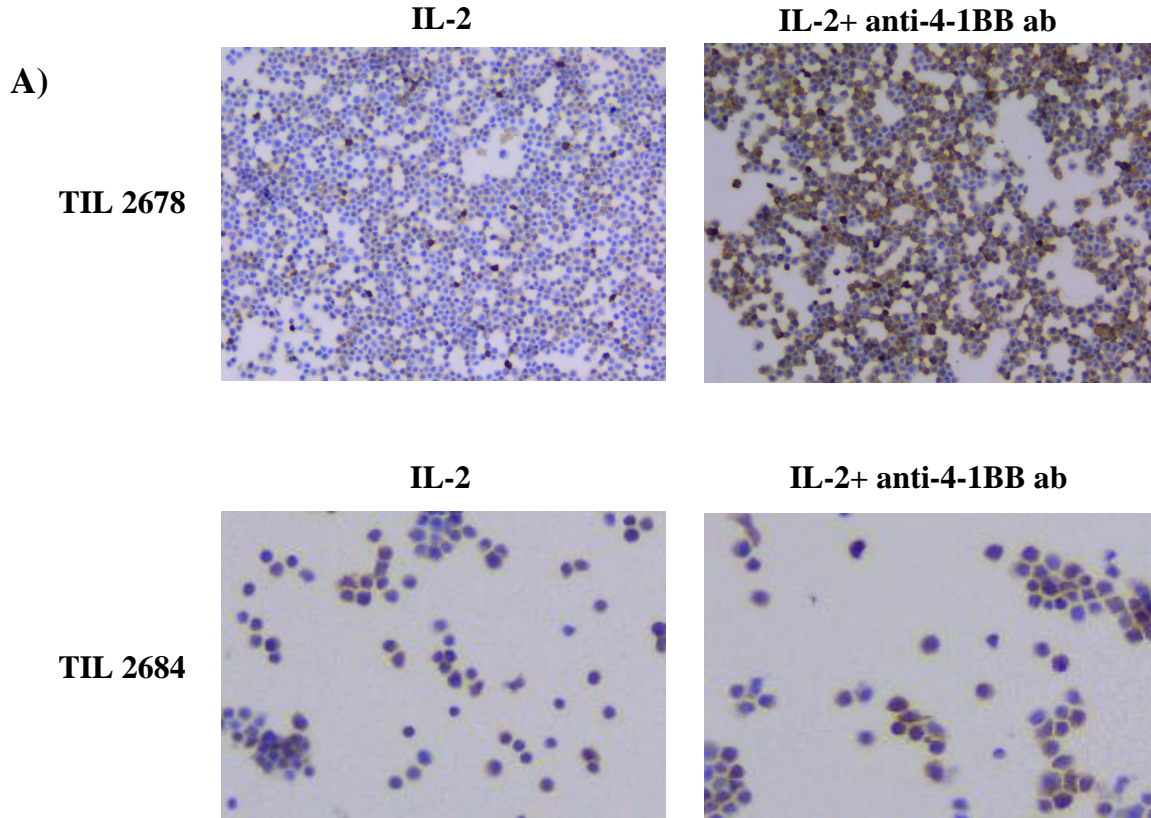
**Figure 2.4. Anti-4-1BB agonistic antibody increases TIL expansion *in vitro*.**



**Activating the 4-1BB pathway increases the CD8<sup>+</sup> TIL expansion.** We wanted to determine whether the cells that were growing out of the fragment were CD8<sup>+</sup> TIL. We therefore set up melanoma fragments with or without the addition of 10µg/ml anti-4-1BB antibody, as described earlier. After 1 week, we collected the cells and supernatant and prepared the samples for cytopsin. The cells were then cytopsun onto glass slides and stained for CD8. We found that the cells grown with IL-2 and the anti-4-1BB antibody contained more CD8<sup>+</sup> cells, compared to the cells that were grown in IL-2 alone (**Figure 2.5A**). After the TIL were grown with or without the anti-4-1BB antibody for 3 weeks, the TIL were stained using flow cytometry for CD3, and CD8 surface markers. In 48 independent TIL lines, we saw the TIL expanded with the addition of anti-4-1BB antibody had more CD8<sup>+</sup> TIL than the TIL grown with IL-2 alone (**Figure 2.5B**).

**Figure 2.5. CD8<sup>+</sup> TIL percentage is increased with the addition of anti-4-1BB antibody to TIL cultures.** We investigated whether activating the 4-1BB pathway could augment the percentage of CD8<sup>+</sup> TIL in the cultures. After 1 week after the fragments were set up, we conducted ICC and found that the TIL expanded with IL-2 and anti-4-1BB antibody exhibited an increase in CD8<sup>+</sup> cells (**A**). In a larger scale (48 independent TIL samples), we found the TIL expanded with the anti-4-1BB antibody exhibited increased percentages of CD8<sup>+</sup> TIL compared to IL-2 control (**B**).

**Figure 2.5. CD8<sup>+</sup> TIL percentage is increased with the addition of anti-4-1BB antibody to TIL cultures.**



**Modulation of melanoma TIL effector phenotype, function, and antigen specificity by activating the 4-1BB pathway.** Melanoma tumors were resected from patients, cut up into multiple fragments and placed in a 24 well plate with IL-2, culture media, and with or without the anti-4-1BB antibody. After 3 weeks, the TIL were stained using flow cytometry for effector-memory markers, markers of effector cytotoxic T lymphocytes (CTL), and effector function was measured using an ELISA assay to measure Interferon (IFN)-gamma secretion. In 48 independent TIL lines, we unexpectedly found that cells grown with the anti-4-1BB antibody had a decreased percentage of CD27<sup>+</sup> TIL in the CD8<sup>+</sup> subset (**Figure 2.6A**). Cells that lose CD27 and CD28 expression are considered late stage terminally differentiated effector cells that do not survive or proliferate in the long term (72, 75, 76, 120). When we investigated the expression of CD70 in the TIL, we found an increase in CD70 expression in the TIL expanded with the anti-4-1BB antibody (**Figure 2.6B**). It has been demonstrated that in an activation-intrinsic event, CD27 expression is decreased in T cells due to either persistent stimulation or the interaction with CD70 in an activation-independent situation (121). Further studies need to be done to investigate this further. When we analyzed the expression of CD28 in the CD8<sup>+</sup> TIL subset in 48 independent TIL samples, we did not detect a difference in CD28 expression in all experimental groups (**Figure 2.6C**).

In order to determine whether the TIL that down-regulated CD27 were still able to expand, the TIL initially grown with or without the anti-4-1BB antibody underwent the secondary expansion (REP). We found that despite the decreased CD27 expression during the initial TIL isolation and expansion, the TIL grown with



anti-4-1BB antibody during the initial expansion expanded at similar levels as the TIL that were not initially grown with the anti-4-1BB antibody (**Figure 2.7A**). This indicated that although the pre-REP TIL down-regulated CD27 expression, they were not terminally differentiated and were still able to expand during the REP. After the 2 week expansion (REP), the post-REP TIL were phenotyped using flow cytometry for CD8, CD27, and CD28 cell surface markers (**Figure 2.7B-D**).

We next investigated the expression of molecules that are associated with effector CTL and killing function on the TIL. We found an increased expression of the intracellular expression of the cytolytic granule Granzyme B (**Figure 2.8A**). We also investigated the expression of the T-box transcription factor Eomesodermin (Eomes). Eomes is a regulator of Perforin, Granzyme B and IFN-gamma in CD8<sup>+</sup> effector T cells (122, 123). In 19 independent TIL samples, we found that the TIL grown with the anti-4-1BB exhibited an increased expression of Eomes in the CD8<sup>+</sup> subset (**Figure 2.8B**).

The expression of 4-1BB on antigen-activated CD8<sup>+</sup> T cells and the detection of a significant frequency of 4-1BB<sup>+</sup> CD8<sup>+</sup> TIL within tumors and during tumor fragment culture (**Figure 2.1**), suggests that in addition to enhancing the numerical expansion of CD8<sup>+</sup> TIL through 4-1BB co-stimulation, addition of agonist anti-4-1BB may also increase the tumor reactivity of the TIL grown out from the treated fragments. We tested this question in different ways. First, we analyzed the anti-tumor reactivity of bulk TIL cultures grown out from tumor fragments after 3 weeks against autologous or HLA-A-matched allogeneic tumor lines by measuring the extent tumor-specific IFN-gamma release after a 24-h tumor:TIL co-culture assay.

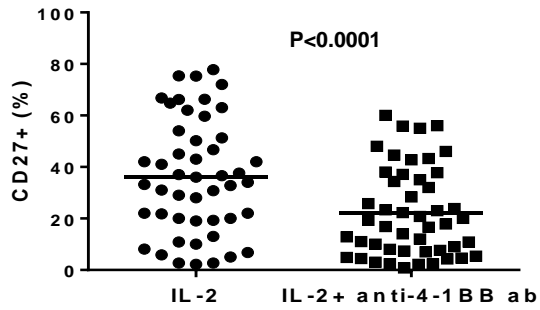
We found that provision of 4-1BB co-stimulation to the tumor fragments yielded TIL with markedly higher anti-tumor reactivity after 3 weeks of culture (**Figure 2.9A**). Second, we performed Intracellular Cytokine Staining (ICS) for IFN-gamma analysis of TIL isolated after 3 weeks from fragment cultures and found a significant increase in the frequency in IFN-gamma<sup>+</sup> CD8<sup>+</sup> T cells in TIL isolated from fragment cultures treated with anti-4-1BB antibody (**Figure 2.9B**). We also measured degranulation using flow cytometry staining for CD107a. We found that the TIL expanded with IL-2 and anti-4-1BB exhibited an increase in CD107a<sup>+</sup> in the CD3<sup>+</sup>CD8<sup>+</sup> subset (**Figure 2.9C**). In addition, in some HLA-A0201<sup>+</sup> patients, we stained TIL that grew out from the fragments for CD8 and MART-1 TCR expression using MART-1 peptide tetramers and found a marked increase in the frequency of MART-1-specific T cells when anti-4-1BB was added to the tumor fragment cultures (**Figure 2.9D**). Finally, we performed a secondary expansion of TIL isolated from fragment cultures treated with or without added anti-4-1BB using the REP and analyzed the post-REP cells for anti-tumor reactivity. Here too, we found an enhanced anti-tumor reactivity of the post-REP product when 4-1BB co-stimulation was provided in the initial tumor fragment cultures (**Figure 2.9E**).

**Figure 2.6. Activation of the 4-1BB pathway augments CD27 and CD70**

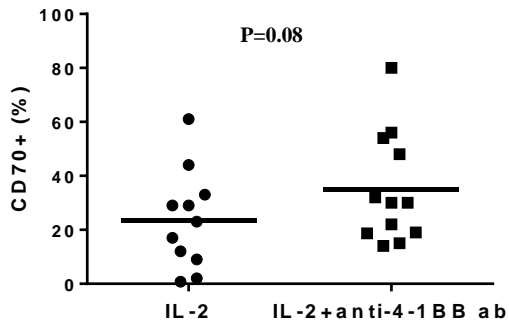
**expression.** Fragments from melanoma tumors were set up in a 24 well plate with or without anti-4-1BB antibody. 3 weeks after the initial set-up, the TIL were harvested and flow cytometry was done to analyze effector-memory markers and cytolytic granule markers. CD27 expression in the CD8<sup>+</sup> subset was down-regulated in the TIL grown with the anti-4-1BB antibody as compared to the TIL grown with IL-2 alone (A). When we investigated the expression of CD70, we found the TIL expanded with anti-4-1BB antibody exhibited an increase in CD70 expression (B). We found no statistical significance in the expression of CD28 in the CD8<sup>+</sup> subset in the different conditions (C).

**Figure 2.6. Activation of the 4-1BB pathway augments CD27 and CD70 expression.**

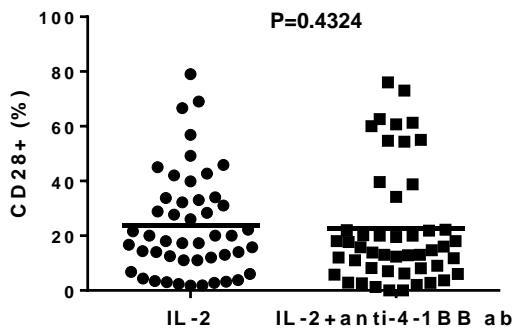
**A)**



**B)**

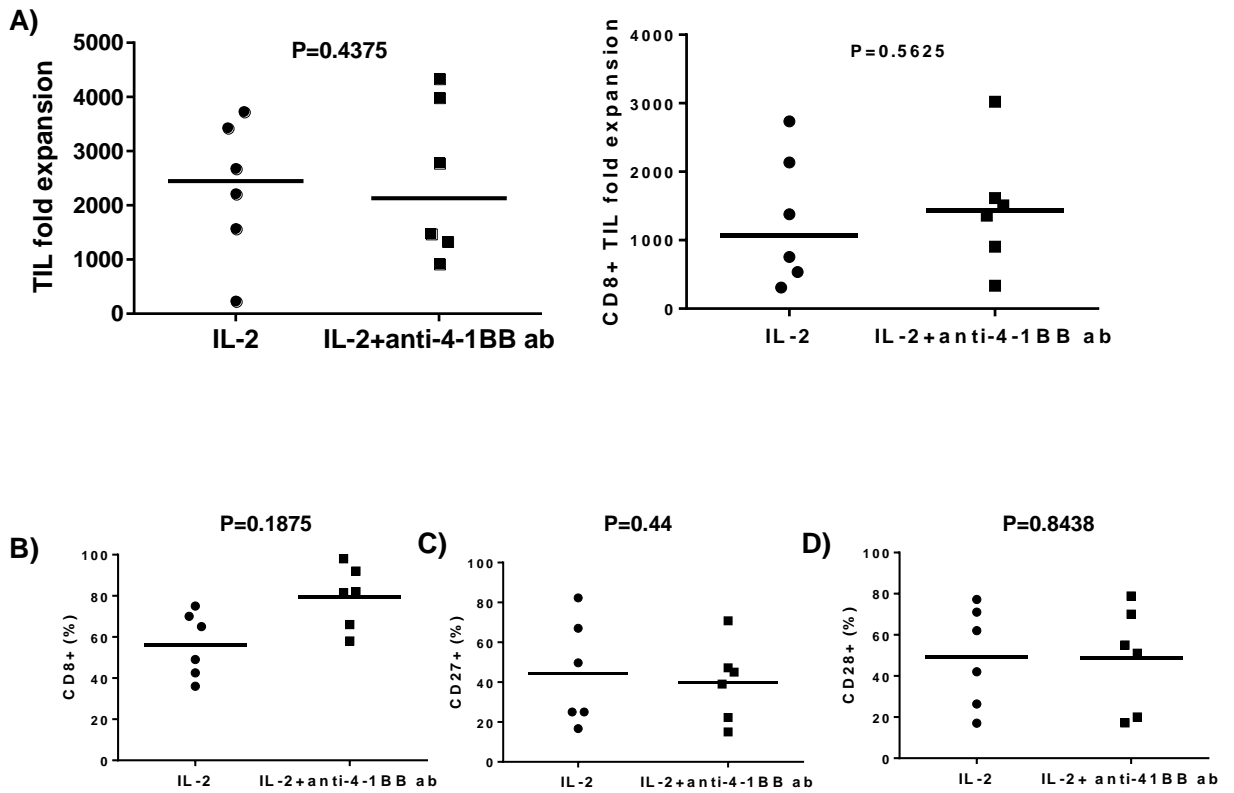


**C)**



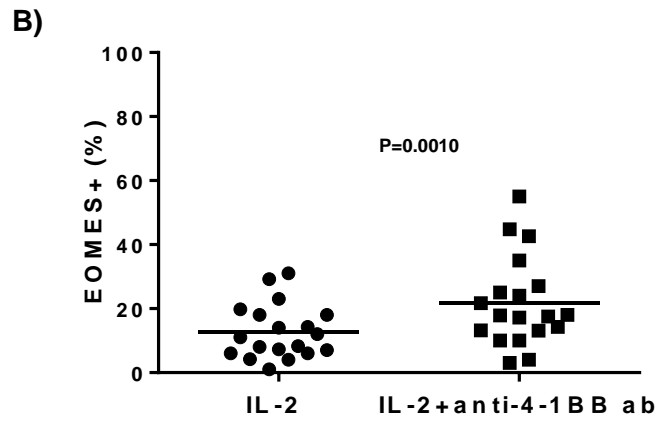
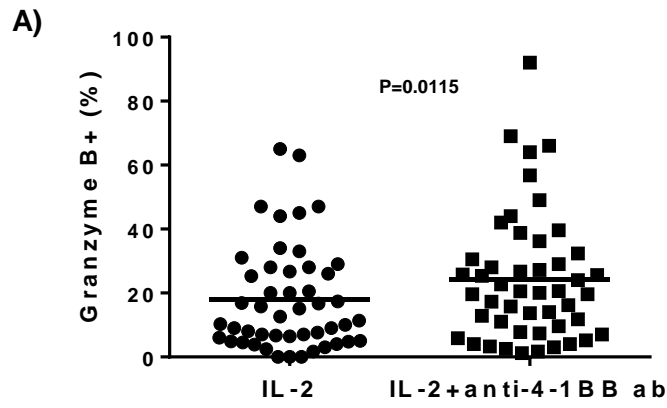
**Figure 2.7. TIL initially expanded with anti-4-1BB antibody continue to expand during secondary expansion in the REP.** After TIL were initially expanded with or without anti-4-1BB antibody, the pre-REP TIL were then subjected to the REP. After the 2 week expansion, phenotype analysis and cell counts were conducted. Post-REP TIL initially expanded with anti-4-1BB antibody exhibited increased CD8+ expression (**A**) and similar levels of CD27 (**B**) and CD28 (**C**). Total (**D**) and CD8 (**E**) fold expansion was not disrupted despite the loss of CD27 at the pre-REP level in the TIL expanded with anti-4-1BB antibody.

**Figure 2.7. TIL initially expanded with anti-4-1BB antibody continue to expand during secondary expansion in the REP.**



**Figure 2.8. Activation of the 4-1BB pathway augments effector cell phenotype and function.** We found the expression of the cytolytic granule marker Granzyme B (A) was increased in the TIL expanded with IL-2 and anti-4-1BB antibody as compared to TIL expanded with IL-2 alone. Eomes is a regulator of Granzyme B, Perforin, and IFN-gamma. When we measured the expression of Eomes using flow cytometry, we found the TIL expanded with the anti-4-1BB antibody exhibited increased Eomes expression in the CD8<sup>+</sup> subset (B).

**Figure 2.8. Activation of the 4-1BB pathway augments effector cell phenotype.**

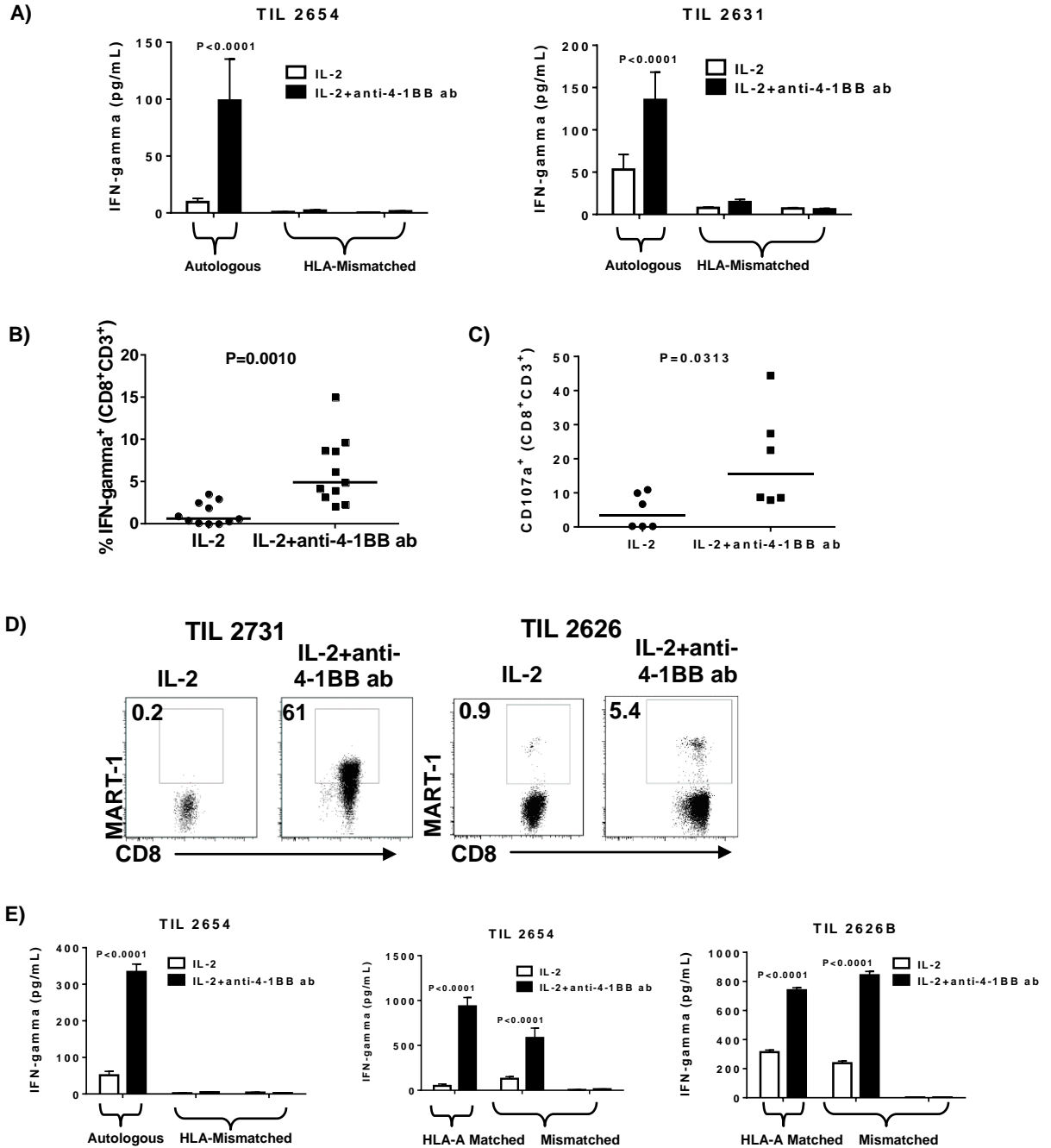




**Figure 2.9. Increased tumor reactivity in TIL expanded with anti-4-1BB antibody.** After the fragments were set up with IL-2 alone or IL-2 plus anti-4-1BB antibody and expanded over a 3 week period (pre-REP), the TIL were harvested and set up at a 1:1 ratio with autologous (**A**) tumor cells. Supernatants were collected after 24 hours and IFN-gamma secretion was measured. We demonstrated in 2 independent pre-REP TIL that IFN-gamma secretion was increased in the pre-REP TIL expanded with IL-2 and anti-4-1BB compared to TIL expanded with IL-2 alone (**A**). After 3 week in culture with or without the anti-4-1BB antibody, the TIL were set up at a 1:1 ratio with HLA-A matched tumor lines. Using flow cytometry, we measured the amount of IFN-gamma<sup>+</sup> cells in the CD3<sup>+</sup>CD8<sup>+</sup> subset (**B**). We found that the TIL expanded with anti-4-1BB antibody exhibited an increase in IFN-gamma<sup>+</sup> cells (**B**). In addition, we also used flow cytometry to determine the amount of degranulation after setting up the TIL at a 1:1 ratio with HLA-A matched tumor lines (**C**). We found that the TIL grown with the anti-4-1BB antibody exhibited an increase in CD107a<sup>+</sup> cells in the CD3<sup>+</sup>CD8<sup>+</sup> subset (**C**). Using flow cytometry, we measured the percentage of MART-1<sup>+</sup> specific T cells on the CD3<sup>+</sup> CD8<sup>+</sup> TIL initially expanded with IL-2 alone or IL-2 with anti-4-1BB antibody. We found that the TIL expanded with the anti-4-1BB antibody exhibited an increase in antigen specific population, compared to the TIL expanded with IL-2 alone (**D**). The pre-REP TIL that were initially expanded with or without the anti-4-1BB antibody then underwent the secondary expansion (REP). After the 2 week expansion, the post-REP TIL were harvested and set up at a 1:1 ratio with the autologous tumor (**E left**) or HLA-matched (**E middle and right**) tumor cells. 24 hours later, the supernatants were

collected and IFN-gamma secretion was measured using ELISA. The post-REP TIL that were initially expanded with anti-4-1BB antibody exhibited an increase in Interferon-gamma secretion compared to the TIL initially expanded with IL-2 alone (E).

**Figure 2.9. Increased tumor reactivity in TIL expanded with anti-4-1BB antibody.**



## 2.3 Discussion

Although ACT has been shown to be an effective therapy for later stage melanoma patients that have failed first and second line therapies, investigators are still trying to find innovative ways to improve the therapy and overall response rates. Our results demonstrate a novel approach using an agonistic anti-4-1BB antibody to expand the melanoma TIL during the initial isolation from the tumor fragment. Not only were we able to grow more TIL, allowing for a potential increase the amount of patients eligible for secondary expansion, but we were able to grow these large numbers of TIL in a shorter amount of time (2-3 weeks compared to the traditional 4-5 weeks needed). We found that the anti-4-1BB antibody not only expands TIL in a shorter amount of time, but these TIL were mainly CD8<sup>+</sup> that expressed cytolytic granule marker Granzyme B, and were tumor reactive against autologous tumors or HLA-A matched tumor lines.

Another important finding that we have consistently seen using the anti-4-1BB antibody during the melanoma expansion is that the anti-4-1BB antibody specifically expands CD8<sup>+</sup> TIL. This is exciting for us because we have found that the more CD8<sup>+</sup> TIL infused into melanoma patients, the better the patient's clinical response. However, it is important to understand the mechanism behind this phenomenon.

## **CHAPTER 3**

**Understanding the role of 4-1BB activation during initial TIL  
isolation from metastatic melanoma tumor fragments**

### 3.1 Rationale and Hypothesis

Our initial observation was that activating the 4-1BB pathway using an agonistic anti-4-1BB antibody increased the TIL proliferation, expansion, CD8<sup>+</sup> output and effector phenotype and functionality. In our second Aim, we wanted to gain a deeper insight as to how the anti-4-1BB antibody was working to induce a better TIL product during the initial expansion. We observed that TIL expanded using the enzymatic digest approach exhibited no difference in total cell growth when expanded with anti-4-1BB antibody. However, when the TIL were expanded from melanoma fragments, the anti-4-1BB antibody greatly enhanced the outgrowth of the TIL in a shorter amount of time, as compared to when the TIL were grown with IL-2 alone. This initial observation prompted us to **hypothesize** that the environment (other cells and cytokines) within the melanoma fragment was playing a role during the initial TIL isolation.

When the 4-1BB pathway is activated, in our case, by using an agonistic antibody, it signals through the TRAF complex to activate the NFκB pathway, as well as other key signaling pathways, such as PI3K/AKT, which can directly control the expression of anti-apoptotic genes, such as Bcl-xL, and bcl-2 (87, 89).

We examined other possible mechanisms of action and found that resident dendritic cells (DC) in the tumor fragments survive for considerable periods of time and also express 4-1BB. These tumor fragment resident DC also activate NFκB, and up-regulate certain maturation markers with 4-1BB agonist addition. In addition, we examined whether ongoing HLA class I antigen presentation occurs in the early tumor fragment cultures that may enhance the output of CD8<sup>+</sup> TIL. We found that

addition of a blocking anti-HLA class I antibody reduced the output of CD8<sup>+</sup> TIL, especially with anti-4-1BB, suggesting that continual antigen presentation occurs *ex vivo* in these early tumor fragment cultures that was not considered before.

STAT5 binding sites have been identified in promoter region of 4-1BB and 4-1BB expression can be modulated by IL-2R signaling through STAT5 activation (124). Therefore, we investigated whether 4-1BB activation augmented the STAT5 pathway in our initial TIL cultures. We found that when the TIL were expanded with anti-4-1BB, the pSTAT5 expression was increased, compared to the TIL grown with IL-2 alone. Bcl-6 has also been important for the maintenance of CD8<sup>+</sup> T cells (122, 123) and when we investigated the expression of bcl-6 in our TIL, we found that the TIL grown with the anti-4-1BB antibody exhibited an increase in bcl-6 expression.

In conclusion, activating the 4-1BB pathway during the initial TIL isolation induces the TIL to become more activated, and also the DCs to become more active and secrete more pro-inflammatory cytokines that may activate the TIL, causing their migration out of the tumor fragment at a faster rate than the TIL without the anti-4-1BB antibody. Pro-inflammatory factors can also promote long-term survival of antigen-specific T cells *in vivo*.

## 3.2 Results

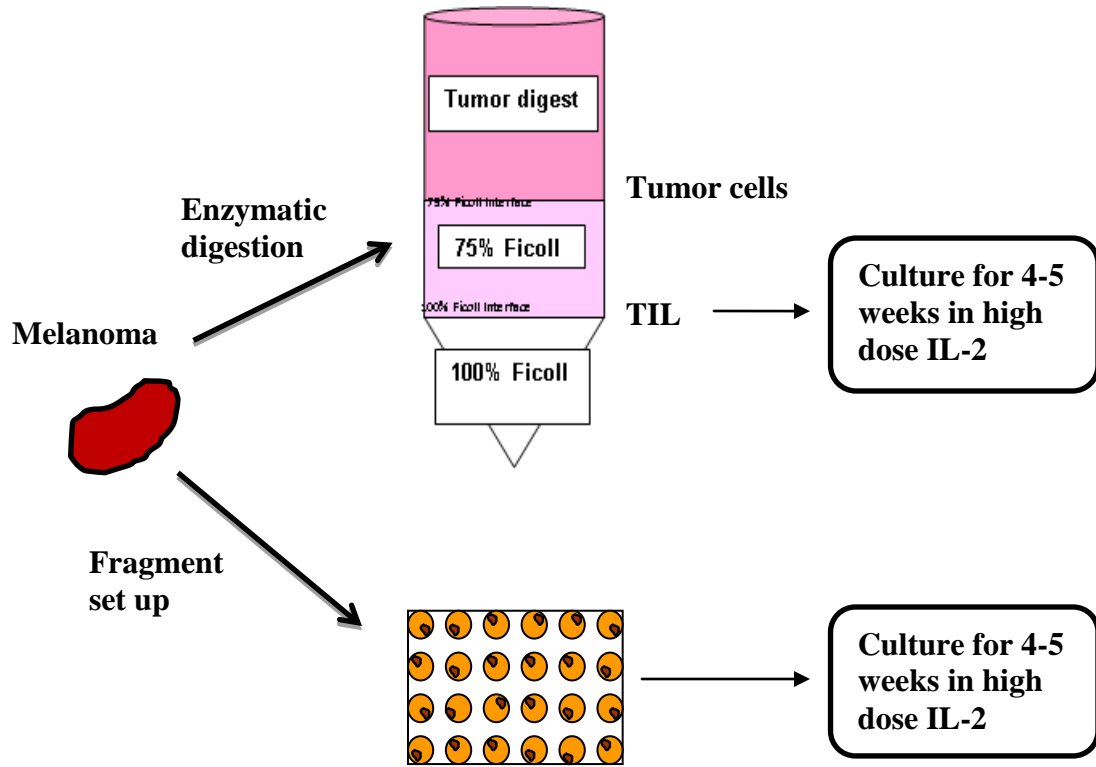
### **TIL require additional activation to respond to anti-4-1BB antibody during initial TIL isolation.**

When we set up our initial TIL cultures, we can either set up fragments from the melanoma tumor or we can conduct an enzymatic digest using collagenase, hyaluronidase, and Ficoll (**Figure 3.1**). The fragment set up includes cutting the melanoma fragment into multiple fragments, expecting the TIL to grow out from the fragment by adding IL-2 over a 4-5 week period. The T cells are not only in contact with other T cells, but the cells within the melanoma fragment. However, when an enzymatic digest is done using Ficoll, the TIL are separated into a single layer (**Figure 3.1**). The enzymatic digest helps the TIL to grow separately from the rest of the cells within the tumor, making the T cells more prone to induce activation by T-cell to T-cell interaction. When we first conducted our experiments, we set up the TIL cultures using the enzymatic digest protocol in parallel to the fragment set up. Surprisingly, we found that when we set up the TIL cultures using the enzymatic digest, there was no difference in TIL proliferation (**Figure 3.2A**), compared to the TIL that were expanded with the anti-4-1BB antibody (**Figure 3.2B**).



**Figure 3.1. Process of TIL isolation from melanoma tumors.** Melanoma tumors are surgically resected from metastatic melanoma patients. The tumors are then cut up and enzymatically digested using Collagenase, Hyaluronidase, DNase I, and Ficoll. This approach results in the physical separation of the TIL from the tumor cells. The other approach to isolate the TIL from the melanoma tumor is cutting up the melanoma tumor into multiple fragments. The TIL are in direct contact with other TIL as well as the other factors within the microenvironment that may influence their outgrowth from the fragment. The TIL are then expanded over a 4-5 week period with high-dose IL-2 and media.

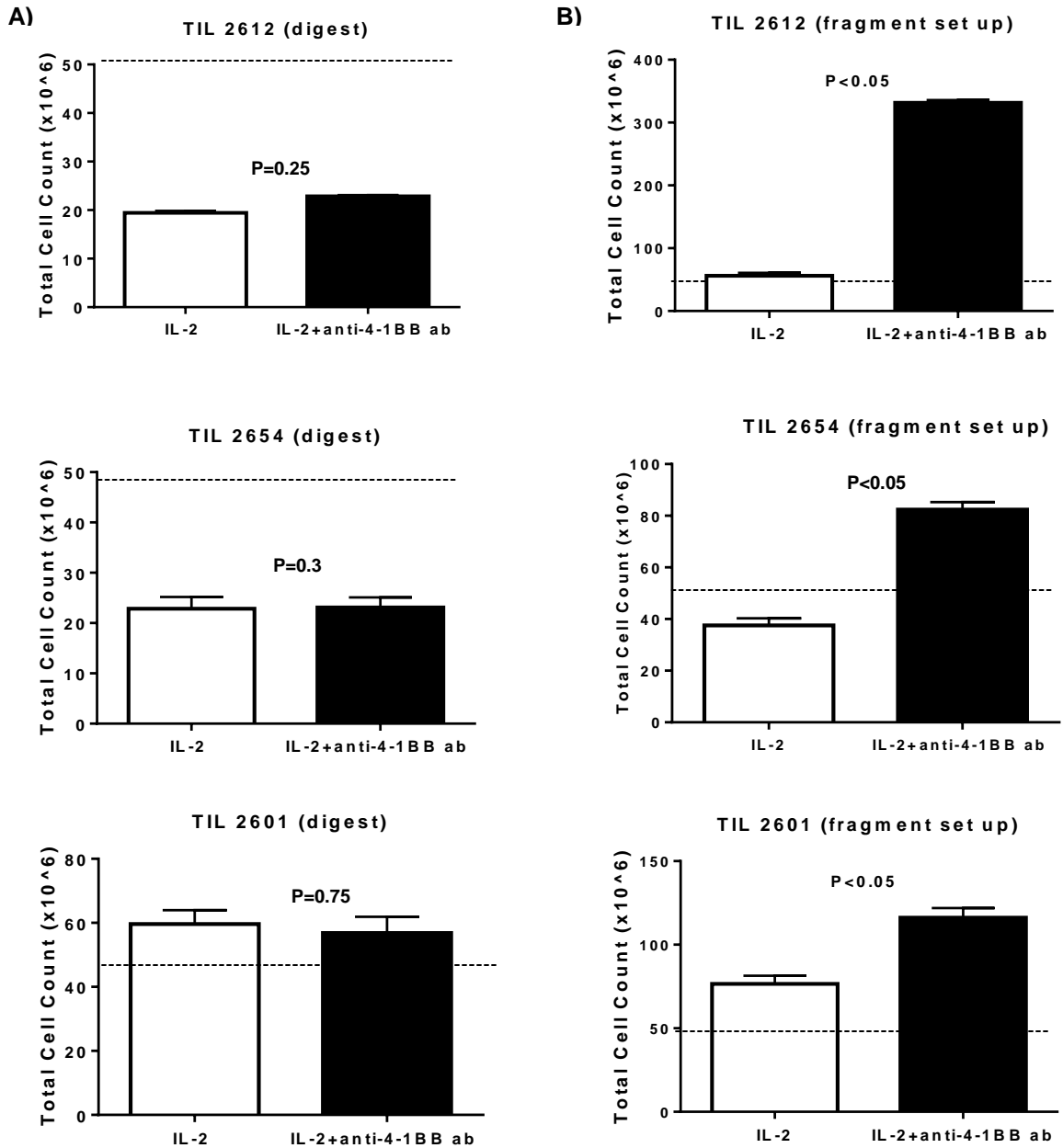
**Figure 3.1. Process of TIL isolation from melanoma tumors.**



**\*Figure adapted from Marie Forget (Radvanyi Lab)**

**Figure 3.2. TIL isolated using enzymatic digest do not expand better with the anti-4-1BB antibody.** The TIL were initially isolated from the melanoma tumor using 2 different methods. The first method uses enzymes to digest the tumor and subsequently separates the TIL layer (**A**). The second method involves cutting up the melanoma fragment in 3-5mm<sup>2</sup> pieces and placing one fragment per well in a 24-well plate (**B**). The TIL isolated from both methods were subsequently placed in a 24-well plate and fed with IL-2±anti-4-1BB antibody and media over a 3 week period. After the 3 week period, the viable TIL were counted. We found that the TIL isolated using the enzymatic approach exhibited no difference in TIL growth when expanded with or without the anti-4-1BB antibody (**A**). However, when the melanoma tumor was cut up into multiple fragments, the TIL expanded much better when grown with the anti-4-1BB antibody compared to IL-2 alone (**B**). The dotted line represents the 50x10<sup>6</sup> TIL threshold needed to be eligible for the TIL to undergo the secondary expansion (REP).

**Figure 3.2. TIL isolated using enzymatic digest do not expand better with the anti-4-1BB antibody.**



**Proposed hypothesis for the role of the anti-4-1BB antibody within the melanoma fragment.** Our finding that the TIL grow better with the anti-4-1BB antibody when fragments are set up prompted us to form a hypothesis that the environment and/or cells within the melanoma fragment were playing a key role in activating and making the TIL more prone to proliferate and expand with the anti-4-1BB antibody (**Figure 3.3**). We hypothesized that when the anti-4-1BB antibody was added to the fragments, the TIL were becoming more responsive and activated, proliferating more and secreting more IFN-gamma either through direct TCR interaction or cytokines secreted by other cells within the melanoma fragment that were also getting activated by the anti-4-1BB antibody (**Figure 3.3**). When IL-2 alone was given, the cells within the fragment may or may not become activated, become anergic or undergo apoptosis, resulting in the TIL not being as activated or responsive (**Figure 3.3**).

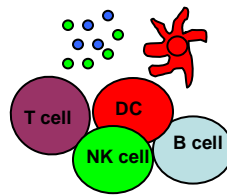
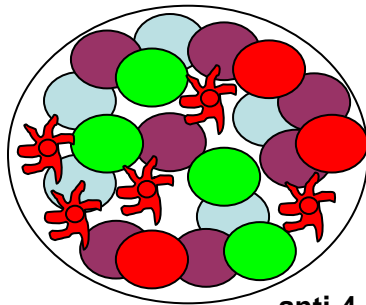
**Figure 3.3. Proposed role for 4-1BB activation within the melanoma fragment.**

We proposed that cells or other factors, such as cytokines, within the melanoma fragment activate the TIL to proliferate and migrate out of the tumor. Some cells within the fragment express 4-1BB and may be responsive to the anti-4-1BB antibody, further activating the TIL to migrate out of the tumor.

Figure 3.3. Proposed role for 4-1BB activation within the melanoma fragment.

## Hypothesis

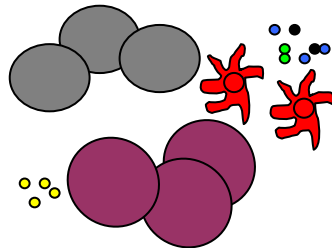
Melanoma Tumor fragment



TIL activated by other cells or cytokines secreted by other cells within fragment

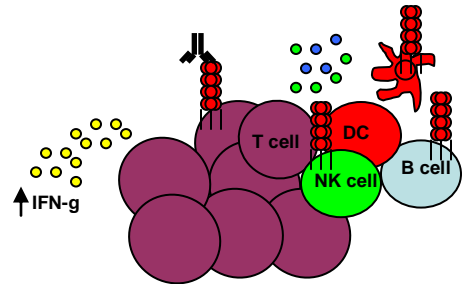
-anti-4-1BB ab

Cells (including TIL) within fragment may or may not become activated, may undergo apoptosis or become anergic



+anti-4-1BB ab

Increase activation/function of cells in fragment that could then further activate TIL



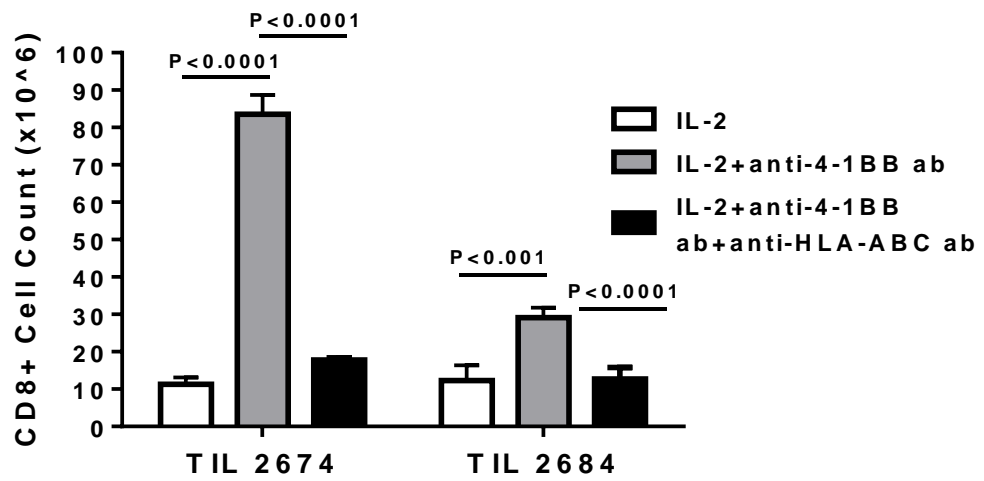
### **Blocking the MHC class I inhibits CD8<sup>+</sup> TIL expansion during initial TIL**

**isolation.** We first wanted to test our hypothesis by determining whether the T-cell receptor (TCR) interaction was crucial in inducing the TIL to expand during their initial isolation from the melanoma fragment. We tested this by adding an anti-human HLA-ABC blocking antibody that recognizes a non-polymorphic epitope shared by the products of HLA-A, B, and C loci ([www.ebioscience.com/human-hla-abc-antibody-biotin-w6-32.htm](http://www.ebioscience.com/human-hla-abc-antibody-biotin-w6-32.htm)) during the initial TIL isolation. When we added the anti-HLA-ABC blocking antibody, we found that the CD8<sup>+</sup> TIL expansion was greatly decreased, compared to the TIL grown with IL-2 alone or IL-2+anti-4-1BB antibody (**Figure 3.4**).



**Figure 3.4. Blocking the MHC class I inhibits TIL recovery during initial TIL isolation.** The melanoma tumor was surgically resected and cut up into multiple fragments. The fragments were then placed in an anti-HLA-ABC blocking antibody (anti-HLA ab) at a concentration of 80µg/ml for 3 hours at 37°. After, the fragments were then given anti-4-1BB antibody or IL-2 alone. The cultures were subsequently fed with additional anti-MHC antibody once more and all cultures were fed over a 3 week period with media, IL-2± anti-4-1BB antibody. After, the viable cells were counted and stained for CD3 and CD8. When the anti-HLA-ABC antibody was added to the cultures, the CD8<sup>+</sup> cell count was greatly decreased compared to the IL-2+anti-4-1BB condition.

**Figure 3.4. Blocking the MHC class I inhibits TIL recovery during initial TIL isolation.**



#### **4-1BB is expressed on different cell subsets in the melanoma fragments.**

We next wanted to investigate whether the TIL were being activated by other cells within the fragment. The melanoma tumors were cut up into multiple fragments and placed in culture with IL-2. After 1 week, the fragment was mechanically disaggregated, filtered, and stained for different immune cell subsets (Dendritic cells, NK cells, B cells and T cells) that are known to express 4-1BB (*110*). We found that the expression of 4-1BB was in the CD8<sup>+</sup> TIL subset (**Figure 3.5A and B**) as shown in 2 representative TIL lines (**Figure 3.5A**) and in 7 TIL patient samples (**Figure 3.5B**). OX40 was also expressed in the CD8<sup>+</sup> subsets (**Figure 3.5A**) as demonstrated in 2 TIL lines (**Figure 3.5A**) and in 5 TIL patient samples (**Figure 3.5B**), but to a lesser extent than 4-1BB.

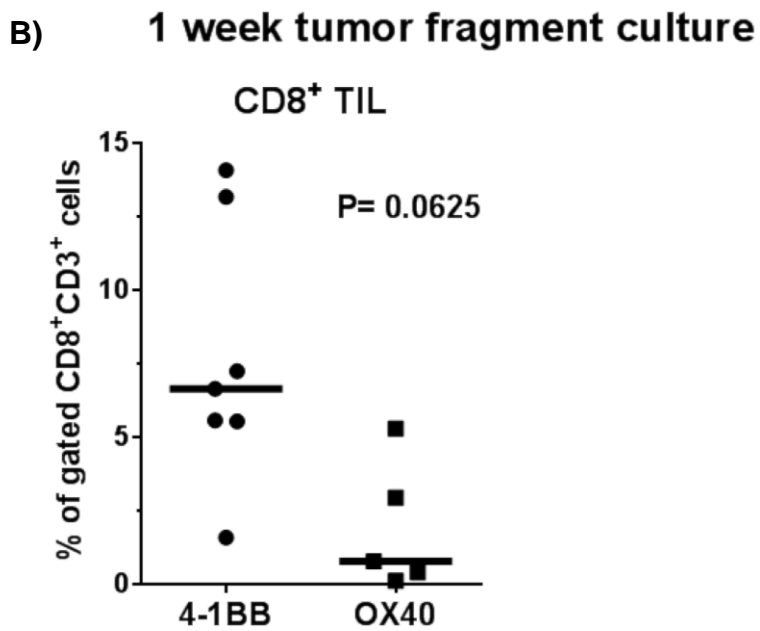
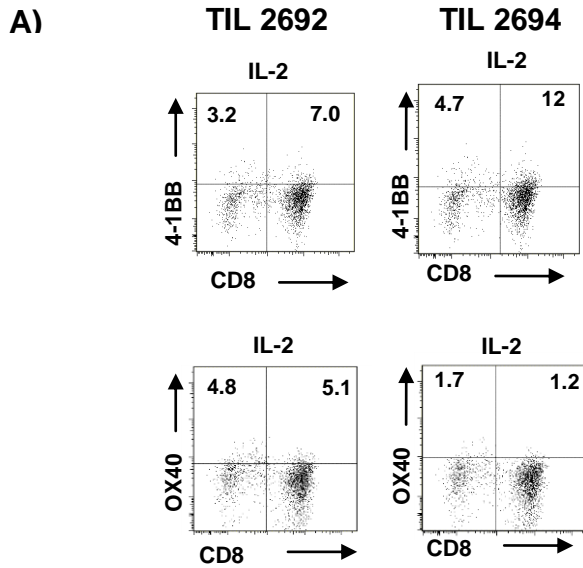
4-1BB can be expressed on Dendritic cells (DCs), Natural Killer (NK) cells, B cells and T cells (*110*). Therefore, we targeted these main populations in the melanoma tumor fragment to determine if they expressed 4-1BB. We found that the population of B cells was less than 0.5% in the total population (**Figure 3.6A**). When we looked at the CD3<sup>-</sup> population, we did not find any statistically significant changes in the NK cell markers or 4-1BB expression in the cultures expanded with or without anti-4-1BB antibody (**Figure 3.6B**).

When we determined the expression of 4-1BB in the DC subset, we found that indeed, the DCs did express 4-1BB (**Figure 3.7**) as shown in 2 independent TIL lines. Interestingly, we found that in the cultures where anti-4-1BB antibody was added, when we gated on dendritic cells, there was an increase expression of MHC-II, CD80 and CD86 expression, and the DCs expressed 4-1BB (**Figure 3.8 and Figure 3.9**). It

has been demonstrated that DCs within a hypoxic microenvironment have decreased activation and maturation markers and down-regulation of pro-inflammatory cytokines (125). However, treatment with an anti-4-1BB antibody increased the expression of pro-inflammatory cytokines and maturation markers in the DCs that were in the hypoxic environment (125). 4-1BB has been shown to be expressed on DCs (113). In another study, when 4-1BB was ligated on the DCs using an agonistic antibody, this resulted in the DCs secreting more pro-inflammatory cytokines and the increased ability to stimulate T cells (113, 126). In addition, we also found that the addition of anti-4-1BB antibody to the cultures increased pro-inflammatory cytokines IFN-gamma, TNF-alpha, and IL-6 (**Figure 3.10**). 4-1BB was also found to function as a survival factor in DCs. DCs that were 4-1BB<sup>-/-</sup> had an impaired survival rate, resulting in decreased levels of anti-apoptotic molecules, such as bcl-2 and bcl-xL, as compared to 4-1BB<sup>+/+</sup> DCs (113, 126).

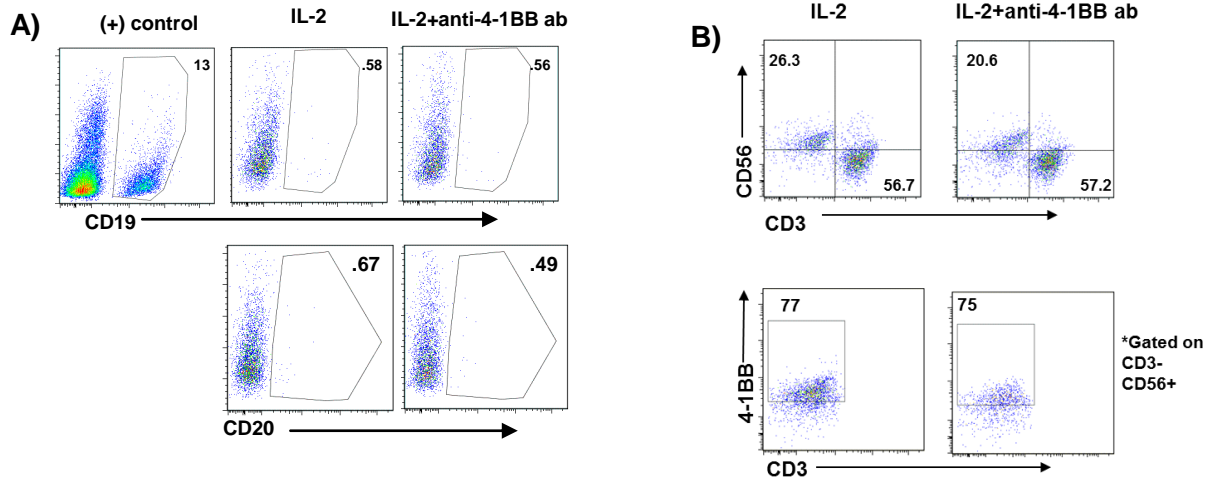
**Figure 3.5. Expression of 4-1BB in the T-cell population within the melanoma tumor fragment.** We set up the melanoma tumor fragments with IL-2 over a 7 day period. We saw that on day 7, the 4-1BB was still expressed in the CD8<sup>+</sup> subsets within the melanoma tumor fragment as demonstrated in 2 TIL patient samples (**A**) and in 7 TIL patient samples (**B**). We found another TNFR super family member OX40 was also expressed in the CD8<sup>+</sup> subsets, as demonstrated in 2 TIL patient samples (**A**) and in 5 patient TIL samples (**B**), but to a lesser extent than 4-1BB.

**Figure 3.5. Expression of 4-1BB in the T-cell population within the melanoma tumor fragments.**



**Figure 3.6. Staining for B cells and NK cells in melanoma fragment.** The melanoma tumor was cut up into multiple fragments, placed in a 24-well plate with media and IL-2± anti-4-1BB antibody. Each condition (IL-2 or IL-2+anti-4-1BB antibody) contained 4 individual fragments to ensure sufficient cell numbers for flow cytometry staining. On day 7 of the cultures, the 4 fragments were pooled from the IL-2 alone condition, mechanically disaggregated, and stained for CD3, 4-1BB, CD56, CD19, and CD20. The same method was applied to the IL-2+anti-4-1BB condition in parallel. There was a very small percentage of B cells after 7 days (**A**). Although there was an NK cell population in the fragments, there was no difference in the expression of 4-1BB or the expression of CD56 in the NK population when the fragments were grown with or without anti-4-1BB antibody (**B**).

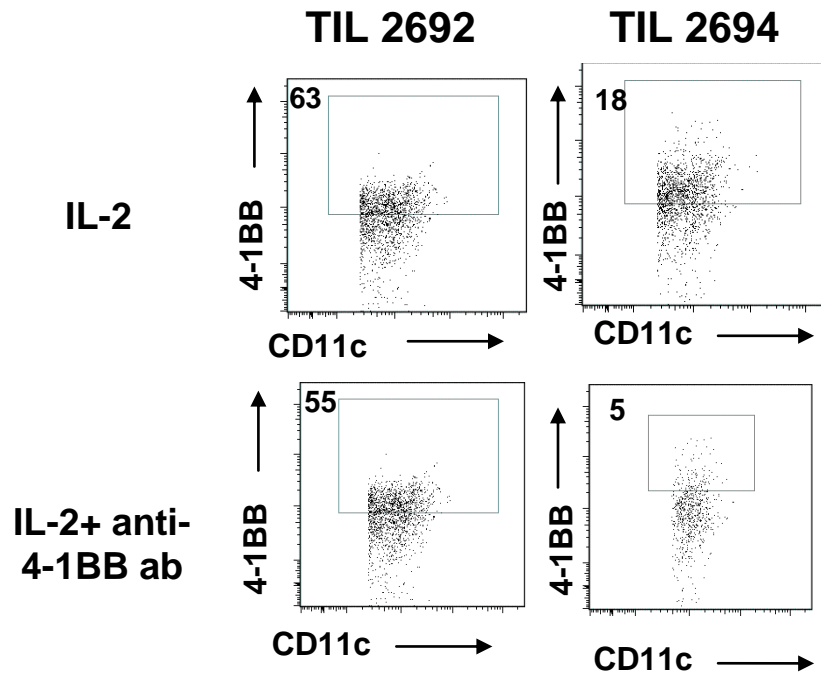
Figure 3.6. Staining for B cells and NK cells in melanoma fragments.





**Figure 3.7 Expression of 4-1BB in DCs in melanoma fragment.** The melanoma fragments were set up with or without addition of anti-4-1BB antibody. In 2 representative TIL samples, we found that 4-1BB was expressed in DCs within the melanoma fragments.

**Figure 3.7: Expression of 4-1BB in DCs in melanoma fragment.**



#### **4-1BB ligation induces activation of dendritic cells within melanoma fragment.**

Since we observed that 4-1BB was expressed on dendritic cells, we wanted to investigate whether 4-1BB ligation via the anti-4-1BB antibody could induce activation of the DCs. Studies have previously showed that dendritic cells within a hypoxic environment are susceptible to undergo apoptosis. However, when co-stimulation was provided to these DCs using an agonistic anti-4-1BB antibody, the DCs were able to secrete more pro-inflammatory cytokines and increase their activation markers. DCs isolated from 4-1BB<sup>-/-</sup> mice survived less than DCs isolated from 4-1BB<sup>+/+</sup> mice.

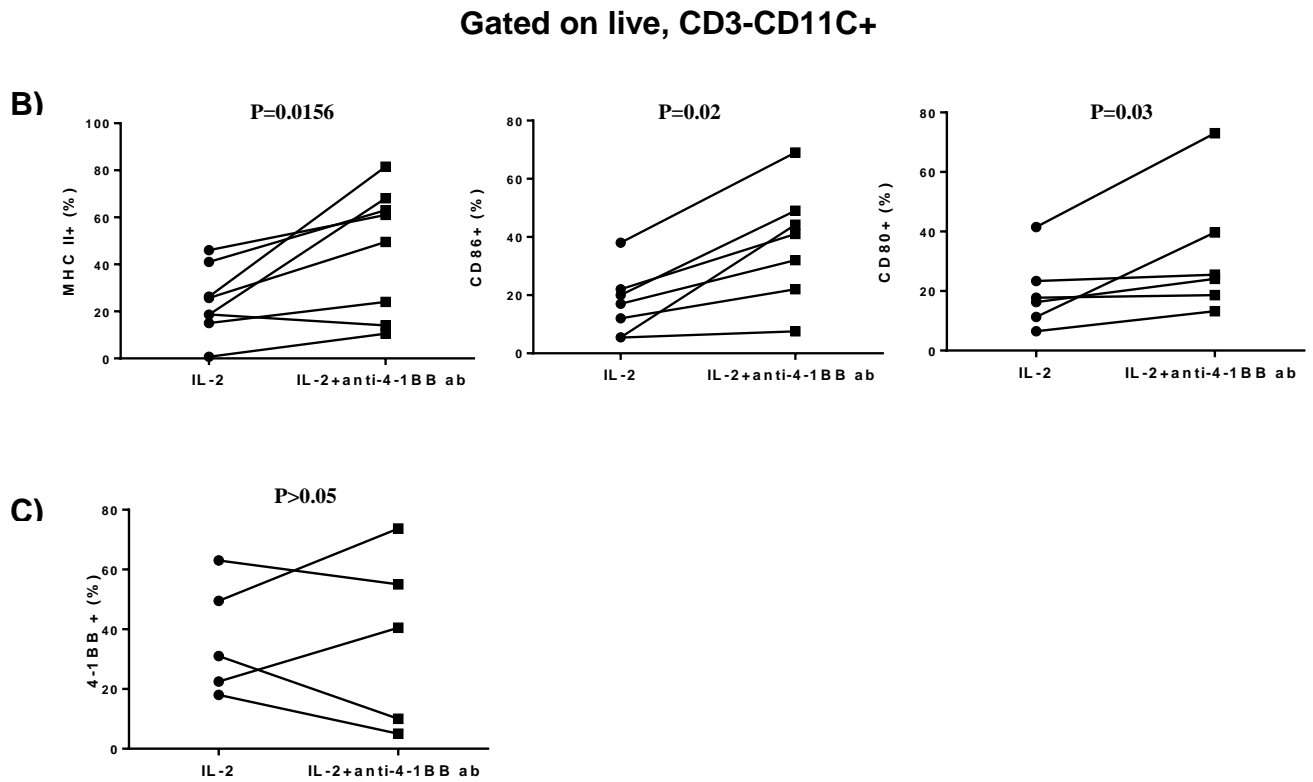
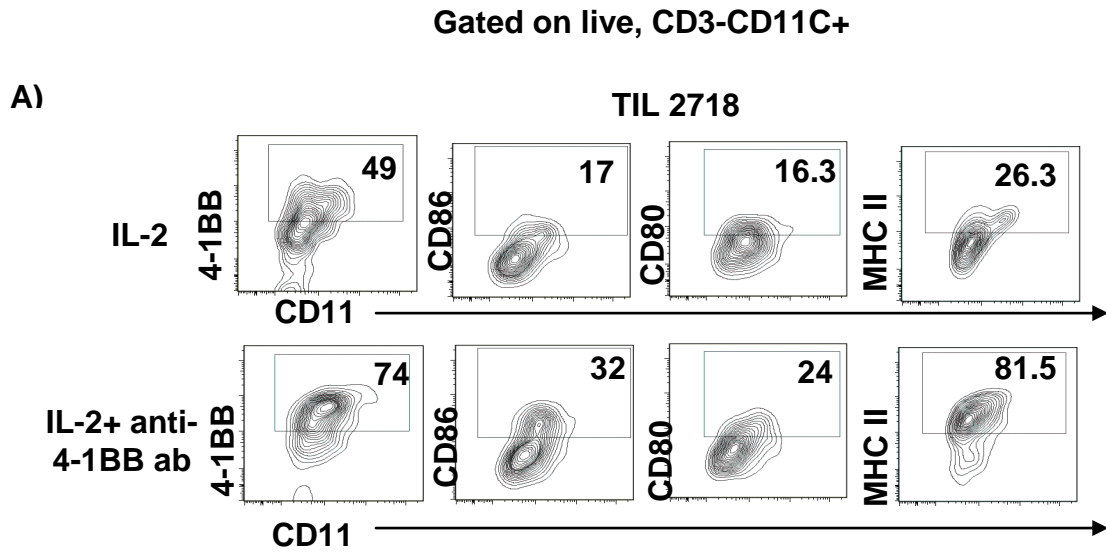
For our studies, melanoma tumors were surgically extracted from patients. The tumors were then cut up into multiple fragments and placed in culture with media, IL-2±anti-4-1BB antibody. The fragment was mechanically disaggregated, filtered, and stained for CD3, CD11c, MHC II, CD80, CD86, and 4-1BB. We show in 1 representative TIL flow cytometry contour plot that the DCs express 4-1BB and that adding the anti-4-1BB antibody to the culture increased DC activation markers (**Figure 3.8A**). When we looked at the activation markers in multiple patient samples, activation of 4-1BB via the anti-4-1BB antibody increased activation markers in the DCs within the fragment (**Figure 3.8B**). In **Figure 3.8C**, we demonstrate in 5 independent TIL lines, that DCs express 4-1BB at varying levels. Patient TIL and tumors vary from patient to patient, therefore we decided to determine whether the 4-1BB ligation induced the activation markers in DCs in a number of TIL lines.

We also investigated whether adding the anti-4-1BB antibody during the initial cultures was inducing the DCs to secrete more pro-inflammatory cytokines. We

conducted a Meso -Scale Discovery (MSD) assay and found that when anti-4-1BB antibody was added to the cultures, there was an increase in IFN-gamma, Interleukin-6 (IL-6), and TNF-alpha (**Figure 3.9**).

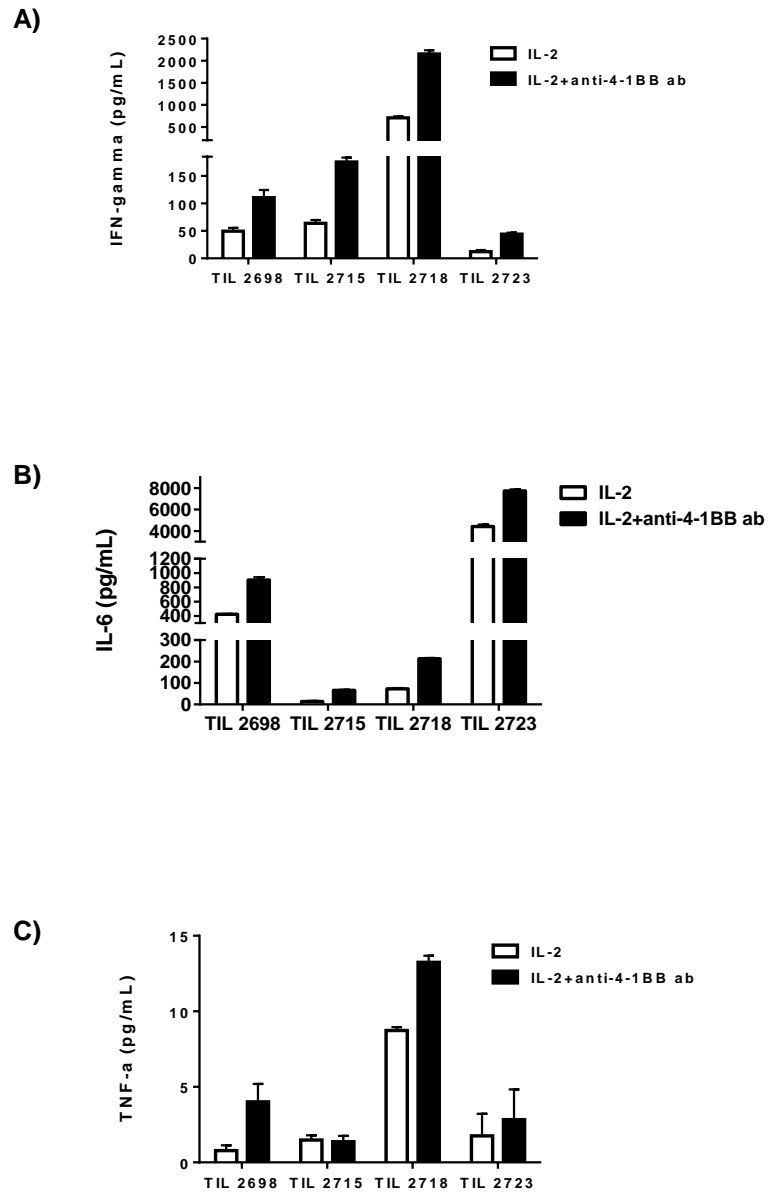
**Figure 3.8. Increased activation markers in DCs when 4-1BB pathway is activated.** The melanoma tumor was surgically removed and cut up into multiple fragments and placed into culture with TIL media and IL-2± anti-4-1BB antibody in a 24-well plate. Each condition contained 4 individual fragments that were pooled on day 7. After 7 days, the 4 fragments per condition were pooled, mechanically disaggregated, filtered, and stained for MHC-II, CD11c, CD80, CD86, 4-1BB, and a viability dye using flow cytometry. We found that the cultures that anti-4-1BB antibody was added induced an increase in MHC-II, CD80 and CD86.

**Figure 3.8. Increased activation markers in DCs when 4-1BB pathway is activated.**



**Figure 3.9. Increased pro-inflammatory cytokines after addition of 4-1BB antibody during initial TIL expansion.** The melanoma tumor was cut up into multiple fragments and placed in culture with IL-2±anti-4-1BB antibody. After 1 week, the supernatant from the two different conditions was collected and Meso Scale Discovery (MSD) was performed to measure the cytokines secreted in the cultures. The secretion of Interferon-gamma (**A**), IL-6 (**B**), and TNF-alpha (**C**) were increased in the cultures where anti-4-1BB antibody was added.

**Figure 3.9. Increased pro-inflammatory cytokines after addition of 4-1BB antibody during initial TIL expansion.**





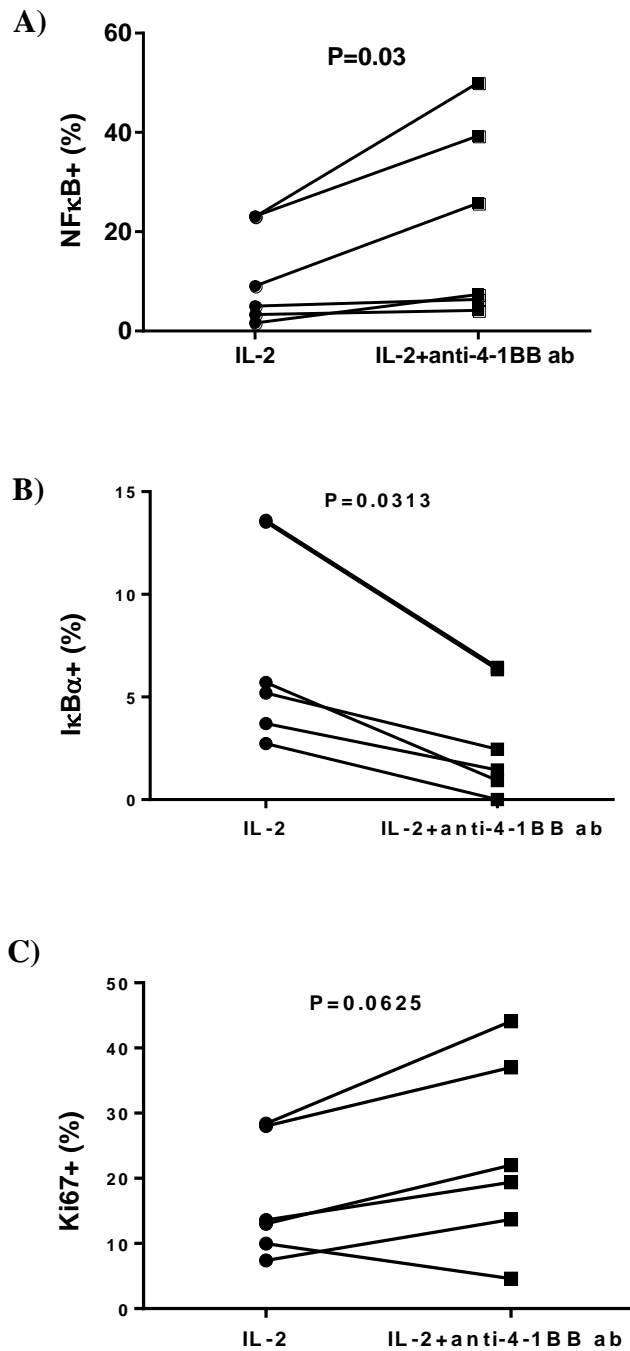
**Activating the 4-1BB pathway increases NF $\kappa$ B translocation in TIL and DCs within the melanoma fragment.**

4-1BB signals through the TRAF complex to activate the NF $\kappa$ B pathway, which can then directly control the expression of anti-apoptotic genes, such as Bfl-1, Bcl-xL, and bcl-2 (**Figure 1.6**). The fragments were set up as previously described, with or without the anti-4-1BB antibody. After 1 week, the fragments were collected, mechanically disaggregated between 2 glass slides, filtered, and stained for NF $\kappa$ B (p65) and I $\kappa$ B $\alpha$  in the TIL (**Figure 3.10A and B**) and DC subsets (**Figure 3.11**) using flow cytometry. In addition, the TIL proliferation was also measured using Ki67 (**Figure 3.10C**). We found that the addition of anti-4-1BB antibody to the fragments induced NF $\kappa$ B (p65), while decreasing I $\kappa$ B $\alpha$  in both the TIL (**Figure 3.10A and B**) and DC subsets (**Figure 3.11**). We also observed that the TIL were proliferating more when the anti-4-1BB antibody was added to the cultures (**Figure 3.10C**).

**Figure 3.10. Modulation of NFκB in T cells within melanoma fragment with 4-1BB pathway activation.** Melanoma tumors were surgically resected from late stage melanoma patients. The tumors were then cut up into multiple fragments and placed in a 24-well plate with IL-2±anti-4-1BB antibody. Each condition contained 4 individual fragments to ensure sufficient numbers of cells would be collected for staining. After 1 week, the 4 fragment per condition were pooled and mechanically disaggregated, filtered, and stained for CD3, CD8, NFκB (p65), IκBα, and Ki67 using flow cytometry. When the anti-4-1BB antibody was added to the cultures, the T cells within the melanoma fragment exhibited increased NFκB and proliferation (Ki67) (**A and C**). The increased NFκB correlated with the decreased IκBα expression seen in the TIL expanded with the anti-4-1BB antibody (**B**).

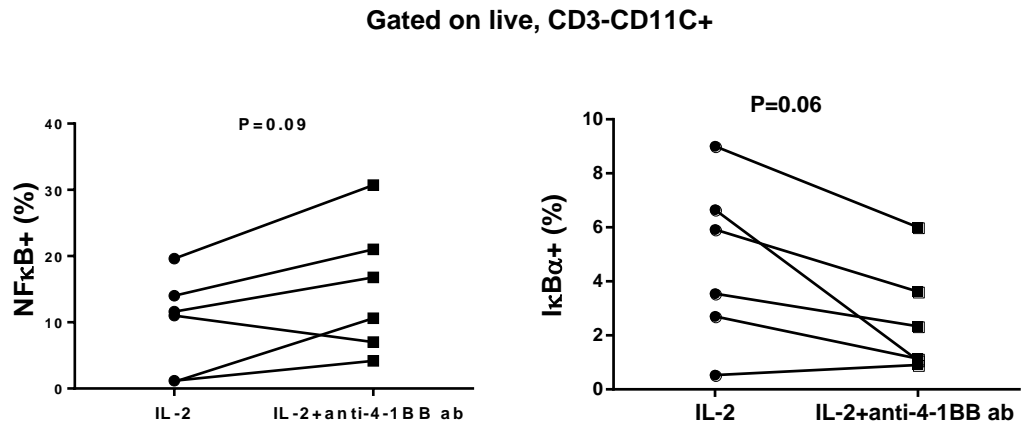
Figure 3.10. Modulation of NFκB in T cells within melanoma fragment with 4-1BB pathway activation.

Gated on live, CD3+, CD8+



**Figure 3.11. Modulation of NF $\kappa$ B in DC subset with melanoma fragment after 4-1BB activation.** After the melanoma fragments were set up for a week with or without anti-4-1BB antibody, the fragment was collected, mechanically disaggregated, filtered, and stained for NF $\kappa$ B (p65) and I $\kappa$ B $\alpha$  using flow cytometry. 4-1BB ligation can induce signaling through the NF $\kappa$ B pathway and we wanted to investigate whether this is occurring in the DC subset within the melanoma tumor. We found that when we added the anti-4-1BB antibody to the cultures, NF $\kappa$ B (p65) was increased in parallel to I $\kappa$ B $\alpha$  decreasing in the DCs.

**Figure 3.11. Modulation of NFκB in DC subset with melanoma fragment after 4-1BB activation.**



**Addition of anti-4-1BB antibody to the cultures decreases 4-1BB expression but increases NFκB translocation in the T cells migrating out of the melanoma tumor fragment.**

When we looked early on in the culture (1 week), we found that the cultures grown with the anti-4-1BB antibody exhibited more cell proliferation in the TIL (specifically CD8<sup>+</sup> TIL) migrating out of the tumor (**Figure 2.4A**). We wanted to determine whether the expression of surface and intracellular 4-1BB was modulated when the 4-1BB pathway was activated. We set up the melanoma fragments with IL-2 ± anti-4-1BB antibody over a period of 7 days. On day 2, 4, and 7, we collected the cells that had migrated out of the fragment, filtered them, and stained for CD3, CD4, CD8, 4-1BB, and a viability dye using flow cytometry. We found that the expression of surface 4-1BB was decreased in the CD4<sup>+</sup> and CD8<sup>+</sup> subset when anti-4-1BB antibody was added to the cultures over the period of 7 days (**Figure 3.12A top**) and in 4 independent TIL on day 7 (**Figure 3.12A bottom**). Interestingly, when we stained for 4-1BB in the intracellular compartment, we found that the expression was increased when the TIL were grown with the anti-4-1BB antibody compared to IL-2 alone (**Figure 3.12B**).

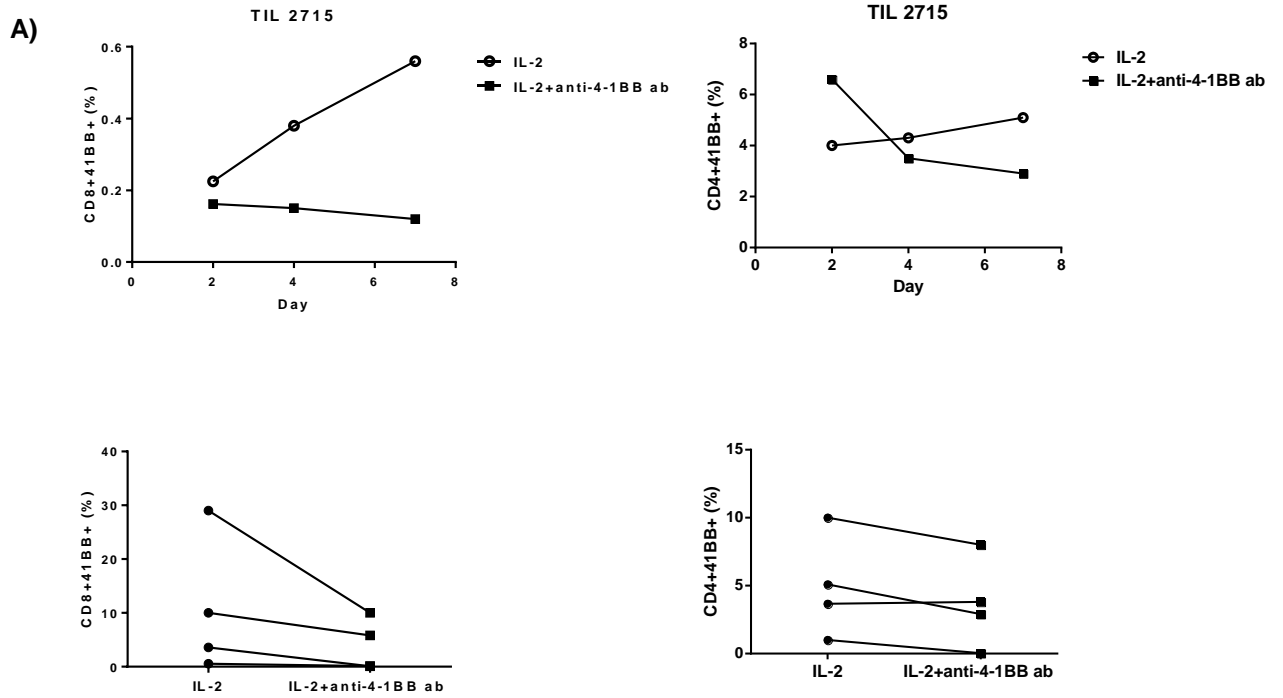
It has been previously demonstrated that when 4-1BB is ligated, it can signal through the TRAF complex to activate the NFκB pathway, which can then directly control the expression of anti-apoptotic genes, such as Bfl-1, Bcl-xL, and bcl-2 (**Figure 1.5**). The fragments were set up as previously described, with or without the anti-4-1BB antibody. After 1 week, the cells and supernatant were collected and prepared for cytospin. The samples were then cytospun onto glass slides and stained

for NF $\kappa$ B (p65). We observed that the TIL grown with IL-2 and the anti-4-1BB antibody exhibited more NF $\kappa$ B translocation in the nucleus as compared to the TIL grown in IL-2 alone (**Figure 3.13A and Figure 3.13B**). When we looked at NF $\kappa$ B (p65) activation in the TIL using flow cytometry, we found that the TIL exhibited an increase in NF $\kappa$ B and a decrease in I $\kappa$ B $\alpha$  in the CD3<sup>+</sup>CD8<sup>+</sup> subset (**Figure 3.13C**).

**Figure 3.12. Expression of 4-1BB on the T cells migrating out of the tumor.** The melanoma fragments were cut up and placed in TIL media, IL-2± anti-4-1BB in a 24-well plate over a period of 7 days. After 2, 4, and 7 days, the fragment was removed, mechanically disaggregated, filtered and stained as mentioned earlier. After the fragment was removed, the remaining supernatant and cells that migrated out of the tumor fragment were collected, filtered, and stained for a viability dye, CD3, CD8, CD4, and 4-1BB expression using flow cytometry. The cultures that were expanded with IL-2 and IL-2+anti-4-1BB antibody exhibited 4-1BB expression in the CD4<sup>+</sup> and CD8<sup>+</sup> subsets over the 7 day period (**A top**). On day 7, we measured the 4-1BB expression in 4 independent TIL lines in the CD8<sup>+</sup> and CD4<sup>+</sup> subsets (**A bottom**).

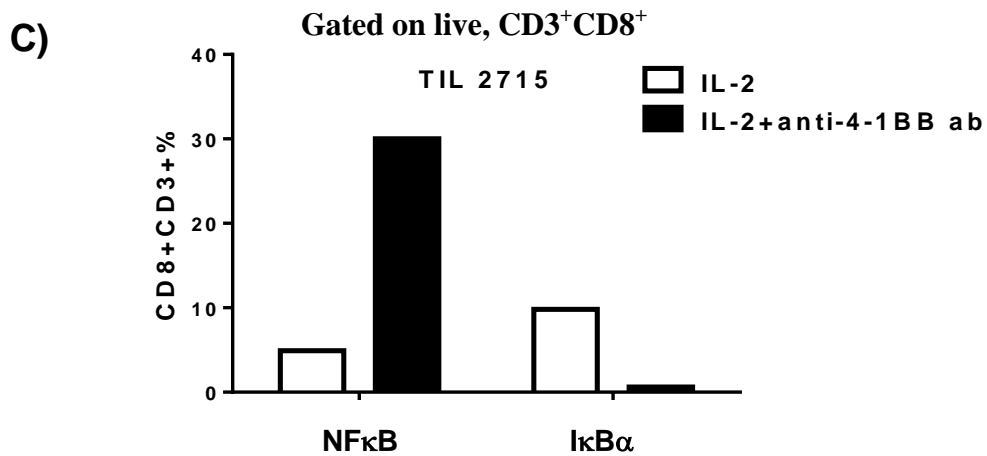
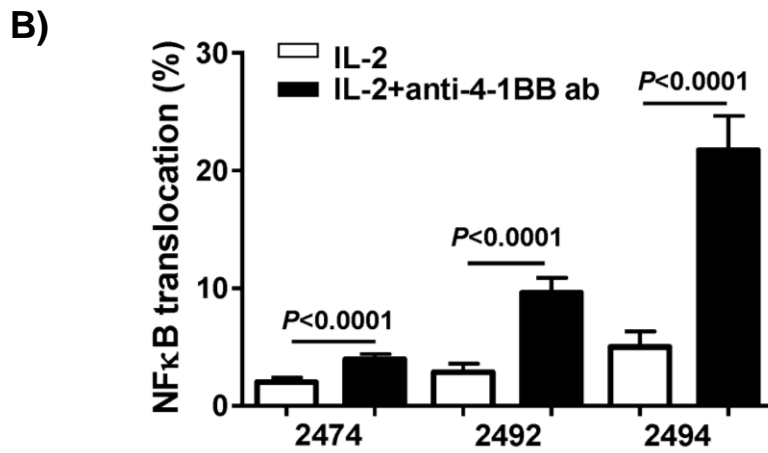
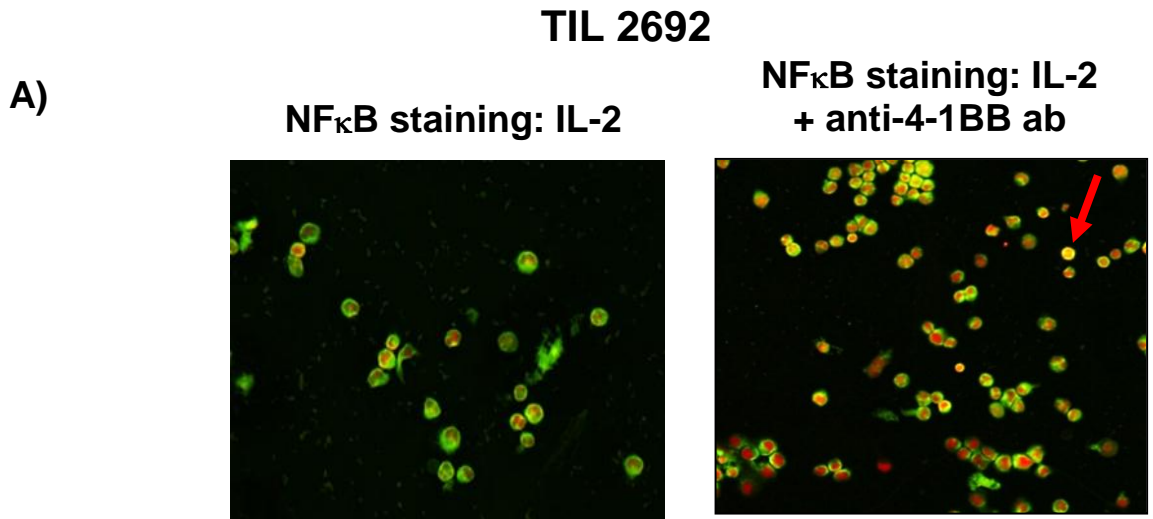


**Figure 3.12. Expression of 4-1BB on the T cells migrating out of the tumor.**



**Figure 3.13. Increased NFκB translocation in TIL grown with the anti-4-1BB antibody.** Melanoma fragments were set up as described in the Methods section with or without anti-4-1BB antibody. After 1 week, the cells were collected and cytospin and Immunocytochemistry were done. Staining for NFκB (p65) revealed more translocation in the nucleus of TIL grown with IL-2 and the anti-4-1BB antibody compared to TIL grown with IL-2 alone. We used Vectra Intelligents slide analysis system (Nuance software) (**A**) to observe the translocation of NFκB (red is nucleus; green is NFκB; and yellow is overlay (translocation)). The red arrow indicates one area where translocation occurred. Quantification of NFκB translocation in 10 different areas per sample is demonstrated in 3 independent TIL samples (**B**). When we determined NFκB (p65) activation using flow cytometry, we found NFκB increased in the CD3<sup>+</sup> CD8<sup>+</sup> subset and a decrease in IκBα in the CD3<sup>+</sup>CD8<sup>+</sup> subset when the TIL were expanded with IL-2+anti-4-1BB antibody compared to IL-2 alone (**C**).

**Figure 3.13. Increased NFκB translocation in TIL grown with the anti-4-1BB antibody.**



**4-1BB ligation modulates pAKT and increases pSTAT5 in the CD8<sup>+</sup> subset during the initial TIL expansion.**

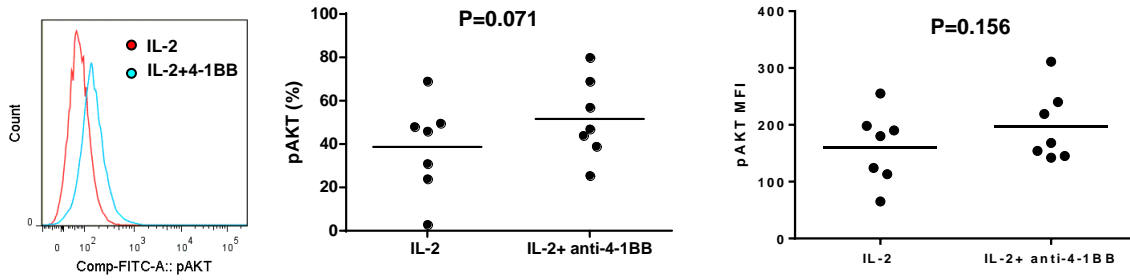
Since we found that activating the 4-1BB pathway was inducing more NFκB translocation in the TIL, we decided to investigate other signaling pathways and molecules that may play a role in the TIL activation. It has previously been reported that using an agonistic antibody of 4-1BB (3H3) promoted the activity of Akt in CD8<sup>+</sup> T cells (127). In addition, co-stimulation by 4-1BB has been shown to inhibit T-cell apoptosis and induce Bcl-xL via PI3K and AKT/protein kinase B (128). We therefore decided to investigate whether activating the 4-1BB pathway to expand melanoma TIL was activating the AKT pathway. We indeed found that activating the 4-1BB pathway was increasing pAKT in the CD8<sup>+</sup> TIL subset (**Figure 3.14**).

Since we have previously demonstrated that activating the 4-1BB pathway induces increased levels of anti-apoptotic molecules, such as bcl-2 (86, 90), we decided to investigate what could be regulating the bcl-2 expression. It has been reported that activation of 2 primary pathways, the JAK/STAT pathway and the PI3K/AKT pathway results in increased levels of bcl-2. We next wanted to focus on the STAT pathway and specifically focus on STAT5. We investigated STAT5 because it has been demonstrated that bcl-2 is a target of STAT5, there have been STAT5 binding sites identified in the promoter region of 4-1BB, and TNFR super family members 4-1BB, OX40, and GITR have their expression modulated by IL-2R signaling through the activation of STAT5 (124). We found that expanding the TIL with the anti-4-1BB pathway increased the pSTAT5 level in the CD8<sup>+</sup> subset (**Figure 3.15A**).

Since we add IL-2 to our cultures, we wanted to investigate whether the TIL expanded with anti-4-1BB were more responsive to IL-2. Therefore we measured CD25, which is the IL-2 receptor alpha chain. We found that when anti-4-1BB antibody was added to the TIL, there was an increase in CD25, compared to when IL-2 alone was added (**Figure 3.15B**).

**Figure 3.14. The addition of the anti-4-1BB antibody modulates pAKT in the CD8<sup>+</sup> subset during the initial TIL expansion.** The melanoma tumor was cut up into multiple fragments and placed in a 24-well plate with media and IL-2±anti-4-1BB antibody. After 3 weeks in culture, the cells were harvested and stained for CD3, CD8, and pAKT using flow cytometry. The percentage and Mean Fluorescence Intensity (MFI) of the CD8<sup>+</sup> subset was slightly increased in the cells expanded with anti-4-1BB antibody but was not statistically significant.

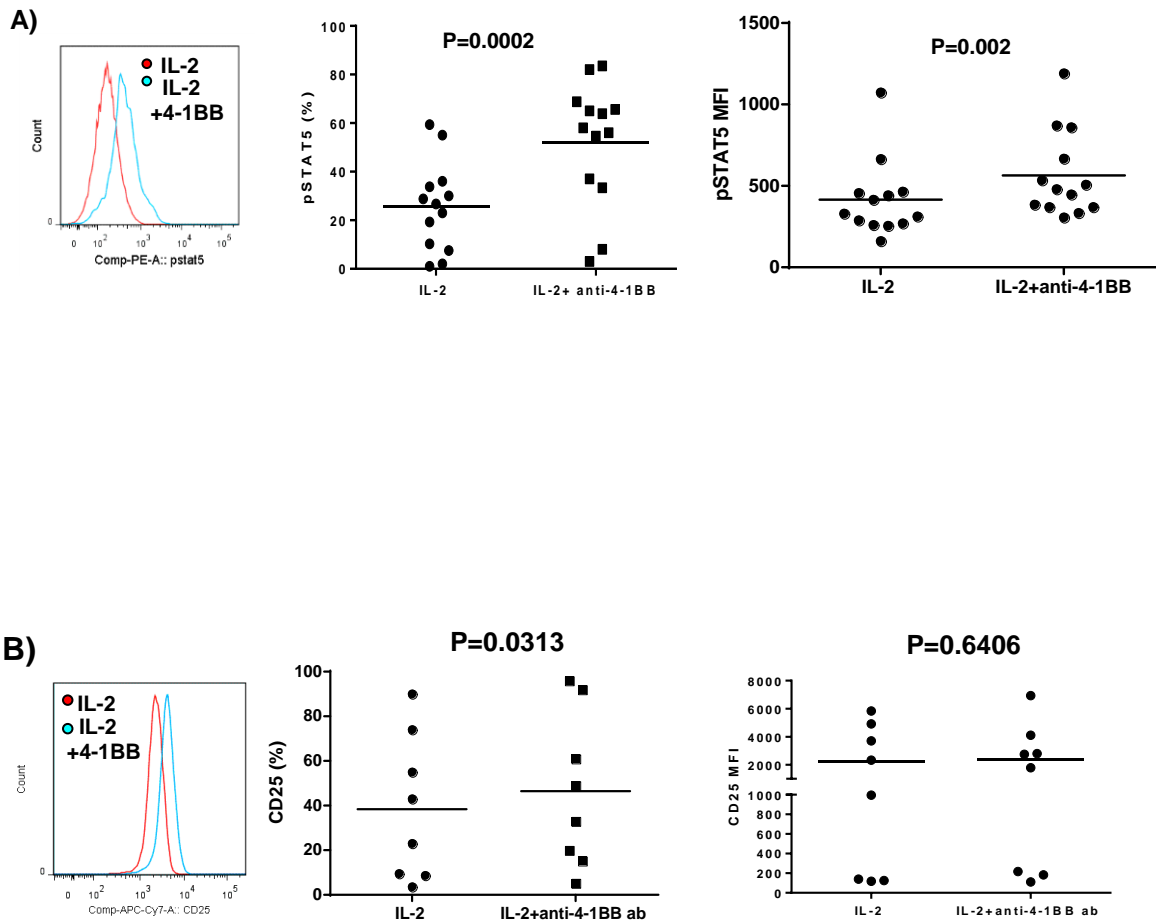
**Figure 3.14. The addition of the anti-4-1BB antibody modulates pAKT in the CD8<sup>+</sup> subset during the initial TIL expansion.**



**Figure 3.15. Activating the 4-1BB pathway during initial TIL expansion increases pSTAT5 and CD25.** The TIL were initially expanded with or without anti-4-1BB antibody for 3 weeks. After, the TIL were harvested and stained for CD3, CD8, pSTAT5 (**A**) and CD25 (**B**). The TIL expanded with anti-4-1BB antibody exhibited an increase in (**A**) STAT5 and CD25 (**B**) percentage and MFI in the CD3<sup>+</sup>CD8<sup>+</sup> subset.



**Figure 3.15. Activating the 4-1BB pathway during initial TIL expansion increases pSTAT5.**



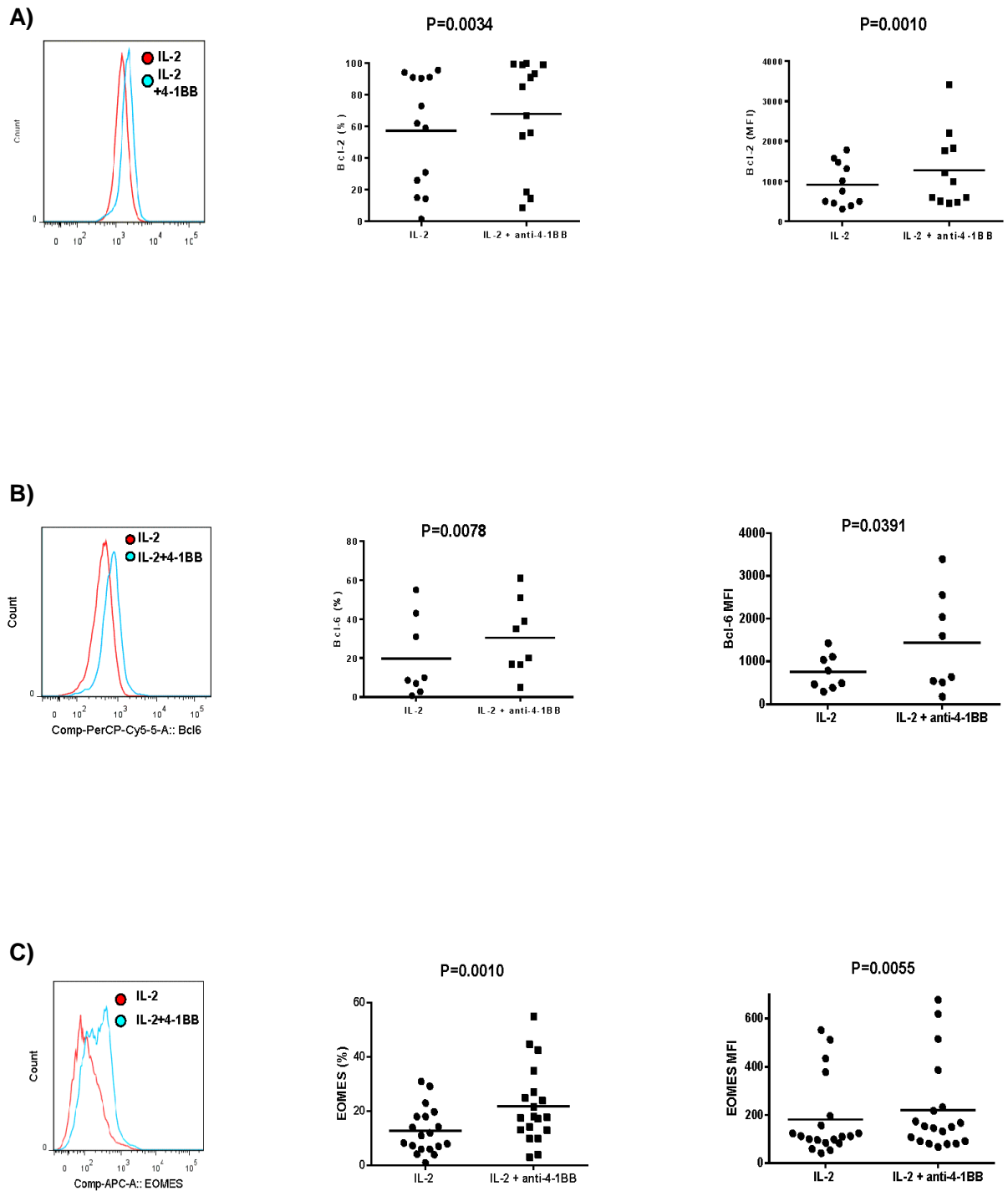
**Increased bcl-2, bcl-6 and Eomes expression in TIL initially expanded with anti-4-1BB antibody.**

Bcl-2 is an anti-apoptotic molecule that is associated with cell survival. When we measured the expression of bcl-2 in the TIL expanded with anti-4-1BB antibody, we observed an increase in the bcl-2 (**Figure 3.16A**). Since we have found that activating the 4-1BB pathway increased Eomes expression (**Figure 2.8B and Figure 3.16C**) and induce the expansion of CD8<sup>+</sup> TIL, we wanted to further investigate this. Bcl-6 and Eomes have been implicated to be major regulators in the differentiation and subsequent survival status of memory cell (*129, 130*). Bcl-6 has been determined to be crucial for the maintenance of activated CD8<sup>+</sup> cells during memory stage (*129, 130*). In addition, it has been demonstrated that Bcl-6 controls Granzyme B expression in CD8<sup>+</sup> T cells and controls the generation and maintenance of CD8<sup>+</sup> T cells (*129-131*). Specifically in B cells, STAT3 and STAT5 have been shown to regulate Bcl-6 (*132*). When we examined the level of bcl-6 after expanding the TIL with or without the anti-4-1BB antibody for 3 weeks, we found that the expression of bcl-6 was increased in the CD8<sup>+</sup> subset when we activated the 4-1BB pathway (**Figure 3.16B**).

**Figure 3.16. 4-1BB ligation induces the expression of bcl-2, bcl-6, and Eomes.**

The TIL were expanded from the melanoma fragment with IL-2±anti-4-1BB antibody for a period of 3 weeks. After, the TIL were then stained for CD3, CD8, Eomes, bcl-2 and bcl-6. We found that the TIL grown with the anti-4-1BB antibody exhibited an increased bcl-2 (**A**), bcl-6 (**B**), and Eomes (**C**) percentage and MFI in the CD3<sup>+</sup>CD8<sup>+</sup> subset compared to the TIL expanded with IL-2 alone.

**Figure 3.16. 4-1BB ligation induces the expression of bcl-2, bcl-6 and Eomes.**



## 3.2 Discussion

When we initially isolate the TIL from the melanoma fragment, there are 2 ways this can be done. The first way is to cut up the melanoma tumor into multiple fragments and place in media and IL-2 over a period of 4-5 weeks. The second way to isolate the TIL is to use enzymatic digestion. This allows the T cells to become physically separated from the tumor cells and other cells within the fragment. The TIL are placed in media with IL-2 over a period of 4 weeks. We initially isolated the TIL using both methods in parallel. Interestingly, we found that only the TIL expanded from the melanoma fragment grew better when the anti-4-1BB antibody was added. This prompted us to investigate whether the anti-4-1BB antibody was having an effect on the other cells within the melanoma fragment as 4-1BB is expressed on a number of cells (T cells, DCs, B cells, and NK cells).

We found that activating the 4-1BB pathway induced an increase in pro-inflammatory cytokines. Pro-inflammatory factors can induce the proliferation of antigen-specific cells. The antibody may also be inducing the dendritic cells to become more activated, secreting pro-inflammatory cytokines and stimulate the T cells within the melanoma fragment, inducing the T cells to migrate out of the tumor faster and proliferate better (**Figure 3.8 and 3.11**).

When 4-1BB is ligated, it signals through a TRAF complex to initiate downstream signaling pathways that result in the increase of anti-apoptotic molecules, such as bcl-2 and bcl-xL. We first investigated the modulation of the AKT pathway in the CD3<sup>+</sup> CD8<sup>+</sup> subset. After 3 weeks in culture, we harvested the TIL, stained for pAKT and found that although there appeared to be an increase in pAKT, it was not

statistically significant. This may be due to the low number of TIL lines (N), but further work needs to be done to investigate this. 4-1BB expression is modulated by IL-2R signaling through STAT5 activation and STAT5 binding sites identified in promoter region of 4-1BB (124).

Bcl-6 has been determined to be crucial for the maintenance of activated CD8<sup>+</sup> cells during memory stage (129, 130). Bcl-6 and Eomes are important regulators of the differentiation of memory cells and the subsequent survival of these cells (122, 123, 129, 130). We determined the expression of bcl-6 and Eomes after 3 weeks, we found that the TIL expanded with the anti-4-1BB antibody exhibited an increase in bcl-6 and Eomes.

Our data suggests that very early during the initial TIL isolation and expansion, the TIL need to be activated in order to proliferate better and migrate out of the tumor faster with IL-2+anti-4-1BB antibody. When the TIL are grown with IL-2 alone, there is a higher possibility that the TIL will not reach the  $50 \times 10^6$  total TIL count needed to undergo secondary expansion as compared to when the TIL are grown with IL-2 + anti-4-1BB antibody. The TIL that receive IL-2 alone may not be getting activated through other T cells or through the DCs as they are when 4-1BB ligation is occurring (**Figure 3.17**).

Our results indicate that tumor fragments placed in culture to expand TIL for adoptive cell therapy are not static tissues, but small, dynamic tumor microenvironments that can be manipulated to alter the yield and phenotype of TIL being expanded for cell therapy as well as enrich for tumor reactivity and improved memory phenotype. The use of 4-1BB co-stimulation enhancement in this system can

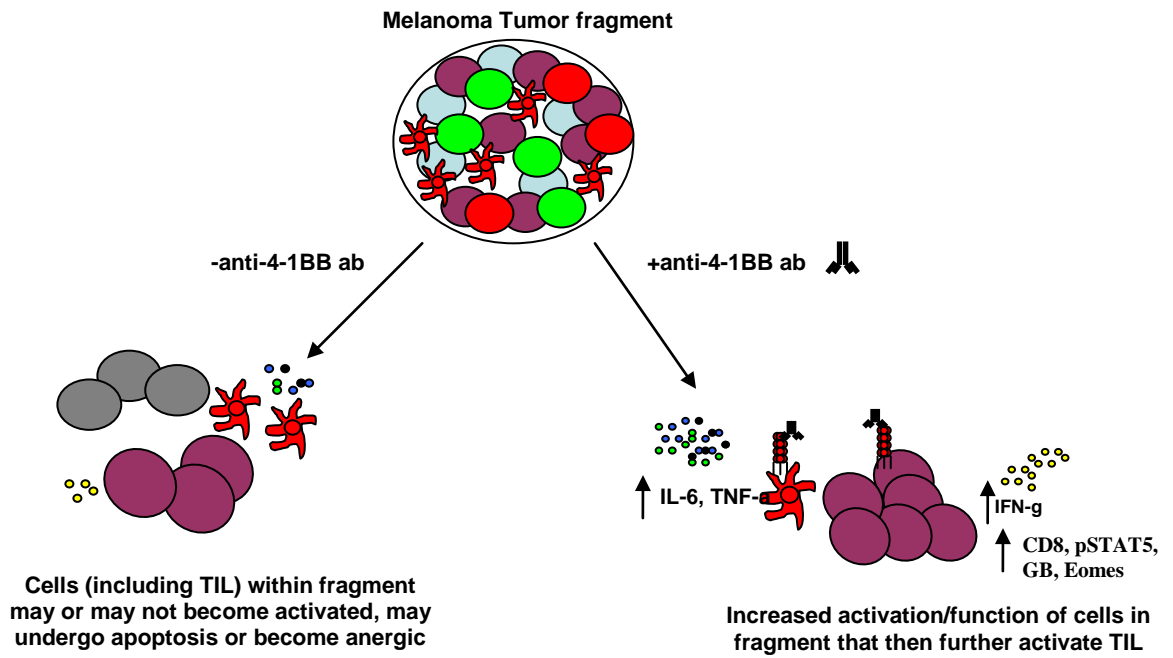
be the first of many ways to manipulate these *ex vivo* tumor microenvironments to develop protocols to expand optimally active TIL for adoptive cell therapy.

**Figure 3.17. Proposed model for 4-1BB activation during initial TIL expansion.**

We observed that the TIL initially isolated and expanded from the melanoma fragment were expanding much faster and to a greater extent when 4-1BB ligation was occurring. Since we did not observe this trend when we isolated the TIL using an enzymatic digest, we hypothesized that the environment within the fragment may be playing a role to expand the TIL when 4-1BB ligation is occurring since many cells can express 4-1BB. We observed that when the anti-4-1BB antibody was added to the cultures, the DCs had an increase in MHC-II, CD80 and CD86. 4-1BB ligation is also known to increase the cytokines secreted by DCs that may also activate T cells. We believe that 4-1BB ligation is activating the DCs and either directly activating the TIL or indirectly through cytokines, resulting in the TIL to proliferate more and migrate out of the fragment at a faster rate than when 4-1BB ligation goes not occur.



**Figure 3.17. Proposed model for 4-1BB activation during initial TIL expansion.**



## **CHAPTER 4**

### **Co-stimulation Through 4-1BB/CD137 Improves the Secondary Expansion and Function of CD8<sup>+</sup> Melanoma Tumor-infiltrating Lymphocytes for Adoptive T-cell Therapy**

## 4.1 Rationale and Hypothesis

Adoptive T-cell therapy (ACT) has emerged as the most powerful immunotherapy for stage IIIc-IV metastatic melanoma. ACT involves the use of expanded tumor-infiltrating lymphocytes (TIL). CD8<sup>+</sup> T cells have been found to be critical in mediating tumor regression during ACT. TIL expansion occurs in two phases: the first phase initially expands TIL from excised tumor pieces with IL-2 and this phase is followed by a rapid expansion protocol (REP) in which the TIL are activated with an anti-CD3 antibody in the presence of feeder cells and IL-2 and expanded to high numbers over a 2 week period. Although the REP yields billions of highly-differentiated cytolytic CD8<sup>+</sup> T cells, the post-REP TIL exhibit a loss of critical co-stimulatory molecules CD28 and CD27, are hyporesponsive to further proliferative signals and are sensitive to activation-induced cell death (AICD) (90, 133). We do not have a state of the art standard protocol for expanding the TIL during secondary expansion that will result in the optimal TIL necessary (mainly CD8<sup>+</sup> T cells) that will persist in the patient, while killing the tumor. We have previously observed that CD8<sup>+</sup> TIL that down-regulate CD28 have the capacity to up-regulate alternative co-stimulatory molecules of the TNFR family, such as 4-1BB (90, 133). We **hypothesized** that since the TIL expressed 4-1BB, the activation of the 4-1BB co-stimulatory pathway would have an effect when added at the initiation of the TIL REP. 4-1BB was activated using an agonistic anti-4-1BB antibody added during the initiation of the TIL REP with anti-CD3 and the cells expanded with IL-2 for two weeks. We found 4-1BB co-stimulation increased the CD8<sup>+</sup> T-cell recovery and significantly enhanced the anti-tumor killing activity of the TIL product. This was

associated with increased expression of CTL-related factors, such as Granzyme B, Perforin, and Eomes. However, this improved CTL phenotype was not associated with KLRG1 expression and CD28 loss. TIL receiving 4-1BB co-stimulation during the REP also had higher bcl-2 gene expression and increased resistance to apoptosis. This was associated with longer persistence of the cells *in vivo* following adoptive transfer into NOD/SCID  $\gamma c^{-/-}$  (NSG) mice. Our findings suggest that augmenting TNFR co-stimulation through 4-1BB co-stimulation during melanoma TIL expansion significantly improves the phenotype and function of tumor-reactive CD8<sup>+</sup> CTL without driving senescence and preserving certain memory T-cell properties. This approach may greatly improve TIL persistence and anti-tumor activity *in vivo* after adoptive transfer into patients

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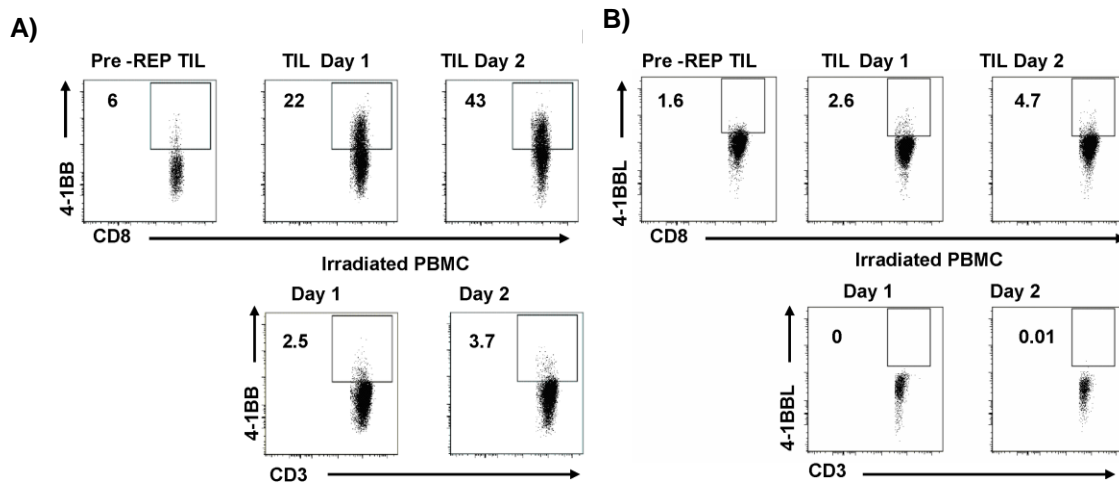
## 4.2 Results

### Induction of 4-1BB expression without induction of 4-1BB ligand after initiation of the REP

We were first interested to determine whether 4-1BB is induced on CD8<sup>+</sup> T cells early during the REP and whether the PBMC feeder cells or the TIL themselves express appreciable levels of the ligand for 4-1BB (4-1BBL) as a possible endogenous source of 4-1BB co-stimulation for the CD8<sup>+</sup> T cells. For these experiments TIL were labeled with CFSE to distinguish them from the feeder cells before being activated by anti-CD3 in the REP. 4-1BB is up-regulated on T cells 24-48 hours after activation. Thus, we analyzed CD8, 4-1BB and 4-1BBL expression on the live TIL (CFSE<sup>+</sup>) and feeder cells (CFSE<sup>-</sup>) 1-2 days after REP initiation. Live cells were gated and the CD8<sup>+</sup>CFSE<sup>+</sup> population and the live CFSE<sup>-</sup> lymphocyte population analyzed for 4-1BB and 4-1BBL expression. We found that in each case a significant frequency of the CD8<sup>+</sup> T cells in the TIL (40%-60%) had induced 4-1BB expression relative to their corresponding pre-REP cells, while little or no 4-1BB expression was found in the remaining live feeder cells (**Figure 4.1A**). However, no appreciable 4-1BBL was expressed in either sub-population (**Figure 4.1B**). Thus, CD8<sup>+</sup> TIL induce 4-1BB expression after REP initiation, but little or no 4-1BBL is expressed by either the TIL or the feeders.

**Figure 4.1. 4-1BB is expressed on CD8<sup>+</sup> TIL within the first 2 days of REP initiation.** Pre-REP TIL were stained for the expression of CD8, 4-1BB, and 4-1BBL, as shown. The REP was then set up with the TIL being labeled with CFSE prior to being added to the flask for the TIL expansion in order to be able to distinguish the TIL from excess of irradiated PBMC feeder cells added. On day 1 and day 2 of the REP, the cells were harvested from the flasks and stained for the expression of CD3, CD8, 4-1BB and 4-1BBL. For analysis of the TIL, the CFSE<sup>+</sup> viable cells were gated, and for the feeders, the CFSE<sup>-</sup> viable feeders were analyzed. We found that the TIL up-regulated 4-1BB on the CD8<sup>+</sup> subset, while the PBMC feeder cells had much less 4-1BB expression (**A**). In contrast, both the CD8<sup>+</sup> TIL and the PBMC feeders expressed only low levels of 4-1BBL (**B**). No 4-1BBL expression was detected on the CD4<sup>+</sup> TIL on day 1 or day 2, or in the pre-REP cells (data not shown).

**Figure 4.1. 4-1BB is expressed on CD8+ TIL within the first 2 days of REP initiation.**



### **Anti-4-1BB antibody increases CD8<sup>+</sup> percentage and recovery during the REP**

The results above indicate that although CD8<sup>+</sup> TIL up-regulate 4-1BB expression, there is no endogenous source of ligand activating 4-1BB co-stimulation. Thus, we tested the effects of an exogenous source of ligand by adding an agonistic anti-4-1BB mAb (BMS-663513) on the yield of CD8<sup>+</sup> T cells during the REP. In the first set of experiments we tested the effects of different concentrations of anti-4-1BB added on day 0 (at the time of REP initiation). Addition of increasing concentrations of anti-4-1BB (0-1,000 ng/ml) resulted in an increasing frequency of CD8<sup>+</sup> T cells with a decrease in the percentage of CD4<sup>+</sup> T cells, as shown in the flow cytometry dot plots of two representative TIL lines (**Figure 4.2A**). This was manifested in an increased yield of CD8<sup>+</sup> T cells at the end of the REP (**Figure 4.2B**) with a maximum frequency and yield of CD8<sup>+</sup> T cells found at 500 ng/ml of mAb. Next, we determined the optimal day of addition of anti-4-1BB to maximize the frequency and yield of CD8<sup>+</sup> T cells during the REP. Two TIL lines were tested by adding anti-4-1BB either on day 0, 1, 2, 3, or 5 of the REP. As shown in **Figure 4.3**, addition of the mAb on day 0 was optimal in both TIL lines tested. Thus, in all subsequent experiments anti-4-1BB was added at a dose of 500 ng/ml on day 0 of the REP. As a control, we also tested the effects of an agonistic anti-CD28 mAb added to the REP at this same dose in comparison to anti-4-1BB. CD28 is expressed on most pre-REP CD4<sup>+</sup> and CD8<sup>+</sup> TIL. However, as opposed to anti-4-1BB, addition of an agonistic anti-CD28 antibody did not increase the yield of CD8<sup>+</sup> T cells (**Figure 4.4**).

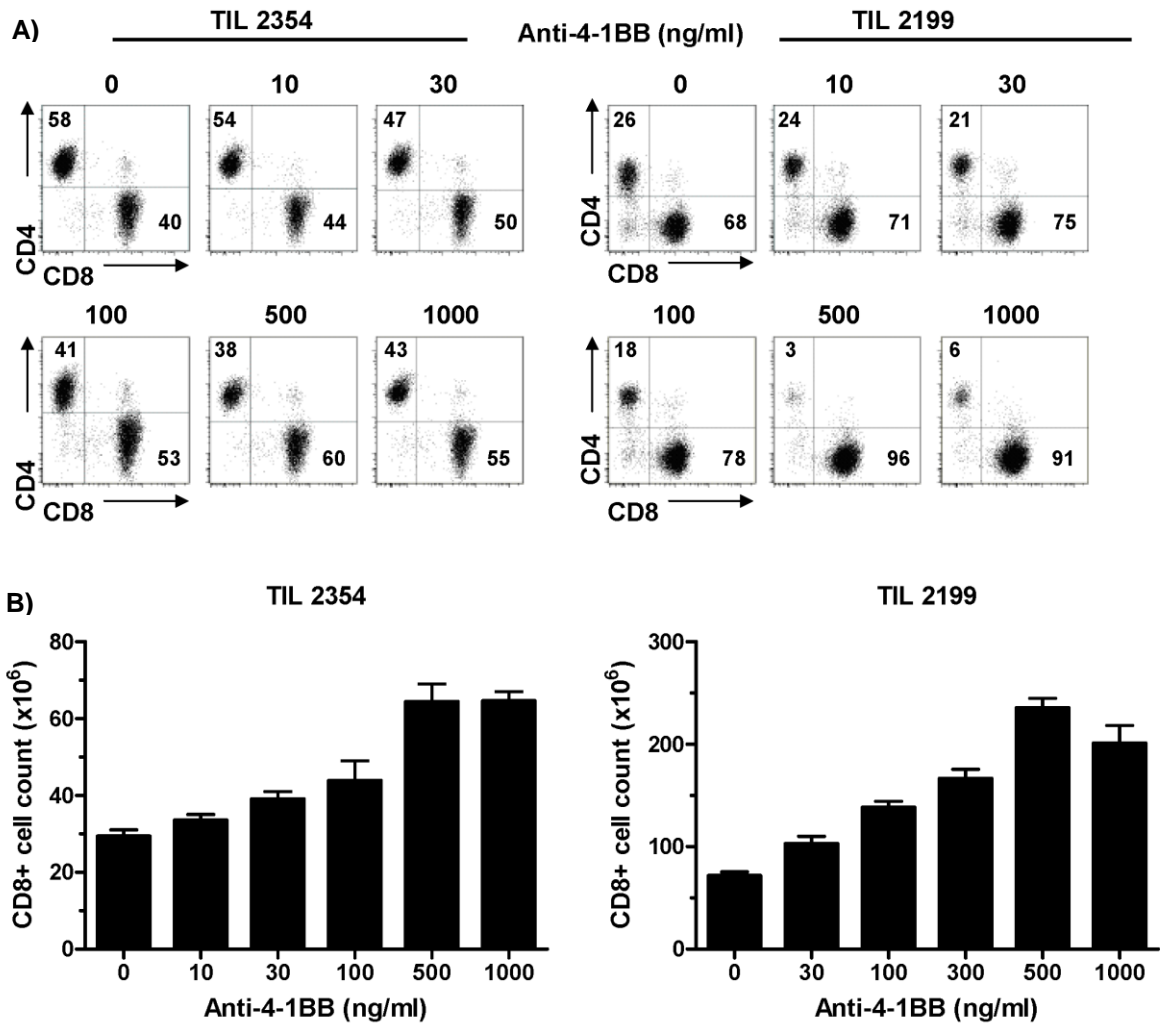
In order to determine how reproducible the effects of 4-1BB co-stimulation were, we performed experiments with TIL from 34 different patients. An equal



number of pre-REP TIL from each patient ( $0.13 \times 10^6$  cells) were activated in a REP with anti-4-1BB added on day 0 (“4-1BB REP”) or without anti-4-1BB (“Control REP”) as control. As shown in **Figure 4.5A**, the anti-4-1BB REP significantly increased the frequency of CD8<sup>+</sup> T cells recovered after the REP. Comparison of the yield and expansion of total CD8<sup>+</sup> T cells for each TIL line also found a significant increase with the addition of anti-4-1BB (**Figure 4.5B and 4.5C**). In contrast the frequency and yield of CD4<sup>+</sup> T cells exhibited an opposite trend (data not shown). The stimulation of the 4-1BB pathway, however, did not alter the total T-cell yield (**Figure 4.5B right panel**) or total TIL fold expansion (**Figure 4.5C right panel**). Thus, 4-1BB co-stimulation at the initiation of the REP reproducibly increases the frequency and yield of CD8<sup>+</sup> T cells for adoptive cell therapy.

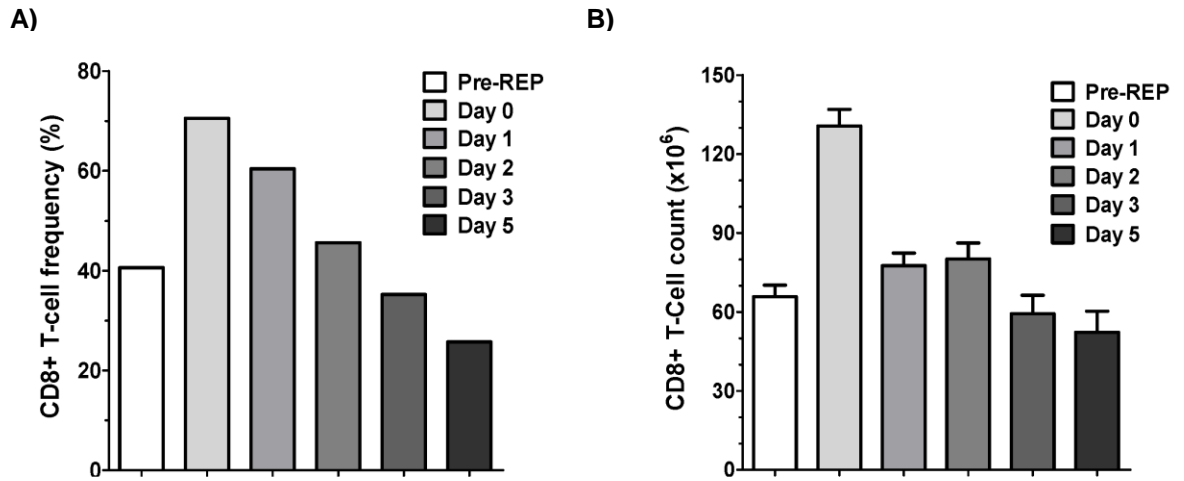
**Figure 4.2. Addition of anti-4-1BB antibody to the REP increased the percentage of CD8<sup>+</sup> TIL.** Anti-4-1BB antibody was added to the TIL on day 0 of the REP at the indicated concentrations. All other conditions were the same in each culture. After 14 days the cells were harvested and stained for CD4 and CD8 expression and viable cell counts were done using a hemocytometer following Trypan Blue staining. 4-1BB co-stimulation during the REP increased the frequency of CD8<sup>+</sup> T cells in a dose-dependent fashion in a representative TIL lines #2354 and #2199 (**A**). The total yield of CD8<sup>+</sup> T cells after the REP with different doses of anti-4-1BB is shown in two independent TIL lines (#2354 and #2199) (**B**). A dose-dependent increase in CD8<sup>+</sup> T-cell yield was noted, with 500ng/ml of anti-4-1BB antibody being optimal. The results of triplicate cell counts  $\pm$  standard deviation are shown.

**Figure 4.2. Addition of anti-4-1BB antibody to the REP increased the percentage of CD8<sup>+</sup> TIL.**



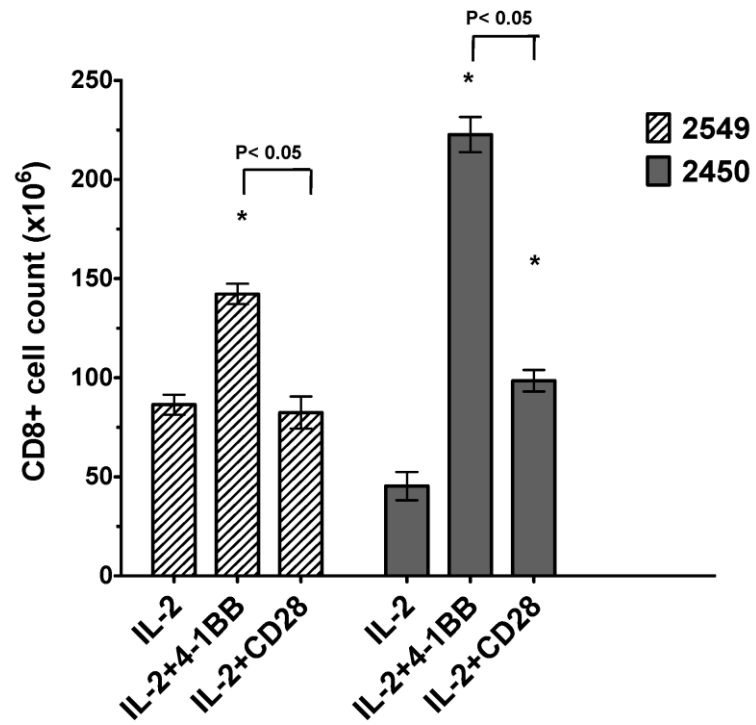
**Figure 4.3. The optimal day to add the anti-4-1BB antibody was day 0 of the REP for CD8<sup>+</sup> TIL expansion.** The TIL were subjected to the REP with or without 500 ng/ml of the anti-4-1BB antibody added on different days of the REP (Day 0, 1, 2, 3, or 5), as indicated. On day 14 of the REP, the post-REP TIL were analyzed for the expression of CD8 on the viable population by flow cytometry. The highest increase in CD8<sup>+</sup> T-cell frequency was observed when anti-4-1BB antibody was added on day 0 of the REP (**A**). Addition of anti-4-1BB on Day 0 also resulted in the highest change in the total yield of CD8<sup>+</sup> T cells after the REP (**B**). The results shown are the average of triplicate cell counts after the REP  $\pm$  standard deviation. A two-way ANOVA found that the Day 0 CD8<sup>+</sup> T-cell count was significantly higher ( $p < 0.05$ ) than in the pre-REP TIL as well as for all other time points of anti-4-1BB addition (**B**).

**Figure 4.3. The optimal day to add the anti-4-1BB antibody was day 0 of the REP for CD8<sup>+</sup> TIL expansion.**



**Figure 4.4. Comparison of the addition of agonistic anti-4-1BB and agonistic anti-CD28 to the TIL REP.** Melanoma TIL from 2 patients were subjected to the REP with or without addition of anti-4-1BB (500 ng/ml) or anti-CD28 (500 ng/ml) added during the REP initiation. Post-REP TIL were harvested, counted, and stained for the expression of CD8, CD27, and CD28. Gating was done on the viable cells. Addition of anti-4-1BB antibody increased the yield of CD8<sup>+</sup> T cells over the control (IL-2) REP significantly more than addition of anti-CD28. An average of 3 independent cell counts are shown with bars indicating standard deviation. Statistical analysis was done using a two-way ANOVA with Bonferroni post-tests. An asterisk above the bar indicates a p-value of <0.05 relative to the control (IL-2) REP. In each case anti-4-1BB induced a significant increase in CD8<sup>+</sup> T-cell yield over anti-CD28.

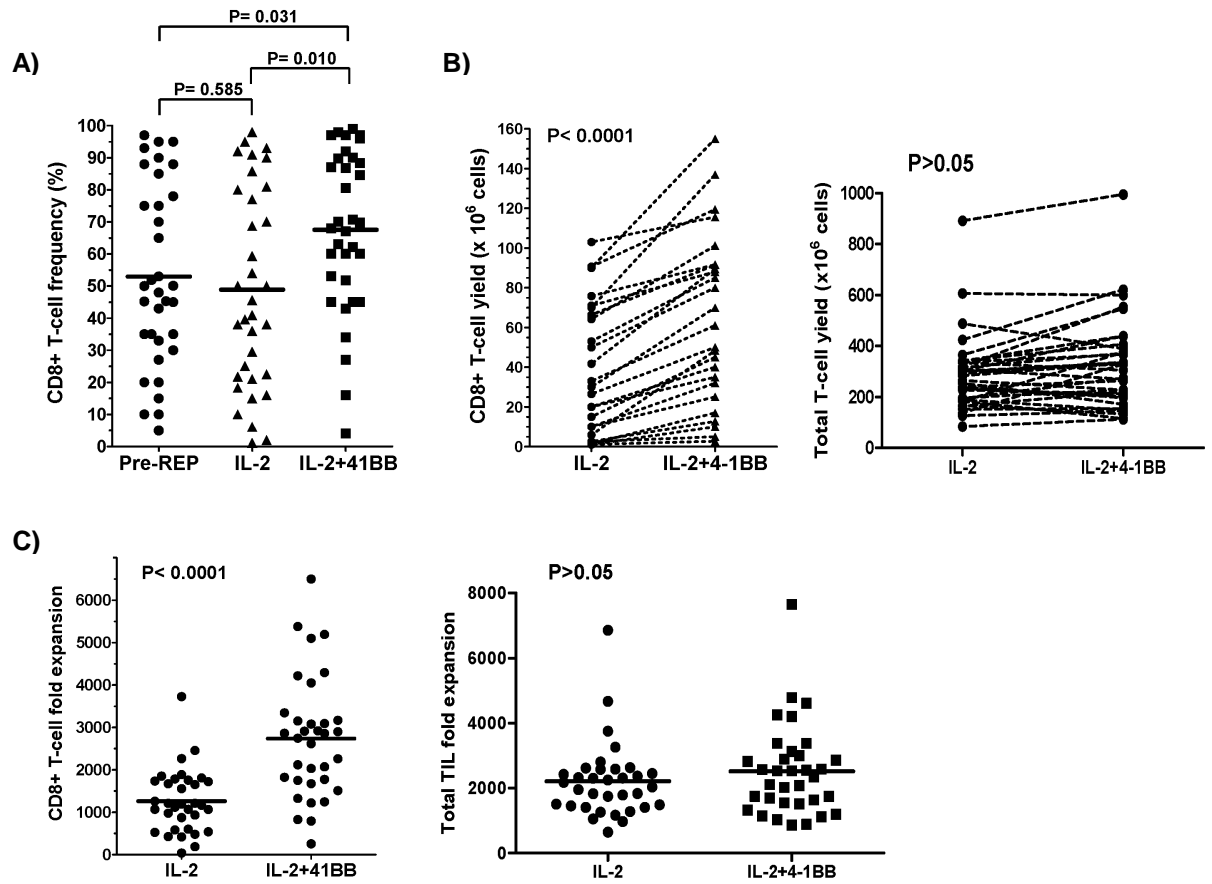
Figure 4.4. Comparison of the addition of agonistic anti-4-1BB and agonistic anti-CD28 to the TIL REP.



**Figure 4.5. Addition of 4-1BB antibody in the REP increases CD8<sup>+</sup> T-cell frequency and yield in a large cohort of patient samples (n= 34).** The effects of addition of an optimal dose of anti-4-1BB (500 ng/ml), as determined previously, were tested in rapid expansions of TIL from 34 separate patient tumors. Addition of anti-4-1BB significantly increased the frequency of CD8<sup>+</sup> T cells in the final TIL product in the patient population (**A**). CD8<sup>+</sup> T-cell yield (**B**), and the fold expansion of CD8<sup>+</sup> cells (**C**), was significantly increased when anti-4-1BB was added to the REP over the patient population. However, 4-1BB stimulation did not alter the total T-cell yield (**B**) or the total TIL fold expansion (**C**). Statistical analysis was done using the Wilcoxon signed rank test using biological relevance occurring when p< 0.05.



Figure 4.5. Addition of 4-1BB antibody in the REP increases CD8<sup>+</sup> T-cell frequency and yield in a large cohort of patient samples (n= 34).



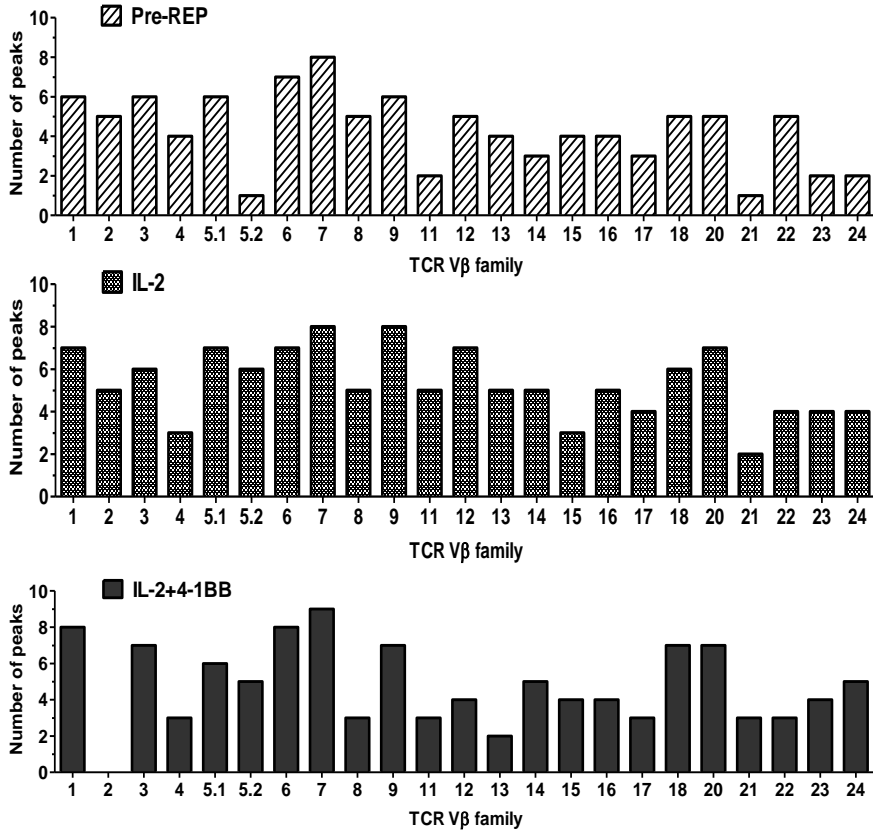
#### **4-1BB co-stimulation during the REP does not restrict the TCR V $\beta$ repertoire.**

We wanted to address the possibility that although 4-1BB co-stimulation enhances the outgrowth of CD8<sup>+</sup> T cells during the REP, it may lead to an oligoclonal expansion of certain CD8<sup>+</sup> T-cell clones restricting the CD8<sup>+</sup> T-cell repertoire after the REP. To test this, we sorted CD8<sup>+</sup> T cells after rapid expansion with or without anti-4-1BB from 2 independent patient samples and performed V $\beta$  spectratyping on isolated RNA. Analysis of the number of detected major V $\beta$  subtypes and the number of CDR3 region lengths in each represented V $\beta$  family revealed that, although some random gains and losses of V $\beta$  CDR3 peaks occurred in either situation, the TCR repertoire of the 4-1BB-costimulated post-REP CD8<sup>+</sup> T cells remained as diverse as in REPs that did not receive 4-1BB co-stimulation with no evident skewing towards any specific V $\beta$  family evident (**Figure 4.6**).

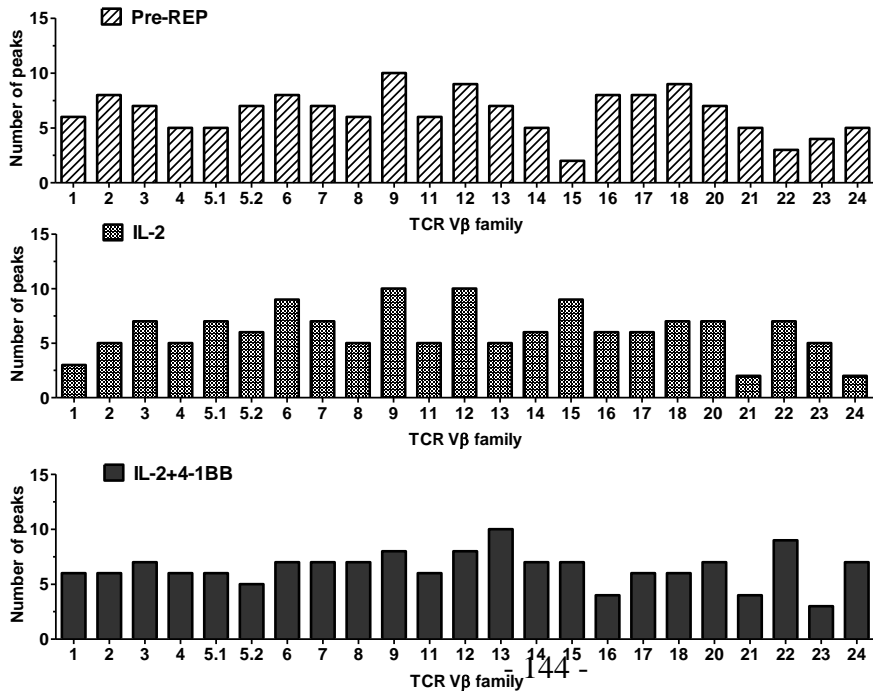
**Figure 4.6. TCR V $\beta$  repertoire is not restricted in the post-REP TIL that received 4-1BB co-stimulation.** RNA was isolated from pre-REP TIL. These TIL then underwent the REP with or without the addition of the anti-4-1BB antibody. RNA was isolated on the post-REP TIL and V $\beta$  spectratyping analysis was done on pre-REP and the post-REP TIL. In 2 representative TIL lines 2549 and 2550, we found that the TIL isolated from the IL-2 or IL-2+4-1BB REP retained a diverse TCR V $\beta$  repertoire without any increased oligoclonality.

Figure 4.6. TCR V $\beta$  repertoire is not restricted in the post-REP TIL that received 4-1BB co-stimulation.

**TIL #2549**



**TIL #2550**



#### **4-1BB co-stimulation during the REP preserves CD28 expression in CD8<sup>+</sup> T cells**

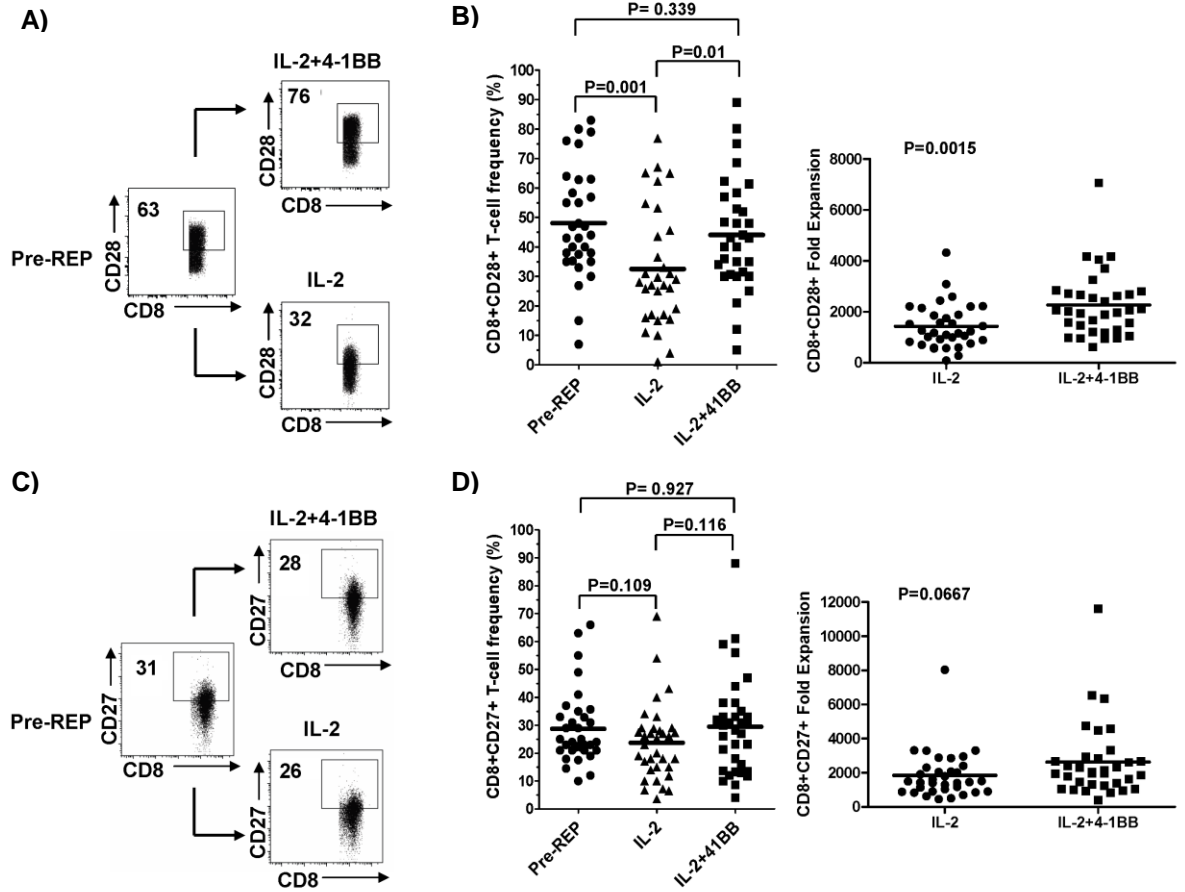
We have previously reported that many CD8<sup>+</sup> TIL down-regulate CD28 expression after the REP and that these cells became hyporesponsive to re-stimulation with melanoma antigens such as MART-1 and were more susceptible to cell death. The remaining CD8<sup>+</sup>CD28<sup>+</sup> T cells had a superior survival and responsiveness to antigenic re-stimulation. Preservation of CD28 expression was also previously shown to be associated with longer telomere length and *in vivo* persistence of transferred TIL. CD27 is another effector-memory marker that can be down-modulated. Studies at the National Cancer Institute (Bethesda, MD) have demonstrated that in melanoma patients receiving ACT, the total number of CD8<sup>+</sup>CD27<sup>+</sup> TIL administered to patients was associated with improved clinical responses. Thus, we went on to investigate the effects of the anti-4-1BB mAb in modulating both the extent of CD28 and CD27 expression.

An example of the flow cytometry analysis of one TIL line is shown in Figure 3A and 3C. A significant loss of surface CD28 expression occurred in the CD8<sup>+</sup> TIL subset during the Control REP, while in the 4-1BB REP the frequency of CD28<sup>+</sup> cells in the CD8<sup>+</sup> subset remained stable (**Figure 4.7A**). In contrast, levels of CD27 expression did not decrease during the Control REP relative to pre-REP levels and anti-4-1BB did not appreciably alter CD27 expression (**Figure 4.7C**). In order to confirm these results, we analyzed the 34 separate patient TIL lines used previously for changes in CD28 and CD27 frequency and fold expansion in the CD8<sup>+</sup> TIL subset (**Figure 4.7B and D**). Plotting the pre-REP, Control REP and 4-1BB REP found that the frequency of CD28<sup>+</sup> TIL significantly decreased from an average of around 50%

to 30% over the entire TIL sample set, a situation that was mostly reversed when anti-4-1BB was added to the REP (**Figure 4.7B**). The left hand graph in **Figure 4.7B** shows that this preservation of CD28 expression was not driven by a few lines that highly increased CD28 expression during the 4-1BB REP, but that in most individual TIL lines a loss of CD28 occurred that was regained with anti-4-1BB , as seen by the “V-shaped” pattern. As before, CD27 frequency levels and fold change over the 34 TIL lines tested was not significantly changed during either the Control or the 4-1BB REP from pre-REP levels in the CD8<sup>+</sup> TIL subset (**Figure 4.7D**).

**Figure 4.7. Addition of anti-4-1BB antibody maintained CD28 expression on CD8<sup>+</sup> T cells during the REP in a large cohort of patient samples (n= 34).** TIL from a representative patient was subjected to the REP with or without the addition of anti-4-1BB antibody and the cells stained for CD8 and CD28 (**A**) or CD8 and CD27 (**B**) and analyzed by flow cytometry both before the REP (pre-REP) and after the control (IL-2) REP or REP with anti-4-1BB (IL-2+4-1BB). Viable cells were gated and the frequency of CD28<sup>+</sup> (**A**) or CD27<sup>+</sup> (**C**) in the CD8<sup>+</sup> subset analyzed. The change in CD28 or CD27 expression in the CD8<sup>+</sup> T-cell subset during the REP with or without added anti-4-1BB was determined in 34 independent TIL lines (**B** and **D**). The left hand panels in **B** and **D** show the overall median difference in the frequency of CD28<sup>+</sup> or CD27<sup>+</sup> cells in the CD8<sup>+</sup> subset over the entire patient population. The right hand panels in **B** and **D** show the fold expansions in the CD28<sup>+</sup> or CD27<sup>+</sup> populations. Statistical analysis was done using Wilcoxon signed rank sum test using biological relevance occurring when  $p < 0.05$ .

**Figure 4.7. Addition of anti-4-1BB antibody maintained CD28 expression on CD8<sup>+</sup> T cells during the REP in a large cohort of patient samples (n= 34).**



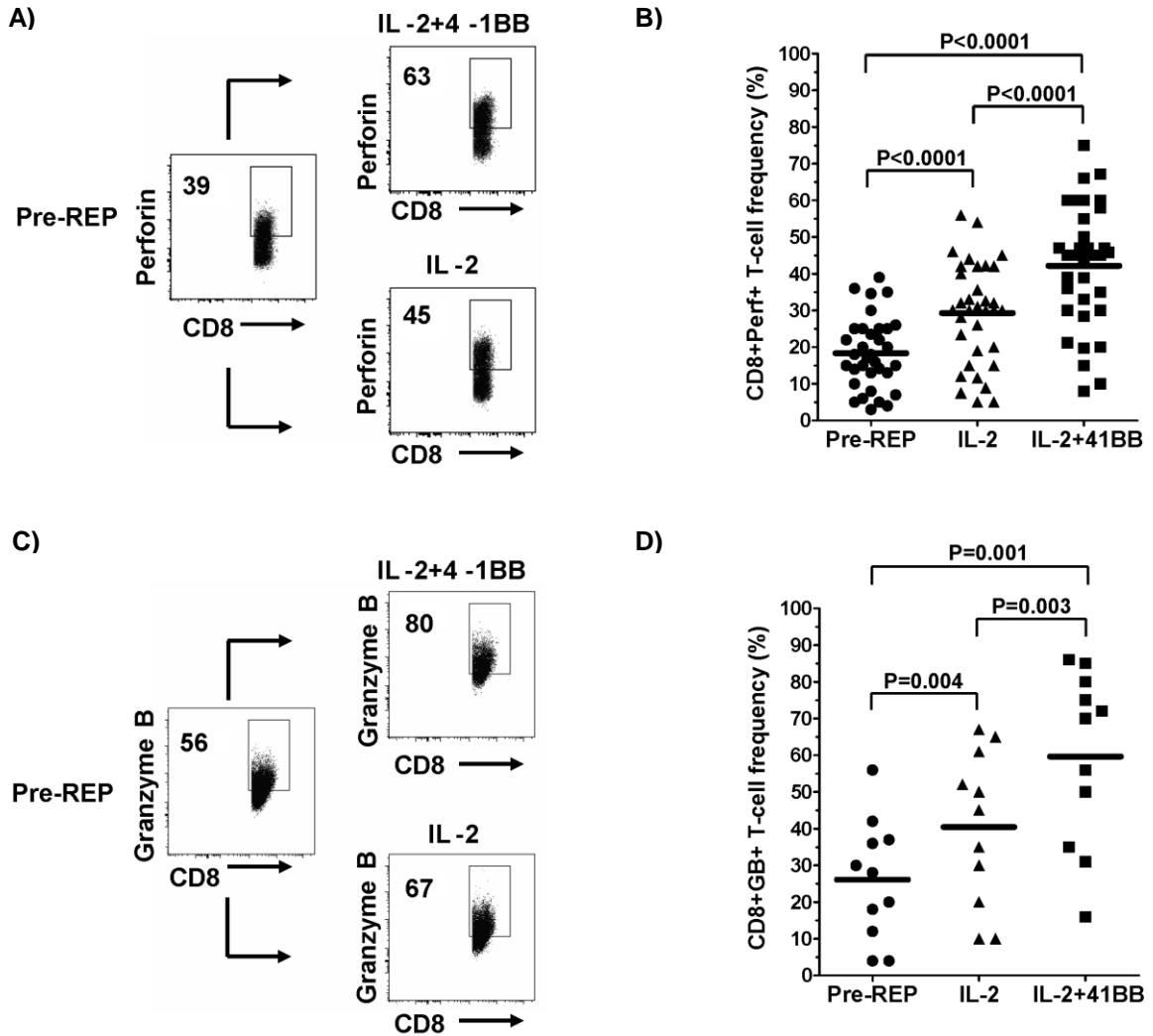


### **Addition of 4-1BB antibody to the REP increases CD8<sup>+</sup> effector phenotype.**

A number of hallmarks are used to differentiate CD8<sup>+</sup> T cells that gain effective cytotoxic T lymphocyte (CTL) or killing function. These include the gain in intracellular expression of cytolytic granule molecules, such as perforin (Perf) and granzyme B (GB). Increased expression of the T-box transcription factor eomesodermin (Eomes), helping drive Perf expression, can also be seen. In some cases, later stage or more highly differentiated effector CD8<sup>+</sup> T cells gain expression of an NK marker called killer cell lectin like receptor subfamily G member 1 (KLRG-1), a marker usually associated with senescent, end-stage CD8<sup>+</sup> T cells with low proliferative capacity. Using flow cytometry analysis as before, we analyzed changes in Perf, GB, Eomes, and KLRG-1 expression in the CD8<sup>+</sup> TIL subset. Perf changes were monitored in the 34 separate patient TIL lines, while GB was determined in a subset of 17 of these lines (**Figure 4.8B and D**). While both Perf and GB frequencies increased during the REP, addition of anti-4-1BB induced significantly higher frequency of Perf and GB CD8<sup>+</sup> T cells (**Figure 4.8A and C**). Eomesodermin and KLRG-1 expression changes were studied in 10 different TIL lines. No significant differences however were found between the Control REP and 4-1BB REP in each case, although Eomes did show a tendency to increase at variable levels in many of the TIL lines (**Figure 4.9**).

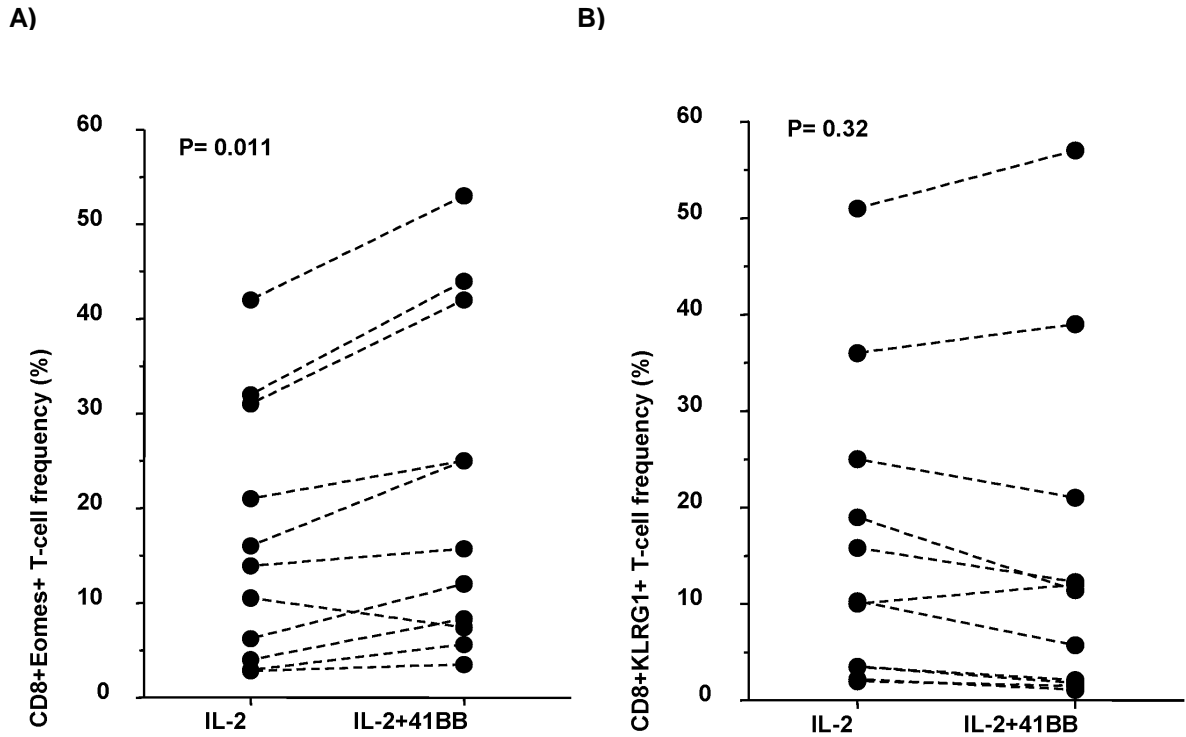
**Figure 4.8. Addition of anti-4-1BB antibody increased Granzyme B (GB) and Perforin (Perf) expression in CD8<sup>+</sup> T cells during the REP in multiple patient samples.** TIL from a representative patient was subjected to the REP with or without the addition of anti-4-1BB antibody and the cells stained for CD8 and Perf (**A**) or CD8 and GB (**B**) and analyzed by flow cytometry both before the REP (pre-REP) and after the control (IL-2) REP or REP with anti-4-1BB (IL-2+4-1BB). Viable cells were gated and the frequency of Perf<sup>+</sup> (**A**) or GB<sup>+</sup> (**C**) in the CD8<sup>+</sup> subset analyzed. The change in Perf or GB expression in the CD8<sup>+</sup> T-cell subset during the REP with or without added anti-4-1BB was determined in larger cohort of patients TIL samples (**B** and **D**). 4-1BB co-stimulation during the REP significantly increased Perf expression in CD8<sup>+</sup> T cells in 34 separate TIL line tested (**B**) and increased GB expression in 11 separate TIL lines tested (**D**). Statistical analysis was done using Wilcoxon signed rank sum test using biological relevance occurring when  $p < 0.05$ .

**Figure 4.8. Addition of anti-4-1BB antibody increased Granzyme B (GB) and Perforin (Perf) expression in CD8<sup>+</sup> T cells during the REP in multiple patient samples.**



**Figure 4.9. Increased expression of Eomes in TIL isolated after the REP with anti-4-1BB antibody, with no significant change of KLRG-1 expression.** The TIL subjected to the REP with or without the anti-4-1BB antibody were stained for CD8 and the expression of T-box transcription factor Eomes (**A**) and Killer cell lectin like receptor subfamily G member 1 (KLRG1) (**B**). 4-1BB co-stimulation during the REP led to an increase in Eomes<sup>+</sup> (**A**) in the CD8<sup>+</sup> population (n=21). However, there was no difference in expression of KLRG-1 (**B**) in the CD8<sup>+</sup> population (n=11). Statistical analysis was done using the Wilcoxon signed rank test with biological relevance occurring when  $p < 0.05$ .

**Figure 4.9. Increased expression of Eomes in TIL isolated after the REP with anti-4-1BB antibody, with no significant change of KLRG-1 expression.**



### **Analysis of cytokine secretion and anti-tumor CTL activity.**

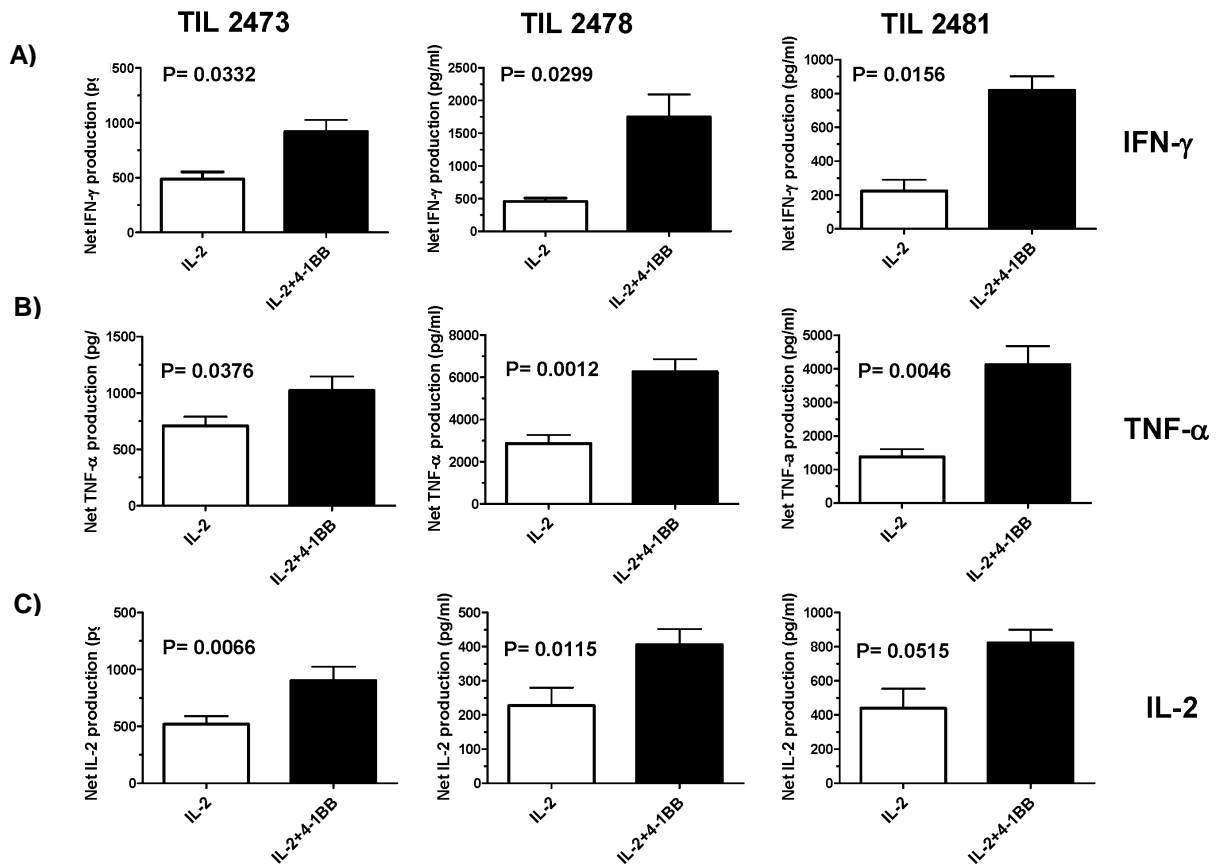
The TIL REP generates CD8<sup>+</sup> T cells with increased effector function. We determined the effector activity of the TIL by testing their ability to produce cytokines in response to TCR stimulation post-REP and their ability to kill tumor cells or targets pulsed with melanoma antigen peptides. As before, TIL from different patient pre-REP lines were rapidly expanded with or without added anti-4-1BB antibody. The post-REP TIL were then sorted by FACS to isolate the CD8<sup>+</sup> subset, washed, and rested for 5-6 h, and then assayed for cytokine production or CTL activity. As shown in **Figure 4.10**, sorted CD8<sup>+</sup> T cells from REP cultures including anti-4-1BB secreted significantly higher amounts of IFN- $\gamma$ , TNF- $\alpha$ , and IL-2 following CD3 activation. CTL activity was determined using a previously published assay measuring capsase 3 cleavage in target cells by flow cytometry. **Figure 4.11** shows the results of post-REP CTL assays on three different HLA-A0201<sup>+</sup> patient TIL lines using an HLA-A0201-matched melanoma cell line target (cell line 624) and an HLA-A-unmatched melanoma target (cell line 938), or T2 target cells pulsed with a an HLA-A0201 MART-1 peptide epitope. We found that the sorted CD8<sup>+</sup> T cells from the anti-4-1BB-treated REP cultures had higher levels of specific killing activity than cells from control REP cultures (**Figure 4.11**). Levels of non-specific killing were low in all cases (**Figure 4.11**). Staining with an HLA-A0201 MART-1 peptide HLA tetramer found that the frequency of MART-1-specific cells in was only slightly higher in the post-REP TIL from the 4-1BB costimulate expansions (**Figure 4.12**) suggesting that the enhanced killing activity (at least in the case of MART-1 antigen)

is not due to a higher frequency of melanoma antigen-specific CD8<sup>+</sup> T cells after the REP with anti-4-1BB.

**Figure 4.10. TIL isolated after rapid expansion with anti-4-1BB antibody displayed an increased ability to secrete IFN- $\gamma$ , TNF- $\alpha$ , and IL-2 after TCR re-stimulation.** Melanoma TIL rapidly expanded with or without the addition of the anti-4-1BB antibody were re-stimulated with anti-CD3 antibody in 96-well plates. Culture supernatants were collected after 24 hours and assayed using anti-cytokine beads for IFN- $\gamma$ , TNF- $\alpha$ , and IL-2 using a Luminex-100 system. Results from 3 different TIL lines comparing the control (IL-2) group with the IL-2+4-1BB group are shown for IFN- $\gamma$  (**A**) TNF- $\alpha$  (**B**), and IL-2 (**C**). In each case the net production of the cytokines was calculated by subtracting the control wells (no anti-CD3) from the wells that had anti-CD3. The averages and standard deviation of triplicate wells are shown in each case. A paired student's t-test was used to determine statistical significance between groups with  $p < 0.05$  indicating statistical significance.



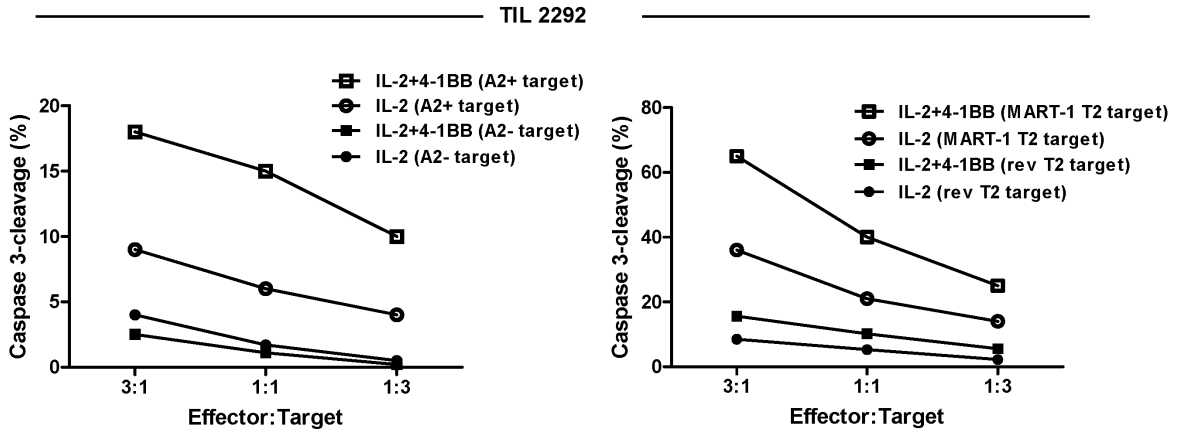
**Figure 4.10. TIL isolated after rapid expansion with anti-4-1BB antibody displayed an increased ability to secrete IFN- $\gamma$ , TNF- $\alpha$ , and IL-2 after TCR re-stimulation.**



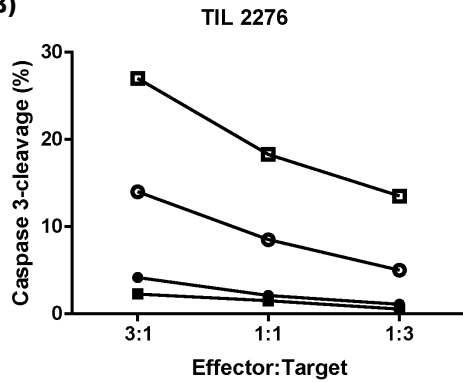
**Figure 4.11. Addition of anti-4-1BB antibody to the REP led to increased post-REP TIL tumor-specific CTL activity.** Melanoma TIL from HLA-A0201<sup>+</sup> patients with a significant population of CD8<sup>+</sup>MART-1 tetramer<sup>+</sup> cells were rapidly expanded with or without anti-4-1BB as before. The post-REP TIL were sorted by FACS for CD8<sup>+</sup> T cells and assayed for tumor antigen-specific CTL activity using a flow-cytometry-based assay that measures caspase-3 cleavage in target cells. The results of three different patient TIL lines are shown. The top panels (**A**) show the CTL activity of TIL #2292 using the melanoma cell line 624 (HLA-A0201<sup>+</sup>) and the control HLA-A-unmatched line 938 as targets (left side), or MART-1 peptide-pulsed T2 target cells as targets (right side). The bottom panels (**B**) show the CTL activity of two other HLA-A0201<sup>+</sup> TIL lines (#2276 and #2122) against 624 or 938 cells with similar results. In all cases (**A** and **B**) the levels of non-specific killing were markedly lower.

**Figure 4.11. Addition of anti-4-1BB antibody to the REP led to increased post-REP TIL tumor-specific CTL activity.**

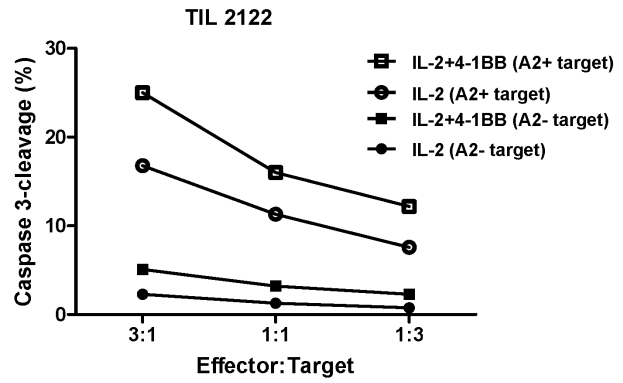
**A)**



**B)**

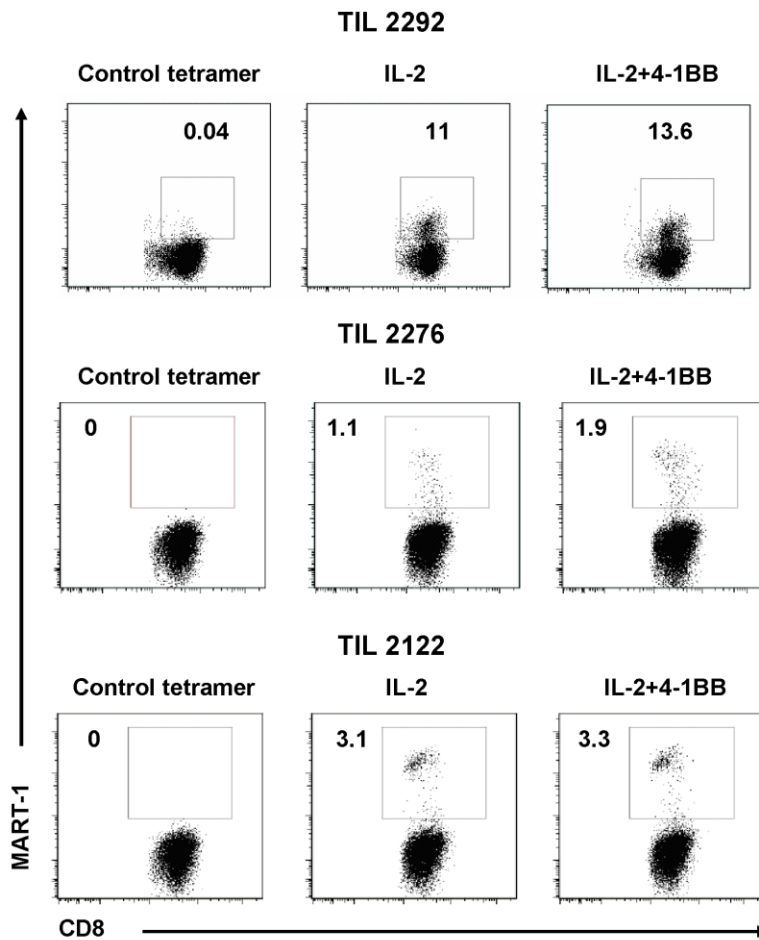


**C)**



**Figure 4.12. 4-1BB stimulation does not increase the frequency of MART-1-specific cells.** TIL were expanded with or without the anti-4-1BB antibody. Post-REP TIL were stained for CD8 and MART-1 tetramer using flow cytometry. The TIL were gated on the live population and analysis of the both types of post-REP TIL found that the percentage of CD8<sup>+</sup> MART-1-specific cells was similar in 3 representative TIL lines

**Figure 4.12. 4-1BB stimulation does not increase the frequency of MART-1-specific cells.**



**Increased bcl-2 expression and post-REP cell survival in TIL rapidly expanded with 4-1BB co-stimulation.**

4-1BB has been shown to prevent apoptosis by up-regulating anti-apoptotic molecules, such as bcl-xL and bcl-2 . We have previously shown that 4-1BB protects post-REP TIL from activation-induced cell death . Therefore, we wanted to determine whether the addition of anti-4-1BB during the REP resulted in any change in expression of the major bcl-2 family members that are anti-apoptotic (bcl-2 and bcl-xL) or pro-apoptotic (bim). We also looked at the levels of survivin, a member of the inhibitor of apoptosis family that has also been shown to be induced by TNF-R family signaling, such as OX40 . Post-REP TIL from two patients were isolated as before and subjected to real-time quantitative PCR analysis. Interestingly, bcl-2 and not bcl-xL was consistently up-regulated in TIL that received 4-1BB co-stimulation during the REP, while bim was not altered significantly (**Figure 4.13A**). We also found a significantly higher expression of the Survivin gene in the 4-1BB costimulated TIL, although this was more nominal than with bcl-2 (**Figure 4.13A**). We also confirmed the increased expression of bcl-2 using flow cytometry. We found an increased expression in the bcl-2 mean fluorescence intensity (MFI) in TIL isolated from the 4-1BB REP compared to the control REP (IL-2) (**Figure 4.13B**). We also analyzed the cell survival ability of post-REP TIL when re-cultured without cytokine or with added IL-2 for 5 days by determining the recovery of viable CD8<sup>+</sup> cells and their level of apoptosis. IL-2 therapy is given immediately after TIL adoptive transfer into patients and, thus, we were interested in how the cells respond to IL-2 following the REP. The cells were harvested and washed three times to

remove any remaining cytokine in the REP and replated. Remarkably, CD8<sup>+</sup> TIL from both patients that received 4-1BB co-stimulation during the REP exhibited a 3-4- fold increase in cell number with or without added IL-2 (200 IU/ml), while control REP CD8<sup>+</sup> TIL only further expanded with additional IL-2 and had a reduction in the number of cells when no exogenous IL-2 was provided (**Figure 4.13C**). This improved yield of TIL from 4-1BB REP cultures when post-REP cells were re-plated with or without added IL-2 was also reflected in a lower percentage of apoptotic (Annexin V<sup>+</sup>) cells (**Figure 4.13D**). Although the data from the four separate TIL lines were not statistically significant due to the different intrinsic apoptosis sensitivities of each line, there was a clear trend towards a decrease in Annexin V<sup>+</sup> cells in each case with TIL from the 4-1BB REP cultures (IL-2+4-1BB) versus control REP cultures (IL-2) when the post-REP cells were re-plated without IL-2 or with added IL-2 (**Figure 4.13D**).

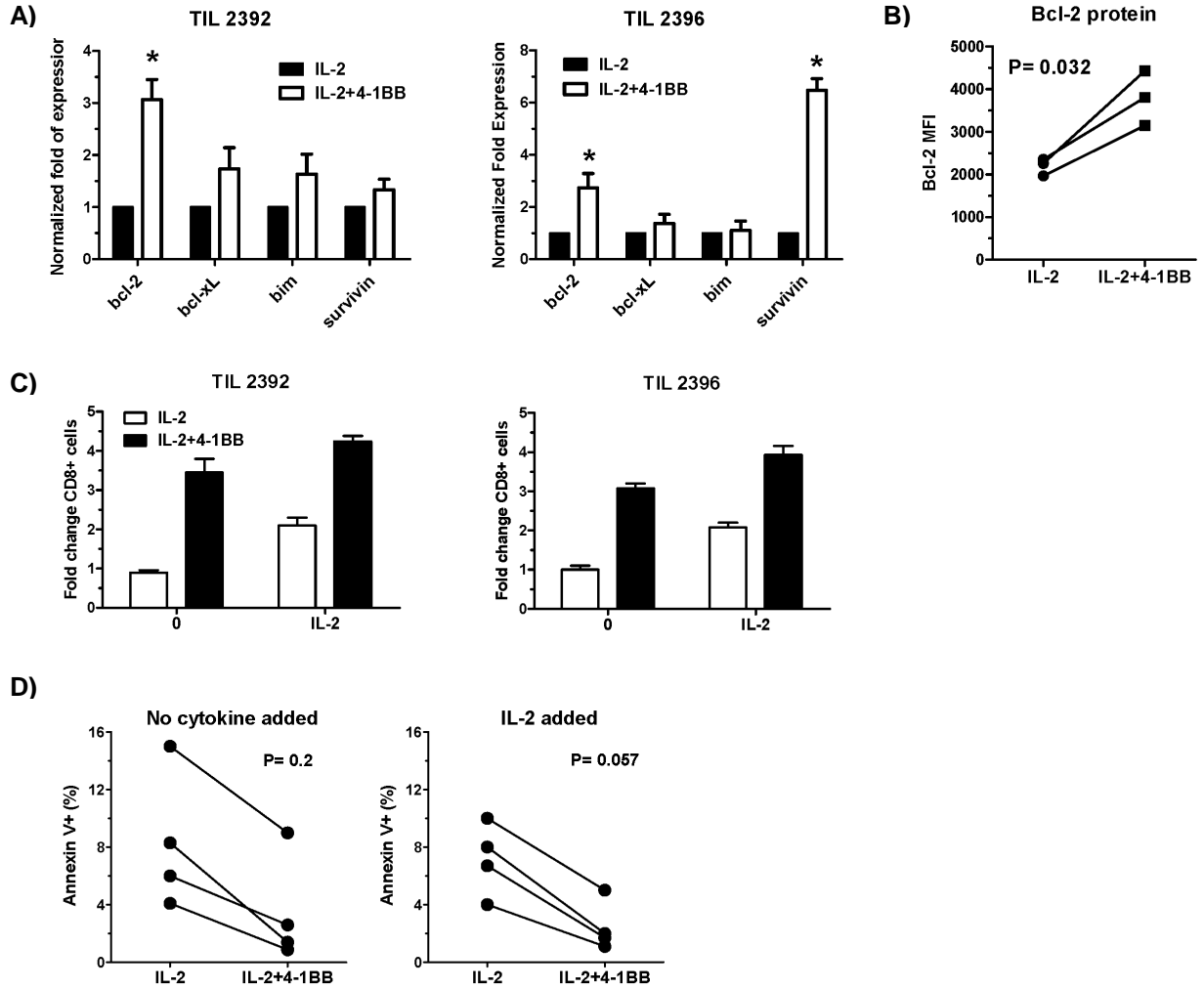
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**Figure 4.13. Increased expression of anti-apoptotic molecules and improved survival in post-REP TIL that received 4-1BB co-stimulation during the REP.**

RNA from post-REP TIL that were rapidly expanded with or without anti-4-1BB were subjected to quantitative real-time PCR (qRT-PCR) analysis for *bcl-2*, *bcl-xL*, *bim*, and *survivin* (**A**). The results of two representative patient TIL lines are shown (#2392 and #2396). Each PCR reaction was ran in triplicate with a CV of <5% for all assays. The results with the IL-2+4-1BB REP were normalized against the levels of gene expression in the control (IL-2) REP, as indicated with the 1-fold expression for the control REP for each gene. The increase in *bcl-2* expression in the IL-2+4-1BB group was confirmed in three different TIL samples by intracellular staining for *bcl-2* using flow cytometry (**B**). The post-REP TIL were also analyzed for their ability to survive and further expand when re-cultured with or without IL-2 (100 U/ml) for 5 days, as described in the Materials and Methods (**C** and **D**). Viable cell counts together with flow cytometry analysis for CD8 expression was performed to calculate the fold change in CD8<sup>+</sup> T cells after the re-culturing period (**C**). TIL from 4-1BB REP (IL-2+4-1BB) or control REP (IL-2) cultures from 4 different patients were also subjected to staining with Annexin V and 7-AAD after re-plating without or with 200 IU/ml IL-2 for 5 days and the frequency of Annexin V<sup>+</sup> 7-AAD<sup>-</sup> (apoptotic cells) were determined (**D**).



**Figure 4.13. Increased expression of anti-apoptotic molecules and improved survival in post-REP TIL that received 4-1BB co-stimulation during the REP.**

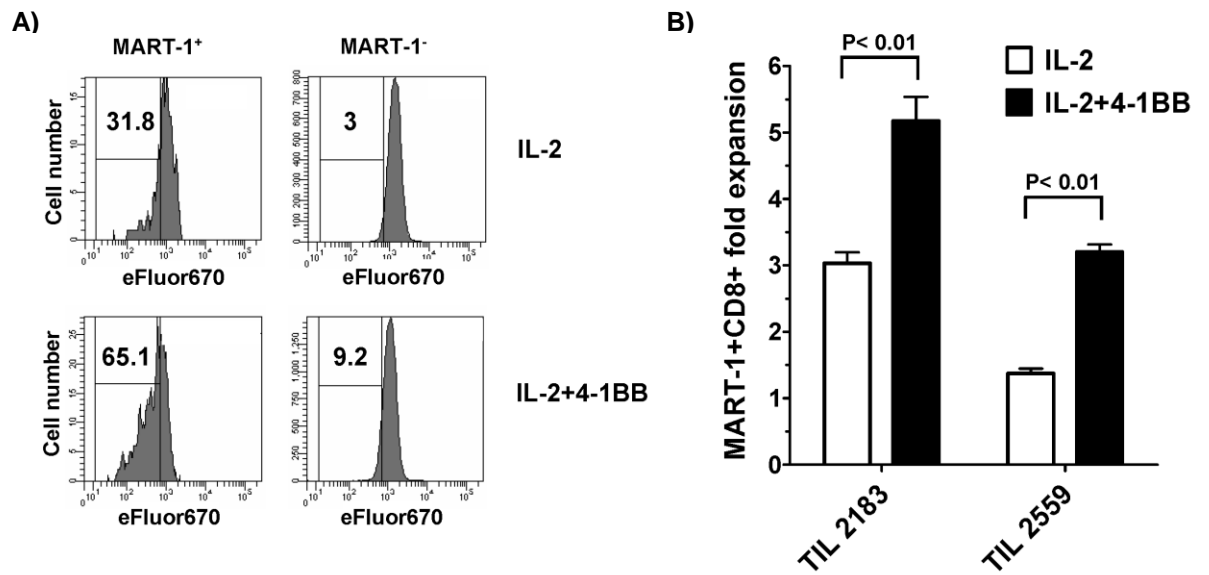


**4-1BB co-stimulation during the REP enhances responsiveness of MART-1 specific TIL to antigen re-stimulation.**

Previously, we reported that MART-1-specific CD8<sup>+</sup> T cells that lose CD28 become hypo-responsive to antigenic re-stimulation by peptide-pulsed DC following the REP. We were therefore interested in whether additional 4-1BB co-stimulation during the REP yields more CD8<sup>+</sup> T cells that may be more responsive to antigenic re-stimulation following the REP. CD8<sup>+</sup> TIL from HLA-A0201<sup>+</sup> patients that had a significant population of MART-1 peptide tetramer<sup>+</sup> CD8<sup>+</sup> T cells were rapidly expanded with or without 4-1BB co-stimulation. Similar starting numbers of CD8<sup>+</sup> MART-1 tetramer<sup>+</sup> TIL were used (data not shown). The cells were labeled with eFluor670 dye and re-stimulated with HLA-A0201<sup>+</sup> MART-1 peptide-pulsed DC, as previously described and cell counts were done using trypan blue and a hemocytometer. As shown in **Figure 4.14A**, CD8<sup>+</sup> TIL isolated from 4-1BB co-stimulated REP cultures had an enhanced response to MART-1 peptide re-stimulation, as shown by the increased number of cell divisions measured by eFluor670 dilution in the CD8<sup>+</sup> MART-1 tetramer<sup>+</sup> gated cells (**Figure 4.14A**). **Figure 4.14B** shows the results of two experiments with HLA-A0201<sup>+</sup> post-REP TIL re-stimulated with HLA-A0201<sup>+</sup> MART-1 peptide-pulsed DC. In both cases, the fold increase in gated CD8<sup>+</sup>MART-1 tetramer<sup>+</sup> cells was significantly higher in the samples that originally received anti-4-1BB in the REP (IL-2+4-1BB).

**Figure 4.14. CD8<sup>+</sup> MART-1-reactive TIL provided 4-1BB co-stimulation during the REP have a greater proliferative response to re-stimulation with MART-1 peptide.** TIL from HLA-A0201<sup>+</sup> patients were rapidly expanded with or without added anti-4-1BB antibody. The cells were labeled with eFluor670 dye and re-stimulated with HLA-A0201<sup>+</sup> MART-1 peptide-pulsed DC, as previously described . The post-REP TIL were labeled with the cell division dye eFluor670 (Invitrogen) and re-stimulated with HLA-A0201-matched mature DC pulsed with MART-1 peptide for 7 days. The cells were harvested and stained for CD8 and MART-1 tetramer and analyzed by flow cytometry for the frequency of divided (eFluor670<sup>low</sup>) in the gated CD8<sup>+</sup> MART-1 tetramer<sup>+</sup> subset. One representative experiment out of two is shown. As shown in **A**, CD8<sup>+</sup> TIL isolated from 4-1BB costimulated REP cultures (#2183) had an enhanced response to MART-1 peptide re-stimulation, as shown by the increased number of cell divisions measured by eFluor670 dilution in the CD8<sup>+</sup> MART-1 tetramer<sup>+</sup> gated cells. TIL isolated from REP cultures that received anti-4-1BB also exhibited an increase in the fold expansion of CD8<sup>+</sup> MART-1 tetramer<sup>+</sup> cells following re-stimulation of the post-REP TIL with MART-1 peptide pulsed DC as shown in experiments with two separate TIL lines (#2183 and #2559) (**B**).

**Figure 4.14. CD8<sup>+</sup> MART-1-reactive TIL provided 4-1BB co-stimulation during the REP have a greater proliferative response to re-stimulation with MART-1 peptide.**



**Determining the role of post-REP TIL expanded with or without anti-4-1BB using NSG mice.**

We next wanted to investigate the role of the TIL expanded with anti-4-1BB antibody using an *in vivo* mouse model. We chose to use the NOD scid IL2 receptor gamma chain knockout mice (NSG) mice because these mice do not have T cells, B cells, or NK cells and can be used as a host for transferred human cells without inducing rejection to the transferred cells. We expanded the TIL with or without anti-4-1BB antibody for 2 weeks. During the 2 week period, we harvested the human melanoma cell line WM35, which is an amelanotic line that is derived from radial growth phase and is an A2<sup>+</sup> cell line. We injected 2x10<sup>6</sup> WM35 cells subcutaneously into the mice. After 1 week, 25x10<sup>6</sup> post-REP TIL were injected intravenously through the tail vein of the mice. IL-2 was given to the mice through intraperitoneal injections of 20,000-25,000 IU, 2 times per day on days 0-3 to help the transferred T cells persist. We conducted 2 independent experiments using different post-Rep TIL lines. For our first experiment, we found that the post-REP TIL that were expanded with the anti-4-1BB antibody exhibited an increase in CD45<sup>+</sup> cells (leukocytes), and particularly CD3<sup>+</sup> CD8<sup>+</sup> T cells in the lung, blood, and spleen (**Figure 4.15**). The tumor size also decreased in size when the mice were injected with the post-REP TIL that were expanded with the anti-4-1BB antibody (**Figure 4.16**).

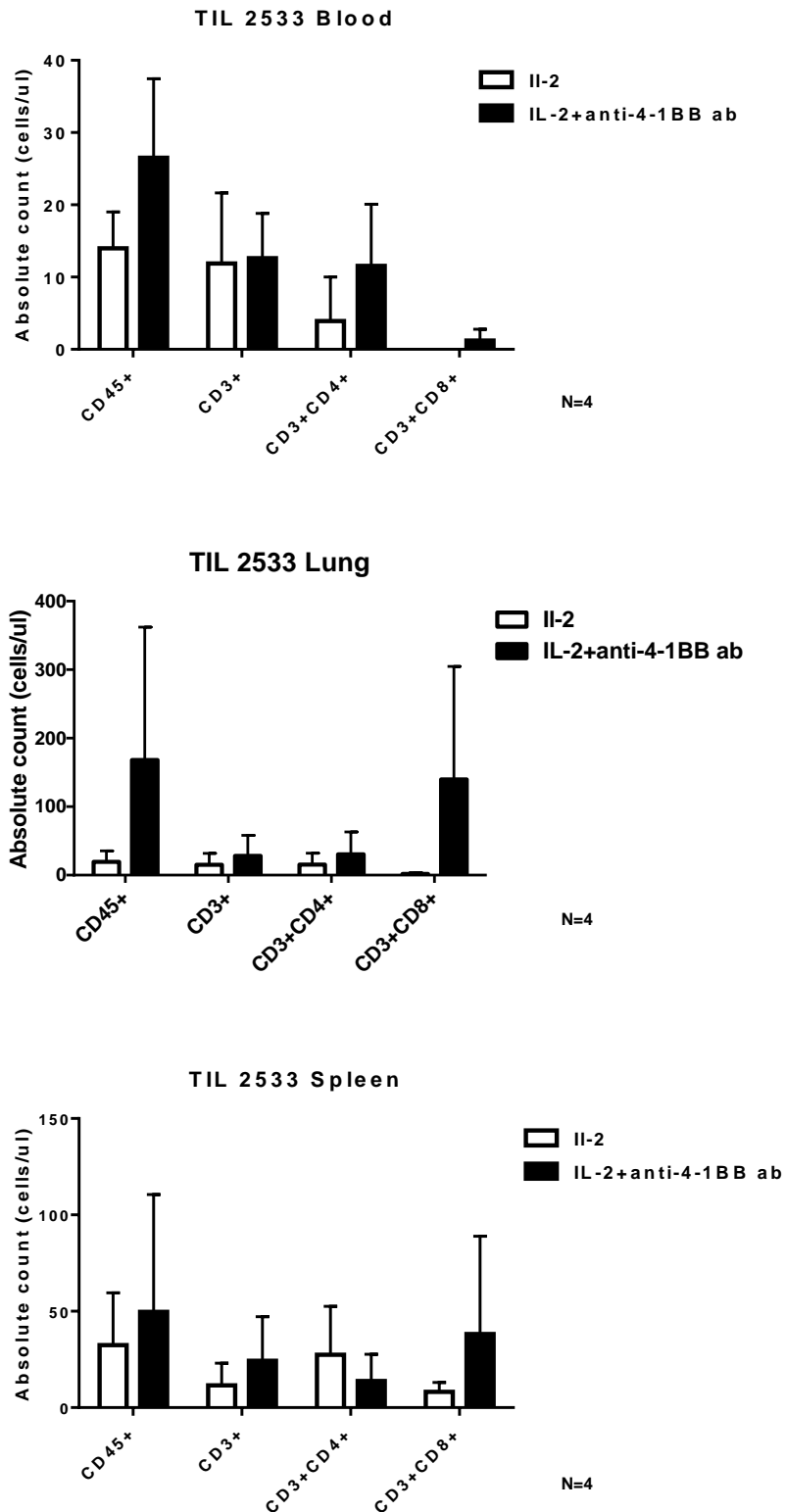
In our second mouse study, we used the same strain of mice (NSG) and melanoma tumor cell line (WM35), but a different post-REP TIL line. This time, the TIL line was 2559 as compared to TIL 2533 that was used in our first experiment. We injected the melanoma cell line subcutaneously into the mice. During this time, the

TIL were being expanded with or without the anti-4-1BB antibody. The post-REP TIL were subsequently injected I.V. into the mice and IL-2 was given. As we saw before, the post-REP TIL expanded with the anti-4-1BB antibody during the REP exhibited better persistence than the post-REP TIL expanded with IL-2 alone (**Figure 4.17**). When we measured the tumor size on different days, we observed that the mice that received the TIL expanded with anti-4-1BB antibody clearly had tumor regression, compared to the mice who received TIL expanded in IL-2 alone (**Figure 4.18**).

Our data suggests that not only are we able to expand a better TIL product with increased CD8<sup>+</sup>, with increased CTL function and phenotype, but we are also able to produce TIL that are able to persist *in vivo* using the NSG mouse model and subsequently kill the tumor.

**Figure 4.15. Persistence of TIL *in vivo* using an NSG mouse model.** Using an NSG mouse model, we injected human melanoma cell line WM35 into the mice. During this time the TIL were undergoing the REP with or without the addition of anti-4-1BB antibody. After 1 week of the melanoma cell line being injected into the mice, the post-REP TIL that were expanded with or without anti-4-1BB antibody were subsequently injected. Over a period of 7 days, any tumor change was measured using calipers. On day 7, the mice were sacrificed and the TIL persistence was measured in various organs and the blood. We found that in the blood, lung, and spleen, the post-REP, particularly the CD3<sup>+</sup> CD8<sup>+</sup> that were expanded with anti-4-1BB antibody exhibited better persistence than the post-REP expanded with IL-2 alone.

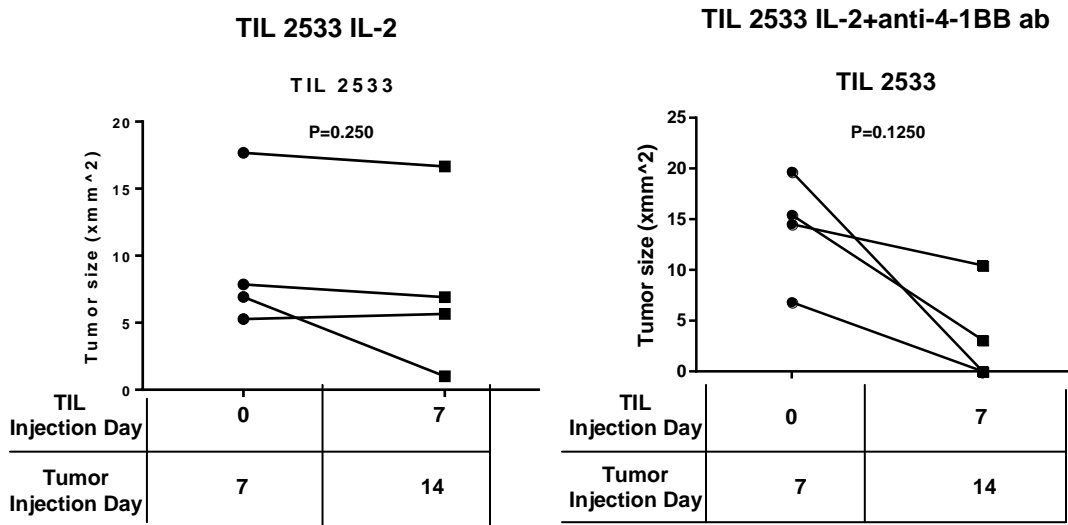
Figure 4.15. Persistence of TIL *in vivo* using an NSG mouse model.





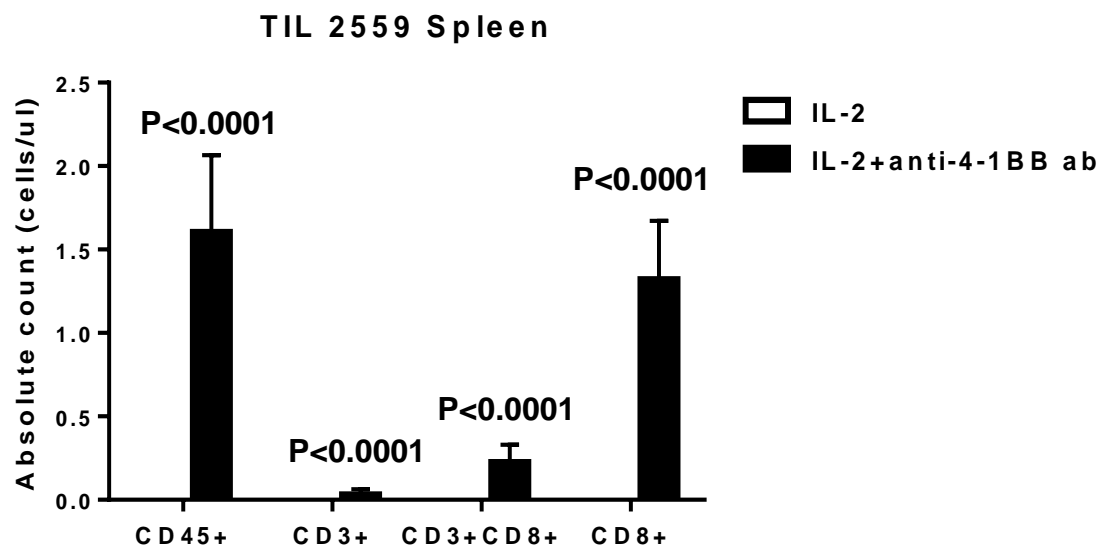
**Figure 4.16. Reduced tumor size in NSG mice when injected with TIL expanded with IL-2+anti-4-1BB antibody.** The NSG mice were injected with the human melanoma cell line WM35. After 1 week, post-REP TIL expanded with or without the anti-4-1BB antibody were injected into the tumor bearing mice. On day 7 of the TIL injection, the tumor size was measured and the mice were sacrificed to measure persistence of the TIL in different organs and in the blood. We found that the mice that received the post-REP TIL expanded with the anti-4-1BB antibody exhibited better tumor regression than the mice that were injected with our control TIL.

**Figure 4.16. Reduced tumor size in NSG mice when injected with TIL expanded with IL-2+anti-4-1BB antibody.**



**Figure 4.17. TIL expanded with IL-2+anti-4-1BB antibody persist better *in vivo* than TIL grown in IL-2 alone.** In our second *in vivo* experiment, we used the TIL line 2559. The mice were challenged with the human melanoma cell line WM35. After 1 week, the post-REP TIL 2559 that were expanded with or without the anti-4-1BB antibody were injected into the mice. The tumor burden was measured over a period of 1 week. After 1 week, the mice were sacrificed. We found that in the spleen of the mice, the post-REP TIL that were expanded with anti-4-1BB antibody were persisting better than the control TIL.

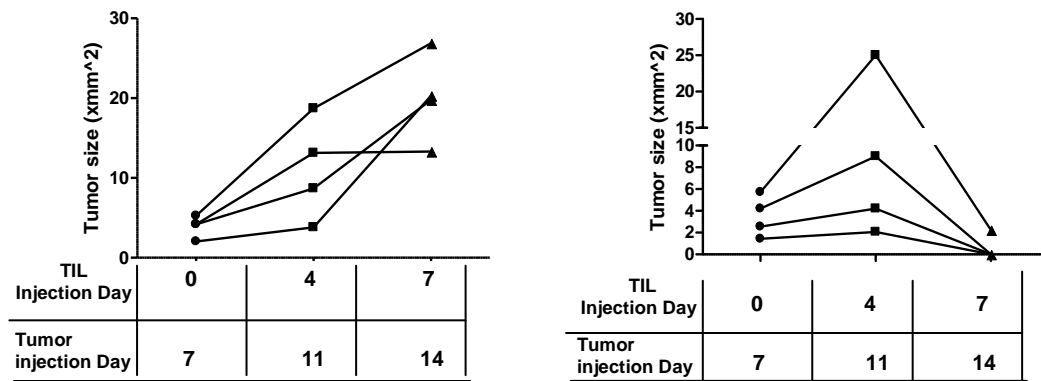
**Figure 4.17. TIL expanded with IL-2+anti-4-1BB antibody persist better *in vivo* than TIL grown in IL-2 alone.**



**N=4**

**Figure 4.18. TIL expanded with the anti-4-1BB antibody induce tumor regression.** The NSG mice were challenged with a human melanoma cell line WM35. After 1 week, post-REP TIL that were expanded with or without anti-4-1BB antibody were injected into the mice. Over 1 7 day period, the tumor size was measured using calipers. We observed that the mice that received the post-REP TIL expanded with IL-2 alone did not exhibit a decrease in tumor size over the 7 day period. However, the mice that received the post-REP TIL that were expanded with anti-4-1BB antibody exhibited tumor regression.

**Figure 4.18. TIL expanded with the anti-4-1BB antibody induce tumor regression.**



### 4.3 Discussion

Although adoptive T-cell therapy (ACT) is the most effective therapy for late stage metastatic melanoma patients that have other first and second line therapies, there are still improvements to be made to the therapy. The current therapy does not preferentially expand TIL that will be able to persist and induce a long-term tumor regression *in vivo*. The current protocol expands billions of TIL during the REP, but the final TIL product that is infused may be sub-optimal to treat all patients. We have found that the more CD8<sup>+</sup> TIL infused, the better clinical responses patients have (38). However, the post-REP TIL lose critical co-stimulatory molecules such as CD27 and CD28 (90, 133). The post-REP TIL are also susceptible to AICD and hypo-responsive to antigenic re-stimulation (90, 133). Although other co-stimulatory molecules, such as those from the TNFR super family, have been demonstrated to increase CTL activity in CD8<sup>+</sup> T cells, the role of these molecules, such as 4-1BB and OX40 have never been studied in expanding TIL from metastatic melanoma patients.

We have previously demonstrated that pre- and post-REP TIL express 4-1BB and OX40. 4-1BB was preferentially expressed in the CD8<sup>+</sup> subset, whereas OX40 was preferentially expressed in the CD4<sup>+</sup> subset (90). Therefore, we wanted to investigate what role activating the 4-1BB pathway would have during the secondary expansion of the TIL. Overall, our data demonstrates that activating the 4-1BB pathway during the REP expands the CD8<sup>+</sup> population that contain an increased cytolytic phenotype and function. In addition the TIL expanded with the anti-4-1BB

antibody exhibited an increase in the level of the anti-apoptotic molecule bcl-2 and appeared to persist more in the NSG mouse model.

We would also like to investigate the role of OX40 in expanding the TIL during the REP since it is also expressed on the TIL. Preliminary studies done in our lab suggest that using an agonistic anti-OX40 antibody preferentially expands the CD4<sup>+</sup> TIL. The specific role of CD4<sup>+</sup> TIL used for ACT for melanoma patients has yet been to be determined and needs to be further investigated.



## **CHAPTER 5: OVERALL DISCUSSION AND FUTURE DIRECTIONS**

## 5.1 Overall Discussion

Avoiding immune destruction has recently been pioneered as a hallmark of cancer (134). New approaches augmenting the immune system have recently been utilized to treat cancer. Immunotherapy uses the patient's immune system to treat their malignant disease. In the last few years, the FDA approved 2 new drugs for the treatment of melanoma (135, 136). One of the drugs, ipilumab (anti-CTLA-4) specifically modulates the immune system to treat melanoma patients (136). Anti-CTLA-4 specifically blocks the CTLA-4 molecule that inhibits T-cell activation, allowing co-stimulation through CD28 and CD80/86 to occur, inducing T-cell proliferation and survival (136). Using this approach led to an insight into using antibodies to modulate the immune system. In our study, we wanted to investigate an approach using an agonistic antibody to stimulate T-cell responses. 41BB was first discovered in the 1980s and has since been demonstrated to induce effector CD8<sup>+</sup> CTL that induce anti-viral and anti-tumor responses (87, 89, 93-95). In 2008, 4-1BB was found to be in the top 20 agents to impact cancer research (137). Since we found that 4-1BB was expressed on freshly isolated CD8<sup>+</sup> cells, we wanted to investigate whether activating the 4-1BB pathway using an agonistic anti-4-1BB antibody would affect our TIL cultures. Although using ACT with TIL has resulted in a 50% response rate for treating metastatic melanoma patients, we believe that by modifying the way we expand the TIL, we can make an impact on the overall therapy.

By activating the 41BB pathway using an agonistic anti-41BB antibody during the initial TIL isolation, we observed that the TIL were proliferating better, and expanding CD8<sup>+</sup> CTL that exhibited more Granzyme B and IFN-gamma

secretion in comparison to expanding the TIL with IL-2 alone. Activating the 4-1BB pathway increased the number of patients that would be eligible for secondary expansion in just 3 weeks compared to the 4-5 weeks that is traditional needed to initially expand the TIL.

Although our initial finding could potentially impact the amount of patients eligible for secondary expansion and decrease the culture period for the initial isolation and expansion of the TIL, we wanted to investigate the possible mechanism behind our findings. Our initial observations were found by using TIL isolated and expanded from the melanoma tumor fragment. However, another approach used to isolate TIL from the melanoma tumor uses enzymes to digest the tumor, followed by Ficoll to separate the TIL into a single layer. This approach is useful in preventing any negative suppressor cells or factors that may impact the expansion of the TIL. However, using this approach does not always result in expanding the TIL to the 50<sup>6</sup> needed to undergo the secondary expansion and we have found to have better success in expanding TIL by setting up multiple fragments from the melanoma tumor instead. When we set up parallel experiments using the enzymatic digest alongside the fragment set up, we found that activating the 4-1BB pathway expanded the TIL more than the traditional IL-2 when we expanded the TIL from the melanoma fragments. We found no difference when we expanded the TIL from the enzymatic digest with or without 4-1BB ligation. This prompted us to investigate the possible mechanism behind this observation.

As mentioned earlier, the major difference between using the enzymatic digestion method and the fragment set up is that enzymatic digestion method

physically separates the TIL from the rest of the cells within the tumor, compared to the fragment set up that allows the TIL to be in contact with other cells within the microenvironment. We hypothesized that 4-1BB ligation during the initial TIL isolation from the melanoma fragment activates not only the T cells, but other cells within the microenvironment. 4-1BB is expressed on a number of cells, such as T cells, NK cells, B cells, and dendritic cells. When we investigated if activating the 4-1BB pathway augmented the other cells in the microenvironment, we found that 4-1BB was indeed expressed on the dendritic cells within the melanoma fragment and activating the 4-1BB pathway increased activation markers on the dendritic cells. The dendritic cells also exhibited increased secretion and expression of IL-6 and TNF-alpha. We hypothesized that the T cells within the fragment were becoming activated either directly through the TCR or indirectly by cytokines secreted by other cells (dendritic cells). When we blocked the MHC class I, we found a significant decrease in expansion of CD8<sup>+</sup> T cells that migrated out of the melanoma fragment compared to when we did not block the MHC class I. Our findings indicated that the addition of the anti-4-1BB antibody is activating the dendritic cells and the T cells, resulting in the T cells becoming more activated, proliferating more and migrating out of the tumor at a faster rate. Providing dendritic cells co-stimulation using an anti-4-1BB antibody has resulted in inducing their survival through increased pro-survival molecules, such as bcl-2 and bcl-xL. There are a number of factors to consider about the activation of the dendritic cells by the 4-1BB ligation. One point to consider is that the level of initial activation differs from dendritic cell to dendritic cell and from area to area of the tumor fragment. We cut the melanoma tumor into multiple

fragments, but each fragment can be independent of the other fragment, depending on the cells within the microenvironment. The levels of surface expression of 4-1BB and subsequent activation from the 4-1BB ligation may differ, depending what cells are within the environment. Nevertheless, we have found that the dendritic cells within the tumor microenvironment do express different levels of 4-1BB and become more activated with 4-1BB ligation.

When we added the anti-4-1BB antibody to the TIL undergoing the secondary expansion (REP), we preferentially expanded the CD8<sup>+</sup> TIL that demonstrated an enhanced effector phenotype and function. In addition, when we injected the post-REP TIL into an NSG mouse, we found that the TIL expanded with the anti-4-1BB antibody persisted longer and induced an anti-tumor effect. This data shows promise of using the anti-4-1BB antibody to expand CD8<sup>+</sup> TIL during the REP. We have previously demonstrated that the more CD8<sup>+</sup> TIL infused in the patient, the better clinical response the patients undergo. However, the current REP protocol does not utilize methods to preferentially expand more CD8<sup>+</sup> than CD4<sup>+</sup> TIL and most of the time, the CD4<sup>+</sup> TIL outgrow the CD8<sup>+</sup> population during the REP.

Overall, we believe that utilizing an agonistic anti-4-1BB antibody to provide co-stimulation to the TIL during the different stages of the ACT can provide a better TIL product (**Figure 5.1**). We can add the anti-4-1BB antibody to the fragments during the initial TIL isolation and expansion. We have demonstrated that by doing this, we not only activate the TIL, but also the dendritic cells that are in the microenvironment that could then directly (through TCR) or indirectly (cytokines)

activate the TIL. Subsequently, the 4-1BB activation will induce the CD8<sup>+</sup> TIL to proliferate and migrate out of the fragment and induce translocation of NFκB.

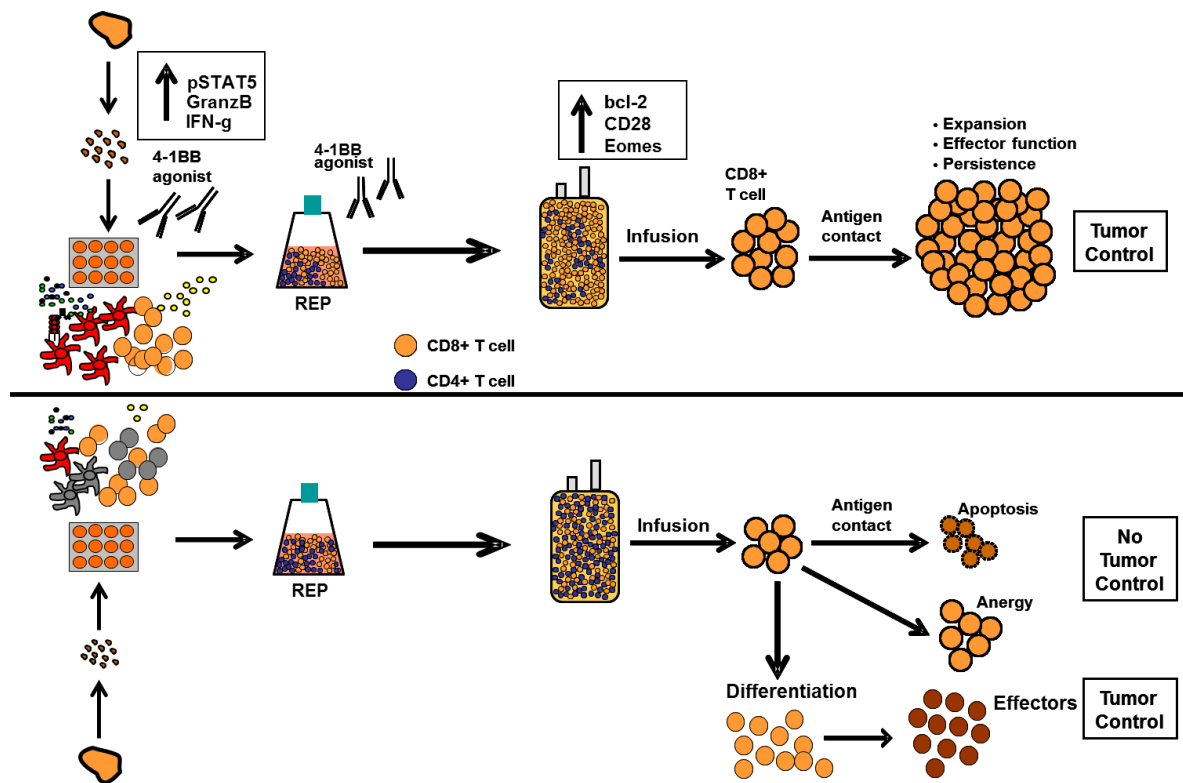
During the REP, as mentioned previously, the CD4<sup>+</sup> TIL can outgrow the CD8<sup>+</sup> TIL. We would like to infuse mainly CD8<sup>+</sup> TIL since this is the population that has been shown to induce clinical responses in melanoma patients undergoing ACT using TIL. When we added the anti-4-1BB antibody during the secondary expansion phase we found that the 4-1BB ligation increased the expansion of the CD8<sup>+</sup> TIL, increased the effector phenotype and function, and increased the anti-apoptotic molecule bcl-2. 4-1BB activation during the TIL expansion also maintained the expression of a critical co-stimulatory molecule CD28, that is normally down-regulated after the REP. We previously found that CD28<sup>-</sup> post-REP TIL are hypo-responsive to antigenic stimulation and susceptible to AICD (90, 133).

We believe these qualities (improved effector phenotype, function, and maintenance of CD28 expression, and increased anti-apoptotic molecule bcl-2, would produce a TIL product that is able to persist *in vivo* and maintain tumor control (Figure 5.1).

**Figure 5.1. Proposed model for the role of 4-1BB co-stimulation during TIL expansion and TIL therapy *in vivo*.** The schematic shows the different stage of TIL expansion through therapy starting from the initial expansion from tumor fragments (pre-REP stage) followed by the REP (rapid expansion) stage generating the final infusion product. Past expansion protocols have not provided enough co-stimulation to CD8<sup>+</sup> TIL, especially through 4-1BB, critical at this stage of effector-memory differentiation. We have found that providing 4-1BB co-stimulation during the REP improves the expansion of tumor-reactive CD8<sup>+</sup> T cells that maintain CD28 expression and up-regulate cell survival molecules that are more responsive to antigenic re-stimulation. These cells would have an improved capacity to expand and persist and control tumor *in vivo* after transfer. In contrast, the traditional expansion approach can lead to the overgrowth of CD4<sup>+</sup> T-cells or the expansion of CD8<sup>+</sup> cells that lose CD28, are low in bcl-2 and other survival and memory markers, and are hypo-responsive to antigenic re-stimulation. These cells are postulated to have poorer persistence with many of them undergoing apoptosis or anergy *in vivo*. We also propose that 4-1BB agonists at low dose may provide a further boost to the infused TIL, perhaps even rescuing cells that would have been otherwise deleted after antigen contact *in vivo* due to lack of adequate co-stimulation.

**Reprinted with permission from: Chacon JA, Pilon-Thomas S, Sarnaik AA, Radvanyi LG. Oncoimmunology. 2013 Sep 1;2(9):e25581.**

**Figure 5.1. Proposed model for the role of 4-1BB co-stimulation during TIL expansion and TIL therapy *in vivo*.**





## 5.2 Future Directions

Although we have demonstrated one way in which the TIL can become activated within the tumor microenvironment, further studies need to be carried out in understanding the role of negative cells and factors that could cause the TIL to undergo apoptosis and not migrate out of the tumor fragment. We did not investigate the role of the melanoma tumor cells, but they could play a key role in suppressing TIL proliferation. Other negative cells and factors within the tumor microenvironment could be T regulatory cells, Myeloid Derived Suppressor Cells, Transcription growth factor- $\beta$  (TGF-  $\beta$ ), and PD-1, PD-L1. These cells and factors could potentially decrease the TIL from migrating out of the tumor and proliferating. Investigating ways to overcome these negative barriers within the tumor microenvironment would allow researchers to find new approaches to isolate and expand the TIL during the initial phase of ACT. By targeting negative factors within the tumor fragment, this could potentially lead to expanding TIL from more patients and an increase in the patients eligible to undergo the secondary expansion phase.

In addition, our initial observation using anti-CTLA-4 to initially expand the TIL was that anti-CTLA-4 preferentially expanded the CD4<sup>+</sup> TIL. However, studies have suggested that the combination of anti-CTLA-4 and anti-4-1BB mediate the rejection of B16 melanoma tumors in a mouse model (138). Combining anti-4-1BB with other co-stimulatory molecules, such as OX40 could have potential in expanding the TIL during the initial TIL isolation and/or during the secondary expansion. We have previously observed that using an agonistic anti-OX40 antibody (Dr. Andrew Weinberg lab) to expand the TIL during the REP expanding the CD4<sup>+</sup> TIL, compared

to the anti-4-1BB antibody that expanded a CD8<sup>+</sup> TIL population. Although the specific role of CD4<sup>+</sup> TIL has not been thoroughly investigated, there are a number of antigens that contain HLA class II-restricted epitopes, such as tyrosinase, NY-ESO-1 and MAGE-1 (48, 60, 66). Clinical trials have been conducted and have shown promising results using antigen-specific CD4<sup>+</sup> cells to treat metastatic melanoma patients (48, 60, 66). These trials demonstrate that CD4<sup>+</sup> T cells do play a role for the treatment of melanoma. The exact role of CD4<sup>+</sup> TIL needs to be elucidated and thoroughly investigated. There could be a number of different subsets of CD4<sup>+</sup> T cells (Th1, Th2, and Th17) within the TIL population (67, 139). These different subsets could play key roles in ACT and secrete different cytokines that could modulate a tumor response.

The standard protocol to expand TIL used during ACT is to use the cytokine IL-2. However, IL-2 can drive the TIL towards differentiation (76). Further studies should investigate whether using lower doses of IL-2 with or without addition of anti-4-1BB antibody can induce a similar effector phenotype and function, without decreasing the levels of CD27 in the CD8<sup>+</sup> subset during initial TIL isolation. Studies have suggested that resting the TIL after expansion can induce an increase in CD27 levels (121, 140) but future work needs to be done to investigate whether this occurs in our cultures after 4-1BB activation. IL-15 is another member of the common gamma chain family. IL-15 has also been shown to induce 4-1BB on CD44<sup>high</sup> CD8<sup>+</sup> memory cells (141).

During the secondary expansion phase of the TIL (REP), irradiated PBMCs from 6 normal donors act as 'feeder cells.' The feeder cells secrete cytokines and are

a mix of different cells within the population of normal donor PBMCs. However, sometimes we encounter technical issues with the feeder cells. Although we pool 6 normal donor PBMCs, and irradiate them prior to culturing these cells with the TIL, the TIL do not always expand. The exact mechanism of what exactly the feeder cells are doing to activate the TIL is still not clear. We presume that the anti-CD3 crosslinks with the Fc portions on the feeder cells and this activates the TIL. However, we do not know for sure if this is occurring. The feeder cells may also be a source for some growth factors, anti-oxidants, and co-stimulatory factors for TIL expansion; although we found that the feeder cells had very little expression of 4-1BB and 4-1BBL (90). We did not dissect the different subsets of cells that are within the feeder population. In addition, this may be difficult to do since we pool 6 normal donor PBMCs to set up the REP. We pool 6 normal donor PBMCs in order to have a large heterogeneous population of feeder cells. It would be interesting to determine what subsets within the PBMC population and/or cytokines initiate the outgrowth of the TIL. In addition, there may be some suppressor factors in the PBMCs that may induce poor expansion of the TIL. This needs to be further investigated.

Another factor that needs further investigation is the persistence of the TIL *in vivo*. Our preliminary data used an NSG mouse model to investigate the persistence and anti-tumor activity of the TIL expanded with or without the anti-4-1BB antibody. Our data demonstrated that the TIL expanded with the anti-4-1BB antibody exhibited better persistence and anti-tumor activity. However, the differentiation stage of T cells may influence where they traffic *in vivo*. Naïve and central memory T cells traffic primarily to the secondary lymphoid structures spleen and lymph nodes

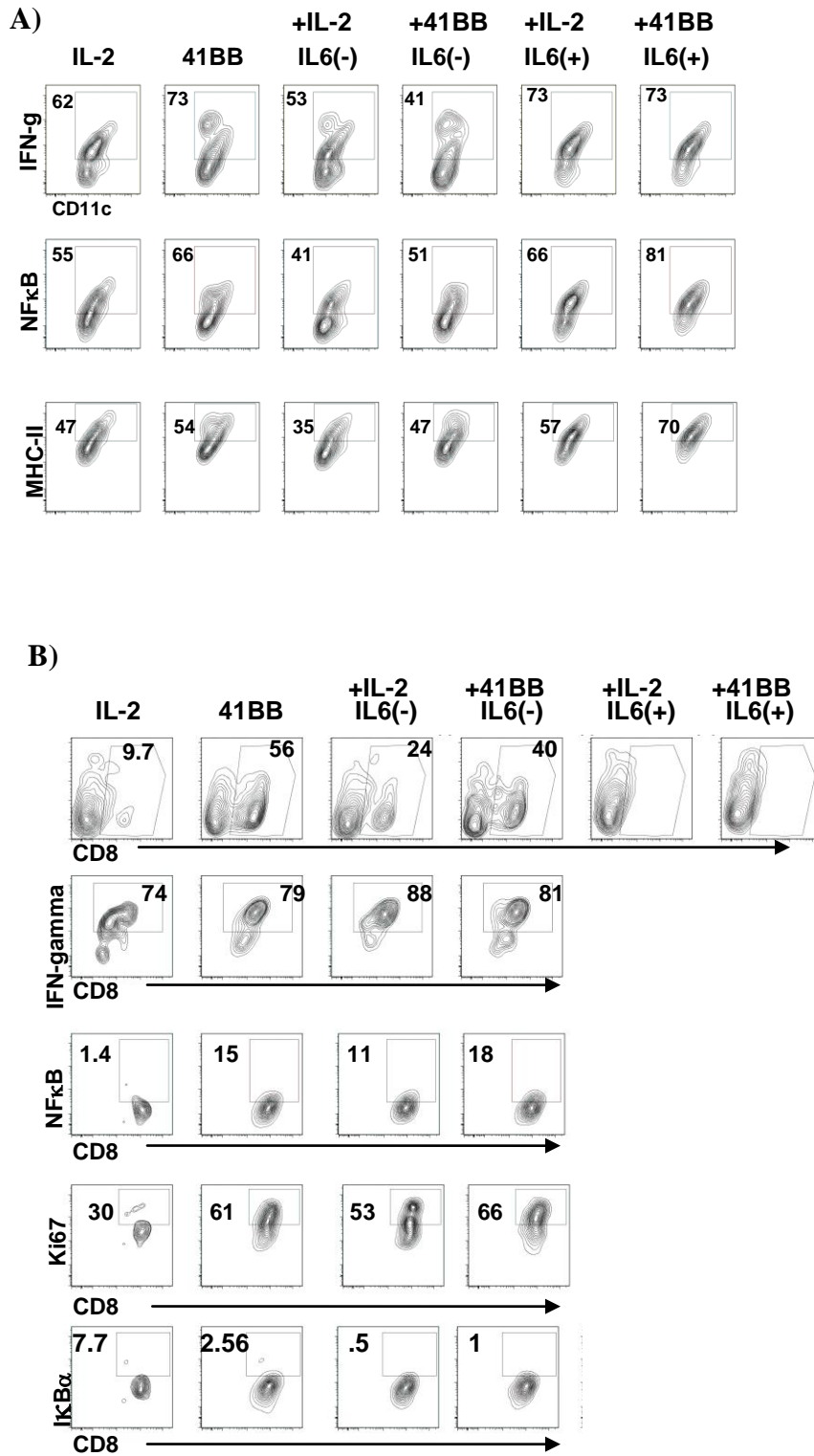
seeking out antigen, whereas effector memory T cells traffic to peripheral places, such as the lung and blood (73, 74, 78, 142, 143). Studies also suggest that central memory cells are the cells that persist *in vivo* (73, 74, 78, 142, 144), however it is the effector cells that initiate the anti-tumor responses. It would be interesting to determine if expanding the TIL with the anti-4-1BB antibody for a shorter period of time during the initial isolation period or in a lower dose of IL-2 helped the TIL persist *in vivo*. Our mouse studies ended after only a week but it would be interesting to see if we can detect the persistence of the TIL after a longer period. In addition, studies directly injecting the anti-4-1BB antibody into mice or humans have shown promise. However, the concentration of the anti-4-1BB antibody needs to be carefully monitored as continuous injections of anti-4-1BB antibody in a clinical trial treating melanoma patients induced toxicity of the liver, resulting in the trial to shut down (145, 146). This was believed to be caused by NK cells in the liver that expressed high levels of 4-1BB (145, 146). In our previous studies, we have also found that even using anti-4-1BB antibodies in an *in vitro* system has to be carefully monitored. We found that when 10ng/ml of anti-4-1BB was coated on a plate, the anti-4-1BB protected post-REP TIL from activation-induced cell death (AICD) (90). However, when we used a higher concentration (100ng/ml), we found that the anti-4-1BB antibody reversed its protective effect against AICD in the post-REP TIL (90). As mentioned previously, a number of cells within the body express 4-1BB. Therefore, when the anti-4-1BB antibody is given to mice or patients systemically, it may have off-target effects on cells that express high levels of 4-1BB, creating too much co-stimulation.

We observed that when we initially set up the fragments with or without the anti-4-1BB antibody, the cells that received the anti-4-1BB antibody exhibited an increase in pro-inflammatory cytokines (**Figure 3.9**). IL-6 is an important cytokine that has been demonstrated to regulate dendritic cell differentiation (*147*). Therefore, we wanted to investigate what the role of IL-6 was in the melanoma tumor fragment. We set up initial experiments investigating what the effects would be if we neutralized or activated IL-6. The addition of IL-6 (IL-6+) abrogated the CD8<sup>+</sup> percentage. The effect of IL-6 had an opposite effect in the dendritic cells within the fragment. The activating IL-6 antibody (IL-6+) had a positive effect on the dendritic cells, increasing the IFN-gamma producing cells, NFκB, and MHC-II (**Figure 5.2A**). We found that when IL-6 was neutralized, (IL-6 (-)), this increased the TIL to increase percentage of CD8, IFN-gamma, NFκB, Ki67, and decreased IκBα (**Figure 5.2B**). We believe that the effect of IL-6 could be a two-edged sword. Although IL-6 seems to play a positive role on the dendritic cell population, it appears to abrogate to the CD8<sup>+</sup> T-cell subset. Other cells within the microenvironment secrete IL-6, such as myeloid derived suppressor cells (MDSCs). MDSC could also play a role in suppressing the T cells from migrating out of the fragment. MDSCs can inhibit T cells through blocking the translation of the T-cell zeta chain (*148, 149*), and promoting T-cell apoptosis. It would be interesting to investigate the role of anti-4-1BB antibody in the MDSC and determine whether the MDSCs are the main population secreting IL-6 and whether anti-4-1BB antibody could augment their suppressive factors. Another population that may be affecting the T cells are macrophages. There are 2 subsets of macrophages, M1 and M2. M1 macrophages are pro-inflammatory producing TNF-

alpha, IL-23, and IL-6 (148, 149). On the other hand, M2 macrophages are anti-inflammatory, and become activated by IL-6 production. It would interesting to investigate whether the different subsets of macrophages are present within the melanoma tumor fragment, how they would affect the TIL, and what role 4-1BB activation may have on the macrophage subsets, if they are present within the melanoma tumor fragment.

**Figure 5.2. Modulation of Dendritic cells and T cells within melanoma fragments with IL-6.** The melanoma tumor was cut up into multiple fragments and placed in culture with IL-2±anti-4-1BB antibody. In addition, a neutralizing (-) or activating (+) IL-6 cytokine was added to the cultures. When we investigated the effect of IL-6 in the dendritic cell subset, we found that adding an activating IL-6 cytokine (+) increased the dendritic cell IFN-gamma production, NFκB, and MHC-II (**A**). We found that neutralizing IL-6 (-) increased the proliferation, CD8 percentage and NFκB within the T cells subset. This effect was reversed when an activating IL-6 cytokine was added to the cultures (**B**).

**Figure 5.2. Modulation of Dendritic cells and T cells within melanoma fragments with IL-6.**





## **CHAPTER 6: MATERIALS and METHODS**

## **Materials and Methods**

### **Reagents**

A fully human and purified IgG4 monoclonal antibody (mAb) against human CD137 (BMS-663513; Lot 6A20377) was kindly provided by Bristol Myers Squibb (BMS; Princeton, NJ) through a Materials Transfer Agreement . BMS-663513 (henceforth called anti-4-1BB) was stored at 4°C at a concentration of 14.9 mg/ml and was certified to have <0.5 EU/mg endotoxin level, >95% purity, and <5% high molecular weight species. Human recombinant IL-2 (Proleukin™) was generously provided by Prometheus Therapeutics and Diagnostics (San Diego, CA). Flow cytometry reagents were obtained from BD Biosciences, eBioscience, or BioLegend.

### **Isolation and expansion of TIL from human melanoma patient tumors**

Melanoma tumors were surgically resected from stage III-IV melanoma patients. The tumor was then cut up into 3-5mm<sup>2</sup> fragments using a sterile scalpel. The fragments were placed in a 24 well plate with TIL culture media (TIL-CM) and 6,000 IU/ml Interleukin-2 (IL-2) in order to help the TIL grow out of the fragment. The TIL-CM contained RPMI 1640 with Glutamax (Gibco/Invitrogen; Carlsbad, CA), 1X Pen-Strep (Gibco/ Invitrogen; Carlsbad, CA), 50µm 2-mercaptoethanol (Gibco/ Invitrogen; Carlsbad, CA), 20µg/ml Gentamicin (Gibco/ Invitrogen; Carlsbad, CA), and 1mM pyruvate (Gibco/ Invitrogen; Carlsbad, CA). Each well had 1 fragment and the fragments were kept in culture for a period of 3 weeks. After the 3 week period, the TIL were referred to as Pre-Rapid Expansion (pre-REP) TIL. For our control cultures, the fragments were placed in 6,000IU/ml IL-2 with a fully-human IgG4 Isotype control (Eureka therapeutics ET904).

### **Rapid expansion protocol (REP)**

The REP was performed in upright T-25 flasks by activating  $1.3 \times 10^5$  pre-REP TIL with  $26 \times 10^6$  allogeneic, irradiated (5,000cGy) PBMC feeder cells with 30 ng/ml OKT3 (anti-CD3; Abbott Labs, Abbott Park, IL) in 1:1 mixture of TIL-CM and AIM-V (Invitrogen). Exogenous anti-4-1BB was added to some of the flasks at different concentrations on day 0 of the REP. On day 2 of the REP, 6,000 IU/ml IL-2 was added to each flask. The TIL were expanded for another 12 days. On days 5, 7, 9, and 12 of the REP, the TIL were diluted as necessary with a 1:1 ratio of TIL-CM and AIM-V with a final concentration of 6,000 IU/ml IL-2 to keep the cells between  $1-2 \times 10^6$ /ml.

### **Fragment flow cytometry staining**

The melanoma fragment was cut up into  $3-5 \text{ mm}^2$  pieces and mechanically disintegrated using glass slides, re-suspended in 2 ml sterile FACS Wash Buffer (FWB) and washed for 5min at 1400rpm. The FWB contained 1X Dulbecco's Phosphate Buffered Saline (D-PBS) and 1% Bovine Serum Albumin (BSA (Sigma Aldrich)). The fragment was then stained for fluorochrome-conjugated monoclonal antibodies that recognized surface markers CD11c, CD80, CD86, 4-1BB, 4-1BBL, HLA-DR, CD3, CD8, CD56, CD19, CD20, CD4, (eBioscience, BD Biosciences, and BD Pharmingen) and live/dead fixable dye (Molecular Probes by Life Technologies; Lot 1413034) in 0.1ml FACS Stain Buffer (FSB) for 25 minutes on ice. The FSB contained 1X D-PBS, 1% BSA, and 5% goat serum. The cells were then washed in FWB for 5 minutes at 1400rpm and fixed in 1X D-PBS, 1% para-formaldehyde

solution. The cells were then acquired using a BD FACScanto II flow cytometer machine using FACSDiva software.

### **TIL flow cytometry staining after 1 week in culture**

One week after the fragments were set up with or without the anti-4-1BB antibody, the fragment was mechanically disaggregated, filtered, and washed with FWB for 5 minutes at 1400rpm. The cells that grew out of the fragment were also collected and re-suspended in 2ml FWB and washed for 5min at 1400rpm. The cells were then stained for fluorochrome-conjugated antibodies that recognized surface markers CD3, CD8, CD56, CD4, CD11c, HLA-DR, CD80, CD86, CD56, and 4-1BB (eBiosciences, BD Pharmingen, and BD Biosciences). A live/dead fixable dye (Molecular Probes by Life Technologies; Lot 1413034) was also used to gate out dead cells when acquiring the samples. The samples were stained in FWB for 25 minutes on ice. After, the samples were washed in 2ml FWB for 5 minutes at 1400rpm and fixed in 1X D-PBS, 1% para-formaldehyde solution. The samples were acquired using a BD FACScanto II flow cytometer machine.

### **TIL staining using flow cytometry after 3 weeks in culture**

Fragments from melanoma tumors were set up as described earlier with or without the addition of agonistic anti-4-1BB antibody. After 3 weeks in culture, the TIL were harvested and stained for surface and intracellular markers using flow cytometry. Briefly, pre-REP TIL were harvested, counted, and washed with 2ml FWB for 5 minutes at 1400rpm. The cells were stained with surface markers CD3, CD4, CD27, CD28, and CD8 (eBiosciences, BD Pharmingen, and BD Biosciences). The viability dye AmCyan Aqua (Molecular Probes by Life Technologies) was also used to help

gate out dead cells while acquiring our samples. The cells were stained in FSB for 25 minutes on ice. After, the cells were washed with 2ml FWB and BD Cytotfix/Cytoperm Fixation and Permeabilization Solution (BD Biosciences Cat: 554722) was added to the samples for 20 minutes at room temperature in the dark. After, the cells were washed with 1X BD Perm/Wash Buffer (Cat: 554723) for 5 minutes at 1400rpm, followed by intracellularly staining for Granzyme B, Perforin and EOMES (eBiosciences, BD Biosciences) in 1X Fixation Buffer for 25 minutes on ice. After, the samples were washed with FWB for 5 minutes at 1400rpm and fixed in 1X D-PBS, 1% para-formaldehyde solution. The samples were acquired using a BD FACScanto II flow cytometer machine. In some cases, harvested cells after 3 weeks of culture were stained for cell surface markers together with a MART-1 peptide HLA-A0201 tetramer (Beckman-Coulter) to enumerate the frequency of CD8<sup>+</sup> MART-1-specific T cells.

#### **Cytospin and Immunocytochemistry staining for surface and nuclear markers**

The fragments were set up as previously mentioned earlier with or without anti-4-1BB antibody. After 1 week in culture, the cells were collected and washed twice in 1X D-PBS for 5 minutes at 1400rpm. After, the cells were re-suspended in 1ml 4% para-formaldehyde for 20 minutes at room temperature. The cells were washed again in 1X D-PBS and re-suspend in 1ml and placed at 4°C. After, the cells were cytospun onto glass slides at 750 rpm for 3 minutes using Shandon Cytospin II centrifuge. The samples for Ki67 (Dako) and NFκB (p65) (BD transduction laboratories) (nuclear staining) were placed in 0.2% PBS Triton-X solution for 10 min at room temperature (RT). After, the nuclear staining samples were washed in 1X D-PBS 3 times. All

samples were then placed in 3% H<sub>2</sub>O<sub>2</sub>/methanol for 10 minutes at RT to block endogenous peroxidase. The samples were then washed 3 times in 1X D-PBS and 2.5% normal horse serum (Vectastain Universal Elite ABC Kit) was added to all slides for 30 minutes in a humid chamber at RT. After, the primary antibody was added and kept at 4°C overnight. The next day, a biotinylated secondary antibody was added to the samples for 30 min at RT (Vectastain Universal Elite ABC Kit). The slides were then washed 3 times in D-PBS and peroxidase conjugated avidin biotin complex ABC Reagent (Vectastain Universal Elite ABC Kit) was added for 30 min at RT. After, the samples were washed again with D-PBS and 3,3-Diaminobenzidine (DAB) was added. The samples were then counterstained and covered. Analysis was done using Leica Application Suite (LAS) V4.2 software (Leica Microsystems). For NFκB (p65) staining, analysis was also done using the LAS V4.2 software, but additional analysis was conducted using Vectra Intelligents Slide Analysis System (Vectra, Perkin Elmer) Nuance software 3.0.1.2, using composite coloring style “Fluorescence.”

### **Ki67 staining using flow cytometry in early cultures**

The melanoma tumor was cut up into 3-5mm<sup>2</sup> fragments and placed in media, IL-2 ± anti-4-1BB antibody. After 1 week, the cells that migrated out of the fragment were collected and washed twice in FWB for 5 minutes at 1400rpm at 4°C. The cells were subsequently stained for cell surface markers CD3 FITC (BD Biosciences; Catalog number 555916; Lot 17763) and CD8 Pacific Blue (BD Pharmingen; Catalog number 558207; Clone RPA-T8) on ice for 25 minutes. During this time, the fixation buffer (BD Bioscience; Catalog 554655) was kept in a 37°C water bath and the Perm Buffer

III (BD Phosflow; Catalog 558050; Lot 2128930) was kept at -20°C. The flow cytometry tubes were labeled and 1ml fixation buffer was added to each tube. After, the TIL were added to each tube, re-suspended, and placed at room temperature for 10 minutes. Then, the cells were spun in FWB for 5 minutes, 4°C, 1400 rpm. Afterwards, the supernatant was aspirated and 100ul Perm Buffer III was added to each tube while vortexing the tube. The tubes were then placed in the dark at room temperature for 5 minutes. After, the cells were washed in 1 ml stain buffer for 5 minutes at 1400 rpm at 4°C. The Ki67 antibody (BD Pharmingen; Catalog 561284) was then added to each tube and the tubes were subsequently left in the dark at room temperature for 1 hour. The cells were then washed with 3 ml FWB for 5 minutes at 1400rpm at 4°C. The cells were then re-suspended with 300µl staining buffer and the samples were acquired using a BD FACScanto II flow cytometer machine.

#### **NFκB, and IκBα staining using flow cytometry in early cultures**

The melanoma tumor was cut up into multiple fragments, placed in TIL-CM, IL-2 ± anti-4-1BB antibody. After 1 week, the fragment and the cells that migrated out of the tumor fragment were harvested. The fragments were mechanically disaggregated and filtered. The fragments and the cells that migrated out of the fragment were washed with FWB and subsequently stained for cell surface markers CD3 FITC (BD Biosciences; Catalog number 555916; Lot 17763) and CD8 Pacific Blue (BD Pharmingen; Catalog number 558207; Clone RPA-T8) on ice for 25 minutes. During this time, the Perm Buffer III was placed at -20°C and the fixation buffer was placed in a 37°C water bath. After the surface staining was done, the cells were washed in 2ml FWB two times. The supernatant was aspirated and 1 ml fixation buffer was

added to each tube and the tubes were placed at room temperature for 20 minutes. After 20 minutes, the cells were washed with FWB for 5 minutes at 1400rpm, 4°C. The supernatant was aspirated and 100µl Perm Buffer III was added to each tube while vortexing each tube. The tubes were then placed on ice for 30 minutes and after washed twice in 1ml FWB for 5 minutes, 1400rpm, 4°C. Afterward, the cells were then stained using NFκB (p65) (BD Pharmingen; Catalog number 560335) and IκBα Alexa 647 (BD Pharmingen; Catalog number 560817) and left in the dark at room temperature for 1 hour. The cells were then washed with 3ml FWB for 5 minutes, 1400rpm, 4°C, then re-suspended in 300µl FWB and samples were subsequently acquired using a BD FACScanto II flow cytometer machine.

### **Staining for Interferon-gamma and degranulation (CD107a) in the TIL after 3 week culture period**

Melanoma tumors were surgically resected, cut up into multiple fragments, and placed in culture with TIL media, IL-2, with or without the addition of an anti-4-1BB antibody. After 3 weeks, the TIL were set up at a 1:1 ratio with HLA-matched melanoma tumor lines in a 96-well plate for 1 hour at 37°C. After, Golgi-Stop (BD Biosciences Catalog 554724) was added to each well and the cultures were left at 37°C for a 5 hour period. During this time, CD107a antibody (BD Biosciences) was also added to each condition. After 5 hours, the cells were harvested, washed with FWB for 5 minutes at 4°C. The cells were then stained for surface markers CD3 and CD8 for 25 minutes at 4°C. After, the cells were washed with FWB again, re-suspended in fixation buffer (added while vortexing the tubes) and incubated for 15 minutes at room temperature. After, the cells were washed with FWB and re-



suspended in Cytotfix/Cytoperm buffer and incubated at room temperature for 20 minutes. The cells were then washed with FWB and stained for IFN-gamma for 25 minutes in 4°C. The cells were then washed again and the samples were acquired using a BD FACScanto II flow cytometer machine.

### **Intracellular phospho-staining in pre-REP TIL**

The melanoma tumors were cut up into multiple fragments and placed in a 24-well plate with TIL-CM, IL-2 ± anti-4-1BB antibody. After 3 weeks, the TIL were harvested, washed twice in media for 5 minutes at 1400rpm at 4°C. After, 250,000 TIL per well were placed in a 48-well plate and kept at 37°C for 3-4 hours. During this incubation period, fixation buffer (BD Bioscience; Catalog 554655) was kept in a 37°C water bath and the Perm Buffer III (BD Phosflow; Catalog 558050; Lot 2128930) was kept at -20°C. After the 3-4 hour incubation period, 200 IU/ml IL-2 was added to the cultures. As a control, half the cultures were not given IL-2. The plate was then placed in 37°C for 20 minutes. The flow cytometry tubes were labeled and 1ml fixation buffer was added to each tube. The TIL were then added to the tubes, re-suspended and placed in 37°C incubator for 10 minutes. After 10 minutes, the cells were spun for 5 minutes at 1400rpm at 4°C. After the wash, the supernatant was aspirated and 1ml Perm Buffer III was added to the tubes, re-suspended and placed in 4°C for 20 minutes. The cells were then washed twice in 1ml stain buffer for 5 minutes at 1400rpm, 4°C. After, the antibodies (CD8, pSTAT5, CD3, pAKT) were added to the tubes to a total volume of 100ul and left in the dark for 30 minutes at room temperature. After 30 minutes, the cells were washed with 3ml stain buffer for 5

minutes, 1400rpm at 4°C, then re-suspended with 300ul stain buffer and the samples were acquired using a BD FACScanto II flow cytometer machine.

### **Staining for bcl-6 in pre-REP TIL**

The fragments were set up as previously described with TIL-CM, IL-2 ± anti-4-1BB antibody. After 3 weeks, the TIL were harvested, washed with 2ml FWB at 1400rpm for 5 minutes, and after stained for surface makers CD3 FITC and CD8 Pacific Blue for 25 minutes on ice. During this time, the Perm Buffer III was kept at 20°C and the fixation buffer was kept in a 37°C water bath. After the 25 minute incubation period, the cells were washed twice in FWB and the cells were fixed in 200ul fixation buffer for 10 minutes at 37°C. The cells were then washed in 2ml 1X-DPS at 4°C, 1400rpm for 5 minutes. The supernatant was then aspirated and the cells were permeabilized using 100ul of the pre-chilled Perm-Buffer III. The cells were then incubated on ice for 30 minutes. After, the cells were washed twice with BD stain buffer and after re-suspended in 100µl FWB containing bcl-6 PerCP-Cy5.5 (BD Pharmingen; catalog number 562198) for 45 minutes at room temperature. The cells were subsequently washed and acquired using a BD FACScanto II flow cytometer machine.

### **Measurement of pro-inflammatory cytokines using Meso-Scale Discovery assay**

The melanoma tumors were surgically resected. The tumors were then cut up into multiple fragments and placed in culture with TIL media, IL-2± anti-4-1BB antibody. After 4 days, the supernatant was collected from each condition. The pro-inflammatory cytokines were measured using a V-plex custom human cytokine kit (Meso-Scale Discovery, Rockville, MD).

## **IFN-gamma measurement using Enzyme Linked Immunosorbent Assay (ELISA) for pre-REP TIL**

After 3 weeks in culture, the pre-REP TIL were co-cultured at a 1:1 ratio with autologous or HLA-matched target cells and placed at 37°C overnight. The samples were added in triplicate with 100µl added to each well in a 96 round bottom plate. The supernatants were collected after 24hr and IFN-gamma secretion was measured using a human IFN-gamma ELISA kit (Thermo Scientific KB132422). A 96-well ELISA plate reader (ELx808, Bio-Tek Instruments Inc., Houston, TX) was used to read the plate.

## **Flow cytometric analysis of post-REP TIL**

Pre-REP and post-REP TIL were washed with FACS Wash Buffer (FWB) that contained Dulbecco's Phosphate Buffered Saline 1X (D-PBS; Gibco/Invitrogen) and 1% Bovine Serum Albumin (BSA). The cells were resuspended in 0.1 ml FACS Staining Buffer (FSB) consisting of 1X D-PBS, 1% BSA, and 5% goat serum and stained on ice for 20 min using fluorochrome-conjugated monoclonal antibodies recognizing the following surface and intracellular markers: CD8, CD4, CD27, CD28, KLRG-1, Eomesodermin, Granzyme B (GB), and Perforin (Perf). The TIL were washed with FWB and re-suspended in 0.3 ml 1X D-PBS, 1% para-formaldehyde solution. The stained cells were analyzed using the BD FACScanto II flow cytometry analyzer using FACSDiva software. The data was later analyzed using FlowJo software (TreeStar).

## **Analysis of 4-1BB and 4-1BBL expression during the REP**

Pre-REP TIL were labeled with 1  $\mu$ M CFSE (Molecular Probes-Invitrogen, Carlsbad, CA) in order to be able to distinguish the TIL from the irradiated PBMC. The REP was then set-up as described above. On day 1 and day 2, the cells were harvested from the REP and stained for CD8, CD3, 4-1BB, and 4-1BBL (BD Biosciences). Gating was done on the viable cell population and then on the CFSE-positive (TIL) or CFSE-negative (feeders) population. The different populations were then analyzed for their expression of 4-1BB and 4-1BBL.

### **Flow cytometric sorting of post-REP CD8<sup>+</sup> TIL**

Post-REP TIL were harvested, washed, and re-suspended at  $25 \times 10^6$ /ml in 2 ml of sterile FSB. The cells were stained using anti-CD8-Pacific Blue on ice for 20 minutes, washed, re-suspended in sterile FWB, and the CD8<sup>+</sup> subset isolated by sorting in a FACSAria sorter (BD Biosciences, San Jose, CA). The sorted cells were washed in cold FWB and rested for 3 hours in order to shed the antibody. Afterwards, RNA was isolated from the sorted TIL for quantitative real-time PCR (qRT-PCR) analysis and/or cytotoxic T-cell assay.

### **Quantitative real-time PCR**

We used quantitative real-time PCR (qRT-PCR) to measure the expression of anti-apoptotic genes Bcl-2 and Bcl-xL or pro-apoptotic gene Bim. RNA was isolated from  $5 \times 10^6$  post-cells on day 14 of the REP (post-REP cells) using the Qiagen RNeasy Mini Kit (Qiagen; Duesseldorf, Germany). By this time no remaining irradiated PBMC feeder cells were left in the cultures. RNA quantity and was determined using a NanoDrop spectrophotometer (Thermo Scientific; Wilmington, DE). The RNA was then subjected to qRT-PCR analysis as previously described. The

following primer sequences were used; *bcl-2*: forward primer: 5'-CAGAAGGGACTGAATCGGAG-3', reverse primer: 5'-TGGGATGTCAGGTCACTGAA-3'; *bcl-x<sub>L</sub>*: forward primer: 5'-TGAGTCGGATCGCAGC TTGG-3', reverse primer: 5'-TGGATGGTCAGTGTCTGGTC-3'; *bim*: forward primer: 5'-ACAGGAGCC CAGCACCCATG-3', reverse primer: 5'-ACGCCGCAACTCTTGGGCGA-3'; and *β-actin*: forward primer: 5'-TTGCCGACAGGATGCAGAA-3', reverse primer: 5'-GCCGATCCACACGGAGTACT-3'.

### **Cytotoxic T-cell assay**

TIL from HLA-A0201<sup>+</sup> patients having a significant pre-REP CD8<sup>+</sup> T-cell population recognizing the Melan-A/MART-1 peptide (ELAGIGILTV), as determined using HLA-A0201-peptide tetramer staining, were subjected to the REP with or without added anti-4-1BB on day 0. The post-REP cells were sorted for CD8<sup>+</sup> T cells and evaluated for their cytolytic function using a caspase-3 cleavage CTL assay as previously described . The target cells included HLA-A0201<sup>+</sup> 624 melanoma cells or MART-1 peptide-pulsed T2 human lymphoma cells. The HLA-A unmatched melanoma cell line 938 and T2 cells pulsed with a human immunodeficiency virus rev peptide (ILKEPVHGV) were used as controls. The target cells were labeled with DDAO-SE (Molecule Probes-Invitrogen) for 15 min at 37°C. The sorted TIL and target cells were incubated at different effector-to-target ratios, as indicated for 3-4 h at 37°C before harvesting and staining for cleaved caspase 3 in the target cells .

### **Multiplex cytokine assay**

Post-REP TIL were harvested and washed twice in TIL-CM to remove any excess IL-2 and plated for 24 h in a 96 well plates pre-coated with or without 10 ng/ml anti-CD3. The supernatants were collected and cytokine secretion was measured using a Luminex-100 system using beads recognizing IL-2, IFN- $\gamma$ , and TNF- $\alpha$ , (Millipore, Billerica, MA). The net cytokine levels after subtraction of control wells without anti-CD3 were determined.

#### **Cell survival assays post-REP**

Post-REP TIL were harvested, washed twice in TIL-CM, and re-cultured in a 24 well plates at 400,000 cells per well in 2-ml TIL-CM with no cytokine or 200 IU/ml IL-2. After 4 days, the cells were harvested and stained with anti-CD8, 7-AAD, and Annexin-V, as described previously . The stained cells were acquired using a FACSCanto II flow cytometer. The samples were analyzed by first gating on the live population. The percentage of CD8<sup>+</sup> cells and AnnexinV<sup>+</sup> was determined. Viable cell counts were performed using a hemocytometer and used to calculate the total number of live CD8<sup>+</sup> T cells in the cultures using the percentage of live CD8<sup>+</sup> T cells found by flow cytometry.

#### **Re-stimulation of post-REP TIL with MART-1 peptide-pulsed dendritic cells**

Post-REP TIL from HLA-A0201<sup>+</sup> patients having at least 0.5% of the CD8<sup>+</sup> T cells staining positive for MART-1 tetramer (ELAGIGILTV) peptide were re-stimulated for 7 days with mature HLA-A0201-matched DC pulsed with the same MART-1 peptide (ELAGIGILTV), as described previously . Dendritic cells (DCs) were generated from adherent monocytes from HLA-A0201<sup>+</sup> normal donors using GM-CSF and IL-4 for 5 days following by incubation with IL-1 $\beta$ , TNF- $\alpha$ , IL-6, and PGE<sub>2</sub>

for 2 days . The mature DCs were isolated, pulsed with MART-1 peptide for 90 min and then washed. Post-REP TIL were isolated and washed in TIL-CM two times and rested in TIL-CM for 6 hours without any additional cytokine. The cells were labeled with the cell division dye eFluor670 (Invitrogen) according to the manufacturer's instructions and washed. The labeled TIL and peptide-pulsed DCs were mixed in 24-wells at a 10:1 ratio ( $2 \times 10^6$  TIL plus  $0.2 \times 10^6$  DCs). IL-2 (100 IU/ml) was added to all cultures to facilitate TIL viability. After 7 days, the cells were stained for CD8 and MART-1 tetramer and analyzed for cell division using a FACSCanto II (BD Biosciences).

### **Polymerase chain reaction (PCR)**

PCR was performed using a panel of optimized primers specific for 24 members of the TCRV $\beta$  family. Briefly, RNA was extracted from  $1 \times 10^6$  pre-REP TIL using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The TIL then underwent the REP with or without the addition of anti-4-1BB. RNA was then isolated the same way on the post-REP TIL. The extracted RNA (1  $\mu$ g) was treated with DNase (Ambion, Austin, TX) to remove contaminating genomic DNA. All of the DNase-treated RNA was used to synthesize cDNA by reverse transcription using the manufacturer's protocol with the SuperScript™ III Reverse Transcriptase (Invitrogen). PCR was then performed by combining 0.5  $\mu$ M of one V $\beta$  primer for each of the different TCRV $\beta$  families with 0.5  $\mu$ M of a C $\beta$  primer, which was used for each of the 24 reactions. The PCR products were visualized on a 1.5% agarose gel.

### **TCR V $\beta$ spectratype analysis**

For TCR V $\beta$  spectratype analysis, PCR products were diluted in nuclease-free water

so that 1.5 ng of the PCR product from each TCR V $\beta$  family was subjected to capillary electrophoresis using an OpenGene™ System (Bayer, Terrytown, NY). Because the positions of the 5'V $\beta$  and the 3'C $\beta$  primers are fixed, variation in length of the PCR fragments within any TCR V $\beta$  family is due to differences in length of the CDR3 regions. Data are presented as fluorescence intensity versus DNA fragment length. The TCR V $\beta$ 10 and V $\beta$ 19 families are pseudogenes and were therefore excluded from analysis. The number of CDR3 sequence peaks were plotted for each major V $\beta$  family.

### ***In vivo* mice experiments**

The melanoma tumor was cut up into multiple fragments and placed in TIL-CM and IL-2 for a period of 4-5 weeks. After, the pre-REP TIL underwent the REP for 2 weeks. The REP was set up with IL-2 alone or IL-2+anti-4-1BB antibody (500ng/ml on day 0) The NOD/SCID $\gamma$ c<sup>-/-</sup> (NSG) mice were kindly provided to us by Dr. Laurence Cooper (UT MD Anderson Cancer Center, Houston TX). Each experiment contained 4, 10-12 week-old female mice per condition. We subcutaneously injected 1x10<sup>6</sup> human melanoma cell line WM35. After 1 week. We intravenously injected 25x10<sup>6</sup> post-REP TIL through the tail vein that were expanded with IL-2 alone or IL-2+anti-4-1BB antibody. During a 7 day period, the tumor sizes were monitored using calipers. After 1 week of the post-REP TIL being injected in the NSG mice, the mice were sacrificed using CO<sub>2</sub> and cervical dislocation. The blood, spleens, and lungs were harvested, filtered, washed with FWB twice and stained for human surface markers, CD45, CD4, CD3, and CD8 Pacific Blue for 10 minutes at room temperature. After, 100 $\mu$ l Cal-Lyse lysing solution was added to each tube. The cells



were incubated in the dark for 10 minutes at room temperature. Prior to acquiring our samples using a BD FACScanto II flow cytometer machine, we added 100µl of AccuCheck counting beads (Invitrogen/Molecular Probes; Product code PCB100) to each tube of lysed cells.

\*The final Absolute Count=(Number of cells counted/ total number of beads counted (A+B) x Number of beads per µl (known concentration)

\*Reprinted from:

[http://tools.lifetechnologies.com/content/sfs/manuals/L20054\\_Rev0809.pdf](http://tools.lifetechnologies.com/content/sfs/manuals/L20054_Rev0809.pdf)

### **Statistical Analysis**

Statistical analysis for comparison of 2 groups was done using the Wilcoxon signed rank test or Student's t test (paired datasets), or the Wilcoxon rank sum test (unpaired datasets). Analysis of experiments with 3 or more treatment groups was done using the one-way or two-way analysis of variance (ANOVA), with Bonferroni post-tests with both tests using biological relevance occurring when  $p < 0.05$ . Statistical analysis was done using Graph Pad Prism (La Jolla, CA).

## CHAPTER 7: REFERENCES

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## **CHAPTER 8: VITA**

Jessica Ann Chacon was born in El Paso, Texas on February 6, 1985, the daughter of Rodolfo O. Hernandez and Corina M. Hernandez. After completing high school at J.M. Hanks High School in El Paso, she attended The University of Texas at El Paso. She received a Bachelor of Science with a major in Biological Sciences and minors in Chemistry and Psychology in May 2007. In August 2007, Jessica entered The University of Texas Health Science Center-Houston, Graduate School of Biomedical Sciences, where she earned her Master of Science degree in Biomedical Sciences,

specializing in Immunology in December 2009, under the mentorship of Dr. Laszlo Radvanyi, Ph.D. In January 2010, Jessica entered the Ph.D program at the University of Texas Health Science Center-Houston, Graduate School of Biomedical Sciences. She carried out this dissertation in the Department of Melanoma Medical Oncology at The University of Texas, M.D. Anderson Cancer Center under the guidance of Dr. Laszlo Radvanyi, Ph.D.

## **CHAPTER 9: PUBLICATIONS**

### **a. peer-review articles**

#### **Submitted:**

1. **Jessica Ann Chacon**, Amod A Sarnaik, Jie Qing Chen, Caitlin Creasy, Charuta Kale, John Robinson, Jeffrey Weber, Patrick Hwu, Laszlo Radvanyi, and Shari Pilon-Thomas. Manipulating the tumor microenvironment *ex vivo* for enhanced expansion of tumor-infiltrating lymphocytes for adoptive cell therapy.
2. Geok Choo Sim, **Jessica Chacon**, Cara Haymaker, Krit Ritthipichai, Manish

Singh, Patrick Hwu, Laszlo Radvanyi. Tumor-infiltrating Lymphocyte (TIL) Therapy for Melanoma: Rationale and Issues for Further Clinical Development. *BioDrugs*. January, 2014

**Published:**

1. **Jessica Chacon**, Shari Pilon-Thomas, Amod Sarnaik, and Laszlo Radvanyi. Continuous 4-1BB co-stimulation signals for the optimal expansion of Tumor-Infiltrating Lymphocytes for Adoptive T-cell Therapy. *OncoImmunology*. 2013 Sept; Vol2 Issue 9: eID: e25581.
2. **Jessica Ann Chacon**, Richard Wu, Pariya Sukhumalchandra, Jeffrey J. Molldrem, Amod Sarnaik, Shari Pilon-Thomas, Jeffrey Weber, Patrick Hwu, and Laszlo Radvanyi. Co-stimulation Through 4-1BB/CD137 Improves the Expansion and Function of CD8<sup>+</sup> Melanoma Tumor-infiltrating Lymphocytes for Adoptive T-cell Therapy. *PLoS ONE*. 2013;8(4):e60031.
3. Laszlo Radvanyi, Chantale Bernatchez, Minying Zhang, Priscilla Miller, **Jessica Ann Chacon**, Richard Wu, Greg Lizée, *et al.* Specific lymphocyte subsets predict response to adoptive cell therapy using expanded autologous tumor-infiltrating lymphocytes in metastatic melanoma patients. *Clin. Cancer Res*. 2012 Dec 15;18(24):6758-70.
4. **Jessica Chacon**, Richard Wu, Marie-Andree Forget, Chantale Bernatchez, Cara Haymaker, Jie Qing Chen, Patrick Hwu, and Laszlo Radvanyi. 2012. Adoptive T-cell therapy using autologous tumor-infiltrating lymphocytes for metastatic melanoma: Current status and future outlook. [\*Cancer J\*](#). 2012;18(2):160-75.
5. Richard Wu, Shujuan Liu, **Jessica Ann Chacon**, Sheng Wu, Yufeng Li, Pariya

- Sukhumalchandra, James Lee Murray, Jeff Molldrem, Patrick Hwu, Hanspeter Pircher, Greg Lizee, and Laszlo Radvanyi. 2011. Detection and Characterization of a Novel Subset of CD8<sup>+</sup>CD57<sup>+</sup> T-cells in Metastatic Melanoma Co-expressing Markers of Early Effector-memory T cells. *Clin. Cancer Research* 2012 May 1;18(9):2465-77.
6. **Jessica Ann Hernandez-Chacon**, Yufeng Li, Richard C. Wu, Chantale Bernatchez, Yijun Wang, Jeffrey Weber, Patrick Hwu, and Laszlo Radvanyi. Co-stimulation through the CD137/4-1BB pathway protects human melanoma tumor-infiltrating lymphocytes from activation-induced cell death and enhances anti-tumor effector function. *J Immunother* 2011;34:236–250.
  7. Dan Li, Yufeng Li, **Jessica A. Hernandez**, Rebecca Patenia, Tae Kon Kim, Jahan Khalili, Mark C. Dougherty, Patrick J. Hanley, Catherine M. Bollard, Krishna V. Komanduri, Patrick Hwu, Richard E. Champlin, Laszlo G. Radvanyi, Jeffrey J. Molldrem and Qing Ma. Lovastatin inhibits T cell proliferation while preserving the cytolytic function of EBV-, CMV- and MART-1-specific CTLs. *Journal of Immunotherapy*. 2010 Nov-Dec;33(9):975-82.
  8. Roberto Carreno, Wells S. Brown, Dan Li, **Jessica A. Hernandez**, Yang Wang, Tae Kon Kim, John W. Craft Jr., Krishna Komanduri, Laszlo G. Radvanyi, Patrick Hwu, Jeffrey J. Molldrem, Glen B. Legge, Bradley W. McIntyre, and Qing Ma. 2E8 binds to the high-affinity I-domain in a metal ion dependent manner: A second generation monoclonal antibody selectively targeting activated LFA-1. *Journal of Biological Chemistry*. 2010 Oct 22;285(43):32860-8.
  9. Yufeng Li, Shujuan Liu, **Jessica Hernandez**, Patrick Hwu, Laszlo Radvanyi.

Rapid expansion of melanoma TIL for adoptive cell therapy leads to loss of CD28 and reduced proliferative potential in the MART-1-specific T cell population. *J Immunol.* 2010 Jan 1;184(1):452-65.

**b. book chapters**

1. **Chacon Jessica**, Haymaker Cara, Bernatchez Chantale, Forget Marie Andree, Sim Geok, Ritthipichai Krit, Kale Charuta and Radvanyi Laszlo. Clinical success of adoptive cell transfer therapy using tumor infiltrating lymphocytes. In *Developments in T Cell Based Cancer Immunotherapies*. Wang, Ascierto, and Stroncek. [In preparation].
2. **Chacon Jessica**, Radvanyi Laszlo, and Hwu Patrick. Adoptive Cell Therapy for the Treatment of Metastatic Melanoma. In: *Targeted Therapeutics in Melanoma*. Gajewski and Hodi: NY, NY, 2012.