DEVELOPMENT OF A T CELL BASED CANCER IMMUNOTHERAPY BY USING THE INDUCED PLURIPOTENT STEM CELL

A Dissertation in
Microbiology and Immunology
by
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of the Requirements
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ABSTRACT

Cancer is one of the leading health issues that has caused tremendous impacts to both affected individuals and the whole society. Conquering cancer is imminent; however, with the currently available therapeutic approaches, it is somewhat difficult to achieve a cure in the clinics. Finding novel and more potent treatments to cancer is urgent.

Following an in-depth understanding of the human immune system, a new concept of cancer immunology has been outlined. It is found that the immune system has its own mechanism to eliminate dysfunction or deregulated self-tissue. However, in many cancer patients, their anti-cancer immune systems were compromised due to different explanations. Supplementing some of the defective immune system components in cancer patients has been shown a promising result in which tumor regression has been observed.

For example, in the clinical trials by using the adoptive T cell transfer (ACT)-based cancer immunotherapy, giving cancer patients either tumor-infiltrating or genetically-engineered T cells have showed significant improvements in the context of tumor-regression and patient-survivals. These studies have suggested a new strategy to treat cancer patients in addition to the currently available ones. However, a major problem of this strategy is the shortage of the T cells for the treatment. In some patients, there are no tumor-infiltrating T cells; in others, their peripheral blood-derived T cells are either anergic or senescent, hence are not suitable for subsequent processing. Exploring new sources of T cells is critical to design a more effective cancer immunotherapy by using the ACT-based approach.
Embryonic stem cells (ESCs) and their derivatives are considered premium candidates for regenerative medicine because of their self-renewable nature and capability of targeted differentiation. In previous work, it was shown that different types of stem cells such as embryonic and hematopoietic stem cells (HSCs) could be induced to develop into both lymphoid and myeloid tissues, under different lineage driving forces. Fitting in this scenario, T cells could also be generated from stem cells by different approaches. Hence, using stem tissue becomes a potential mechanism for getting adequate numbers of cells for T cell-based cancer immunotherapy. Moreover, there is another advantage of using T cells that are directly derived from stem cells; the induced T cells are naïve-phenotyped because they have no access to potential antigens. Based on current models of T cell development, naïve T cells are more persistent than effector T cells. In addition, naïve T cells have the capability to develop into memory stem T cells and central memory T cells, which are substantially longer-lived than any other subtypes of T cells. In summary, stem cells could potentially serve as new sources of large numbers of T cells; however, technical difficulties and ethical concerns have both limited the broad application of stem cells, especially ESCs.

The recent staging of induced pluripotent stem (iPS) cell technology might help change the uncertain fate of using stem cells in both basic research and clinical study. By overexpressing a certain combination of different genetic factors into terminally differentiated somatic cells, it is found that those cells are able to reprogram back into the stem-like cells which highly resemble ESCs in many aspects. This approach could theoretically reprogram any type of somatic cell back into stem cells. This discovery could technically and ethically break the barrier of using embryonic tissues and other
types of somatic stem cells. In the field of regenerative medicine, many reports have shown that iPS cells have become alternative choices to replace ESCs in tissue engineering, which further indicates that iPS cells might be the substitutes of ESCs in generating T cells for cancer immunotherapy.

My doctoral thesis research is part of the big picture trying to understand the possible utilization of iPS cells in cancer immunotherapy. This broad scheme contains many different directions and my work mainly focuses on the T cell, especially the cytotoxic T lymphocytes (CTLs). The hypothesis of my research is that the iPS cell is identical to ESC in the context of T lineage differentiation, and furthermore, the iPS cell can be engineered and induced into antigen-specific T cells to enhance immune surveillance against cancer.

To test this central hypothesis, both in vitro and in vivo T cell differentiation models and xenograft tumor models were used. In the first study, it was found that iPS cells were able to differentiate into conventional T cells after in vitro Notch ligand stimulation. In vitro developed iPS cells expressed general T cell markers and were able to respond to costimulatory signal stimulation in the form of secreting cytokines. Subsequent transfer of partially developed iPS cells into lymphopenic mice could reconstitute their T cell pool. This was the first report to show that iPS cells were similar to ESCs in T lineage differentiation.

A second work showed antigen-specific CTLs were induced from iPS cells partly by T cell receptor (TCR) stimulation. First, iPS-derived CTLs persisted in the animal compared to naïve T cells isolated from transgenic mice. Also, it was shown that in vivo
developed iPS-derived CTLs were able to respond to cognate antigens in terms of secreting cytokines and lysing target cells. Those iPS-derived antigen-specific CTLs had anti-tumor reactivity such as tumor infiltration and reducing tumor burden. Most importantly, it was observed the iPS-derived antigen-specific CTLs could significantly protect mice from tumor challenge. In conclusion, genetically modified iPS cells with certain TCR could develop into corresponding CTLs in vivo and developed CTLs were functional in the context of antigen reactivity and tumor control. This report further explored the possibilities of using iPS cells in cancer immunotherapy.

These two pioneering studies have initially supported our hypothesis however we would like to understand more about the mechanisms of T cell development and the ability of iPS-derived CTLs in targeting naturally occurring tumor antigens. To test these specific aims, additional studies were performed. We hypothesized that both Notch and TCR signals cross-talk in differentiating iPS cells into T cells, and possibly, this process is mediated by transforming growth factor (TGF)-β signaling. At the same time, melanoma and its associated antigens were used to test the ability of the iPS cell-derived CTLs to target a natural tumor antigen since the previously used an artificial antigen. The following studies have been outlined and hopefully will further our understanding for using iPS cells for ACT-based cancer immunotherapy.

In conclusion, this study served as the first exploratory work in proposing an iPS cell-derived and T cell-mediated cancer immunotherapy.
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Brief scheme for the in vivo development experiment design

The in vivo development of iPS/OT-I at different time points

In vivo developed iPS/OT-I cells were MHC-restricted

iPS/OT-I cells were able to develop into antigen-specific T cells

The CD25 and CD69 expressions in different groups of gated CD8+ Vβ5+ cells

Cytokine secretion profiles of iPS/OT-I derived T cells

In vivo killing capabilities of iPS/OT-I derived T cells

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<td>$\alpha$</td>
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<tr>
<td>$\beta$</td>
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<td>$\gamma$</td>
<td>gamma</td>
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<tr>
<td>$\delta$</td>
<td>delta</td>
</tr>
<tr>
<td>$\mu g$</td>
<td>Micro gram</td>
</tr>
<tr>
<td>$\mu L$</td>
<td>Micro liter</td>
</tr>
<tr>
<td>$\mu m$</td>
<td>Micro meter</td>
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<tr>
<td>%</td>
<td>Percent</td>
</tr>
<tr>
<td>&lt;</td>
<td>Less than</td>
</tr>
<tr>
<td>ºC</td>
<td>Degree Celsius</td>
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<td>ACK</td>
<td>Ammonium-Chloride-Potassium</td>
</tr>
<tr>
<td>ACT</td>
<td>Adoptive T cell transfer</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody-dependent cell-mediated cytotoxicity</td>
</tr>
<tr>
<td><em>Ad libitum</em></td>
<td>At one's pleasure</td>
</tr>
<tr>
<td>AFP</td>
<td>Alpha fetoprotein</td>
</tr>
<tr>
<td>alloSCT</td>
<td>Allogenic stem cell transplantation</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>ASC</td>
<td>Adult stem cell</td>
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<td>ATT</td>
<td>Anti-tyrosinase TCR</td>
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<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<table>
<thead>
<tr>
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<td>CAR</td>
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</tr>
<tr>
<td>CCL</td>
<td>CC chemokine ligand</td>
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<td>CCR</td>
<td>CC chemokine receptor</td>
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<td>Abbreviation</td>
<td>Description</td>
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<td>-------------</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein succinimidyl ester</td>
</tr>
<tr>
<td>CMC</td>
<td>Complement-mediated cytotoxicity</td>
</tr>
<tr>
<td>CMP</td>
<td>Common myeloid progenitor</td>
</tr>
<tr>
<td>CLP</td>
<td>Common lymphoid progenitor</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CTLA</td>
<td>Cytotoxic T cell antigen</td>
</tr>
<tr>
<td>cTEC</td>
<td>Cortical thymic epithelial cell</td>
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<tr>
<td>CXCL</td>
<td>CXC chemokine ligand</td>
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<td>CXCR</td>
<td>CXC chemokine receptor</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>DL</td>
<td>Delta-like ligand</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle’s minimal essential medium</td>
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<td>Dimethyl sulfoxide</td>
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<td>DN</td>
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<td>Deoxyribonucleic acid</td>
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<td>DsRed</td>
<td>Discosoma sp. red</td>
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<td>Ethylenediaminetetraacetic acid</td>
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<td>ESC</td>
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<td>ETP</td>
<td>Early thymic progenitor</td>
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<tr>
<td>ex vivo</td>
<td>Out of the living</td>
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<td>Fab</td>
<td>Fragment antigen-binding</td>
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<tr>
<td>FACS</td>
<td>Fluorescent activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>Fc</td>
<td>Fragment, crystallizable</td>
</tr>
<tr>
<td>FcR</td>
<td>Fragment, crystallizable receptor</td>
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<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<td>Description</td>
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<tr>
<td>Flt-3</td>
<td>Fms-like tyrosine kinase 3</td>
</tr>
<tr>
<td>FTOC</td>
<td>Fetal thymic organ culture</td>
</tr>
<tr>
<td>G</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GADPH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-monocyte colony stimulating factor</td>
</tr>
<tr>
<td>G-SCF</td>
<td>Granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>GVL</td>
<td>Graft-versus-leukemia</td>
</tr>
<tr>
<td>H</td>
<td>Hepatocellular carcinoma</td>
</tr>
<tr>
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<td>Hepatocellular carcinoma</td>
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<td>H&amp;E</td>
<td>Hematoxylin &amp; eosin</td>
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<td>HER2/neu</td>
<td>Human epidermal growth factor receptor 2</td>
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<td>Hematopoietic stem cell</td>
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<td>I</td>
<td>Interferon</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>in vitro</td>
<td>In glass</td>
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<td>in vivo</td>
<td>Within the living</td>
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<td>i.p.</td>
<td>Intraperitoneal</td>
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<td>iPS</td>
<td>Induced pluripotent stem</td>
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<td>IRES</td>
<td>Internal ribosome entry site</td>
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<tr>
<td>i.v.</td>
<td>Intravenous</td>
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<td>L</td>
<td>Leukemic inhibitory factor</td>
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<tr>
<td>LIF</td>
<td>Leukemic inhibitory factor</td>
</tr>
<tr>
<td>LMPP</td>
<td>Lymphoid-primed multipotent progenitor</td>
</tr>
<tr>
<td>M</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
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<tr>
<td>MAGE</td>
<td>Melanoma-associated antigen</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MART</td>
<td>Melanoma antigen recognized by T-cells</td>
</tr>
<tr>
<td>MDSC</td>
<td>Myeloid derived suppressor cell</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblasts</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimal essential medium</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MMLV</td>
<td>Moloney murine leukemia virus</td>
</tr>
<tr>
<td>MPP</td>
<td>Multipotent progenitor</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>MSCV</td>
<td>Mouse stem cell virus</td>
</tr>
<tr>
<td>mTEC</td>
<td>Medullary thymic epithelial cell</td>
</tr>
<tr>
<td>NEAA</td>
<td>Nonessential amino acid</td>
</tr>
<tr>
<td>NIC</td>
<td>Notch intracellular</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NSC</td>
<td>Neuronal stem cell</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>pMiG</td>
<td>Plasmid MSCV-IRES-GFP</td>
</tr>
<tr>
<td>pMiDR</td>
<td>Plasmid MSCV-IRES-DsRED</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Real-time quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell park memorial institute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
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</tr>
<tr>
<td>SCF</td>
<td>Stem cell factor</td>
</tr>
<tr>
<td>scFv</td>
<td>Single chain variable fragment</td>
</tr>
<tr>
<td>SCNT</td>
<td>Somatic-cell nuclear transfer</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>SP</td>
<td>Single positive</td>
</tr>
<tr>
<td>T</td>
<td>Central memory T cell</td>
</tr>
<tr>
<td>TCM</td>
<td>Central memory T cell</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>T_EFF</td>
<td>Effector T cell</td>
</tr>
<tr>
<td>T_EM</td>
<td>Effector memory T cell</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TIL</td>
<td>Tumor infiltrating T lymphocyte</td>
</tr>
<tr>
<td>T_M</td>
<td>Memory T cell</td>
</tr>
<tr>
<td>T_N</td>
<td>Naïve T lymphocytes</td>
</tr>
<tr>
<td>TPO</td>
<td>Thrombopoietin</td>
</tr>
<tr>
<td>T_REG</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>TRP</td>
<td>Tyrosinase associated protein</td>
</tr>
<tr>
<td>T_SCN</td>
<td>Memory stem T cell</td>
</tr>
<tr>
<td>TSP</td>
<td>Thymic seeding progenitor</td>
</tr>
<tr>
<td>U</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
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</table>
Acknowledgements

When I got to the point of picking up a road, I have always chosen a tough but promising one. Because I understood that the easiest day was yesterday. I have prepared myself to go to medical school when I was in high school back in China. However, when it approached to officially becoming a physician, I’ve slightly changed my mind and set another goal as to pursue a Ph.D. degree in the United States. This turned out to be a very tough and painstaking choice. Fortunately, I got a lot generous helps from many people to keep my dream moving. The first great person to mention is Dr. Gordon Kauffman, who is the pivotal person getting involved in this process. The second ones are Dr. Richard Courtney and Dr. Jianming Hu. It’s their endeavors that made it impossible for me to start my days here at Hershey. I appreciate them from the deep of my heart for giving me such a precious opportunity.

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Last but not least, I should say thank you to my parents and the late grandparents. They did their best to raise me up, to send me to schools and to teach me how to be a real person. Maybe, they still did not quite understand why I came to the Unites States to pursue this Ph.D. degree. But they never blamed me for not paying them a visit in past years. Instead, their continuous loves and supports never stopped. Their encouragements and expectations have propelled and will keep propelling me to go further.
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Table 3 was partly derived from *J. Vis. Exp.* (63): e3986. Lei F, Haque R, Xiong X and Song J. “Directed Differentiation of Induced Pluripotent Stem Cells towards T Lymphocytes.” (2012), with permission from the Journal of Visualized Experiments.
Chapter I: Introduction

Cancer has been one of the leading health issues since the beginning of human history [1]. To both affected individuals and the whole society, it is a significant burden, in many aspects. Recently, although there are some slight fluctuations, the major trends of cancer morbidity and mortality have continued to increase. According to statistics from both the World Health Organization (WHO) and the U.S. National Institutes of Health (NIH), cancer right now ranks second among the leading causes of human death globally, only behind cardiovascular diseases [2]. Thanks to the significant advancements in both basic and clinical medical sciences over the past decades, the management of cancer patients has been substantially improved. However, there is no cure for the majority of cancers due to their nature of metastasis and mutation. Because of the increased socioeconomic impacts of cancer to our society, finding a cure for cancer has become imminently urgent.

Currently, mainstream cancer managements include surgery, cytotoxic chemotherapy and radiotherapy. Surgery treats cancer patients by physically removing the affected tissues. It is very effective in treating localized, early stage cancer; however, when cancer cells have spread or metastasized to distal organs, the therapeutic efficacy of surgery is significantly diminished. Chemotherapy and radiotherapy basically kill high turn-over rate cells such as cancer cells by disturbing the structures and/or functions of the cancer cells. The efficacies of these therapies depend on the sensitivities of the target cancer cells. For example, certain types of cancer are susceptible to chemotherapy and some others are sensitive to radiotherapy. By administering these treatments to cancer
patients alone or in a combined manner, some successes have been documented. However, as mentioned above, each one of them has certain limitations. For achieving a better outcome, new strategies are needed.

From progress toward understanding the human immune system, some new approaches to target cancer using the immune system have surfaced in recent years. It is well-known that the human body has its own arsenal, the immune system, to eliminate invading pathogens and endogenous dysfunction cells. In past decades, through tremendous efforts, the complicities of the immune system have been unveiled. Through the understanding of the immune system, several new therapeutic approaches have been developed, such as vaccination. Vaccination has substantially decreased the morbidity and mortality caused by a variety of infectious agents. For example, one milestone achievement of vaccination is the worldwide eradication of small pox [3]. Other than vaccines, new pharmaceutical agents have been developed by applying the discoveries in the immune system into the clinical setting. Monoclonal antibody-based drugs and other immune-modulating agents are good examples in this case. However, there are still a lot of unknowns to be addressed; further exploring of our immune system might give new insight in the context of fighting cancer.

The immune system is broadly divided into two major categories: the innate and adaptive immune systems. The innate immune system is relatively limited in terms of antigen recognition pattern compared to the more versatile adaptive immune system and it serves as the first line of host defense. The adaptive immune system is further subcategorized into two components, the humoral and the cellular arms. Both of them have a more stringent and specific targeting mechanism in the context of recognizing
antigens compared to the innate immune system. Meanwhile, from previous studies, it is shown that both humoral and cellular immunity can recognize antigen expressed on self-tissue which is collectively called autoimmunity. Autoimmunity such as rheumatoid arthritis is also claimed as one of several serious health issues in human society. However, the information obtained from the study of autoimmunity suggests that this could provide insight for a new mechanism to target cancer cell, which is also self-derived.

Cancer is the general name of a group of diseases that have a common feature: loss of highly-regulated cell growth control. Therefore, all clinical symptoms and signs in cancer patients are related to the deregulated growth of cancer cells. For instances, the mass effect, local invasion, distal spread and so on. From their names, although different types of cancer cells come from different self-tissue, they are not considered as the invading pathogens by the immune system so they are able to evade immune surveillance. However, not all cancer cells are immunologically invisible. In recent years, many cancer-related or cancer-specific proteins which are termed cancer antigens have been identified from the observation that many new or aberrant proteins are expressed during the process of malignant change. Alpha fetoprotein (AFP) is a good example of a cancer antigen [4]. AFP is normally expressed in human fetus but its expression levels decrease rapidly after birth. However, in some patients with hepatocellular carcinoma (HCC), the serum levels of AFP increase drastically. In the clinic, AFP serves as an important marker to monitor the tumor progression in the HCC patient. This story tells us, it is possible to find some abnormally expressed proteins or antigens in the normal tissue-derived cancer cells. And these characterized cancer antigens would potentially serve as the targets for the recognition of our immune system.
Under this guidance, several monoclonal antibody-based drugs that target mutated cancer proteins have been approved by United States Food and Drug Administration (FDA) and put on the market to treat cancer. For instances, rituximab in treating B cell lymphoma [5], trastuzumab in metastatic breast cancer [6] and bevacizumab in several different types of cancers including colorectal, lung, kidney and brain cancer [7]. The success of antibody-mediated cancer management has opened the door of using the components of our immune system to target cancer. Although the detailed mechanism of antibody-mediated therapy is still not clear, so far complement-mediated cytotoxicity and antibody-dependent cell-mediated cytotoxicity have been shown involved. Subsequently, a dendritic cell-based cancer vaccine Provenge has been approved by the FDA in treating advanced prostate cancer [8]. With these promising results, however, it is observed that some cancer patients do not respond to those anti-cancer therapies. One of the possible reasons is that such therapies could not directly kill the tumor. The final cancer execution needs additional cytotoxic mechanisms such as the presence of activated cytotoxic T lymphocytes (CTLs). This suggests that giving cancer patients CTLs might achieve better results than simply giving them these immune modulating agents.

Actually, many years ago, it has already been observed that a special phenomenon called the graft-versus-leukemic effect developed in many leukemia patients who received allogenic stem cell transplant [9]. Briefly, when donor-derived T cells developed from the engrafted allogenic bone marrow (BM)-derived hematopoietic stem cells (HSCs), they could not only reconstitute the induced lymphopenia but also specifically target and kill the residual leukemic cells, which resulting in a significant cancer regression. This is the first piece of evidence that T cells could recognize self-antigen. By
further studying this phenomenon, it becomes one of the foundations for carrying out an adoptive T cell transfer (ACT)-based cancer immunotherapy.

The other discovery that leads to the staging of the idea to give cancer patients T cells for therapeutic purposes is the isolation of tumor infiltrating T lymphocytes (TILs) in the cancer tissue [10]. In several types of cancer, during pathological evaluations, it is found that inside the tumor tissue there are a certain numbers of T cells residing. This piece of information indicates that there is an active interaction of T cells with the tumor tissue. Further study shows that after re-infusing the isolated and expanded TILs back to the cancer patients, they help control tumor growth. In some patients, a complete response was achieved. Taken together, it suggested that giving cancer patients tumor-reactive T cells might help build up a new strategy for cancer treatment.

Right after these pioneering works in designing a novel T cell-based cancer therapy by using TILs, some studies have shown that TILs could not be unanimously isolated from the tumor tissue [11]. Second, some isolated tumor infiltrating T cells are not reactive to tumor tissue [12]. Therefore, to stride over these hurdles, some groups began to investigate other approaches, such as to genetically engineer T lymphocytes with different antigen-recognition mechanisms so that T cells could be educated to specially kill the antigen-bearing target cells. By using different tumor-antigen targeting mechanisms such as artificial T cell receptor or chimeric antigen receptor, autologous T cells harvested from peripheral blood could be engineered to be tumor-reactive T cells and some therapeutic effectiveness in different clinical trials has been achieved [13-15]. The questions arisen from these studies are autologous T cells are not all healthy from the following observations; in some cancer patients, it is witnessed that their T cell pools are
shrunken or dysfunctioned [16]. An effective T cell-based therapy requires healthy T cells first; therefore it urges researchers to look for new sources of T cells, the key factor in building up a successful T cell-based cancer immunotherapy.

On the way to search for a new source of T cells, stem cells and their derivatives have drawn attention in the notion that every single cell in the body is developed from the stem cell, especially the embryonic stem cells (ESCs). In past years, several groups have announced the successful programming of stem cells into lymphocytes, including the T lymphocytes by a myriad of methods [17]. This gives scientists new ideas to improve the efficacies of ACT-mediated cancer immunotherapy. Owing to their unique properties of self-renewal and division, embryonic and other stem cells could serve as the source to generate an unlimited number of T cells, which could significantly boost ACT-based cancer immunotherapy. The challenge is to find a source for those stem cells. Because of the totipotency of ESCs, they are thought to be the best source of stem cells. However, it is impossible to obtain ESCs in the adult and it is ethically unacceptable to destroy human embryos for therapeutic purposes. Using allogenic HSCs for this situation is a feasibly alternative strategy to replace the ESCs. But the immunogenicity of allogenic tissue caused by major histocompatibility complex (MHC) mismatch should be addressed before carrying out any clinical work. This concern of avoiding immunogenicity also brings out another novel concept of the modern medicine, personalized medicine.

Personalized medicine admits that every single patient is different although they might have the same diagnosis. A specially-designed, individualized management approach should be considered for different patients based on their genetic background and other conditions such as metabolic activities and biocompatibilities of different
pharmaceutical agents. The idea of conducting personalized medicine roots in the awareness that different individuals respond differently to the same treatment or same drugs. For example, penicillin is a broadly used antibiotic used to treat many patients with gram-positive bacteria infection. However, certain people are allergic to penicillin exposure and sometimes death could happen due to the fulminant anaphylactic responses [18]. To avoid this penicillin-induced hypersensitivity, usually a skin-test will be given to exclude those potentially allergic individuals from being mistakenly given the drug. This is the early version of the current concept of personalized medicine, and the current one is more complicated. In the context of cancer treatment, for example, in patients with the broad definition of the breast cancer, some patients might have HER2/neu gene mutation but some might not [19]. Moreover, in androgen-independent prostate cancer, hormone-mediated therapy is futile because there is no hormone receptor expressed on the tumor cells [20]. To treat the patients with either breast cancer or prostate cancer, the pathological profiles of the tumors should be first evaluated to make the anti-cancer regimen more specific. An advantage of treating cancer patients with tumor antigen-specific T cells is its specificity. Only tumor or normal cells that express the relevant antigen will be targeted and other antigen-free tissues will be spared. Using allogenic stem cells to generate tumor antigen-specific T cells is acceptable; however approaches that to lower the impacts of MHC mismatch caused side effects should be considered.

How can adequate numbers of stem cells be easily generated for personalized medicine? In 2006, a ground-breaking report addressed this question above. The discovery of induced pluripotent stem cells (or iPS cells) breaks the deadlock of using human ESCs for both research and therapeutic applications [21]. Through the previous
observation that somatic cells could be reprogrammed back into stem cells by nuclei transfer, it is suggested that certain cellular factors might play key roles in regulating cell fate [22]. Under this hypothesis, scientists screened several known factors that regulate stem cell biology and finally, a combination of four factors: c-Myc, Oct3/4, Klf4 and Sox2 have been shown to switch terminally differentiated somatic cells back into the stem cell stage. Further analysis of these reprogrammed cells shows that they are similar to their natural counterparts—the ESCs in the context of morphology, genetic profiles, and the abilities to develop into the embryo, teratoma as well as fetus. Because of the resemblances of these artificial stem cells to ESCs but they come from different origins, they were given the new name: iPS cells. Since the first report about iPS cells came out in 2006, in as short as seven years, numerous iPS-related studies have been reported worldwide. Indeed, this cutting-edge study has made tremendous contributions to the modern society of biomedical sciences. It is not only a new tool to conduct basic scientific studies but also a therapeutical hopeful for treating many human diseases in the clinic.

In summary, other than improving current approaches, we need to find new strategies to treat cancer. The study of the human immune system suggests that supplementing tumor-reactive T cells might serve this purpose. Moreover, other studies of stem cell biology and T cell development have shown that tumor-reactive T cells could be generated from stem cells. To follow the trends of personalized medicine and to meet the goals of obtaining stem cells easily, iPS cells could serve as the substitutes of ESCs in generating an ideally unlimited number of highly-reactive, tumor antigen-specific T lymphocytes for ACT-based cancer immunotherapy.
One of the long-term goals of our laboratory research is to develop a comprehensive cancer therapy by harnessing immune mechanism and stem cell technology. More specifically, my studies focus on the iPS and T cells. Previously it is shown that both HSCs and ESCs are able to be induced to differentiate in to T lymphocytes in artificial settings such as \textit{in vitro} Notch signal stimulation or fetal thymus organ culture (FTOC). Based on the similarity between iPS cell and ESC, it comes to the central hypothesis of my thesis research that is iPS cells can be induced to develop into tumor antigen-specific CTLs for ACT-based cancer immunotherapy. To investigate this central hypothesis, we have developed several specific aims. Due to the paucity of data about iPS cells, the first thing to look into was the T lineage differential capabilities of iPS cells in comparing to their counterparts of ESCs and HSCs. In completing this specific aim, we have showed that, by using an \textit{in vitro} differentiation system, iPS cells were found similar to ESCs and HSCs in the context of T lineage differentiation. This was the first report showing iPS cells are capable of the T lymphoid lineage development.

We next evaluated the possibility to instruct iPS cells to develop into antigen-specific T cells and to enhance cancer immunosurveillance. To perform this study, a different \textit{in vivo} developmental system and murine tumor model were used. At the conclusion of this work, we further showed that iPS cells were able to develop into antigen-specific T lymphocytes \textit{in vivo} with the educational signals from the corresponding T cell receptor (TCR). Meanwhile, iPS cell-derived T cells could specifically target the antigen-expressing tumor cells and significantly enhance the survival rates of the tumor challenged animals. Following our previous work, this report
was featured as a novel mechanism of generating antigen-specific T cells for the ACT-based cancer immunotherapy.

In the aim of designing a better cancer immunotherapy with high efficiency, we were trying to find the detailed mechanisms that governing the T lineage differentiation as well as to test the efficacies of iPS-derived T cells in targeting naturally occurring tumor-associated antigens. To perform these studies, different approaches and experimental systems were used to test our hypothesis.

It is understood that the molecular network governing the T cell development from stem cell is vast and it is impossible to address everything in a short time. In dissecting this network, it is supported by other groups and our own data that TCR signal and Notch signal play individual roles in determining T cell development from stem cell. Therefore, it came to our hypothesis that Notch and TCR signaling pathways cross-talk during T cell development from stem cell. We have initially found that Notch and TCR played a synergistic role in promoting T cell development from stem cell but more in-depth studies are still needed to support this initial observation.

Second, for evaluating iPS-derived T cells in terms of interacting with naturally occurring cancer and its antigen, we picked the melanoma and its associated antigens as the model system. In this study, it focused on two components, the \textit{in vitro} induction and \textit{in vivo} maturation as well as tumor management. So far, cell lines have been established and characterized; and this work continues.

In conclusion, my thesis research provides the concept-proof work in studying the application of iPS cells in both immunology and cancer treatment. Our works, for the
first time, have showed that iPS cells were suitable to be engineered to become tumor antigen-specific CTLs. Also, iPS-derived, antigen-specific CTLs were able to control tumor growth in the animal model. However, it is only just a start. To finally put this idea into reality, tremendous efforts from all aspects are still required. And, although this process will be also winding and labor-consuming, with the fast-accumulating knowledge in both immunology and cancer biology, it is believed that a comprehensive, personalized cancer immunotherapy by using iPS cells will be available in near future.
Chapter II: Literature Review

A. Cancer Immunology and Immunotherapy

1. Cancer Biology and the Immunological Basis for Cancer Treatment

Cancer is a common name for a group of heterogeneous diseases in which cells exhibit deregulated growth. According to the tissue origin of the cancer cells, it is named differently. For example, squamous cell-derived cancer is generally termed a carcinoma [23] and mesenchymal originated cancer is called a sarcoma [24]. Although usually deregulated or dysfunction cells will be removed and destroyed by the host immune system through different mechanisms, cancer arises from the normal self-tissue, therefore, it can sometimes evade host immunosurveillance. A key concept in cancer biology is the expression of cancer-specific or cancer-associated antigen [25]. The aberrant expression of these cancer-specific or cancer-associated proteins is the foundation to utilize components from host immune system to destroy cancer cells.

A cancer-specific antigen is termed as the unique protein that expressed after the malignant change and not in normal tissue [26]. A cancer-associated antigen is defined as a universally expressed protein in a tissue, whether cells of that tissue are normal or have gone through a malignant change. As mentioned, these types of antigens could be recognized by the host immune system such as T cells, B cells and natural killer (NK) cells to exert a cancer killing subsequently through their unique mechanisms [27].

In the context of tumor antigen recognition, different types of immune cells have unique patterns of antigen targeting. In general, there are two major categories of the host
defense systems: innate and adaptive immune systems [28]. Innate immunity mediates the initial and first line immune responses towards microbial infections. It is also called the native or natural immunity, which has a fixed pattern of recognition that could only recognize certain particles or molecules expressed by the microbes. The members of innate immunity include epithelial barriers, neutrophils, complement, NK cells, and phagocytes. Because of the fixed pattern of this antigen targeting mechanism, it is difficult to engineer those components for attacking tumor cells, which merely express any molecules shared by an invading microbe.

The second line of defense is adaptive immunity; also called specific or acquired immunity [29]. The adaptive immune system consists of lymphocytes and their products such as cytokines and antibodies. As innate immunity recognizes certain structures shared by some classes of microorganisms, the lymphocytes in the adaptive immunity specifically recognize different microbial substances as well as noninfectious molecules by their surface-expressed receptors, namely, B cell receptor (BCR) [30] and T cell receptor (TCR) [31]. As mentioned, those substances or molecules that could be recognized by lymphocytes are collectively called antigens. The recognition patterns are, for instances, B cell recognizes B cell antigen through its BCR and T cell reacts with correspondent T cell antigen through its TCR. These recognition mechanisms could not only respond to invading foreign substances but also to some self-substances; for example, the autoimmune diseases are typically caused by overreacted adaptive immune system [32]. Meanwhile, the advantage of self-reaction is the foundation for designing a potential cancer immunotherapy because cancer cells are basically a group of uncontrolled self-tissues.
Adaptive immunity can be further divided into two subcategories, humoral and cellular immunity. Humoral immunity is mediated by B cells/plasma cells and their secreted antibodies [33]; meanwhile, cellular immunity is mediated by T cells [34]. Generally, humoral immunity plays a role in controlling extracellular microorganisms, and cellular immunity acts in eliminating intracellular microbes. Although the immune system has been categorized into two major types, they are not completely independent. Actually, the innate and adaptive immune systems are interconnected. Dendritic cells (DCs) and other antigen presenting cells (APCs) play a major role in connecting both innate and adaptive immunity [35].

For adaptive immunity in targeting cancer, B cells recognize cancer antigens by their BCRs and secrete antibodies and T cells target cancer by their TCRs. The TCR mediated antigen recognition is different compared to the BCR-involved antigen recognition because TCR can only recognize the MHC loaded antigen peptide. For example, TCRs on CD4⁺ T cells bind to class II MHC molecule (MHC-II) loaded antigen and CD8⁺ T cells recognize with MHC-I presented antigen through their TCRs [36]. This unique pattern of recognition makes the T cell mediated antigen targeting very specific and it becomes the basis for designing an effective approach to conduct precise cancer execution.

Typically, the intrinsically expressed cancer-specific or associated antigen will be further processed by the proteosome before being loaded on the MHC molecules [37]. After proteosome-mediated epitope-processing, the unique antigen epitope will be captured by the MHC molecule and expressed on the surface of the cancer cells. These MHC-loaded antigen epitopes attracts the corresponding TCRs expressed on the T cells.
The binding of the MHC-antigen to TCR will initially activate the T cells and following a second step of costimulatory signal stimulation, the T cells will be fully activated and exert the target cell killing through the cytotoxic effects. This fact serves as a simplified strategy to design a potential T cell mediated cancer killing mechanism.

On the other hand, B cell-mediated cancer antigen targeting is relatively free. No MHC molecules are required in this process. Also, no direct killing mechanism is involved in this process. B cell or antibody-mediated cancer killing is always associated with other mechanisms such as antibody-dependent cell-mediated cytotoxicity (ADCC) [38]. The targeting and killing strategy mediated by NK cells is simple, and because it belongs to the definition of innate immunity, its application in cancer immunity is significantly limited [39].

Taken together, the current understanding of the immune system and cancer biology, especially the identification of a myriad of cancer-specific as well as cancer-associated antigens in different types of cancers have paved the way for the successful development of a novel type of cancer treatment strategy, the cancer immunotherapy [13, 40, 41].

2. Cancer Immunotherapy

Other than the introduction of the cytotoxic chemotherapy in the curative treatment of some metastatic germline tumors and choriocarcinomas more than 30 years ago [42], there has been little progress in the development of a more curative cancer therapy. The only exception in recent years is cancer immunotherapy. Following a comprehensive understanding of the immune system, it is realized that by harnessing certain components of the host immune system, it could help design a better strategy to battle cancer.
Theoretically, any functional components in the immune system could be modulated for using in cancer immunotherapy. However, the anti-tumor efficacies of those immune components may vary depending on the roles they are playing in the immune network. As mentioned above, innate immunity basically recognizes a fixed pattern of structures/substances that are only found on intruding microbes, limiting its application in cancer immunotherapy which focuses on targeting self-tissue. The versatile molecule/substance or antigen recognition patterns of the adaptive immune system could serve as the basis of carrying out a possible cancer immunotherapy in the awareness that compared to the normal tissue tumor cells always express certain types of aberrant proteins or antigens. These tumor-specific antigens could be used to educate the adaptive immune system to be tumor-specific. Through intensive research, several major components of the immune system have been identified, which could provide therapeutic efficacies in cancer treatment, such as antibodies, cytokines, vaccines, and CTLs.

Antibody is the major product of activated B cells or plasma cells. Upon stimulation by a certain antigen, B cells will be activated and produce the relevant antibody that could specifically recognize the antigen. This mechanism plays a significant role in eliminating extracellular pathogens. From the understanding of some autoimmune diseases such as rheumatoid arthritis (RA) [43] and systemic lupus erythematosus (SLE) [44], antibody could also recognize and bind to self-tissue. This also suggests that an antibody could be used in cancer immunotherapy due to this binding property. Typically, an ideal tumor-specific antibody should acquire three major characters: specificity, affinity, and avidity. In search of a more potent antibody, the technique of monoclonal antibody (mAb) has surfaced. Literally, mAb is unanimously identical, which is selected,
engineered, and expanded from a pool of antigen reactive antibodies based on those three criteria listed above. With an identified tumor-associated antigen, the strong binding property of mAbs renders them as a good arsenal for tumor cells [45].

Currently, mAbs have had a significant impact on clinical oncology. The three top-selling anti-cancer drugs on market: rituximab [46], trastuzumab [47], and bevacizumab [48] are all mAbs. Because of the limitations in both \textit{in vitro} and \textit{in vivo} testing systems, the exact mechanisms of mAbs’ antitumor effects are still vague. However, strong data have supported that the immune responses play central roles in the killing of tumor cells, and, in particular, antibody-dependent cell-mediated cytotoxicity (ADCC) is the most important mechanism. In ADCC, after mAbs binding to the tumor cells with their Fab fragments, the Fc fragments on mAbs will bind to the Fc receptor (FcR) expressed on the NK cells, which execute the tumor lysis. This phenomenon has been well recorded in the clinical administration of rituximab [49]. The other possible mechanisms as observed in various studies include a combined anti-cancer action initialed by mAb binding and followed by activation of other members of the immune system such as DCs as well as complement system which mediates the complement-mediated cytotoxicity (CMC) towards tumor cells [50].

To achieve a better therapeutical result in killing tumor cells, mAbs have been further engineered and modified to increase the anti-tumor efficacy. One example is the increase of Fc fragment affinity to FcR [51], and the other includes the generation of bifunctional mAbs which could bind to tumor associated antigen and, at the same time, either recruit T cells to exert a CTL-mediated cytotoxicity or deliver anti-tumor drugs with engineered components on mAbs. As described, antibody mediated cancer therapy
requires a combined action of several components of the immune system, any defects in the system such as NK cell anergy could possibly fail the regimen. Extensive work needs to be done to further elucidate the mechanism of action of mAbs mediated killing of tumor cells, which will significantly benefit the application of mAbs in clinical oncology.

A second category of antibodies have been applied in cancer immunotherapy are the immune-modulating antibodies [52]. One important mechanism in modulating T cell function is the surface expressed T cell costimulatory molecules. In the two steps T cell activation model, without the engagement of stimulating costimulatory molecules, T cells will not be activated even when TCR has already bound to MHC loaded antigen [53].

There are two groups of costimulatory molecules, i.e., stimulatory and inhibitory, in order to maintain homeostasis of the immune system [54]. Stimulatory molecules activate the T cells, but inhibitory molecules shut down and/or inhibit the activation of T cells. In many cancer patients, it is well observed that circulating T cells have developed tolerance and/or anergy due to the stimulatory insufficiency or inhibitory prevalence. Hence, boosting stimulatory and/or blocking inhibitory costimulatory molecules might break the tolerance.

The immune-modulating mAbs either boost stimulatory or block inhibitory costimulation; they have been developed and clinical studies have shown promising outcomes in treating advanced tumor patients. For example, mAbs specifically blocking CTLA-4 ligation, an inhibitory costimulatory molecule highly expressed in several cancer patients, has gained initial successes in treating patients with metastatic melanoma.
and renal cell carcinoma [55]. Further studies on generating other costimulatory molecules-specific mAbs are still extensively undergoing.

Cytokines are a group of small molecules that are secreted by different types of cells. Usually, they play the roles of signal transduction messengers between different cells. In immunology, cytokine is the pivotal element in connecting different groups of cells. Different types of cytokines have different mechanisms of actions. Some are immunoenhancing and others are immunosuppressing. For example, in the superfamily of interleukins (IL), IL-2 is an immunoenhancing cytokine, which boosts the expansion and activation of T lymphocytes. In recent clinical studies, it is shown that administration of IL-2 could significantly inhibit the growth of melanoma and renal cell carcinoma by reversing the tumor-induced T cell tolerance, although it always accompanies with global, massive side effects [56]. In the context of boosting host immune system, other cytokines such as Interferon (IFN)-α and Granulocyte-monocyte colony stimulating factor (GM-CSF) have also been used clinically in the treatment of melanoma and several hematologic malignancies [57]. In contrast, the other IL superfamily members such as IL-6 and IL-10, play the opposite function in which they turn down the activated T cells.

In the intratumor microenvironment, it is found that IL-6 and IL-10 along with transforming growth factor (TGF)-β are highly expressed than in normal setting. Those cytokines are able to silence the TILs; thus, providing a mechanism evading normal immune surveillance [58]. Supplementation of immunoenhancing as well as blockage of immunosuppressive cytokines will possibly facilitate the achievement of a profound efficacy in a combined cancer immunotherapy.
The idea of cancer vaccine was proposed many years ago following the tremendous success of vaccination in the control of many infectious diseases; however, no major advance has been reported until recently. Although microbial components-derived vaccine could elicit strong protective adaptive immune responses due to the activation of the T cell responses, many clinical trials of cancer vaccine by using tumor-specific or tumor-associated antigens failed to arouse both CD4+ and CD8+ T cells to generate a noticeable anti-tumor response [59]. The successful cancer vaccine, Provenge, which has been approved by the FDA for treating advanced hormone-independent prostate cancer, is a DC-based cellular vaccine. Provenge has achieved a 4-month overall survival benefit in the past studies; however, it was not able to cure the patients with advanced prostate cancer [60]. Therefore, the DC-based cancer vaccine could only serve as a supplement to other mainstream of cancer therapies in the aim of getting a cure.

Besides cellular cancer vaccine, peptide-based cancer vaccine has also acquired certain positive results. A combined vaccine approach by using gp100, a peptide which is isolated from melanoma cells plus IL-2 has shown an improvement in patients with metastatic melanoma [61]. Also, No cure in this approach has been documented. In summary, current knowledge in basic immunology is still inadequate to develop an ideal cancer vaccine to render patients with an established anti-cancer immunity as vaccines to microbial infections have achieved. However, it is possible that better understanding of the immune system in future will help the development of a potent cancer vaccine such as polio vaccine.

The above mentioned anti-cancer strategies are indirect approaches, all of them need an executor in killing the tumor cells. The well-known executors in our immune
system are NK cells and CTLs. NK cells are a part of innate immunity and their killing patterns are not antigen-restricted. However, CTLs have a more versatile and precise mechanism of killing that is the TCR-mediated targeting and killing. This property is the basis of carrying out a directed, tumor-specific cancer therapy or the ACT-based immunotherapy. The idea of carrying out ACT-based immunotherapy is derived from the observation obtained in the treatment of hematologic malignancies by allogenic stem cell transplantation (alloSCT) [9]. It is found that the donor T cells are able to target and kill leukemic cells by recognizing the aberrantly expressed leukemic antigens, which is described as the graft-versus-leukemia (GVL) effect in the literature. From this discovery, it is recognized that T cells are able to respond to the autoantigens that are abnormally expressed on the surface of malignant cells, and this provides the first evidence for searching for tumor-reactive T cells.

Another substantial discovery is the identification of TILs in patients with melanoma [62]. Following surgical excision and processing of the melanoma tissue from patients, a group of T lymphocytes was isolated and expanded *ex vivo*. Because of their property of tumor tissue infiltration, they are named as TILs. This heterogeneous group of T cells shows different profiles of phenotype, antigen-reactivity, and functionality; however, after infusing back into the tumor-bearing patients following *ex vivo* expansion and manipulation, they are able to control tumor growth in some melanoma patients [63]. By further combining with preconditioned lymphodepletion which aimed at depleting immunosuppressive regulatory T (T\textsubscript{REG}) cells and myeloid derived suppressor cells (MDSCs) as well as cytokine supplementation in terms of boosting infused TIL persistence, this TIL-based therapy can cure patients with advanced, metastatic
melanoma. The existing problems for using TILs to conduct cancer immunotherapy are also quite clear: first, TILs could only be identified in a few type of cancer such as melanoma but not in other types of carcinomas [10]; second, recovered effector-phenotyped TILs are always short-lived compared to naïve-phenotype T cells [64]; third, because of the heterogeneous properties of TILs, many cells have different efficacies in targeting tumor cells [65]. Addressing these problems may lead to strategies suitable for the treatment to other types of cancers.

One approach that has been recently tested for improving cancer immunotherapy is the genetic modification of T lymphocytes such that they acquire a specific antigen-reactivity [66]. Two major strategies will provide T cells with identical and specific antigen-reactivity: 1) overexpressing a tumor-associated antigen specific TCR [67] and 2) introducing a chimeric antigen receptor (CAR) [68] that recognizes the tumor-specific antigen. Both have achieved substantial success in both artificial animal models and clinical trials in human patients.

The mechanism of generating tumor-specific, antigen-reactive TCR modified T lymphocytes for targeting and killing a tumor is straightforward and similar to the screening of a high-efficacy mAb. The most antigen-reactive T-cell clone is isolated and its recombined, full-length TCR gene is molecularly isolated. This TCR gene bears both antigen-recognition and signal-transduction capabilities and is subsequently introduced into naïve T (T\textsubscript{N}) lymphocytes. This enables them to target and kill the specific antigen-bearing tumor cells through the classical cytotoxic mechanisms [63].
For CAR, the mechanism of tumor targeting and T cell activation is somewhat complicated. A functional CAR is a single chain structure that consists of several different components: an antigen-recognition domain from a mAb, a transmembrane hinge domain and a T cell activation domain. Usually, a single chain variable fragment (scFv) of a tumor-specific mAb serves as the tumor/antigen recognition domain. The intracellular signaling domains of several different T cell activation molecules are recombined to signal T cell activation [69]. In general, when CAR binds the antigen via the scFv region, it activates its modified T cells through the intracellular signaling domain to express Perforin and Granzyme B for tumor cell execution [70]. The advantage of CAR is it is MHC-independent. Because TCR-based antigen recognition is MHC-dependent, the CAR is able to give T cell an extended targeting capability.

The general protocol of generating genetically modified T cells is more or less the same in both scenarios. First step is the most important, that is the identification of tumor-specific antigens and their correspondent TCR as well as CAR. This is the limiting step for carrying out a successful ACT-based immunotherapy by using genetically modified T cells. The expression profiles of the tumor-associated antigen, along with the specificities and affinities of TCR and CAR are both the determining factors. The second step is the isolation and ex vivo expansion of autologous T lymphocytes from the cancer patients to obtain a large number of T cells for the following genetic manipulation. These types of genetic manipulations provide T cells with antigen specificity and reactivity. In this step, either TCR or CAR is genetically introduced into the expanded T cells by a viral vector-mediated transduction. After ex vivo manipulation and characterization, TCR or CAR bearing T cells will be reinfused back to patients. In the very beginning, TCR-
engineered ACT was applied in the treatment of advanced melanoma that was shown to be effective [65]. As documented in recent studies, this regimen has been broadened into other types of tumors, for example, neuroblastoma [71], synovial cell sarcoma [72], leukemia [73], and lymphoma [74]. In the context of CAR-engineered ACT, the most important achievement is the management of B cell lymphoma by targeting CD19 molecule, a common B cell marker [75]. With current promising data and in the light of advancement in tumor associated antigen identification in different types of cancers, additional therapeutic proposals by using TCRs and CARs are under intense investigation.

Although ACT with genetically modified autologous T lymphocytes has gained tremendous therapeutic efficacies and even cure in certain cancer patients, it is still far from perfect [76-78]. The most important result from the use of autologous T cells is that most cells are already terminally differentiated into antigen-experienced effector T (T_{EFF}) cells. These cells are short-lived compared to T_N and memory T (T_M) cells, and _ex vivo_ expansion by cytokines and costimulation causes a sharp reduction in the cell numbers after reinfusion [79]. Second, terminally differentiated T cells have already expressed an original, functional TCR on their surfaces. Overexpression of an additional TCR will possibly cause TCR mispairing, which could greatly reduce the antitumor potency of the engineered T cells [80]. To find alternative sources of undifferentiated or less-differentiated T cells could help solve this problem because precursor cells do not have a mature TCR that would interfere with the introduced TCR, and more importantly, undifferentiated or less-differentiated cells are usually self-renewal capable which could probably provide a significant number of cells for the therapeutic purpose. In our current understanding of system biology, the best candidate that suits this scenario is stem cells.
B. T Cell Development and Phenotypes

1. General T Lineage Lymphopoiesis

The process of T cell development is a complicated multi-stage event that has many types of cells and regulatory factors involved. In mouse and human, the T lineage lymphopoiesis initiates from the HSCs and, depending on the site of lymphopoiesis, the whole process can be further divided into many stages such as BM stage, intra-thymic stage and post-thymic stage. In this section of the literature review, T cell differentiation will be discussed based on their sites of development.

BM is the starting point of the T cell differentiation. As one type of partially committed multi-potent stem cells, HSCs originate from the ESCs in the early stage of embryogenesis. And after hematopoietic determination, ESC-derived HSCs maintain their presence in different hematopoietic organs at different periods of development. In adult, HSCs reside in the BM niches to give rise to all lineages of blood cells. In the BM, HSCs are usually divided into two groups the long-term HSCs \( (\text{lin}^- c\text{-kit}^+ \text{Sca1}^+ \text{CD150}^+ \text{CD48}^- \text{CD34}^- \text{Flt3}^-) \) and short-term HSCs \( (\text{lin}^- c\text{-kit}^+ \text{Sca1}^+ \text{CD150}^+ \text{CD48}^- \text{CD34}^+ \text{Flt3}^-) \). At the same time of maintaining their self-renewal and pluripotency in the BM, HSCs will also give rise to a lesser differentiated or lineage-restricted hematopoietic progenitor cell, the multipotent progenitors (MPPs). MPPs are considered as the direct predecessors of both myeloid and lymphoid lineages. The HSC-derived MPP has lost the ability of self-renewal however still retains the ability of multi-lineage differentiation [81]. At this point, MPPs will go through a more restricted lineage commitment to differentiate into either common myeloid progenitors (CMPs) or common lymphoid progenitors (CLPs)
[82]. The CMPs are able to develop into the downstream myeloid precursors including megakaryocyte/erythrocyte and granulocyte/monocyte [82]. Hematopoiesis is very complicated process and my research is highly focused on the T lineage development from stem cells therefore the detailed myeloid developmental system will not be fully expanded in this review.

In searching for potential candidates of lymphoid precursors in the MPPs, a subgroup of cells called the lymphoid-primed MPPs (LMPPs) has been identified as lin$^-$ c-kit$^+$ Sca1$^+$ CD150$^-$ CD48$^+$ CD34$^+$ Flt3$^+$ in the mouse BM cells [83]. In addition, these LMPPs have lost the ability to develop into any megakaryocyte/erythrocyte lineage precursors but maintained the capability to develop into T lymphocytes, B lymphocytes and granulocyte/monocyte [84]. With the identification of LMPPs in the MPPs population, it is pointed out the possible direction for the search of other precursors in the lymphoid lineage commitment.

T lymphocyte development requires the involvement of the thymus [85]. However, LMPPs reside in the BM as other hematopoietic progenitors. Meanwhile, the thymic components do not acquire the ability to develop into any known T lymphocyte progenitor. Thus fresh T lymphocyte precursors are constitutively needed to maintain the production of mature T cells. This suggests that certain cells have migrated from BM to thymus during the T lymphopoiesis. By carefully analyzing the thymus-entry cells, a small fraction of cells called early thymic progenitors (ETPs) defined as lin$^-$ CD44$^+$ CD117$^+$ CD25$^-$ have been traced. Interestingly, besides T lineage development, ETPs are also able to generate myeloid cells [86]. This suggests that they are the direct successors of the LMPPs because they have not completely committed to the T lineage, and they are
supposed to be the predecessors of the CLPs. In the context of CLPs, they are able to give rise to both B lineage and T lineage cells; hence a bifurcating point to go to either B or T lineage should be present. In addressing this hypothesis, a unique surface molecule of Lyd6 has been proposed to be the marker of B lineage precursor in the CLPs [87]. Normally, Lyd6+ CLPs will commit to B lineage differentiation and Lyd6− CLPs will commit to T lineage. Moreover, another small fraction of cells called the thymic seeding progenitors (TSPs) are considered as the immediate T lymphoid progenitors that do not retain any myeloid potential [88]. These studies have stepwise narrowed down the search for the immediate T lymphoid progenitor. However, it is still challenging because the limitations in different experimental systems and techniques. For example, in the in vivo system, CLPs have lost myeloid potential but they still maintained both lineage potential in the in vitro system [89].

In conclusion, although a significant amount of work has been done to understand the whole process of the T progenitor development from HSCs in the BM, this gradual process involves multiple factors such as cytokines and cell signaling molecules. Due to the limitation of information as well as available techniques, to thoroughly interpret this process is still difficult currently. More substantial progress is still required to detail this complicated cascade.

Thymus is the major place of T cell development. Although the concept of TSP is still controversial because of its rarity, several studies suggest that TSPs are the predecessors of the ETPs [90]. After TSPs entering the thymus and interacting with the thymic epithelial cells, they will convert into the ETPs. Although ETPs can differentiate into myeloid cells in the in vitro culture system, the intra-thymic environment refrains the
myeloid potential of ETPs [91]. With this restriction, ETPs can start the cascade process to develop into mature T cells.

To introduce the intra-thymic T cell development, it is necessary to describe the thymic structures first. Typically, the intact thymus is divided into four major compartments. Each compartment has its own structure and function in the context of supporting the development of T cell in different stages. The four compartments, from the outmost to innermost, are named as subcapsular zone, the cortex, the corticomedullary junction and the medulla. In the context of the composition of these four different structures, the subcapsular zone is mainly occupied by cortical thymic epithelial cells (cTECs); the cortex is composed by a mixture of cTECs, fibroblasts and macrophages; a dense layer of endothelial cells line the corticomedullary junctions; and the inner medulla is filled by a mixture of DCs and medullary TECs (mTECs). The development and maturation of T cells in the thymus follow these spatial sequences from the cortex to subcapsular zone then back to cortex and finally into the medulla, and mature T cells exit thymus through the medullary blood vessels.

The brief process of T cell development is summarized in the figure 1. TSPs seed in the cortex region to develop into ETPs and following double-negative stage 1 (DN1) of the precursor T cells, Thereafter, DN1 stage precursor promotes to DN2 stage then migrates into subcapsular zone to further develop into DN3 and DN4 stages. DN4 stage precursors move back to cortex to keep differentiating into CD4⁺CD8⁺ double-positive (DP) stage precursor T cells and finally either CD4⁺ or CD8⁺ single-positive (SP) mature T cells are developed in the medulla. Fully developed T cells will enter the secondary
**Figure 1: Schematic illustration of intra-thymic T cell development.** This figure showed the simplified T cell development in different areas of the thymus. T progenitors enter the thymus in the corticomedullary junction then travel around the thymus to experience different signals for T cell development. At the end, mature T cells will leave the thymus through the blood vessels located in the medulla.

Legends: cTECs, cortical thymic epithelial cells; mTECs, medullary thymic epithelial cells; DCs, dendritic cells; TSPs, thymus seeding progenitors; DN, double negative; DP, double positive; SP, single positive.
Figure 1
lymphoid organs such as lymph nodes and spleen to experience antigen and play their various roles in the cellular immune system.

The detailed T cell development stage will be introduced based on the cell surface marker changes. Generally, the developmental stages of T cell differentiation are divided based on the surface expressions of CD4, CD8, CD25, CD44, CD117 molecular markers and the rearranged TCRs. In the following paragraphs, intra-thymic T cell development will be described based on these definitions and are presented in Table 1.

DN1 stage is the lineage commitment stage of T lymphocyte development in the thymus. DN 1 cells are a mixture of several types of early stage progenitor cells that reside in the corticomedullary junction. Because of the scarcity of the cell numbers, it is difficult to clearly distinguish these cells however the majority of DN 1 cells express a high level of CD117 [92]. As the early committed progenitors of T lineage differentiation, DN 1 cells are proliferatively robust in the observation that DN 1 cells only take up less than 0.01% of the total thymic T cells pool [92]. In the characterization of DN 1 cells, it is found that actually these cells are still capable of myeloid differentiation. Therefore, the T lineage commitment is not determined by the progenitor cells themselves but by the surrounding environment. Notch signaling pathway has been identified under the searching for this unique, environment-provided factor of the T lineage commitment [93, 94]. Because of its relative importance in T cell development as well as close relationship with our study, the Notch signaling pathway will be specially discussed in the following sections.
Previous studies have shown that conditional knockout of Notch ligands in the thymic tissue abolishes T cell development and promotes an aberrant B cell development in the thymus [93]. Further studies show that Notch ligand DL4 and Notch receptor 1 are the essential mediator of the T lineage commitment of DN 1 cells in the thymus [95, 96]. Other studies have also shown that Notch receptor 1 mediated signaling pathway could exclude the differential capabilities of myeloid lineage, B lymphoid lineage as well as DCs from the TSP/ETP stage [86, 97].

Taken together, when the early T lineage progenitor cells enter the thymus and under the stimulation of the Notch ligands expressed on the TECs, these cells would pick up the T lineage commitment and proceed to the next DN 2 stage for further development.

In DN 2 stage, DN 1 cell derived precursor T cell leave the corticomedullary junction and move up into the thymic cortex and the subcapsular zone. The most significant event in DN 2 stage of T cell development is the initiation of the TCR gene such as TCR-β chain, TCR-γ chain and TCR-δ chain rearrangements mediated by the \textit{Rag} gene [98, 99]. The TCR subtype chain rearrangement is the diversion of T cell subtype development. There are two subtypes of T cells based on the differences of TCR expression, the αβ T cells and γδ T cells. In this DN 2 stage of T cell development, it is found that based on the IL-7 receptor (IL-7Rα) expression, DN 2 cells could further subdivided into IL-7Rα$^{hi}$ DN 2 cells and IL-7Rα$^{lo}$ DN 2 cells. IL-7Rα$^{hi}$ DN 2 cells have been shown will go through the process to develop into γδ T cells and IL-7Rα$^{lo}$ DN 2 cells will maintain their ways to αβ T cells [100]. Following the T lineage development, based on the expression level of CD117, DN 2 cells are divided into DN 2a and DN 2b cells. The major difference between DN 2a and DN 2b stage is the narrowed lineage
commitment. DN 2a cells are still able to differentiate into DCs and NKs however DN 2b cells have completely lost these potentials [101].

In summary to the DN 2 stage, T lineage commitment will be further fortified and two distinct subtypes of αβ and γδ T cells have emerged from this stage after the TCR rearrangement initiation. These further committed precursor cells will move forward to the DN 3 stage for an ultimate T lineage determination.

DN3 stage of T cell development occurs at the subcapsular zone of the thymus. When either precursor αβ or γδ T cells get into the DN 3 stage from DN 2b stage, their fates are finally determined. In this DN 3 stage, the rearrangements of their TCR genes continue to carry out to generate the functional TCR chains respectively [102]. In γδ T cells, their γ chain and δ chain genes rearrangement is Notch signaling independent and their fates are determined solely the γδ chain genes themselves [103].

In αβ T cell, the situation is completely different. It has to pass a β-selection before entering the DN 3b from DN 3a stage. The setup of the β-selection checkpoint aims to test the complete signaling transduction mediated by a formed pre-TCR complex that consists by of TCR-β chain, CD3 molecule and an invariant pTα chain [104]. When αβ T cells pass β-selection, they will keep proceeding for future development and those αβ T cell who fail β-selection will undergo apoptosis. Other than the pre-TCR signal, CXCR4 and Notch1 mediated signals are also mandatory in this β-selection process, although these two signals are playing a cell proliferation role at this point [105, 106]. After going through the β-selection and moving into the DN 3b stage, pre-αβ T cell is ready for the
expression of a fully functional TCR complex as well as other selections processes lined up.

When pre-αβ T cell gets into the DN4 stage, they migrate again across the cortex, corticomedullary junction and towards the inner medulla. Along with process, the CD4 and CD8 genes will be expressed under the control of the pre-TCR signals. Also, the Rag gene will be activated again to mediate the rearrangement of the TCR-α chain gene [107]. When CD4, CD4 and TCR-α chain emerge on the surface of pre-T cell, it officially gets into the DP stage.

Before stepping into the DP stage, the fate of the precursor T cell has been decided. The most important event in this stage of the T cells development is to eliminate the T cells that have defects. Generally, a well-developed T cells should have a functional TCR that can specifically bind to the MHC loaded antigen peptide. Under this selection process, DP cells will interact with the cortex residing cTECs, DCs and fibroblasts in terms of using their TCRs to bind the MHC-peptide complex expressed on the surface of the latter type of cells. Only those DP cells bearing functional TCRs will survive; however, DP cells with defective TCRs will undergo apoptosis. This is called positive selection and only pre-T cells that have a functional TCR as well as a complete signal transduction pathway will survive this process [108]. Positively selected DP pre-T cells will keep moving inwards to the medulla for the final maturation. During this migratory process, DP cells will further commit to either CD4 or CD8 lineage for the final development of SP cells.
Finally, after a long journey traveling through the whole thymus from the first entry, the precursor T cells get into the SP stage with expression of either CD4 or CD8 molecules on their surfaces. Before being released into the circulation and homing to secondary lymphoid organs all around the body, these SP cells will have to go through a last checkpoint, negative selection [108]. Similar to positive selection, the SP cells will interact with certain thymic cells with their TCRs; however, different to the positive selection, the self antigen-reactive T cells will be eliminated by apoptosis but non-reactive or low-reactive cells will survive. The purpose of this negative selection is to minimize the generation of autoreactive T cells from the T lymphopoiesis. Passing the final negative selection means the whole process of T cell development is concluded. The T cells are certified to deploy to their residing secondary lymphoid tissues and organs all around the body.

At this time, the freshly made T cells have not experienced any foreign antigen therefore they are termed naïve T cell (T_N). After migrating out of the thymus, the fate of T_N will be determined by other factors to convert to different subtypes of cells, for example, effector T cells (T_EFF), effector memory T cells (T_EM), central memory T cells (T_CM) and memory stem T cells (T_SCM). Different T cells in different subtypes have different capability in term of antigen-response, proliferation and persistence rates. From T_EFF to T_CM, T_CM and T_SCM, the abilities of antigen-response, proliferation and persistence are drastically increased. Understanding of this information will be beneficial in gaining a maximal therapeutical efficacy for the T cell-based therapy.

The extra-thymic development of the different stages of T cells starts from the interaction of T_N with professional APCs. Upon the ligation of TCR to the MHC-bound
antigens on the APCs, the T_N cell is activated. A second activation signal called costimulation will further activate the T_N cell. After these “two signals” activation, T_N cell will get fully activated in the context of proliferation and differentiation. Some of the activated T_N cells will convert into T_EFF cell [109]. And the other might differentiate into T_SCM, T_CM and T_EM cells. Based on the cell surface markers, it is not difficult to distinguish these different populations. However, due to the short of background information and underdeveloped experimental system, it is somewhat hard to completely address the differential process. Therefore, three major hypotheses about extrathymic T lineage differentiation such as the lineage differentiation model [110], the bifuractive differentiation model [111] and the progressive differentiation model [112] have been proposed by different groups according to their discoveries. It is summarized in figure 2.

The lineage differentiation model suggests that primed T_N cell gives rise to T_EFF cell directly, and then T_EFF cell either goes to apoptosis or goes to develop into T_EM cells. T_CM cell is further developed from certain T_EM cells.

The bifuractive differentiation model suggests that primed T_N cell divides asymmetrically to give rise of different types of cells such as T_EFF, T_EM or T_CM cell.

The third progressive differentiation model proposes the primed T_N cell firstly develops into T_SCM cell then in the cascade of T_CM, T_EM and T_EFF cell sequentially.

Currently, these three different models all have their grounds and according to available knowledge, it is hard to get a consensus how these different types of T cells are formed. However, the discovery of these subtypes of mature T cells has significantly contributed to modern immunology, especially the therapeutic treatment approaches.
Figure 2: The hypothetic models of extra-thymic T cell development. Three proposed T cell differentiation models, *i.e.* the lineage, bifuractive and progressive models were summarized in this figure.

Legends: T\textsubscript{N}, naïve T cell; T\textsubscript{EFF}, effector T cell; T\textsubscript{CM}, central memory T cell; T\textsubscript{EM}, effector memory T cell; T\textsubscript{SCM}, stem cell memory T cell.
Figure 2

The lineage differentiation model

The bifurcative differentiation model

The progressive differentiation model
using T cells. For example, a small fraction of stem-cell like memory T cell or TSCM cell has been recently defined to have the most robust antigen-reactivity, proliferative rates and persistence time; therefore, obtaining TSCM cell in carrying out T cell-based therapy is the optimal choice.

The complete set of T cell development process has been briefly summarized above. For the convenience of characterizing the phenotypes of T cells under different stage, please refer to Table 1 for the surface markers of T cells under different developmental stages.

2. Signaling Pathways Involved in T Cell Development

There is a network of signaling pathways that crosstalk with each other to regulate T cell development at different times and locations. Although a fully-described network is still in vague, the three most characterized ones in the intrathymic T lineage commitment are chemokine, Notch ligands as well as cytokine TGF-β mediated pathways.

As described above, the homing of progenitor T cells as well as intra-thymic development have been linked to some chemokines and their corresponding receptors. Several notable pairs of chemokines and corresponding receptors include the ICAM-I and VCAM-I to P-selectin [113, 114], CCL21 and CCL19 to CCR7 [115], CCL25 to CCR9 [116]and CXCL12 to CXCR4 [117]. These chemokine pathways have been shown to either direct the homing and migrating process during the progenitor T cell development or provide survival signals to support the cell survival in the absence of other signaling ligands such as Notch ligands [117].
Table 1: Markers of T lymphocytes in different stages

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Surface markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long-term HSCs</td>
<td>lin^− c-kit^− Sca1^+ CD150^− CD48^− CD34^− Flt3^−</td>
</tr>
<tr>
<td>Short-term HSCs</td>
<td>lin^− c-kit^− Sca1^+ CD150^+ CD48^+ CD34^+ Flt3^+</td>
</tr>
<tr>
<td>LMPPs</td>
<td>lin^− c-kit^− Sca1^+ CD150^− CD48^− CD34^− Flt3^−</td>
</tr>
<tr>
<td>ETPs</td>
<td>lin^− CD44^− CD117^+ CD25^−</td>
</tr>
<tr>
<td>DN1</td>
<td>CD117^hi CD44^mi CD25^− CD24^−/lo CD27^mi</td>
</tr>
<tr>
<td>DN2a</td>
<td>CD117^hi CD44^mi CD25^− CD24^hi CD27^mi</td>
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<td>DN2b</td>
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<td>DN3a</td>
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<td>DN4</td>
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<td>DP</td>
<td>CD4^+ CD8^+ TCR-β^int</td>
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<tr>
<td>T_N</td>
<td>CCR7^+ CD62L^− CD27^+ CD28^+ CD45RA^− CD45RO^− CD122^+ CD95^−</td>
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<td>T_EFF</td>
<td>CCR7^+ CD62L^+ CD27^+ CD28^+ CD45RA^+ CD45RO^+ CD122^+ CD95^+</td>
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<td>T_SCM</td>
<td>CCR7^+ CD62L^+ CD27^+ CD28^+ CD45RA^+ CD45RO^+ CD122^+ CD95^+</td>
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<td>T_CM</td>
<td>CCR7^+ CD62L^+ CD27^+ CD28^+ CD45RA^+ CD45RO^+ CD122^+ CD95^+</td>
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<tr>
<td>T_EM</td>
<td>CCR7^+ CD62L^− CD27^− CD28^− CD45RA^− CD45RO^− CD122^− CD95^−</td>
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Abbreviations:

HSCs, hematopoietic stem cells;
LMPPs, lymphoid-primed multipotent progenitors;
ETPs, early thymic progenitors;
DN, double negative;
DP, double positive;
T_N, naïve T cell;
T_EFF, effector T cell;
T_SCM, memory stem T cell;
T_CM, central memory T cell;
T_EM, effector memory T cell.
Cytokine signaling pathway also gets involved in the process of the T cell development. The most prominent and well-studied cytokine signaling pathway is the transforming growth factor- β family (TGF-β) mediated pathway. One member of the TGF-β, TGF-β1 has been shown to be a pivotal regulatory cytokine in the in controlling T cell differentiation and homeostasis. The signaling transduction pathway from the ligation of TGF-β to its receptor to the activation of the effector gene involves a series of intracellular transduction cascade including Smads protein and could also involve either cross-talking with the components from other signaling pathway or directly acting on the transcription factors [118]. The mouse with TGF-β1 deficiency develops severe T cell-dependent sterile inflammation and dies early after birth [119]. Also, there are studies showing a profound cross-talk between Notch and TGF-β signaling pathway [120-122].

Although the role of TCR in determining T cell development is still not clear, several previous findings show that, the pre-TCRs signal determines the success of the β-selection [104] and in γδ T cells, the TCR γδ chains together determine the lineage fate of the cells [103]. This suggests that TCR signal plays key function in T cell development.

Furthermore, in recent years, several studies conducted by introducing TCR genes into the HSCs could have the TCR-bearing T cells developed from these HSCs in both in vivo and in vitro systems [123, 124]. Most recently, in a study, a reprogrammed iPS cell from the antigen-specific CD8+ cell could be induced to develop back into that unique T cell bearing the identical TCR once again through the in vitro differentiation system [125]. Taken together, these data strongly suggest that TCR signaling plays a critical role during T cell development.
The most important signaling pathway involved in T cell development is the Notch signaling pathway from the observation that Notch ligand knockout mice failed to develop mature T cells [126]. The Notch signaling pathway was first described in the *D. melanogaster* it provides the key modulator for wing development [127]. In following years, it has been widely accepted that this canonic pathway is actually involved in the developmental processes of many different types of cells and organ, including T cells [127]. The mammalian genome encodes four types of Notch receptors (Notch 1-4) and two families of ligands (Delta-like family and Jagged family). Notch receptor 1 has been shown to play a critical role in HSCs generation in the embryo; however, it is dispensable in HSCs maintenance and homeostasis in adult [128]. The governing mechanisms of the Notch signaling pathway in the hematopoietic system are largely unknown, except for an identified role in T lymphocyte commitment. Previous studies have outlined the possible relationship between Notch signaling and T cell development based on the observation that progenitor T cells express low levels of Notch receptors. Meanwhile thymic stromal cells express a higher number of Notch ligands, especially delta-like 1 and 4 (DL-1 and DL-4) [129]. Later, several studies showed Notch signaling pathway is the critical, indispensable component in T lymphocyte commitment from HSCs [130].

Binding of the Notch receptor with its ligand triggers a sequential proteolytic cleavage of the receptor. First it is cleaved at the S2 site in the juxtamembrane extracellular region, which is followed by cleavage with γ-secretase at the S3 site within the transmembrane region [131, 132]. These serial cleavages activate the pathway by releasing the Notch intracellular (NIC) domain, which traffics to the nucleus to interact with transcription factors to control the gene expression [133].
Through tremendous efforts in understanding the role Notch signaling plays during the T lymphopoiesis, it has been found that it mainly acts on the DN 1 and DN 3 stages of intrathymic development. However, these studies do not exclude the involvement of Notch in other stages of development. Furthermore, it has been shown that DL-4 ligand is more important for directing T cell development than is the DL-1 ligand in the intrathymic environment [95]. Besides these in vivo data, several in vitro 2-dimensional culture systems also support the idea that Notch ligand plays a critical role in T cell development. Unfortunately, because of the lack of information, additional studies are needed to fully explain the complex role that the Notch pathway plays.

3. In Vitro T Cell Development Models

In my thesis research, we focus on both in vivo and in vitro T cell differentiation model. The detailed in vivo T cell developmental process has been described above therefore it is helpful to introduce the two widely used and recognized in vitro experimental models for investigating T cell differentiation and development.

The first one is the fetal thymus organ culture (FTOC) or it is also called the 3D-thymus [134]. The FTOC is developed by Jenkinson group at the University of Birmingham, United Kingdom. The protocol of this technique requires the isolation of murine fetal thymus. The biggest advantage of this system is it completely mimics the physiological environment of intrathymic T cell development. Therefore, it supports a full range of in vitro precursor T cell differentiation. This technique provides an accessible model system to investigate the intrathymic regulation of T cell development.
In addition, it is a unique tool to investigate the development of chimeric T cell progenitors after bleaching the autologous thymic resident lymphoid cells [135].

The second is the OP9-DL1 system we have been actively using in our laboratory to investigate in vitro T cell development [136]. This system was initially developed by Zuniga-Pflucker group at the University of Toronto. Previous data showed that Notch signaling pathway ligand DL-1 plays a critical role in the development of T cells. By combining this Notch ligand with some hematopoiesis-supportive system, a novel in vitro T cell differentiation system could be setup. In searching for this differentiation-supportive tissue, a murine BM-derived stromal cell line OP9 has been identified. The constructed OP9-DL1 system together with cytokines like IL-7 and Flt-3 ligand could obtain a programmed T cell differentiation in the culture dish. One advantage of this 2-D system is that it avoids the use of animals. This system has been shown to support the development of T cells from HSCs, ESCs and other T lineage progenitor cells [17, 136].

C. Stem Cell and iPS cell

1. The Concept of Stem Cells

There are a group of specialized cells in all organism called stem cell or pluripotent cells because of their two unique properties of self-renewal and directed differentiation. Based on differential potencies, the stem cells are categorized into totipotent, pluripotent, multipotent, oligopotent and unipotent with a decreased capability of differentiation potency [137]. Totipotent stem cells could ideally differentiate into any kind of cells including extraembryonic cells in the body; pluripotent stem cells are descendentsof
totipotent stem cells with a slightly limited capability of differentiation but they can still
differentiate into basically all types of somatic cells found in the body.

Upon differentiation, pluripotent stem cells enter the stages of multipotent,
oligopotent and unipotent stem cells. Those cells have further limited potentials of
differentiation and could only differentiate into closely related cell family members [138].
On the other hand, according to the isolation stages of stem cells, it could be divided into
two broad types, the ESCs and the adult stem cells (ASCs). Generally, ESCs are equal to
the pluripotent stem cells and the following multipotent, oligopotent and unipotent stem
cells could be included into the definition of ASCs. The hierarchy of stem cell
differentiation is outlined in figure 3. Because of the plasticity and self-renewal capacity
of the stem cells, especially the ESCs, stem cells are potential candidates in regenerative
medicine [139]. Also, stem cell could be applied in deciphering the mechanisms as well
as progressions of the disease development [140]. However, the usage of human
embryonic tissue has drawn a lot of debates.

The first time of the description of stem cell property or pluripotent is made in 1891
that Driesch observed the development of two complete sea urchins by separating a sea
urchin blastocyst. Several decades later, in the 1960s and 1970s, different groups of
studies about embryo aggregation and blastocyst chimerism further support the notion
that the isolated cells from inner cell mass of the mouse blastocyst acquire the
pluripotency [141-143]. These early work in understanding the pluripotency finally led to
the isolation and characterization of the mouse ESCs in 1981[144]. Following this, in
1998, the Thomson group, for the first time, reported that human ESCs were successfully
**Figure 3: Hierarchy of stem cell differentiation.** The figure showed the hierarchy of stem cell differentiation from pluripotent to unipotent. The T lineage progenitors were specifically highlighted.

Legend: ESCs, embryonic stem cells; iPS, induced pluripotent stem cells; HSCs, hematopoietic stem cells; ASCs, adult stem cells; MPPs, multipotent progenitors; CLPs, common lymphoid progenitors; CMPs, common myeloid progenitors; LMPPs, lymphoid-primed multipotent progenitors; TSPs, thymus seeding progenitors; ETPs; early T progenitors.
Figure 3
isolated [145]. With these pioneering works in the early stage, many studies have been carried out to understand the different aspects of ESCs. Generally, ESCs can be in vitro cultured for a certain period of time and genetically modified without the loss of their pluripotency [146]. Meanwhile, the ESCs are capable to differentiate into all types of cells and tissues found in the body. For example, other than the extraembryonic membranes [147], ESCs are able to differentiate into all three layers: ectoderm, mesoderm and endoderm in the embryo [148]. These three layers of cells could further develop into downstream somatic cells such as HSCs, endothelial cells, neurons, cardiac cells and so on [149-151]. From this point, it is highly suggested that by using ESCs, different types of cells and tissues could be induced under different induction conditions. Potential clinical applications of ESCs become the ultimate driving forces to thoroughly understand the biology of stem cell, especially the ESCs.

As mentioned above, besides the ESCs, there are a group of stem cells found in the adult body which are collectively called the ASCs. For instances, HSCs [152], neuronal stem cells (NSCs) [153], mesenchymal stem cells (MSCs) [154] and their offspring are considered as the ASCs. ASCs are lineage restricted or called the multipotent, oligopotent and unipotent which indicate that they are only able to differentiate into the succeeding cells in the same lineage but are not able to initiate trans-lineage development. Under this principle of lineage differentiation, it is found that HSCs, under normal driven forces, are only able to develop into either myeloid or lymphoid cells such as T cells, B cells, NK cell, neutrophils, monocyte/macrophages, dendritic cells (DCs) and so on. In my thesis research, it is focused on the T lymphoid lineage development; therefore, HSCs will be favored other than the other types of ASCs.
HSCs, a large number of hematopoietic progenitor cells found in the body, are considered as the multipotent ASCs that are able to differentiate into both myeloid and lymphoid lineages of blood cells as well as themselves, or the sister HSCs [155]. Because of the importance of their roles in developing, maintaining and regenerating of the circulating blood, the HSCs are the best characterized cell population among all different types of cells and tissues in the entire human body. Thanks to this in-depth understanding of their biology, HSCs have been widely used in the clinical setting in the context of alloSCT in the treatment of some hematopoietic malignancies such as acute leukemia [156].

HSCs could be first identified in the yolk sac blood island of the early embryo of both mouse and human [157]. In the late fetal stage, HSCs could be isolated from fetal liver tissues. However in the adult, most reside in the BM niches around the body [158]. In mouse, the phenotype of HSCs is characterized as c-Kit$^+$, Thy-1.1$^{lo}$, lineage marker$^{-lo}$, and Sca-1$^+$ [159] and in human, the combination of CD34$^+$, Thy-1$^+$ and lineage markers are used to describe the HSC population isolated from the BM [160]. The isolated HSCs from both mouse and human donors have been shown to develop into different types of blood cells after transplant and reconstitute the recipients blood systems [161, 162]. Following these contributions, the developing process of hematopoiesis has been better described through the efforts by many scientists.

Generally, HSCs will give rise to the common blood progenitor, multipotent progenitor (MPP) cells after several rounds of self-renewal and differentiation [163, 164]. The MPP will keep differentiating into either common lymphoid progenitors (CLP) [165] or common myeloid progenitors (CMP) [82] under different induction conditions. The
CLP will further develop into different lineages of lymphoid cells such as B cells, T cells and NK cells [166]; however, CMP will give rise to granulocytes, erythrocytes, DCs, monocytes/macrophage, and platelets [167]. All this information further supports the concept of administrating alloSCT in the clinical managements of malignant blood-borne abnormalities.

My thesis research is aimed at generating T cells, especially antigen-specific T cells from the iPS cells. Previously, studies have shown that by using several in vitro systems that HSCs could be induced to differentiate into T cells [136, 168]. One involves a unique technique called FTOC and the other involved the simplified the Notch ligand stimulation system. The in vitro development of T cells from HSCs significantly solidifies previous studies and our hypothesis that iPS cells are able to develop into T cells under optimal conditions.

2. The Utilization of Stem Cells

Because of the unique property of stem cells in self-renewal and differentiation, massive efforts in translating basic stem cell research into clinical application have been diligently undergoing. The use of stem cells in clinical science has two major directions, the remolding of disease progression and the supplementing and/or substituting of defect or deficit tissues which is termed regenerative medicine.

In the generative medicine, the most prominent story of the clinical usage of stem cells is the alloSCT in the treatment of malignant blood cancers. This approach has been performed for years and many patients have gained substantial improvements in the
context of overall survival and quality of life. The message obtained from this approach is stem cells could be used in the clinics in treating various diseases including cancer.

Scientists are working on how to modulate stem cells for the treatment of other abnormalities like Alzheimer’s disease [169], Parkinson [170] and neuronal tissue injuries [171]. Although it is still far away from final clinical use, these studies have already shown promising preliminary data which could be further beneficial for its bedside application.

On the other hand, in the remodeling of diseases and drug screening, stem cells have better advantages than conventional cell lines. For example, in drug screening, stem cells could be used to test the toxicity in the desired tissue-specific pattern by some conditioned stem cell differentiation inductions [172]. In the stem cell-based disease remodeling, analyses to the characteristic phenotypes of the disease could be performed in either differentiated or undifferentiated stages [173]. Following the analyses and comparisons, a detailed disease model could be setup. Furthermore, a disease management approach such as drug designing could be subsequently performed by using the well-characterized disease model.

ESCs and its derivative ASCs have reshaped modern biomedical science. However, there are several big problems related to the use of tissue isolated stem cells. One is the ethical issue about using human embryo tissue which had incurred the banning of federal funding during the George Bush administration. The other is the technical problems that come from the isolation of ASCs from adult individuals. To better fuel the stem cell-related studies and applications, novel sources of stem cell are urgently needed.
3. Stem Cells in Immunology and Immunotherapy

HSCs are able to give rise to a full spectrum of blood cells including both myeloid lineage (monocytes/macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/platelets, and DCs) and lymphoid lineage (T lymphocytes, B lymphocytes and NK cells) [174]. HSCs in the adult are found in the BM niches with only a few cells circulating in the peripheral blood. It is possible to drive HSCs to enter the circulation, and this is described as HSC mobilization. By mobilizing HSCs with granulocyte-macrophage colony-stimulating factor and granulocyte colony-stimulating factor (GM-CSF and G-CSF), it is easy to harvest a significant number of HSCs from peripheral blood for clinical applications such as allogenic HSC transplant for the treatment of hematologic disorders and malignancies. Harvested HSCs can be expanded ex vivo via certain cytokines, such as stem cell factor (SCF), IL-3, IL-6, and thrombopoietin (TPO).

Because of their ease of isolation and ex vivo manipulation, HSCs have been widely utilized in both the laboratory and clinic. One of the most prominent applications of HSCs is the alloSCT-based treatment of leukemia, which aims at reconstituting the dysfunctional hematopoietic system in affected individuals [175]. After transplant, the donor-originated HSCs will engraft, expand and further differentiate into both lineages of blood cells, including T cells in the recipient, which is designated as bone marrow chimerism. In some patients, a significant GVL phenomenon could be observed. This observation not only helps foster the idea of conducting a T cell-based cancer immunotherapy but also intrigues scientists to find a fast and efficient mechanism to generate lymphocytes directly from HSCs. Under intensive research, the general
lymphopoiesis is concluded that both B and T lymphoid progenitors arise from HSCs. After stepwise differentiation from HSCs, B progenitors stay and get final mature in the BM; however, T progenitors will have to migrate into thymus to become matured, which indicates that there are significant regulatory events in the thymus that direct T lymphocytes development and maturation.

For the efficient generation of B cells from HSCs, it has been reported that in an *in vitro* BM stromal cell-based system, mature B-lymphocytes could be generated. In terms of T cell differentiation from HSCs; several models have been studied and one of the successful *in vitro* models is the FTOC, which is based on the coculture of HSCs with the isolated fetal thymus. The microenvironment in fetal thymus helps the differentiation and maturation of progenitor T cells that existed in the HSCs pool [176].

Other than the widely reported applications of HSCs in immunology and immunotherapy, the data about using other types of stem cells in the clinical setting are still short.

4. The Concept of induced pluripotent stem cells

Previous observation has indicated that nuclei transfer from stem cell to somatic cell converts the latter into stem cell which is called the somatic-cell nuclear transfer (SCNT) [22]. For instance, Dolly, the sheep was created by transferring nucleus substance into the adult mammary cells [177]. It is suggested that certain molecules are only expressed in stem cells but not in somatic cells which govern the fates of stem cells.

In 2006, a ground-breaking study from Shinya Yamanaka group for the first time showed that terminally developed somatic cells could be reprogrammed into pluripotent stem cells by introducing a combination of four transcription factors (*Oct3/4, Sox2, Klf4,
and \(c-Myc\) [21]. Through the endeavors of Takahashi and Yamanaka in understanding the intrinsic mechanisms in maintaining the pluripotency and self-renewal capability of the ESCs, they narrowed a series of 24 transcription factors to 10 and finally to those four key factors. By an ectopic, retroviral expression of a combination of these four factors, mouse somatic fibroblasts are switched back into their pluripotent status. The converted fibroblast cells resemble ESCs but not their prototypes in both morphology and function, such as genetic profile, stem cell marker expression, embryogenesis, and teratoma formation. This novel type of genetically modified somatic cell is pluripotent stem cell-like but not quite related to any known stem tissues. Therefore, it is named the induced pluripotent stem (iPS) cells. One year later, both Yamanaka [178] and Thompson group [179] reported the generation of human iPS from human somatic fibroblasts.

Following these works, a wide variety of human somatic cells have been tested for their capability of generating iPSCs. So far, human mature B cells [180], stomach [181], liver [181], pancreatic \(\beta\) cells [182], skin-derived melanocytes [183] and keratinocytes [184], adipose-derived stem cells [185], and neural stem cells [186] have been reported are able to switch back into iPSCs. Also, in order to efficiently and safely generate iPSCs from somatic cells, many new improvements in iPSC generation have been reported, such as lentiviral-vector [179] and inducible lentiviral-vector [187] based gene introduction. However, retroviral or lentiviral mediated gene deliveries always have a concern about tumorigenesis.

To avoid the potential problems caused by gene integration, an excisable gene delivery approach such as transposons [188] and \(loxP\)-flanked lentiviral vector [189]
have been introduced. Furthermore, non-integrating strategies such as adenoviral [190] and plasmid vector based gene deliveries [191] have been reported to be feasible.

Recently, several studies of pluripotency induction further indicate that several non-DNA based approaches are also able to generate iPSCs from human fibroblasts; for example, Sendai (RNA) virus-mediated gene delivery [192], direct protein delivery of transcription factors [193], delivery of modified mRNA encoding transcription factors [194], and microRNA based conversion [195]. The advancement of gene delivery techniques eases the safety issues aroused from the oncogenic-prone gene integration method. The second concern in the safety of using iPS cells in clinic is the introduction of potential oncogenes such as c-Myc. To overcome this, different combinations of transcription factors have been studied. In the previously mentioned studies, direct delivery of Oct3/4 and Sox2 proteins into somatic fibroblasts could generate iPS cells, although the efficiency is very low [196].

In summary, this information further confirms the concept of iPS cells and offers new hope and opportunity for modeling human diseases and designing a personalized medicine. The discovery of iPS cells could also avoid the constraints from using ESCs.

For the substitution of iPS cells to ESCs in laboratories and clinics, the most important issue is the similarity of iPS cells to ESCs. Well-described methods to evaluate the pluripotency functions of ESCs are also suitable in determining iPS cells [197]. Because of the relatively young-aged concept of iPS cells, it is understandable that there are still debates among scientists with different thoughts. It is believed that these debates will further promote the maturation of iPS technology in the future. Accordingly,
considerable information is still needed to establish more consistent and effective standards to evaluate the generated iPS before the application of iPS cells in both research and clinical setting.

5. Potential uses of iPS cells

The ultimate goal of studying iPS cells is to find a treatment for many human diseases, including cancer. Similar to the applications of ESCs and other ASCs, there are two approaches of using iPS cells in finding a cure: one is the remodeling of human diseases with patient-specific or disease-specific iPS cells, so that therapeutical strategy could be developed. The other approach is to repair or supplement defective and/or deficient cells or tissue with patient iPS cell-derived ones. Although only a few years have passed since the debut of iPS technology, many applications of iPS have already been reported in the literatures. In terms of remodeling human diseases and drug screening with iPS cells, the first study is done in patients with the familial dysautonomia, a genetic disorder of the peripheral nervous system, which characterized as extensive autonomic nervous system defects and small-fiber sensory neuron dysfunctions [198]. With the successful remodeling with affected tissue-derived iPS cells, the cellular and molecular properties of this disease have been carefully evaluated and a tentative management approach has been shown effective. This pioneering research unveils the new stage of iPS cell-based research in human diseases.

So far, iPS cells have been applied in major systems, including cardiologic, hematologic, metabolic, musculoskeletal, and neurological system. Approximately, nearly 50 different types of diseases have been reported by iPS cell remodeling, and some
of them have shown promising results; for instance, familiar dysautonomia, Rett’s syndrome [199] [200], type 2 long QT syndrome [201] and retinitis pigmentosa [202].

With the use of iPS technology together with further unveiling of disease mechanisms, additional iPS-derived models will be setup to benefit the exploration of effective therapeutical managements.

The other iPS-derived treatment option is regenerative medicine. It has been tried for a long time to find an optimal substituting mechanism to replace failed human cells, tissue, and organs. Although allogenic transplantation has been successfully carried out in past decades, concern about using allogenic tissue involves the post-transplant donor rejection or side-effects following immunosuppressant administration that aims at reversing or suppressing the tissue rejection. In clinics, physicians have been trying hard to balance these two events in post-transplant patients. Thanks to the development of iPS technology, it is suggested that iPS cell derived cells and tissue might help address the difficulties in finding suitable organs and battling immune rejections.

It is impossible to summarize all the advanced developments of iPS science here. To date, cardiomyocytes [203], chondrogenic cells [204], endothelial progenitor cells [205], male germ cells [206], hepatocytes [207], neural progenitor cells [208], osteocyte progenitors [209], pancreatic β cells [210], retinal pigment cells [211], and various types of cells in the hematologic system are derived from different types of iPS cells. The continuous advancements in comprehending stem cell biology will keep expanding the pool of differentiated cells from both natural and induced pluripotent cells for tissue engineering. However, currently all iPS-related works are limited in animal studies.
Clinical studies and following patient treatments are still not applicable until safety and efficacy issues are completely addressed in the pre-clinic setting.

In summary, the extensive researches in the iPS cell field provide the solid background to support our study in directing iPS cell to differentiate into T lymphocytes.

**D. Melanoma**

1. Melanoma and Its Associated Antigens

   From its name, the melanoma is developed from the normal skin-residing melanocyte after the malignant change. It is the most lethal type of skin cancer in the human. However, because of its superficial location of original occurring site, it is one of the most studied malignant cancers in the context of designing an effectively and comprehensive immunotherapy. Due to the identification of several highly specific melanoma-associated antigens and the discovery of TILs in the tumor tissue, it is an excellent model to study the interaction of tumor and host anti-cancer immunity. In our study of modulating a personalized cancer immunotherapy, this well-characterized interaction system has been adopted to facilitate our study. To specify the rationales for our proposed studies, the background information about melanocyte as well as melanoma will be stated in the following.

   Melanocyte, the pigmentation cell in higher vertebrate that generally resides in the skin, actually is not categorized as the genuine cutaneous tissue. From its origin, it belongs to the neuronal tissue histologically. The progenitor of melanocyte is derived from the neural crest formed in the gastrulation [212], and during the embryogenesis, it migrates outwards to the superficial skin where it resides. After differentiation and
migration, the melanocyte resides in the epidermal layer, or more specifically in the stratum basale of the skin [213]. The major role of melanocyte is to synthesize the pigment molecule in the skin—melanin—which is the critical component to protect the underlying cells from the photodamage caused by the ultraviolet (UV) radiation, especially the UV-B [214]. Because of presence of this unique melanin in the melanocyte, its synthetic pathway components could serve as the unique melanocyte associated antigens which are not present in other types of cells and tissues.

The biosynthetic pathway of melanin starts from tyrosine and ends with the eumelanin. The biochemical pathway of melanin synthesis is beyond the scope of our study however certain enzymes or proteins that highly involved in this pathway need to be specially addressed because of their unique expression patterns that only could be found in the melanocyte and melanoma. These enzymes or proteins are considered as the melanocyte or melanoma-associated antigens in the context of pursuing a targeting mechanism in the melanoma therapy. Among those antigens, the most important ones are tyrosinase [215], tyrosinase related protein -1 (TRP-1) [216] and TRP-2 [217]. These three major melanogenic enzymes are found unanimously expressed in both normal melanocyte and malignant melanoma but not those non-melanin producing cells. Actually, it is found that these melanogenic enzymes are highly expressed and are able to illicit some effective cellular immune responses towards them. Therefore, these enzymes are able to serve as the tumor-associated antigens for constructing an immunotherapy. In past decades, many studies have been carried out to target these melanoma-associated antigens.
Other than those melanogenic pathway enzymes, other antigens associated with either melanocyte or melanoma have also been discovered. For instances, melanoma antigen recognized by T-cells 1 (MART-1) [218], melanoma-associated antigens (MAGEs) [219] and gp100 [41] and etc. As mentioned, these melanoma associated antigens are the ground for outlining an effective melanoma, T cell-based immunotherapy. Therefore, it will be systemically introduced in the following section.

Normally, the development and regeneration of melanocyte are well regulated; however, under certain circumstances, such as the mutation of certain house-keeping genes, cells will go through malignant changes and melanoma formation is expected. In melanoma, the most involved pathway in the tumorigenesis of melanoma is the B-raf pathway. It is found that almost 50% of all melanoma patients have different mutations in the Raf protein [220]. Raf family proteins are the serine/threonine kinases that consisted by three major members, A-raf, B-raf, and C-raf [221]. As far as B-raf is concerned, there are more than 50 patterns of mutation have been identified [222]. And the most noted one is the B-raf$^{V600E}$ mutation in which the valine is replaced by a glutamic acid. B-raf$^{V600E}$ mutation has contributed to over 80% of all B-raf mediated melanoma development [223]. In recent years, from this discovery, many pharmacological agents that specifically target this B-raf$^{V600E}$ mutation have been developed and some of them have been put on market, such as the vemurafenib (PLX4032) in the treatment of late stage melanoma patients [224]. However, due to the high turn-over rates of the melanoma cells, the drug resistance is established shortly after the drug administration therefore the long-term survival rates of the patients could not be effectively achieved. Usually, the B-raf$^{V600E}$ mediated
activation is transduced through the mitogen-activated protein kinase (MAPK) signaling cascade, which regulated the cell cycle control and related cell proliferation [225].

Beside this major player in the melanoma development, other minor but also lethal mutations are also reported playing roles in the conversion of melanoma. C-Raf mutation has been shown to cause malignant changes via the MAPK pathways [226]. In addition, any mutations involved in B-raf downstream signaling such as the PI3K/AKT [227], KIT [228] and MEK/ERK [229] pathways could also lead to the development of melanoma. Accordingly, specific drugs in targeting these abnormal mutations have been developed but due to safety and efficacy issues, many are not suitable for the clinical purposes.

At the same time of developing pharmacological managements to melanoma patients, a novel immunotherapy of tackling melanoma is staging. As indicated before, certain types of proteins which are collectively defined as the melanoma associated antigen have been identified in past decades. MAGEs, MART-1, gp100, tyrosinase, TRP-1 and TRP-2 are among the most well characterized melanoma-associated antigens. The discovery of these antigens is the foundation to outline this novel melanoma immunotherapy.

MAGEs or melanoma-associated antigens are a large family consisting of two major types, MAGE-I and MAGE-II [230]. In the MAGE-I subfamily, there are MAGE-A, MAGE-B and MAGE-C groups of proteins. So far, only the MAGE-D group of proteins has been placed in the MAGE-II subfamily. In melanoma immunotherapy, the MAGE-I family has drawn a lot of attention because of its wide expression in a variety of malignant tumors, including melanoma and in germ-line cells but not in normal adult
tissue. A MAGE-A derived, human leukocyte antigen (HLA)-A*0201-restricted epitope that is relatively conserved in different members of this subgroup has been characterized, and this peptide epitope could illicit a strong CD8+ T cell response for target cells [231]. Because MAGE-I is only expressed in tumor cells, it may be possible to target these cells via this epitope and the corresponding T cell interactions. Further, in melanoma, a MAGE-A3 derived adjuvant immunotherapy has been in Phase III clinical trials in treating melanoma and non-small cell lung cancer [232]. In addition to MAGE family, other melanoma-associated antigens have also been characterized such as MART-1.

MART-1, melanoma antigen recognized by T-cells 1 (also known as Melan-A) is firstly identified by two different groups [218, 233]. MART-1 gene encodes a type III signal-anchor protein that has 118 amino acid residues. It is mainly localized in the endoplasmic reticulum and the trans-Golgi network, and it is also detectable in the early development stages of the melanosome [234, 235]. Although the detailed function of MART-I is still not clear, it acts as a strong immunogen for arousing a significant T cell response. Analyses of the tumor infiltrating T lymphocytes have shown that the majority of HLA-A2+ CD8+ T cells are MART-1 responsive. Furthermore, these cells express a memory phenotype in terms of CD45RO+ and CD45RAlow and CCR7[236]. This information indicates MART-I could be a good targeting antigen for melanoma-specific immunotherapy. Other than MART-1, other melanoma-associated antigens have also been described.

Gp100, a type-I transmembrane glycoprotein of 100kD, is highly homologous to the melanocyte-specific protein, Pmel17 [237]. Previously, a mAb that recognize gp100 has been widely used as a diagnostic tool to detect malignant melanoma [238]. Recently, it
has been demonstrated that gp100 could be processed and presented as a T cell antigen to induce the CD8+ T cell responses [239]. Meanwhile, CD8+ T cells not only recognize the gp100 expressed on the melanoma cells but also on the melanocytes [240]. Therefore, gp100 is categorized into the melanocyte-lineage or melanoma-associated antigen.

Tyrosinase is the key enzyme in the biosynthesis of melanin in the normal melanocyte and melanoma. It is a single chain type I membrane glycoprotein which catalyses the critical rate-limiting hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-dopa), which is rapidly converted into L-dopaquinone [241]. Besides its regulatory function in melanin synthesis, it is also a very important antigen that could be presented by MHC molecule and recognized by CD8+ T cells [242]. Due to its natural presence in melanocyte and melanoma, it is suggested that tyrosinase could serve as a melanoma-associated antigen to design the immunotherapy.

TRP-1, tyrosinase related protein-1, is the homolog of the tyrosinase in the melanosome of the higher animal, the major function of TRP-1 is to correctly traffic tyrosinase to the melanosome and stabilize the enzymatic activity of the tyrosinase [243]. As a melanocyte associated protein, similar to tyrosinase, it is found that after processing and presentation, TRP-1 could arouse a robust CD8+ T cell response [216]. According to its antigenicity, it could also be grouped into the melanoma-associated antigen for immunosurveillance purposes.

TRP-2, tyrosinase related protein-2, is also the other homolog of the tyrosinase in the melanosome of the higher animal. In terms of its functions, TRP-2 is involved in the detoxification processes taking place within melanosome [244]. In addition to the T cell
response that MHC-I loaded TRP-2 peptide could induce, TRP-2 based vaccines have been show effective in mediating the anti-melanoma immunity in the mouse model [245]. As summarized, TRP-2 is considered as a melanoma-associated antigen and can be used to screen for TRP-2 specific TCR or mAb for future melanoma management.

In summary, under different situations, the melanoma-associated antigen listed above could be processed to design a melanoma-specific cancer immunotherapy. In our study, through the collaboration with other groups, the corresponding TCRs that target either tyrosinase or TRP-2 have been chosen. In comparing to our collaborators system by using the mature T cells, our system could take the advantages of stem cells to get a significant larger number of T cells. These melanoma-associated antigens provide a powerful tool to target melanoma by the immune mechanisms. In addition, there are other therapeutical options in the field melanoma immunotherapy.

2. Immunotherapies Targeting Melanoma

The classical management of melanoma includes surgery, chemotherapy and radiation therapy. Because of various reasons, it is difficult to achieve an advanced therapeutical response. Surgery is only able to cure melanoma patients in the very early stage of the disease development; however, when the metastatic foci have occurred, it is barely useful to perform the surgical treatment. Chemotherapy and radiation therapy are able to control tumor growth in the early stage through different cytotoxic mechanisms, but when drug resistant or radioresistant tumor cells evolved in the long run, the therapeutical responses would get significantly compromised. Also, it always comes with several serious side-effects after administrating those cytotoxic therapies because of their
non-specific targeting. In the treatment of melanoma, it is urgent to raise the bar to obtain a specifically curative approach. Immunotherapy to melanoma has been shown promising, from the results acquired in recent studies.

Many strategies in immunotherapy have been tested to treat melanoma in past decades, different immune system components have been utilized as well. These successful studies involved the cytokines, antibodies, cancer vaccines and, the most important one, T cell-based immunotherapy. Clinical studies in treating melanoma by those listed approaches have gained solid ground in the past and possibly it will play a pivotal role in instructing the future direction of the melanoma therapy.

The melanoma immunotherapy could be divided into two major categories, the humoral immunity-mediated therapy and cellular immunity-mediated one. Cytokine, antibody and vaccine-based melanoma immunotherapies are in the first category. The simplified strategies of melanoma immunotherapy were summarized in figure 4.

The most prominent trial in cytokine-based immunotherapy is the administration of IL-2 in the management of metastatic melanoma [56]. Previously, it is found that IL-2 is the T cell growth factor therefore it comes to the hypothesis that IL-2 can promote the T cell activity in the cancer patients whose T cell-mediated anti-cancer immune responses are severely impaired [246]. According to this guidance, in some IL-2 based clinical trials in treating metastatic melanoma, 5-10% of the patients experienced a complete response and another 10% of a partial response after the treatment. Furthermore, 70% of the IL-2 responsive patients have been observed that no further tumor progression has recurred
Figure 4: The schematic illustration of melanoma immunotherapy. The melanoma immunotherapy was briefly divided into two categories, the humoral based and the cellular based immunotherapy. The humoral immunotherapy was composed by cytokines, antibodies and vaccines. And the cellular immunotherapy was based on the T cells but simply divided into two subtypes. One was the TILs based and the other was the genetic engineering of the peripheral T cells based.
Figure 4

Humoral Immunotherapy

- Cytokines
- Vaccines
- Antibodies

Cellular Immunotherapy

- TILS
- Peripheral T cells
- Ex vivo Expansion
- Genetic manipulation
- TCR-T
- CAR-T

Legends
- Treatment
- Cell isolation
- Ex vivo processing
Together with other promising results obtained, the FDA approved the high-dose IL-2 in the treatment of metastatic melanoma in 1998. Although high-dose IL-2 has therapeutical merit, it always comes with severe side effects.

The second type of immunotherapy by harnessing the humoral immunity is the antibody, especially the mAbs. Studies have shown that costimulatory signals are important in the context of activating resting and naïve T cells. Two major groups of costimulatory molecules have been characterized thereafter. One is the T cells activating molecules and the other plays the T cell suppressing function. It is found that either boost the activating or silence the suppressing costimulatory signals could greatly promote the T cell activation. In searching for these signals, cytotoxic T cell antigen-4 or CTLA-4 (CD-152) has been identified [248]. CTLA-4 is expressed on the T cells that could bind to its ligands B7.1 (CD80) and B7.2 (CD86) that expressed on the antigen presenting cells. The biology of CTLA-4 has been shown is the inhibition of T cell activation. This signaling pathway is mediated by SHP-2 and PP2A and also the PI3K, although the importance and consequence of this interaction are unknown [249]. With the discovery of CTLA-4, it is suggested that blocking this molecule could promote the T cell activation. In certain cancer, including melanoma patients, the T cell pool has been shown seriously dysfunctioned and some of the T cells have a very high expression of CTLA-4 molecules on the surface. This gives another rationale for developing the antagonistic antibodies to block the CTLA-4 in the aim of activating T cells. Ipilimumab or the anti-CTLA-4 mAb has been developed under these circumstances. In the following studies as well as clinical trials, ipilimumab has been shown effective. Especially in the treatment of metastatic melanoma, it has been observed to prolong the median survival time by 3.7 months.
compared to other treatment approaches [250]. However, only a small fraction of the patients are able to achieve a complete response. It is difficult to use ipilimumab alone for the metastatic melanoma treatment.

Cancer vaccine, as the other branch of the humoral immunity, has also been proposed in the treatment of metastatic melanoma. As introduced above, gp100 is a melanoma-associated antigen that could arouse certain anti-melanoma immunity. The gp100 derived peptide vaccine, gp100:209-217(210M) has resulted in recruiting a very high level of circulating T cells that are capable of recognizing and killing melanoma cells [251]. A recent study has even shown that by combining with IL-2, the gp100-derived melanoma vaccine could achieve a better clinical response in the treatment of metastatic melanoma compared to either IL-2 or vaccine alone [252].

These humoral immunity mediated melanoma therapy have acquired some breakthrough in raising the bar of targeting melanoma. The nature of humoral immunity determines that the presence of the final cellular immunity is mandatory in killing melanoma cells. In this scenario, supplementation of melanoma-reactive T cells fits the goal. Actually, in the past, better therapeutical achievements have been obtained in using melanoma fighting cells compared to the cytokine, antibody or vaccine-based studies. This approach is generalized as the ACT-based immunotherapy but according to the origins and preparations of T cells, it is usually divided into two categories. The first one is the administration of expanded TILs and the second is the genetically engineered T cells.
TILs are found residing in the tumor tissue, including melanoma. In the melanoma patients, TILs could be obtained from the excised tumor tissue and they could be further expanded \textit{ex vivo} in the laboratory. After re-infusing the ex vivo expanded TILs back into the patients, it is observed that certain tumor regression and extended survival have been attained [62]. The major drawback of this approach is that TILs could not be isolated from every patient and every tumor, a more generalized ACT-therapy is favored.

As introduced in the previous paragraph, many melanoma associated antigens have been described; therefore it is possible to conceive a new mechanism to kill melanoma cells by targeting these antigens expressed on the tumor cells. Following the discovery of these antigens, their corresponding TCRs have been isolated and characterized from tumor-reactive T cell populations. The concept of applying melanoma-specific TCRs in the immunotherapy is based on the ectopic expression of these TCRs in the mature T cells. Generally, TCRs are subcloned into retrovirus or lentivirus based vector to mediate the final gene integration in the recipient T cells. After gene integration, TCR genes are transcribed and expressed on the cell surface together with their original TCRs expressed on the recipient T cells. These overexpressed ectopic TCRs have been shown are able to recognize the MHC-bond melanoma-associated antigens that expressed on the tumor cells. In this approach, melanoma-attacking T cells are made by using the autologous T cells that isolated and expanded from the peripheral blood [253, 254]. In the literature, TCRs against MART-1, tyrosinase, MAGEs, gp100, TRP1 and TRP2 have been shown effective in the laboratory as stated above. In the clinical setting, ACT-therapy by using anti-MART-1 TCR has gained a complete regression of tumor in certain patients for at least 6 years [63]. Although it has also caused some side-effects originated from non-
specific attack of other normal melanocytes. The promising results indicate that this is one of the potential directions in targeting not only melanoma but also other tumors.

The third category of ACT-based therapy by using autologous T cells is called the CAR expressing T cells or CAR-T. The rationale of design CAR-T is very simple. Because TCR-mediated antigen targeting mechanism requires the participation of MHC molecule, the CAR can bypass this complicate process through combining a monoclonal antibody-derived antigen targeting domain with several T cell activation signal domains such as CD3 zeta, CD28 and 4-1BB [69]. CAR-T in treating melanoma has been reported by targeting the GD-2 molecule expressed on the tumor cells [255]. As an alternative or additive approach, CAR-T can also be proposed in the future treatment cancers.

Because of the superficial location and high mortality of melanoma, it has drawn a lot of attentions from both basic and clinical scientists. In the past decades, different areas of studies have been extensively carried out and in the context of finding a new treatment strategy, the melanoma immunotherapy has staged. In comparison of the different types of immunotherapy, these ACT-based melanoma immunotherapies have shown an increased efficacy than other humoral immunity-based immune modulating mechanisms. However, the biggest challenge in this ACT-based immunotherapy is the source of the T cells. Patient-derived T cells are the optimal candidates for the ACT-therapy. However, in many senior patients, the T cells are senescent and sometimes dysfunctioned therefore are not suitable for laboratory processing. In the searching for a new source of T cells for future immunotherapy, the stem cells, especially iPS cells have been noticed. By taking these experimental melanoma models, my thesis study focuses on figuring out these possibilities.
Chapter III: Materials and Methods

A. Animals

C57BL/6J, B6.129S7-\textit{Rag}^{1\text{-}tm1Mom}/J (Rag 1-deficient), C57BL/6-Tg (TcraTcrb) 1100Mjb/J (OT-I TCR transgenic) mice were obtained from The Jackson Laboratory. The mice were maintained and bred in the animal facility at the Penn State College of Medicine. All mice were grouped at 4-5 per cage and food and water were given \textit{ad libitum}. No experiments were conducted until mice were acclimated for at least one week.

All experimental procedures were carried out in compliance with the regulations of the Pennsylvania State University College of Medicine Animal Care committee in accordance with guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care and National Institutes of Health.

B. Cell lines and Medium

All cell lines were maintained at 37°C and 5% CO$_2$.

1. iPS cell line

iPS-MEF-Ng-20D-17 cell line, which was induced from mouse embryonic fibroblasts (MEF) by retroviral transduction of \textit{Oct}3/4, \textit{Sox}2, \textit{Klf}4 and \textit{c-Myc} factors was kindly gifted by Dr. Shinya Yamanaka at the Institute for Frontier Medical Sciences, Kyoto University through the RIKEN cell bank [256]. iPS cells were maintained on irradiated SNL76/7 feeder cells in Dulbecco’s Modified Eagle’s Minimal Essential medium (DMEM) supplemented with 15% (v/v) heat-inactivated fetal bovine serum
(FBS) (Hyclone), 0.1mmol/L nonessential amino acid (NEAA), 1mmol/L L-glutamine (both from Invitrogen), 0.1mmol/L β-mercaptoethanol (Sigma) and 100U/mL penicillin-100μg/mL streptomycin (Invitrogen).

2. SNL76/7 cell line

SNL76/7 cell line was obtained from the American Type Culture Collection (ATCC). It is clonally-derived from a STO cell line that expresses leukemic inhibitory factor (LIF) at an abundant level [257]. This cell line was used as a feeder layer to support the growth of iPS cells. SNL 76/7cells were maintained in DMEM supplemented with 10% heat-inactivated FBS 100U/mL penicillin-100μg/mL streptomycin (Invitrogen). When cells reached confluency in culture, they were trypsinized by 0.05% trypsin-EDTA (Invitrogen) and pellet down for irradiation. Irradiation of SNL76/7 cells was performed in a 60Co irradiator with a dose of 5000 Rads. After irradiation, cells were resuspended in FBS with 10% (v/v) dimethyl sulfoxide (DMSO) and then cryopreserved in liquid nitrogen for future use. Irradiated SNL76/7 (irSNL76/7) cells were usually seeded on 0.5% gelatin pre-coated culture dishes one day prior to any iPS culture.

3. OP9 cell line

OP9 cell line was obtained from ATCC. OP9 cell line, a bone marrow stromal cell line, was established from newborn op/op mouse calvaria [258]. They were normally maintained in α-Minimal Essential Medium (α-MEM) (Invitrogen) in the presence of 20% FBS and 100U/mL penicillin-100μg/mL streptomycin (Invitrogen).
OP9-DL1 cell line was obtained from Dr. Juan Carlos Zuniga-Pflucker at Department of Immunology, University of Toronto. OP9-DL1 cell line has been shown specifically support the T lineage direction of differentiation from stem cells [136]. Briefly, these cells were normally maintained in α-Minimal Essential Medium (α-MEM) in the presence of 20% FBS.

5. E.G7-OVA cell line

E.G7-OVA cell line was obtained from ATCC. It was derived from the C57BL/6 mouse lymphoma cell line EL4 by inserting a single copy of the plasmid containing chicken ovalbumin sequence (OVA). It synthesizes and secretes OVA constitutively and its surface expresses a MHC-I restricted H-2Kb molecule loaded OVA\textsubscript{257-264} peptide [259]. This MHC-peptide moiety could be recognized by OT-I TCR. Generally, E.G7-OVA cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen) supplemented with 10% FBS and 100U/mL penicillin-100μg/mL streptomycin (Invitrogen).

6. B16-F10 cell line

B16-F10 cell line was obtained from ATCC. It was derived from the melanoma of C57BL/6 mouse. B16-F10 cells express the tyrosinase-related protein 2 (TRP2), a melanocyte differentiation antigen that could be commonly found in melanocytes and melanoma [217]. Normally, B16-F10 cells were maintained in the DMEM supplemented with 10% FBS and 100U/mL penicillin-100μg/mL streptomycin (Invitrogen).

7. Platinum-E cell line
Platinum-E (Plat-E) retroviral packaging cell line was a kind gift from Dr. Toshio Kitamura at the Department of Hematopoietic Factors, Institute of Medical Science, University of Tokyo. Plat-E was generated based on the 293T cell line by expressing a Moloney Murine Leukemia Virus (MMLV)-based retroviral packaging constructs gag-pol and env under the EF1α promoter [260]. This cell line was used to perform the packaging of pseudovirus for following retroviral transduction. Plat-E cells were maintained in DMEM supplemented with 10% FBS and 100U/mL penicillin-100μg/mL streptomycin (Invitrogen).

C. Vectors and genes

Mouse stem cell virus (MSCV)-based retroviral vector expressing green fluorescent protein (GFP) tag MSCV-IRES-GFP (pMiG) was kindly provided by Dr. David Baltimore at California Institute of Technology [261]. Retroviral vector of pMiDR that express red fluorescent protein tag was constructed by replacing the GFP gene in pMiG with the red fluorescent protein DsRed gene. OT-I TCR gene linked by a 2A sequence was a gifted by Dr. Dario A. A. Vignali at St. Jude Children’s Research Hospital. TRP-2 TCR Vα17 Vβ11 genes were gifted by Dr. Arthur A. Hurwitz at the National Cancer Institute (NCI) [262]. 2A sequence linked anti-tyrosinase TCR (ATT) was a generous gift from Dr. Richard A. Morgan at the Surgery Branch, NIH [242]. Notch ligand Delta 1 and Notch ligand Delta 4 were generously gifted by Dr. Freddy Radtke at Swiss Institute for Experimental Cancer Research.

D. Generation of retroviral constructs

1. Generation of MiDR vector
MiDR vector was constructed based on the backbone of pMiG vector. To make MiDR vector, the IRES-GFP fragment of pMiG was replaced by IRES-DsRED fragment. Double nuclease digestion (EcoRI and SalI, New England Biolabs) was performed to generate this vector. (Figure 5)

2. Generation of OT-I-MiDR

OT-I fragment was cut from the OT-I MiG vector and inserted into the MiDR vector by double nuclease digestion (BamHI and EcoRI, New England Biolabs).

3. Generation of TRP2-MiDR

TRP-2 TCR Va17 chain and Vβ11 chain genes were subcloned in pcDNA3.1 vector separately. To make a picornavirus 2A peptide linked single fragment which has an equal expression of both genes, Va17 and Vβ11 genes were amplified by polymerase chain reaction (PCR) with a 2A sequence introduced in. Amplified Va17 and Vβ11 were inserted into the MiDR vector (BglII and EcoRI, New England Biolabs). PCR primer designs please refer to Table 2.

4. Generation of ATT-MiDR

ATT gene Va chain and Vβ chain was originally subcloned into pMSGV1 vector with a 2A sequence linker. To make ATT-MiDR, PCR was performed to amplify ATT gene and it was inserted into the MiDR vector subsequently by double nuclease digestion (BglII and XhoI, New England Biolabs). PCR primers please refer to Table 2.

5. Generation of DL1/DL4-MiG
Figure 5: The schematic presentation of the generation of MiDR retroviral vector.

Generally, the MiDR vector was constructed by replacing the IRES-GFP fragment in the backbone of MiG vector with the IRES-DsRED fragment.
Figure 5

Diagram showing the structures of pMiGR, pMiRII, and pMiDR with highlighted genetic elements and restriction sites.
Table 2: The Primers of PCR and qRT-PCR assays

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Directions</th>
<th>Primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRP-2 Vα17</td>
<td>F</td>
<td>5’-ATTAGATCTCCACCATGCTGATTCTAAGCCTGTTG-3’</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5’-AATTCCGGAACACTGGACCACACGCCTCAG-3’</td>
</tr>
<tr>
<td>TRP-2 Vβ11</td>
<td>F</td>
<td>5’-AATTCCCGGACGACACTTCTCTCTGTTAAAGC AAGCAGGAGACGTGGAAGAAAAACCCCGTCCCATG GCCCCAGGCTCTTTCTGTCTG-3’</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5’-TAAGAATTCTCGGAATTTTTTTCTTGGAC-3’</td>
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<tr>
<td>ATT TCR</td>
<td>F</td>
<td>5’-ATTAGATCTCCACCATCTCCATGGACTCATAC-3’</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5’-TATCTCGAGGACTACGTCGCCGCTCATGAAAT TC-3’</td>
</tr>
<tr>
<td>DLL1</td>
<td>F</td>
<td>5’-AAATTCTCGAGCACCACCTGGGCGTGAGCGGCCTA-3’</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5’-TTGCTTTAACAGGAGGAGTTTCGTTGCGTAACTTCCACCTCAGTCTAACA -3’</td>
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<tr>
<td>DLL4</td>
<td>F</td>
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</tr>
<tr>
<td></td>
<td>R</td>
<td>5’-TTTGAAATTCTTATACCTCTTGCGGACATACACAC TC-3’</td>
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<tr>
<td>OT-1 Vβ 5</td>
<td>F</td>
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<tr>
<td></td>
<td>R</td>
<td>5’-GCGAAGGCAGCAATTATGCTG-3’</td>
</tr>
</tbody>
</table>

A: Adenine; T: Thymine; G: Guanine; C: Cytosine;  
5’: 5 prime; 3’: 3 prime; F: Forward; R: Reverse.
DL1 and DL4 genes were subcloned into pMiG2 vector when they arrived. To make a polycistronic fragment co-express both DL1 and DL4 genes equally, DL1 and DL4 genes were amplified by PCR with a 2A linker introduced in between. Amplified fragment was inserted into pMiG vector by double nuclease digestion (XhoI and EcoRI, New England Biolabs). Primer designs for PCR are listed in table 2.

E. Retroviral transduction protocol

Plat-E packaging cells are used to generate pseudovirus that were used for the following retroviral transduction. $3 \times 10^6$ Plat-E cells were seeded on a 100mm culture dish one day prior to transfection. On day 0, Plat-E cells were transfected with different retroviral vector plasmid by using GeneJamma (Stratagene) transfection reagent. On day 1, $1 \times 10^6$ iPS cells were seeded into one well of a 0.1% gelatin (Sigma) precoated 24-well plate. On day 2, pseudovirus-containing supernatant from Plat-E culture were collected and passed through a 0.4 μm filter to exclude potential contaminants. Transduction was performed under the condition of 32°C centrifuge at 1400rpm for 1 hour in the presence of 5 µg/ml polybrene (Sigma). After centrifuge-based transduction, cells were placed in 32°C, 5% CO₂ incubator over night. On day 3, the day 2 transduction procedure was repeated as described above. At the same time, a 6-well plate will be precoated with irradiated SNL76/7 (irSNL76/7) feeder cells for future use. On day 4, transduced iPS cells were trypsinized off, centrifuged at 400g for 5 min and seeded on precoated irSNL76/7 feeder cells. At confluency, transduced cells would be trypsinized off, centrifuged at 400g for 5 min and processed for either flow cytometric analysis or cell sorting. Figure 6 showed the generalized scheme of retroviral transduction.
Figure 6: The schematic presentation of the protocol for retroviral transduction.

The general protocol of the retroviral-mediated gene transduction is summarized as below.
Figure 6

Pre-transfection Plat-E cells

48 hours

Post-transfection, pseudovirus producing Plat-E cells

2x 32°C 1400rpm spin for 1 hour

iPS cells
F. Cell sorting

Cell sorting was performed in the flow cytometry core facility at the Penn State College of Medicine. Two types of cell sorter, MoFlo high-performance cell sorter (Dako Cytomation) and FACSaria high-speed cell sorter (Becton Dickinson) were used to perform cell sorting. Cell sorting was carried out based on either internal fluorescent protein expression or fluorochromes conjugated antibodies that specifically recognize the targeted cell surface markers. Sorted cells were centrifuged down and re-seed either on plain culture dish or feeder cells precoated culture dishes.

G. In vitro differentiation protocol

In vitro differentiation system is used to evaluate the T lineage differentiation potential of iPS cells. A confluent layer of different OP9-derived feeder cells (for example OP9, OP9-DL1, OP9-DL4 and OP9-DL1/DL4 cells) was prepared before the start of the experiment. At Day 0, 5×10^4 iPS cells were seeded on a 100mm culture dish containing confluent OP9-derived cell monolayer in 20% FBS α-MEM media. At Day 3, culture media were changed with fresh ones. At Day 5, cells were trypsinized off and centrifuged at 400g for 5 min before incubating on a fresh 100mm culture dish for 30 minutes in 37°C incubator to exclude excessive feeder cells. Floating cells were collected and counted, 1×10^5 cells were transferred to a fresh culture dish containing confluent OP9-derived feeder cell monolayer in 20% FBS α-MEM media. Cytokine mouse Fms-like tyrosine kinase 3 ligand (mFlt-3L) (final concentration: 5ng/mL) were added in the culture at this time. At Day 8, loosely attached cells were gently pipette down. The OP9-derived feeding layer would be washed with 10mL PBS one more time to get the
maximal recovery of partially differentiated iPS cells. After harvesting cells from the coculture, cells were centrifuged at 400g for 5 min and resuspended in 20% FBS α-MEM media supplemented with mFlt-3L (5ng/mL) and mouse interleukin 7 (mIL-7) (1ng/mL). At the end, cells were transferred into a 6-well culture plate coated with confluent OP9-derived feeder cells. Usually iPS cells recovered from one 100mm culture dish would be transferred into one well of the 6-well plate. From day 10, culture media were changed in every other day (20% FBS α-MEM media supplemented with mFlt-3L (5ng/mL) and mIL-7 (1ng/mL). At the same time, culture plates coated with OP9-derived feeder cells will be changed in 4-6 days depending on the growth of the feeder cells. At the different time point of in vitro differentiation, iPS-derived precursor T cells were harvested for different studies. The simplified sketch is described in Figure 7.

**H. Adoptive transfer**

This aims at introducing either differentiated or non-differentiated iPS cells into mice to monitor their development. Briefly, iPS cells were trypsized off, centrifuged at 400g for 5 min and incubated on a fresh culture dish in 37°C for 30 minutes to exclude feeder or partially differentiated cells. After that, floating cells were collected and passed through 70 μm nylon strainer to exclude cell clumps which might cause pulmonary embolism to mice. Those cells were washed three times in cold PBS as well to exclude FBS which might cause anaphylaxic reactions in mice. At the end of processing, cells were resuspended in phosphate buffered saline (PBS) with a concentration of 1.5×10^7 cells/mL and they were maintained on ice before injection. Before doing intravenous (i.v.) injection through the tail vein, mice were placed under an infrared light to dilate their tail vein. After vein dilatation, 200 μl cell suspensions or 3×10^6 cells were adoptively
Figure 7: Simplified sketch of the in vitro differentiation system. This system is used to induce iPS cells for the T lineage differentiation *in vitro*. 
Figure 7

OP9-DL1 cells → iPS/TCR cells → Characterizations

D5 Co-culture → D22 and following days Co-culture
transferred into a 4-6 week old mouse through its tail vein. Adoptive transfer of lymphocytes isolated from different strains of transgenic mice also followed this protocol.

I. *In vitro* proliferation/activation assay

*In vitro* proliferation assay is designated at testing the function of iPS cell derived precursor T cells in terms of receiving costimulatory stimulations. One day before proliferation/activation assay, a 24-well plate was pre-coated with anti-CD3 antibody (BD Biosciences) (final concentration: 4 μg/mL, in PBS) at 4°C over night. At day 22 of *in vitro* differentiation, iPS cells derived precursor T cells were harvested from culture and washed with cold PBS before stimulating with plate-coated anti-CD3 and soluble anti-CD28 antibodies (BD Biosciences) (final concentration: 4 μg/mL). After that, cells would be incubated in 37°C, 5% CO₂ incubator for 40 hours and then Befeldin A (Sigma) ((final concentration: 5 μg/mL) would be added into culture for another 4 hours. At the end of incubation, cells were processed for intracellular staining.

J. Isolation of lymphoid organ and tissue from mice

1. Isolation of lymph nodes and spleen

Mice were euthanized by cervical dislocation and then fixed on dissecting board. Superficial lymph nodes such as cervical, axillary, brachial and inguinal were removed first and placed into chilled RPMI-1640 medium supplemented with 10% FBS. Following this, peritoneal cavity was opened to access to mesenteric, renal, lumbar and caudal nodes. After collecting all visible lymph nodes, spleen was finally removed and place into chilled medium.
2. Isolation of thymus

Mice were euthanized by cervical dislocation and then fixed on dissecting board. Thoracic cage was opened and both lobes of thymus were removed and placed into chilled RPMI-1640 medium supplemented with 10% FBS.

3. Isolation of tumor infiltrating T lymphocytes (TILs)

At the late stage of tumor challenge, mice were sacrificed and tumors were removed from peritoneal cavity from different groups of mice. Tumors were cut into pieces; one piece were put into a cryovial and placed on dry ice immediately for immunofluorescent study, the other half will be fixed in formaldehyde for histological study and a third piece were preserved in chilled RPMI-1640 medium conditioned with 10% FBS for isolating TILs. Tumors were mechanically breakdown and single cells were collected for the isolation of TILs after red blood cell lysis. TILs were determined by flow cytometric analysis.

K. Preparation of single cell suspension from mouse lymphoid organs

Mouse lymphoid organs such as spleens, thymi and lymph nodes were collected in complete RPMI-1640 medium. Tissues were mechanically broken down via a 70 μm nylon cell strainer (BD Biosciences) to generate single cells. After breaking down the tissue, red blood cells (RBCs) were lysed by ACK lysis buffer (Lonza) and mononucleocytes were collected for future use.

L. Ex vivo peptide stimulation
Ex vivo peptide stimulation is used to evaluate the antigen reactivity of antigen-specific T cells that derived from iPS cells. Mice bearing in vivo developed iPS-derived T cells were sacrificed and spleen, lymph nodes were removed from animals. Single cell suspension was made through mechanical breakdown. RBCs were lysed by using ACK lysis buffer and mononucleocytes were collected and washed twice in cold PBS. CD8+ T cells were isolated by using magnetic beads-based CD8+ T cell isolation kit (Miltenyi Biotec). Isolated CD8+ T cells were then mixed with irradiated splenocytes isolated from naïve C57BL/6J mice in the ratio of 1: 10 and pulsed with 0.5μmol/ml OVA\textsubscript{257-264} peptide (GenScript) for 40 hours. Thereafter, Brefeldin A was added into the culture for another 4 hours. At the end of coculture, cells were processed for intracellular staining.

M. Xenograft tumor inoculation

My study focused on the xenograft model of tumor challenge and two tumor xenografting methods were used.

1. Intraperitoneal (i.p.) xenograft

4×10\textsuperscript{6} tumor cells were inoculated intraperitoneally through this procedure. E.G7-OVA cells were harvested from culture and washed three times in PBS. At the end of wash, cells were resuspended in cold PBS in a concentration of 8×10\textsuperscript{7} cells/ml. 50μl cell suspension or 4×10\textsuperscript{6} cells were injected into the peritoneal cavity.

2. Subcutaneous (s.c.) xenograft

Different number of B16-F10 tumor cells was inoculated subcutaneously depending on different experimental settings. Briefly, B16-F10 cells were harvested from culture
and washed three times in PBS. At the end of wash, cells were resuspended in cold PBS in different concentrations. Before injection, the flanks of mice were shaved to obtain an ideal visibility for both injection and further tumor size monitoring. 50μl cell suspension was injected into the subcutaneous tissue.

**N. Tumor size measurement and volume calculation**

Tumors were measured by using a caliper. The largest and the shortest diameters of the tumor were recorded. In the context of tumor volume, it was estimated by calculating based on this following formula: tumor volume = the shortest diameter² × the largest diameter × 0.5 [263].

**O. Peritoneal lavage**

At day 20 of tumor challenge, mice were sacrificed and peritoneal cavity lavage was performed by using cold PBS. Peritoneal lavage recovered tumor cells were counted by using hematocytometer.

**P. Isolation of HSCs**

Isolation of HSCs from OT-I transgenic mice served as the control of OT-I TCR transduced iPS cells in the context of *in vivo* development. Generally, the OT-I transgenic mice were euthanized by cervical dislocation and then fixed on the dissecting board. Their tibia and femur bones were collected and preserved in chilled RPMI-1640 medium with 10% FBS. The bones were trimmed and flushed with 10mL complete RPMI-1640 medium using a syringe and a 25G needle in a 100mm culture dish. Cells were resuspended by gently pipetting with the syringe and a 25G needle.
the cells, RBCs were lysed by ACK lysis buffer and mononucleocytes were collected for HSC isolation. A CD117 magnetic beads-based mouse HSC isolation kit (Miltenyi Biotec) was used to isolate the HSCs. Isolated mouse HSCs were seeded on iSNL76/7 feeder cells for maintenance and proliferation.

**Q. In vivo killing assay**

*In vivo* killing assay is used to evaluate the antigen reactivity and cytotoxic function of *in vivo* developed iPS cells. Splenocytes from naïve C57BL/6J mice pulsed with peptide were used as the targets for *in vivo* killing assay. Spleens were isolated and processed to single cells. Single splenocytes were labeled with carboxyfluorescein succinimidyI ester (CFSE) as target cells. Cells labeled with 5μmol/ml CFSE (CFSE<sup>hi</sup> cells) were pulsed with 10μg/ml OVA<sub>257-264</sub> peptide and cells labeled with 0.5μmol/ml CFSE (CFSE<sup>lo</sup> cells) were not be pulsed and served as internal control. A mixture of 2.5×10<sup>6</sup> CFSE<sup>hi</sup> cells plus 2.5×10<sup>6</sup> CFSE<sup>lo</sup> cells was adoptively transferred by *i.v.* injection into indicated recipient mice. After 16 hours, splenocytes from those mice were isolated and CFSE+ cells were analyzed by flowcytometry.

**R. Cell surface marker assessment**

Briefly, for various types of cells, cell surface staining could provide direct information on certain marker expression. For conducting surface staining, Fc receptor (FcR) blocker 2.4G2 (BD Biosciences) was added to block nonspecific binding of the antibodies. After 20 minutes FcR blocking in 4°C, different types of antibody that conjugated with different types of fluorochrome were incubated for additional 20 minutes in the dark at room temperature. At the end of incubation, cells were washed three times
with PBS to exclude excessive antibodies before flow cytometric analysis. The list of commercial antibodies used in my study is listed in Table 3.

**S. Intracellular staining for IL-2 and IFN-γ**

At the end of incubation, cells were harvested, washed and blocked by Fc receptor blocker. Processed cells were stained for different surface markers such as CD8 and TCR Vβ chain by using fluorochrome conjugated antibodies first. After cell surface staining, cells were fixed by using 4% formaldehyde (BioLegend) and permeabilized by using permeabilizing buffer (BioLegend). After permeabilization, intracellular molecules like IL-2 and IFN-γ will be stained by using fluorochrome conjugated antibodies. Before final flowcytometric examination, cells were washed three times in cold PBS to exclude excessive antibodies.

**T. Immunofluorescent staining**

Immunofluorescent study was used to directly detect TILs that developed from iPS cells. Tumor tissues were preserved in dry ice and later in -80°C refrigerator immediately after removing from sacrificed mice. After sectioning and mounting on micro slides, they were preserved in -20°C before use. Tissue sections were air dried for 15 minutes before a 15-minute cold acetone fixation. After fixation, the sections were air dried for another 15 minutes before a 5-minute PBS wash. After wash, slides were placed in a moist chamber and the tissue sections were covered with 30μl 3% bovine serum albumin (BSA) (Sigma) in PBS for 30 minutes to block non-specific binding. At the end of blocking, blocking buffer was blotted off and tissue sections were covered with a 50μl mixture of phycoerythrin (PE)-anti-TCR Vα2 antibodies (BD Biosciences) and fluorescein
Table 3: Fluorescent and convention antibodies used in the study

<table>
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<tr>
<th>Name</th>
<th>Clone Name</th>
<th>Company</th>
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<tbody>
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<td>BD Biosciences</td>
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<td>93</td>
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isothiocyanate (FITC)-anti-OVA antibodies (Rockland Immunochemicals) diluted in 3% BSA in PBS. The slides were incubated in a moist chamber for another 2 hours and at the end of incubation, slides were washed three times in cold PBS and mounted with a water-based mounting media before fluorescent microscopic examination.

U. Histological sample preparation

For performing histological study, tumor tissues were fixed in 10% neutral buffered formalin (Fisher Scientific) after excised from animal. The tissue processing and hematoxylin & eosin (H&E) staining were performed at the Pathological Core Facility of Penn State College of medicine.

V. Microscopic study

1. Light Microscopy

Light microscopy was used to monitor the morphological changes of iPS cell clones under the in vitro differentiation. Pictures were taken at various time points of differentiation. Light microscopy was also used to exam the histological profiles of tumor tissue. Images were obtained by Nikon Eclipse 80i microscope and NIS-Elements AR 3.10 software (Nikon Instruments Inc.).

2. Fluorescent Microscopy

Fluorescent microscopy was utilized to check the fluorescent protein tag (such as GFP and DsRED) expression in transduced iPS cells and to evaluate the tumor infiltrating T lymphocytes in immunofluorescent study. Fluorescent images were
obtained by Nikon Eclipse 80i microscope and NIS-Elements AR 3.10 software (Nikon Instruments Inc.).

**W. Flow cytometric analysis**

Flowcytometric analysis was performed by using either FACSCalibur or FACS LSR II (Becton Dickinson) flow cytometer. Forward-angle light scatter and 90 light scatter profiles were used for detecting samples. Electronic gates were set around the live cells and at least 20,000 events were collected per sample. Dot plots and histograms were analyzed by FlowJo software (TreeStar, Inc.; Ashland, OR). The total number of cells per sample was determined as: (percentage of specific cell type in sample) × (number of viable cells in sample).

**X. PCR, RT-PCR and qRT-PCR assays**

1. **PCR**

PCR assay was used to amplify gene of interests or detect the retrovirally incorporated gene of interests. For conducting the amplification of gene-of-interest, high-fidelity Platinum® Pfx DNA Polymerase (Invitrogen) was used. For conducting the general purpose of gene detection, Qiagen PCR master mix (Qiagen) was used.

2. **RT-PCR**

RT-PCR assay was used to detecting the presence of transcribed gene products. SuperScript® III One-Step RT-PCR System with Platinum® Taq (Invitrogen) was used in our RT-PCR assay. PCR and RT-PCR cycling conditions were various depending on
the melting temperature (Tm) of different primers. PCR and RT-PCR were performed by using GeneAmp PCR system 9600 (PerkinElmer Inc.)

3. qRT-PCR

Real-time quantitative PCR (qRT-PCR) was performed using primer sets specific for mouse genes, and an internal standard, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), in an ABI PRISM 7900 sequence detector. A total of 1 μg cDNA was applied in a 10 μL PCR mix using a QuantiTect SYBR Green PCR kit (Qiagen). The change in fluorescence of SYBR Green dye in every cycle was monitored and calculated using ABI 7900 system software and the threshold cycle (Ct) for each reaction. All values were normalized to GDPDH mRNA levels.

Y. Statistical analysis

One-way ANOVA was used for the statistical analysis between groups and significance was set at 5%. Kaplan–Meier analyses were used to determine percentage of survival based on death of the animals due to tumor growth in the peritoneal cavity. All statistics were calculated using GraphPad Prism (San Diego, CA)
Chapter IV: Results

A. iPS cells are capable of T lineage differentiation

The concept of iPS cells was brought out in 2006 by Yamanaka group [21]. In 2008, when we first started working on iPS cells, there was very limited information about iPS cell available. Several reports have shown that hematopoietic stem cells (HSCs) and embryonic stem cells (ESCs) are capable of differentiating into T lymphocytes under different in vitro or in vivo scenarios [17, 136, 176, 264-266], at that time, no information has been published regarding the differentiation of iPS cells into the hematopoietic lineage, especially the T lymphocyte lineage. To use iPS cells for generating abundant T cells for cancer immunotherapy, the first question we wanted to investigate was whether iPS cells could behave like other naturally occurring stem cells to differentiate into T lymphocytes or not.

A.1 iPS cells were capable of differentiation into lymphocytes

To investigate this question, both general iPS culture and in vitro differentiation systems were setup. iPS cell line iPS-MEF-Ng-20D-17 was generated by the Yamanaka group by introducing 4 reprogramming factors into MEFs [256]. We obtained the cells from Dr. Yamanaka through the RIKEN cell bank. Upon receiving the cells, a culture was established by using the irradiated SNL76/7 (irSNL76/7) cells as feeder cells. The reason for switching from using conventional MEFs was that SNL76/7 cells are able to produce LIF, a key cytokine that maintains the pluripotency of ESCs [267]. Generally, after recovering from liquid nitrogen, it took 5-6 days for the iPS cells to reach confluency on the irSNL76/7 feeder layer. Theoretically, iPS cells could be generated.
from any kind of somatic cells through the reprogramming protocol. To distinguish the successfully reprogrammed cells from their precursors, a GFP marker was introduced, together with those reprogramming factors, under a different Nanog promoter. The Nanog gene is a stem cell marker that universally expressed in ESCs and early embryos, and it functions to maintain their pluripotency [268, 269]. The GFP signal in iPS cells indicated that the somatic precursor had been successfully reprogrammed into pluripotent cells. Figure 8 shows GFP expression in the iPS colony that on the feeder cell layer.

iPS cells were maintained on irSNL76/7 cells and passed when they reached confluency. Because of the differentiating nature of stem tissue, fluorescent activated cell sorting (FACS) was used to select and enrich the GFP+, stem cell-phenotyped iPS cells after several passages.

When the iPS culture system was established, the T lineage differentiation capability was tested by an in vitro system as described in the materials and methods [136]. Briefly, iPS cells were processed and collected from culture. To start the designated T lineage differentiation, at Day 0, $5 \times 10^4$ iPS cells were added on the monolayer of OP9-DL1 bone marrow stromal cells which overexpress the Notch signaling pathway ligand DL-1. OP9 bone marrow stromal cell line has been established to support a multi-lineage differentiation of HSCs including the B lineage differentiation [270]. From the understanding of the T lineage development, precursor T lymphocytes were developed from HSCs in the bone marrow and migrated out in the thymus where they achieved their final maturation through several processes such as positive selection and negative selection [271]. Previous studies showed that Notch signaling pathway ligands, especially DL-1 and DL-4, were highly expressed in the thymic
Figure 8: The iPS colony on the feeder cell layer and their GFP expression. iPS cells were routinely maintained on the irradiated mouse embryonic fibroblast (MEF) derived the cell line SNL-76/7, which overexpresses the leukemia inhibitory factor (LIF). GFP expression was used as a marker to track to pluripotency of the iPS cells. The figure shows a single iPS colony that composed by several hundred and thousands of iPS cells.
Figure 8

Bright field

GFP channel
microenvironment and has been observed to play a significant role in T lymphocyte development [93, 272, 273]. The rational of this system was OP9 bone marrow stromal cells would support the initial development of stem cells, in my case the iPS cells, to early hematopoietic precursor cells and later on the Notch signal would further drive the precursor cells into T lineage direction. After five days of coculture with OP9-DL1 cells, the iPS cells differentiated into flat, mesoderm-like colonies which were morphologically different compared to their undifferentiated, dome-shaped colony on irSNL76/7 feeder cells. On Day 12, the mesoderm-like colonies had disappeared and lymphocyte-like colonies had emerged which looked completely different to their previous morphologies and resembled grape clusters. Interestingly, on Day 22, the lymphocyte-like cells were fully spread in the culture plate on top of the OP9-DL1 feeder cells. Figure 9 showed the morphological changes of iPS cells under T lineage differentiation.

In addition to the morphological changes of iPS cells under T lineage differentiation, the absolute cell numbers also significantly increased following coculture with OP9-DL1 cells. Starting as $5 \times 10^4$ cells on culture, on Day 7, the cell numbers increased 5 fold to approximately $2.5 \times 10^5$, a recovery rate of 500%. On Day 14, the cell recovery rate reached to 100,000%, i.e. $5 \times 10^7$ cells and on Day 21, the cell numbers kept increasing as higher than 1000 fold. Figure 10 showed the absolute cell number changes in different days of in vitro differentiation.

The other changes of iPS cells after coculturing with OP9-DL1 cells were their markers of stemness. At different time points, cells were recovered and their stem cell
Figure 9: The morphological changes of iPS cells under T lineage differentiation. iPS cell differentiation in the presence of Notch signaling. Figure showed the morphology of the iPS cell under Days 0, 5, 12, and 22 of differentiation. Data were representative of three independent experiments.
Figure 9
Figure 10: The cell number changes in different days of *in vitro* differentiation. Cell recovery ratios of iPS cell-derived precursor T cells were shown in this figure. Numbers of iPS cell-derived cells present on Day 0 were assigned a value of 100%, and numbers surviving on Days 5, 12, and 22 were used to calculate the percentage recovery relative to day 0. Because of the variations of cell numbers in different experiments, the cell number changes were plotted as recovery rates. Data represented the mean ± SEM.
markers such as CD117 and CD45 were checked by flow-cytometry. It was found that those markers disappeared following the T lineage differentiation through Notch signaling stimulation. Figure 11 showed the trend of stem cell maker changes of iPS cells in the T lineage differentiation.

Taken together, these data strongly indicated that iPS cells were under lymphocytic, especially T lymphocytic through the stimulation from OP9-DL1 cells. However, these morphological changes could not rule out other possibilities such as B lineage or myeloid lineage differentiation. Further evidences were required to support our hypothesis that iPS cells were following T lineage differentiation on the OP9-DL1 cells through the Notch signaling pathway stimulation. To determine whether these differentiated iPS cells were truly under T lineage direction, the study of T lymphocyte marker expression on differentiated iPS cells were performed.

**A.2 iPS cells differentiated into T lymphocytes**

The T cell surface markers used in this study were CD3 and TCR-β chain molecules. These two molecules are specifically expressed on T lymphocytes, and thus, their presence on differentiated iPS cells suggests that iPS cells differentiated into T lymphocytes on OP9-DL1 cells. After 12 days of coculture with OP9-DL1 cells, iPS cells lost their stem cell marker CD117 but expressed CD44 and CD25 on their surface, and this resembles the DN 1 and DN 3 stages of T lymphocyte development in the thymus. Figure 12 shows the surface marker profiles of iPS cells on day 12. From Day 18 on, the majority of iPS derived cells started to express both CD4 and CD8 markers of the CD4⁺CD8⁺ DP population and CD4⁻CD8⁺ SP population.
Figure 11: The cells surface marker changes during the differentiation. The changes in hematopoietic marker expression (CD117 and CD45) on iPS cells were monitored. Expression of each marker was determined at the indicated times. Data represented the mean ± SEM.
Figure 11

[Graph showing % expressing of GFP+ over days for CD117 and CD45]
Figure 12: The surface expression of CD25 and CD44 on iPS cells on day 12 coculture. Mouse iPS cells were cocultured with OP9-DL1 cells for the T lineage differentiation, on Day 12, the surface expression of CD25 and CD44 of iPS cell-derived cells were analyzed by flow cytometry, cells were gated on CD45+ population. A shows the Day 0 control and B shows the Day 12 cells. Data were representative of three independent experiments.
Figure 12

A

B

CD44

CD25

2.58% 1.46%

0.05%

20.8% 75.5%
CD3 and TCR-β chain were also expressed on the surface of iPS-derived cells. Figure 13 shows the day 22 profiles of iPS derived T lymphocytes. Further analysis of iPS cells on Day 18 of coculture with OP9-DL1 cells by gating on the CD45+ population showed that CD45+ cells were composed of three major groups: CD4+CD8+ DP population, CD4-CD8- DN population and CD4-CD8+ SP group. The majority of CD3+ cells were located in CD4-CD8+ SP group; however, there were approximately 5% of CD4+CD8+ DP population showed CD3+. Figure 14 shows the Day 18 profiles of iPS cells under T lineage differentiation. This information suggested that CD3+TCRβ+ cells were generated from CD4+CD8+ DP population. These CD4+CD8+ DP were also found in the thymus and were considered as the progenitor T lymphocytes, which have large nuclei with dense heterochromatin and thin rims of cytoplasm. Collectively, these data indicated that iPS cells were able to differentiate into T lymphocytes.

**A.3 iPS cell-derived T lymphocytes were functional in terms of cytokine secretion**

The iPS cell-derived lymphoid cells have been shown morphologically similar to T lymphocytes and expressed T lymphocyte specific markers. However, classical T lymphocytes bear other functions such as activation and cytokine secretion. To further investigate iPS-derived lymphoid cells were truly functional T lymphocytes T lymphocyte activation assay was performed. In doing this study, on Day 22, iPS cell-derived T lymphocytes were harvested from the OP9-DL1 coculture and stimulated by plate-bound anti-CD3 Abs and soluble anti-CD28 Abs for 40 hours in 37°C, 5% CO2 incubator. Four hours before the termination of stimulation, Brefeldin A was added into culture. After that, cells were processed and intracellular staining for IFN-γ and IL-2 was
Figure 13: The day 22 profiles of iPS derived T lymphocytes. On Day 22 of coculture, iPS-derived cells were isolated for the analysis of surface CD4 and CD8 as well as CD3 and TCR-β expressions. Figure A shows the expression of CD4 and CD8; figure B shows the expression of CD3 and TCR-β. Data were representative of three independent experiments.
Figure 14: The surface marker profiles of iPS cells on day 18 of coculture. On Day 18 of coculture, iPS-derived cells were isolated for the analysis of surface CD45 expressions. A, CD45\(^+\) cells were gated to further analyze for the CD4 and CD8 expressions. B, CD4\(^-\)CD8\(^-\) and CD4\(^+\)CD8\(^+\) cells were subsequently gated to check the CD3 expression which is plotted in C. Data were representative of three independent experiments.
performed subsequently as described in Materials and Methods. It was found that only CD3+CD4+CD8+ cells derived from iPS cells but not CD3+CD4+CD8+ cells are able to produce IFN-γ and IL-2 (Figure 15). This information showed that when iPS derived T lymphocytes passed the DP stage to SP stage of development, they acquired the function of cytokine secretion. These data suggested that under the T lineage differentiation stimulated by Notch signaling ligands, iPS cells acquired both morphological and functional properties of conventional T cells. However, our studies were carried out in the in vitro system of T lineage differentiation; no thymic selection, which is critical in T lymphocyte differentiation and development, was present. A large number of iPS cell-derived lymphocytes were still in their progenitor stage in the context of expressing both CD4 and CD8. In the coming study, it was designed to evaluate the ability of these progenitor T lymphocytes to continue their development into fully mature T lymphocytes.

**A.4 iPS cell-derived progenitor T lymphocytes were able to continue their development in vivo and restored the T lymphocyte pool in the lymphopenic mice**

To evaluate the continuing differential capability of iPS cell-derived progenitor T lymphocytes, the lymphopenic mouse model Rag1−/− mice were used. Owing to the knockout of Rag1 gene, which is pivotal in the TCR and BCR recombination process, there were no endogenous T lymphocytes or B lymphocytes in the Rag1−/− mice. On Day 22 of differentiation, iPS cell-derived CD3+ cells were isolated from co-culture and adoptively transferred into Rag1−/− mice through the tail vein. After four weeks of in vivo development, mice were euthanized and single cells were recovered from spleens and lymph nodes by mechanical separation. The T cell surface markers on those single
Figure 15: Cytokine secretion from iPS-derived T cells. On Day 22 of coculture, iPS-derived cells were harvested and stimulated with plate-coated anti-CD3 and soluble anti-CD28 for 40 h at 37 °C at 5% CO₂. IL-2 and IFN-γ were analyzed by intracellular staining, after gating on live CD4⁺CD8⁺ or CD4⁻CD8⁺ T cells. Left panel shows the IL-2 expression and right panel shows IFN-γ expression. Data were representative of three independent experiments.
cell suspensions were checked by flow cytometry. It was found that both CD4$^+$ and CD8$^+$ SP cells could be recovered from the spleens and lymph nodes of transferred \textit{Rag1$^{-/}$} mice. And their relative percentages were similar to wild type C57BL/6J mice (Figure 16). Further gating on either CD4$^+$ or CD8$^+$ cells showed that both populations were CD3$^+$ TCR-$\beta^+$ (Figure 17). These experiments directly indicated that iPS cells derived, \textit{in vitro} differentiated T progenitors could further develop into mature T lymphocytes \textit{in vivo}.

\textbf{A.5 Summary and conclusion}

Since the introduction of iPS cell in 2006, there was little information available about its differentiation capabilities. There were studies reporting the successful programming of iPS cells into functional cardiomyocytes [274, 275]. However, no study has been documented to investigate iPS cells’ ability in hematopoietic lineage, especially T lymphoid lineage differentiation. We were the first group to investigate this question. Based on previously published data, both ESCs and HSCs are able to differentiate into T lymphocytes with the Notch signaling stimulation [17, 136], as well as the resemblance of iPS cells to ESCs both morphologically and functionally, it comes to our hypothesis that iPS cells are able to differentiate into T lymphocyte as their naturally occurring counterparts. Studying the T lineage differentiation capability has two major impacts to our scientific community. First, it would help us fully understand the property of iPS cells; second, it could help provide a new source of getting large numbers of naïve lymphocytes for the application in personalized medicine. In this first part of my thesis research, it was clearly shown that by using the simple \textit{in vitro}, Notch signaling pathway mediated differentiation induction system, iPS cells could be able to turn on
Figure 16: iPS-derived T cells reconstitute the *Rag1*-deficient mice. Mouse iPS cells were cocultured with OP9-DL1 for 22 days thereafter iPS cell-derived CD3$^+$ T cells were injected *i.v.* into *Rag1*-deficient mice. After 4 weeks, single-cell suspensions from lymph nodes and spleen were analyzed for expression of CD4 and CD8 by flow cytometry. Single-cell suspension from C57BL/6J was assessed as a control. Data were representative of three independent experiments.
Figure 16
**Figure 17: Further analysis of the iPS-derived, reconstituted CD4^+ T cells.** As introduced in Figure 16, Mouse iPS cells were cocultured with OP9-DL1 for 22 days thereafter iPS cell-derived CD3^+ T cells were injected *i.v.* into *Rag1*-deficient mice. After 4 weeks, single-cell suspensions from lymph nodes and spleen were analyzed for expression of CD4 and CD8 by flow-cytometry. The CD4^+ T cell populations were further analyzed for the CD3 and TCR-β expressions. Data were representative of three independent experiments.
Figure 17
their differentiation towards hematopoietic lineage and further into T lineage. In my observation, following the Notch ligand DL1 stimulation, first, the morphologies of iPS cells changed. Then, the expression of stem cell markers such as CD117 and CD45 have discontinued but T lymphocyte markers as CD4, CD8, CD3 and TCR-β have been detected. Along with the morphological changes, these differentiated cells also acquired the T lymphocyte function in the context of cytokine secretion upon costimulatory signals mediated activation. These *in vitro* observations that iPS cells were able to differentiate towards T lymphoid cell was further supported by the *in vivo* study that *in vitro* differentiated cells could reach a full maturation in the observation that CD4CD8 DP populations disappeared and only CD4 and CD8 SP populations could be identified. Moreover, these iPS cell-derived T lymphocytes reconstituted the lymphoid organs in the lymphopenic *Rag1*−/− mice.

All my data suggested that iPS cells were similar to ESCs and HSCs in terms of T lineage differentiation. This significantly improved our understanding of using stem tissue for clinical studies. Because it helped overcome the ethical and technical obstacles related to the usage of human embryonic and hematopoietic tissues. However, generating a more specific subgroup of T lymphocytes will be more beneficial for designing a personalized medicine. For example, T lymphocytes either isolated from tumor infiltrates or genetically modified to express tumor antigen-specific T cell receptor have been shown being able to control melanoma growth and substantially increase the quality of life of the patients. This gave us a new suggest to use iPS for the personalized medicine that was to generate antigen-specific T lymphocytes from iPS cells.

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B. iPS cells are capable of being programmed into antigen-specific T lymphocytes to promote cancer immunosurveillance

In the first part of my thesis research, we showed that the capability of iPS cells in T lineage differentiation was similar to their natural counterparts such as HSCs and ESCs. This built the foundation of using iPS cells in ACT-based cancer immunotherapy. Previous studies have demonstrated that giving cancer patients with cancer-attacking T lymphocytes could significantly control tumor growth as well as improve patient’s quality of life [63]. Currently, different types of original or engineered cancer-attacking T cells were isolated and ex vivo expanded from cancer patients. The re-infusion of these self-derived T cells would not cause any immune responses. However there was a disadvantage of using autologous, antigen-experienced, effector-phenotyped T cells, which is their short lifespan compared to naïve-phenotyped T cells. In searching for a new source of a large numbers of tumor antigen-specific, naïve phenotyped T cells to avoid any immune responses caused by MHC mismatch, we turned our focus to the iPS cells. iPS cells were to be able to develop into conventional T lymphocytes. Theoretically, they could be further engineered to differentiate into different sub-types of T lymphocytes, such as CD4⁺ T helper cells, CD8⁺ CTLs and so on. Previously, researchers have shown that HSCs could be engineered to be antigen-specific T lymphocytes by introducing an antigen-specific TCR through in vivo and in vitro approaches [123, 124]. This further supported the idea that iPS cells might be suitable of carrying out a more-specified, sub-type T lineage differentiation. Taken together, I came to the hypothesis that iPS cells can be further engineered into antigen-specific T lymphocytes. By doing this study, we could further achieve the long-term goal of
designing personalized cancer immunotherapy through the iPS technology. In short, through our research, it suggests to get a large number of cancer-fighting T cells from a small piece of tissue or a tube of blood.

**B.1 Generation of antigen-specific TCR gene transduced iPS cells**

To test our hypothesis, the very first thing to do is to generate iPS cells that overexpress a certain type of TCR. We acquired a murine, MHC-I restricted TCR that could specifically recognize chicken ovalbumin (OVA) as our model system. The OVA-specific TCR or OT-I TCR is specific for the OVA\textsubscript{257-264} (SIINFEKL) peptide loaded on H-2K\textsuperscript{b} molecule [259]. OT-I TCR can target any cells that co-express both OVA and H-2K\textsuperscript{b} molecules. With this classic OVA system which has been widely used in the research field of immunology [276], we could easily investigate the TCR mediated T lineage development as well as TCR-rendered antigen reactivity of our iPS-derived cells.

In order to generate the OT-I overexpressed iPS cells we used a retroviral mediated gene delivery technique. The advantage of retroviral mediated gene delivery is that the gene-of-interest could be permanently inserted into the host genome although some reports showed retroviral mediated gene integration has the potential to randomly activate certain oncogene [277, 278]. Similar to other TCRs, OT-I TCR is consisted by a separate \(\alpha\) chain and a \(\beta\) chain. Only an assembled TCR \(\alpha\beta\) chain complex has biological activity. In our study, the cloned OT-I TCR-\(\alpha\) and TCR-\(\beta\) chain genes which were linked by a picornavirus P2A sequence were a kind gift from Dr. Dario A. A. Vignali at St. Jude Children’s Research Hospital. Because the iPS cell line we obtained expresses GFP universally as an internal stemness marker, to further distinguish the gene-incorporated
cell, a different red fluorescent protein tag DsRED was used. The retroviral vector used in our study was a mouse stem cell virus (MSCV)-based backbone flanked by an internal ribosome entry site (IRES) fragment separated GFP tag (pMiG) [279]. The MSCV-based IRES-DsRED (pMiDR) vector which expresses the red fluorescent tag DsRED was made by replacing the GFP fragment with the DsRED fragment by endonuclease-based digestion and ligation, which was schemed in Figure 5. The OT-I TCR gene was subcloned into the MiDR vector for retroviral mediated transduction. Retroviral mediated transduction for iPS cell was performed as described in materials and methods. After transduction, cells were re-plated on irSNL76/7 feeder cells for future cell sorting and characterization.

FACS was performed by using the MoFlo cell sorter at the Penn State College of Medicine Flowcytometry Core Facility. For several reasons such as our iPS cells were generated by retroviral transduction, due to different mechanisms such as stem cell is active in shutting down retrovirus and retroviral infection sometimes causes the downregulation of viral binding receptors on host cell, it was difficult to perform a second retroviral transduction [256]. Therefore, a very low transduction efficiency of 3.32% was obtained. However, after several cell sorting by gating on the GFP and DsRED double positive group, enriched iPS cells with transduced OT-I TCR (designated as iPS/OT-I cells) were obtained (Figure 18). At the same time, a vector-only transduced control cell line iPS/MiDR was also established as described above.

The GFP and DsRED expression could be visualized and captured by fluorescent microscopy (Figure 19). Fluorescent protein tag expression was a useful marker to track
Figure 18: FACS enrichment of DsRED$^+$GFP$^+$ iPS/OT-I cells. GFP$^+$ iPS cells (left) were transduced with the retroviral construct MiDR-OT-I TCR, and DsRED$^+$GFP$^+$ iPS cells (middle) were analyzed by flow cytometry and sorted by a high-speed cell sorter (right). Data were representative of three independent experiments.
Figure 18

No transduction  Post transduction  Post cell sorting
Figure 19: Fluorescent profiles of DsRED⁺GFP⁺ iPS/OT-I cells. MiDR-OT-I TCR transduced DsRED⁺GFP⁺ iPS cells were visualized by the fluorescent microscopy. Picture shows a single iPS colony that composed of thousands of individual cells. Data were representative of two independent experiments.
and select the transduced cells but it was an indirect marker. The most direct way to support the success of gene incorporation was to directly detect the gene-of-interest in the genome and evaluate the transcribed mRNA level. To check these, a special pair of primers covering the recombined VDJ region of the OT-I TCR Vβ5 gene was designed. In a PCR assay, the presence of the gene in the genome level was detected in the iPS/OT-I cells (Figure 20). Similarly, the transcribed TCR mRNA was detected by an RT-PCR assay (Figure 21). The primers for these PCR and RT-PCR please refer to Table 2. Although gene integration and expression could be observed, no TCR protein expression was identified in western blot.

In summary, the iPS cells express a certain antigen-specific TCR i.e. the iPS/OT-I cells were established by retroviral transduction and fluorescent activated cell sorting.

**B.2 iPS/OT-I cells were able to differentiate into cytotoxic T lymphocytes in vivo**

With the iPS cell line overexpressing OT-I TCR established, we aimed at studying the role of TCR in T cell differentiation from iPS cells. Previously, we had used an *in vitro* system to investigate the T lymphocyte development. However, this *in vitro* Notch signal stimulation system could only generate progenitor T cells and we still have to introduce those cells into the animal to obtain a full maturation. In bypassing this two-step scenario, we elected to use an *in vivo* system to investigate the development of iPS/OT-I cells. The immunocompetent C57BL/6 mice were used to conduct this study for two reasons: 1) our iPS cell line originated from C57BL/6 mouse; and 2) C57BL/6 mice express a full spectrum of molecules, cytokines, growth factors and microenvironments that played key roles in T cell development.
**Figure 20: PCR analysis of gene integration.** DsRED\(^+\)GFP\(^+\) iPS cells were sorted and total DNA were analyzed for the presence of the recombined VDJ region of the OT-I TCR Vβ5 gene. Lymphocytes from C57BL/6J mouse and iPS cells transduced with vector were used as negative controls. The positive control was lymphocytes isolated from OT-I transgenic mouse. Data were representative of three independent experiments.
Figure 20
Figure 21: RT-PCR analysis of gene transcription. DsRED\textsuperscript{GFP} iPS cells were sorted and total mRNA were analyzed for the presence of the recombined VDJ region of the OT-I TCR V\textbeta{}5 gene. Lymphocytes from C57BL/6J mouse and iPS cells transduced with vector were used as negative controls. The positive control was lymphocytes isolated from OT-I transgenic mouse. Data were representative of three independent experiments.
Figure 21

RT-PCR
The general strategy is summarized in Figure 22. Briefly, early passages of iPS/OT-I or iPS/MiDR control cells were harvested from culture and processed to generate single cell suspensions. Then, iPS/OT-I or control cells were adoptively transferred into individual C57BL/6J mice through their tail vein. 6 weeks were allowed for the T lineage development of iPS/OT-I cells. And after 6 weeks of development, the tumor cells bearing OVA (E.G7-OVA) were inoculated into the peritoneal cavity of different groups of mice. At different time point, T lymphocyte development and function was closely monitored.

In week 2 after cell transfer, there was no statistically significant amount of iPS/OT-I derived T cells detected from the peripheral lymphoid tissue such as spleen or lymph nodes (0.16% compared to 0.15% of the iPS/MiDR control). The presence of iPS/OT-I cells in the peripheral lymphoid organs could be initially detected on week 4 after adoptive transfer as defined by checking the surface expression of both CD3 and TCR Vβ5 in the pooled T lymphocytes (3.55% compared to 0.13% of iPS/MiDR group). This indicated that OT-I TCR plays function in determining T cell development. On week 6, a significant population of iPS/OT-I cells (23.5% of total pooled T lymphocytes) could be detected in the periphery and the percentage of this population kept slightly increasing in week 10 to reach to 25.1% of total CD3+ T cell Data are summarized in Figure 23.

Further analysis of these CD3+ and TCR Vβ5+ double positive cells showed that the majority of them were CD8+ cells. This is consistent to our hypothesis that MHC-restricted TCR determines the development of their corresponding subtype of T lymphocytes. Figure 24 displays that starting from week 4, about 71.3% of iPS/OT-I derived T cells were CD8+ and this population kept expanding to 80.4% in week 6
Figure 22: Brief scheme for the *in vivo* development experiment design. The experimental approaches to investigate the *in vivo* development of iPS/OT-I cells were described in this scheme. In short, $3 \times 10^6$ iPS-derived or control cells are adoptively transferred into different groups of mice respectively. Following adoptive transfer, 6 weeks are allowed for the *in vivo* development and at the end of week-6 $4 \times 10^6$ E.G7 tumor cells are inoculated in the peritoneal cavity.
iPS/TCR cells by i.v. injection → E.G7 challenge by i.p. injection

6 weeks of *in vivo* development

- *In vivo* evaluation of T cell function
- T cell development and persistence
- *Ex vivo* peptide stimulation
- Animal survival
- Tumor infiltrating T cells
Figure 23: The *in vivo* development of iPS/OT-I at different time points. As described above, iPS/OT-I cells were adoptively transferred into C57BL/6J mice. After two to ten weeks, OVA-specific Vβ5⁺ CD8⁺ T cell development was examined. CD3⁺ Vβ5⁺ T cells from pooled lymph nodes and spleen were analyzed by flow cytometry. Data were representative of four experiments.
Figure 23
Figure 24: *In vivo* developed iPS/OT-I cells were MHC-restricted. As described above, iPS/OT-I cells were adoptively transferred into C57BL/6J mice. After two to ten weeks, OVA-specific Vβ5⁺ CD8⁺ T cell development was examined. CD3⁺ Vβ5⁺ T cells from pooled lymph nodes and spleen were analyzed by flow cytometry. CD4 and CD8 expression profiles were further evaluated by gating on CD3⁺ Vβ5⁺ T cells. Data were representative of four experiments.
Figure 24

MiDR-TCR/iPS cells

Week 2  Week 4
40.2%  71.3%

Week 6  Week 10
80.4%  81.5%

CD8
CD4
and 81.5% in week 10 post transfer. To further investigate the antigen-specific T cell development from iPS cell, we used OT-I transgenic mice as positive control.

OT-I transgenic mouse line (C57BL/6-Tg(TcraTcrob)1100Mjb/J) was established by knocking in the OT-I TCR into the recipient mouse genome and cross-bred with Rag\(^{-/-}\) mouse therefore it only has CD8\(^+\) T cells that unanimously express the OT-I TCR (classified as TCR V\(\alpha\)2 and TCR V\(\beta\)5) [280]. HSCs from OT-I mice (HSC/OT-I) were only able to develop into CD8\(^+\) TCR V\(\alpha\)2\(^+\) and TCR V\(\beta\)5\(^+\) T cells. To better understand the function of TCR in determining the T cell development from stem cells, an additional study has been performed. In this study, control iPS/MiDR cells, iPS/OT-I cells or HSC/OT-I were given to recipient mice through their tail vein. 10 weeks later, mice were sacrificed and T cell profiles were checked. In comparison of the T lineage development between iPS/OT-I cell and HSC/OT-I transgenic mice, there was no significant difference. However, the iPS/MiDR cells failed to develop any CD8\(^+\) TCR V\(\beta\)5\(^+\) T cells in the recipient mice and only endogenous V\(\beta\)5\(^+\) populations could be seen, as shown in Figure 25.

This information along with other reports strongly suggests that TCR determined T cell development although detailed mechanisms were still not clear [123, 124].

**B.3 in vivo developed iPS/OT-I derived CTLs were activated upon antigen stimulation**

My initial study detected the presence of iPS/OT-I derived T cells in the peripheral lymphoid tissue. However, the key property of CTLs is the activation upon antigen stimulation and the activated CTLs kill target cells by their cytotoxic mechanisms.
Figure 25: iPS/OT-I cells were able to develop into antigen-specific T cells. Antigen-specific CD8+ T cell development GFP+DsRed+ iPS/OT-I cells were identified after the i.v. injection into C57BL/6J mice. After 6 to 10 weeks, the development of OVA-specific CTLs was determined. Lymphocytes pooled from lymph nodes and spleen were analyzed by flow cytometry for the presence of Vβ5+CD8+ T cells. The percentiles were calculated by dividing Vβ5+CD8+ T cells to total CD8+ T cells. Data were representative of 3 independent experiments.
Therefore, we asked was whether those iPS/OT-I derived T cells were activated upon antigen stimulation. By answering this, two commonly used T cell activation markers CD25[281] and CD69 [282] were used.

In week 10 post cell-transfer and week 4 post E.G7-OVA tumor-challenge, mice were euthanized and lymphocytes were harvested from their spleens and lymph nodes. Single cell suspensions were prepared as described. Flow cytometry study was performed thereafter. By gating on the CD8$^+$ TCR Vβ5$^+$ populations, we further analyzed the CD69 and CD25 expression levels. Due to the presence of some endogenous CD8$^+$ T cells also expressed TCR Vβ5, although the absolute number of these cells was very low, the expression of a significant level of both CD25 (75% of total CD8$^+$ TCR Vβ5$^+$ cells) and CD69 (73.2% of total CD8$^+$ TCR Vβ5$^+$ cells) molecules could be observed in the control mice that received iPS/MiDR cells. In our experimental groups receiving iPS/OT-I cells, there was no significant differences in CD25 (iPS/OT-I: 74.1% of total CD8$^+$ TCR Vβ5$^+$ cells; OT-I/HSCs: 72.4% of total CD8$^+$ TCR Vβ5$^+$ cells) as well as CD69 (iPS/OT-I: 76.7% of total CD8$^+$ TCR Vβ5$^+$ cells; OT-I/HSCs: 72.3% of total CD8$^+$ TCR Vβ5$^+$ cells) expressions could be identified compared to the positive control group which received the HSC/OT-I cells. Figure 26 shows the comparison of CD25 and CD69 in different groups of gated CD8$^+$ TCR Vβ5$^+$ cells.

In this part of our study, we understood that our iPS/OT-I derived CD8$^+$ CTLs were activated after experiencing the OVA antigen expressed on the E.G7-OVA tumor. The expression of activation markers highly suggested that *in vivo* developed iPS/OT-I cells were functional in terms of secreting cytokines upon antigen stimulation. In the following study, we tried to answer this question.
Figure 26: The CD25 and CD69 expressions in different groups of gated CD8⁺ Vβ5⁺ cells. In week 10 after cell-transfer and week 4 after tumor challenge, GFP+DsRed+iPS/OT-I derived OVA-specific Vβ5⁺CD8⁺ T cells were further gated for checking the expression levels of CD25 and CD69 in dark lines; shaded areas indicate isotype controls. Data were representative of three independent experiments.
Figure 26
**B.4 in vivo developed iPS/OT-I derived CTLs were antigen reactive**

Another key feature of CTL activation is the cytokine secretion [283, 284]. When CTLs were activated by antigen stimulation, they would further amplify the immune responses toward the stimulating antigen by secreting cytokines. Two most important cytokines that were secreted by activated CTLs were IL-2 [285] and IFN-γ [286]. In the aim to determine the antigen reactivity of *in vivo* developed CTLs from iPS/OT-I cells, we decided to evaluate the cytokine secretion of those cells.

In this study, in week 10 post cell transfer and week 4 after tumor challenge, lymphocytes from different groups of mice were pooled to perform the *ex vivo* peptide stimulation assay. The detailed protocol was described in materials and methods. Briefly, spleen and lymph nodes were isolated from mice and broken down mechanically to make single cell suspensions. CD8⁺ T cells of different experimental groups were separated and collected by the commercial CD8⁺ T cell isolation kit. In order to check the antigen-specific activation of CTLs, naïve APCs loaded with OVA₂₅₇₋₂₆₄ peptide were added into the culture of CD8⁺ T cells. 40 hours later, Brefeldin A was added into the culture to retain the synthesized cytokine in the cytosol of activated CTLs. 4 hours later, intracellular staining to both IL-2 and IFN-γ was performed. CD8⁺ TCR Vβ5⁺ T cells were gated to check the IL-2 and IFN-γ expression levels. As noted in previous work, there was a small population of endogenous, naturally occurring CD8⁺ TCR Vβ5⁺ T cells in control mice received iPS/MiDR cells, these cells also responded to OVA₂₅₇₋₂₆₄ peptide stimulation in terms of secreting IL-2 (39.5% of total CD8⁺ TCR Vβ5⁺ cells) and IFN-γ (45.2% of total CD8⁺ TCR Vβ5⁺ cells). However, positive percentages of IL-2 secreting cells in iPS/MiDR were significantly lower than either iPS/OT-I derived CTLs (73.7% of...
total CD8^+ TCR Vβ5^+ cells) or HSC/OT-I (74.6% of total CD8^+ TCR Vβ5^+ cells) derived CTLs. In the IFN-γ secreting groups, the positive percentages in either iPS/OT-I derived CTLs (51.2% of total CD8^+ TCR Vβ5^+ cells) or HSC/OT-I (49.9% of total CD8^+ TCR Vβ5^+ cells) derived CTLs were also higher than iPS/MiDR group but not as dramatic as the differences in the IL-2 expression. Figure 27 showed the comparisons of both IL-2 and IFN-γ secretion levels in different groups of mice received different cells.

The results of this study supported that compared to the positive control HSC/OT-I derived CTLs as well as endogenously developed CD8^+ TCR Vβ5^+ T cells, iPS/OT-I derived CTLs expressed the identically functional TCR and could respond to antigen peptide stimulation in terms of secreting both IL-2 and IFN-γ. This *ex vivo* peptide activation assay indicated that CTLs developed from iPS/OT-I cells were antigen-reactive. To state that iPS/OT-I cells could differentiate into functional CTLs, there was still one more question to ask: whether these iPS/OT-I derived CTLs were able to exert cytotoxic function or not.

**B.5 in vivo developed iPS/OT-I derived CTLs acquired cytotoxicity**

One of the most important features of CTLs is their ability to exert cytotoxic effects to kill the target cells [287-290]. Usually, after the TCR recognizes its corresponding antigen loaded on the MHC-I molecule of the target cell, the CTLs will secrete perforin [291], granzyme B [292, 293] and granulysin [294] to lyse the target cells. Previous studies showed that upon the engagement of TCR to MHC loaded peptide, CTLs will secrete perforin to form aqueous channels on the membrane of the target cell.
Figure 27: Cytokine secretion profiles of iPS/OT-I derived T cells. After in vivo development, the antigen reactivity of iPS/OT-I derived CD8^+Vb5^+ T cells were evaluated through ex vivo peptide stimulation. Different groups of isolated cells were co-cultured with antigen-presenting cells pulsed with SIINFELK peptide for 40 hours. Befeldin A was added into culture 6 hours before intracellular staining to cytokine IL-2 and IFN-γ. Antigen-reactive cells were determined by their secretion of IL2 and IFN-γ. The positive population is calculated from mean fluorescent density in comparing to isotype control (Dark lines indicate results; shaded areas indicate isotype controls). Data were representative of 3 independent experiments.
Figure 27

C

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<th>MiDR vector/iPS cells</th>
<th>MiDR-TCR/iPS cells</th>
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Following this, granzyme B and granulysin will be injected into the target cells to degrade the intracellular organelles such as nuclei, mitochondria and etc. This process is collectively called the cytotoxic effect and this gives CD8⁺ T cell another name as the cytotoxic T lymphocyte [295]. To address whether our iPS/OT-I derived T cells had cytotoxicity or not, an in vivo killing assay was used as described in the Materials and Methods.

In short, OVA257-264 peptide pulsed target cells or non-pulsed control cells were labeled with CFSE dye in either high (CFSE hi, target) or low (CFSE lo, control) concentration. The targets and controls were mixed in the 1:1 ratio (50%: 50%) after the staining, and then injected into week 10 post cell transferred mice through their tail vein. In this study, to clearly compare the cytotoxicity of mature CTLs, positive control used was the OT-I CTL were from OT-I transgenic mouse. One day prior to the killing assay, OT-I CTLs were isolated from OT-I mice and adoptively transferred into C57BL/6J mice. 16 hours after the injection of CFSE labeled cells, mice were sacrificed and lymphoid organs were collected respectively to check the presence of CFSE hi and CFSE lo cells by flowcytometry. By comparing the percentage of CFSE hi and CFSE lo cells presented in different groups of mice, it was found that in iPS/MiDR transferred mice CFSE hi cell fraction did not change significantly (49.5%:50.5%). However, in iPS/OT-I transferred mice, the CFSE hi fraction significantly shrank (5.37%: 94.63%) which was similar to the result obtained from OT-I CTL transferred mice (7.52%:92.48%). Figure 28 demonstrated that target cells were lysed by iPS/OT-I derived CTLs.

To date, our data substantially supported our hypothesis that iPS/OT-I cells could develop into fully mature, antigen-specific CTLs in vivo. However, in many cancer
Figure 28: *In vivo* killing capabilities of iPS/OT-I derived T cells. After the *in vivo* development, the antigen reactivity of iPS/OT-I derived CD8^+^Vb5^+^ T cells were further evaluated by the *in vivo* proliferation/cytotoxicity assay. CFSE<sup>hi</sup> (right peaks) and CFSE<sup>lo</sup> (left peaks) target cells were pulsed with OVA<sub>257–264</sub> peptide and the control, respectively, and were injected into mice 10 weeks after iPS cell transfer or 1 day after OT-I CTLs transfer. Data were representative of 2 independent experiments.
Figure 28
patients received ACT-therapy with processed effector CTLs, it was always found that adoptively transferred CTL numbers were shrinking over a period of time [296, 297]. The great advantage of our strategy of using stem cell, especially iPS-derived T cells, for the ACT-based therapy was that these cells were naïve-phenotyped therefore their life spans would be significantly longer than those of the fully differentiated effector CTLs. In the following work, we were very interested to test our hypothesis that iPS/OT-I developed CTLs will persist in the transferred hosts.

**B.6 iPS/OT-I derived CTLs were persistent in transferred mice**

For monitoring the persistence of iPS/OT-I derived CTLs in the lymphoid tissue of transferred mice, two experiments were conducted. In the first experiment, we aimed to monitor the persistence of iPS/OT-I derived CTLs in the presence of E.G7-OVA tumor cells or antigen stimulation. In the second one, no E.G7-OVA tumor cells were xenografted. To mimic the situation of adoptive transfer with effector-phenotyped CTLs for cancer immunotherapy, OT-I CTLs isolated from OT-I transgenic mice were used as positive control in both studies. In the first tumor challenged assay, in week 7 post-tumor challenge, mice were euthanized and lymphoid tissues were collected. In the second experiment without tumor challenge, mice were sacrificed to collect their spleens and lymph nodes in week 13 post cell transfer. Pooled lymphocytes were prepared for flowcytometric analysis. CD8+ TCR Vβ5+ T cell populations were gated on the dot-plots to represent the persistent CTLs. In tumor challenged mice, the numbers of iPS/OT-I derived CTLs (49.8% of total CD8+ cells) were significantly higher than mice receiving OT-I CTLs (13.9% of total CD8+ cells). In tumor-free mice, the population of iPS/OT-I derived CTLs (47.5% of total CD8+ cells) was also higher than mice receiving
OT-I CTLs (6.02% of total CD8^+ cells). Figure 29 showed the differences between iPS/OT-I derived CTLs and OT-I CTLs in the context of long-term in vivo persistence.

The conclusion of this study was quite obvious that iPS/OT-I derived CTLs were able to persist in the lymphoid tissue of the recipient mice. This might be due to the intrinsic property of iPS-derived T cells. Previous studies indicated that naïve-phenotyped T cells that directly differentiated from HSCs or other progenitor T cells could further develop into memory-phenotype which was a key determining factor for the long-term persistence [112, 298, 299]. Also other studies indicated memory T cells were similar to stem cells in self-renewal through the life span of the host [300, 301]. The result from this study appeared to be another piece of evidence to support previous researches.

So far, our studies in understanding the generation of antigen-specific CTLs from iPS cells have gained solid ground in several aspects such as physical presence, antigen reactivity, cytotoxicity and persistence; however, the most important question has yet been addressed. Whether the iPS/OT-I developed CTLs could kill the tumor cells or not.

B.7 iPS/OT-I derived CTLs were able to infiltrate into tumor tissue

Our previous work has demonstrated that internal OT-I TCR signaling together with other external factors could induce the iPS cells to differentiate and develop into OVA-antigen specific CTLs in the animal. Those iPS derived CTLs were functionally identical compared to mature OT-I CTLs that isolated from the OT-I transgenic mice. Furthermore, they were able to persist longer than effector OT-I CTLs by possible mechanism of memory T cell conversion [112].
**Figure 29: In vivo persistence of iPS/OT-I derived T cells.** Adoptive transfer of GFP⁺DsRed⁺ cells into C57BL/6J mice and then mice were subjected to challenge with E. G7 tumor cells. Antigen-specific T cell persistence were checked seven weeks post tumor challenge or 13 weeks without tumor challenge, CD8⁺Vb5⁺ T cells from the pooled lymph nodes and spleen were analyzed by flow cytometry. Data were representative of 3 independent experiments.
Figure 29
With these findings, we were more interested in exploring the possibility of using the iPS cells as an ultimate source of CTLs in the ACT-based cancer immunotherapy. In order to investigate this possibility, we sought to observe the interaction of our iPS/OT-I derived CTLs with the tumor tissue. Lymphocytes infiltrating into tumor tissue has been widely reported [302-304]. Also, in several animal and clinical studies, TILs were harvested, expanded and re-infused back to treat the original cancer which has gained some initial promises [62, 305-307]. This approach of using TILs has been accepted as the foundation of the modern ACT-based cancer immunotherapy. Direct observance of the presence of the iPS/OT-I derived CTLs in the tumor tissue could serve as the first-hand evidence that iPS cells derived CTLs were interacting with tumor tissue.

To check the presence of iPS/OT-I derived CTLs in the tumor tissue, three individual but related investigations have been performed. In the first study, on day 30 to 35 after tumor challenge, a histological examination was conducted. Briefly, tumor tissues were harvested after euthanizing mice and fixed by using formalin buffer. Hematoxylin & eosin (H&E) staining was performed on paraffin embedded and thin-sliced samples. Prepared slides were evaluated under the guidance of a well-trained pathologist: Dr. Neil Christensen. Figure 30 showed the H&E staining of tumors harvested from mice receiving different types of cells or no cells. Compared to the tumor in OT-I CTL-transferred group, the iPS/OT-I transferred groups showed similar profile such as low cellularity of tumor, connective tissue filled extracellular space, and more importantly, the presence of mononucleocytes in the tumor (red arrows). However, in the control groups received iPS/MiDR cells or no cell transfer no such profile has been observed.
Figure 30: Histological examination of the tumor tissue. On day 30 to 35 after tumor challenge, tumor tissues were examined for tumor-reactive T cell infiltration. H&E stainings were performed and inflammatory cells infiltrated in tumor tissues were indicated by arrows. Data were representative of 3 independent experiments.
Figure 30
This initially supported that iPS/OT-I derived CTLs infiltrated as well as killed the tumor tissue. However, on H&E staining, it was impossible to distinguish the origin of those mononucleocytes. More studies were needed to confirm that those cells were iPS cells originated.

At the same time of conducting histological examination, an immunofluorescent study on the tumor sample was also performed. In this work, tumors were excised from mice shortly after euthanasia and placed on dry ice immediately. Thin slices were made and mounted on regular slides. The detailed protocol of staining process could be found in chapter III. In short, two fluorochrome-conjugated antibodies were used in this study, i.e. FITC-anti-OVA and PE-anti-TCR Vα2. Because E.G7-OVA tumor constitutively expressed OVA protein, the FITC-anti-OVA antibody staining would give a greenish background on the immunofluorescent images. Tumor infiltrated iPS/OT-I derived CTLs could be easily distinguished as the red dots on the green background. Figure 31 showed the sharp differences in infiltrating TCR Vα2+ CTLs between iPS/OT-I transferred tumor and iPS/MiDR transferred tumor samples. Mice received OT-I CTLs and no cells served as positive and negative controls respectively. This study shows iPS/OT-I derived OVA-specific T cells are able to infiltrate into tumor tissue.

In addition to those two studies, a third one was performed to directly visualize the percentages of iPS/OT-I derived CTLs in the tumor tissue. In doing this, tumor tissues were collected as previously described but preserved in 10%FBS conditioned RPMI-1640 medium. Tumor tissues were mechanically broken down to make single cell suspensions. Staining different T cell markers such as CD4, CD8, TCR Vα2 and TCR Vβ5 were performed prior to flowcytometric analysis. By gating on the CD8+ population, it was
Figure 31: Immunofluorescent examination of the tumor tissue. On day 30 to 35 after tumor challenge, tumor tissues were examined for tumor-reactive T cell infiltration. Immunofluorescent stains were performed. OVA-specific Vα2⁺ CTLs (red) infiltrated in OVA-expressing tumor tissues (green) were shown. This figure shows iPS/OT-I derived antigen-specific T cells are able to infiltrate into tumor tissue. Data were representative of three independent experiments.
Figure 31
found that the majority of infiltrated cells were TCR Vα2+ TCR Vβ5+ CTLs in iPS/OT-I transferred mice (72.5% of total CD8+ population) and OT-I CTL transferred mice (35.8% of total CD8+ population) whereas almost no TCR Vα2+ TCR Vβ5+ CD8+ T cells could be detected in either iPS/MiDR transferred or no cell transferred mice. Figure 32 showed the representative profiles of the percentages of TCR Vα2+ TCR Vβ5+ in total CD8+ tumor infiltrating T cells from different groups of mice.

Collectively, our results indicate that iPS/OT-I derived CTLs are able to infiltrate into the tumor tissue and we would like to directly observe that whether these cells could kill tumor cells or not.

**B.8 iPS/OT-I derived CTLs were able to control tumor growth and protect animal**

The ultimate goal of ACT-based cancer immunotherapy is to reduce the tumor burden and enhance the life expectancy of affected cancer patients. To better understand whether iPS derived T cells were suitable for cancer immunotherapy or not, tumor cell counting and tumor tissue measurement were performed.

Tumor cell counting was performed at the early stage of tumor challenge. At day 20 post i.p. E.G7-OVA tumor challenge, mice were sacrificed and peritoneal lavages were performed to collect the free tumor cells in the peritoneal cavity. Collected the tumor cells were washed and resuspend in chilled PBS for enumeration. Cell counting was performed with the regular hematocytometer. It was observed that the absolute cell numbers were substantially lower in iPS/OT-I transferred mice (average 3.1×10^6 cells) compared to mice with iPS/MiDR (average 9.0×10^7 cells) or with no cells (average 9.7×10^7 cells). OT-I CTL transferred mice have significantly low numbers of tumor cells
Figure 32: Flowcytometric examination of tumor infiltrating T cells. On day 30 to 35 after tumor challenge, tumor tissues were examined for tumor-reactive T cell infiltration. Single-cell suspensions were made from tumor tissues and subjected to the analysis for the expression of $\text{V}\alpha 2^+$ and $\text{V}\beta 5^+$ by flow cytometry, after gating on the CD8$^+$ population. Data were representative of 3 independent experiments.
Figure 32

The figure shows a comparison of different cell transfer scenarios. The top left quadrant represents 'No cell transfer', the top right quadrant shows 'iPS cells/MiDR-TCR' with a highlighted area indicating 72.5% activation. The bottom left quadrant depicts 'iPS cells/MiDR' with a highlighted area showing 35.8% activation. The bottom right quadrant illustrates 'OT-I CTLs' with a highlighted area.
(average $3.9 \times 10^7$ cells) in comparison to no cell transferred or iPS/MiDR transferred. But they were still more than the tumor cells isolated from iPS/OT-I transferred mice. Figure 33 demonstrates the differences of tumor cells recovered from intraperitoneal cavity of mice in different groups.

Tumor sizes were also checked at the end stage of tumor challenge. This study showed the actual tumor in the peritoneal cavities or tumor volumes that were removed from the peritoneal cavities of different groups of mice. Only iPS/OT-I or OT-I CTL transferred mice could survive to day 50, therefore, the tumors shown in this figure were on the day 50 post tumor challenge. However, tumors of either control iPS/MiDR group or no cell transferred group were on the day 30 post E.G7-OVA inoculation. In Figure 34, the day 50 tumor samples recovered from iPS/OT-I or OT-I CTL transferred mice were visually smaller compared to the day 30 tumor samples excised from control iPS/MiDR transferred or no cell transferred groups. Therefore, this piece of information provided additional supporting evidence that iPS/OT-I derived CTLs could kill tumor cells and restrain tumor growth.

As mentioned above, the ultimate and most important goal of ACT-based cancer immunotherapy is to increase the survival of cancer patients. In our previous works, iPS/OT-I cells have been shown that were able to differentiate into functional antigen-specific CTLs in vivo as well as infiltrate into tumor tissue to kill the antigen-bearing tumor cells. Hence, studying the iPS derived CTLs in protecting mice and prolonging their life spans could provide more insights about applying iPS cells for a potential ACT-based cancer immunotherapy. To evaluate this, mice survival were closely monitored every single day after tumor challenge. Starting from day 30 post E.G7-OVA tumor
Figure 33: Peritoneal lavage recovered tumor cell numbers. On day 20 after tumor challenge, tumor cells in the peritoneal cavity were enumerated. Data represented mean (±SEM) tumor cell counts from 6 individual mice. One-way ANOVA test was used for statistical analyses between 2 groups (*, P < 0.05). Data were representative of 3 independent experiments.
Figure 33
Figure 34: Animal autopsy post-tumor challenge. On day 30 (No transfer and iPS/MiDR) or day 50 (OT-I CTLs and iPS/OT-I) after tumor challenge as described previously, tumor tissues from the peritoneal cavity were displayed. Data were representative of three independent experiments (n=6).
Figure 34
challenge, mice received either control iPS/MiDR cells or no cells succumbed due to heavy tumor burden. The transferred OT-I CTLs could initially protect mouse at the very early stage of tumor challenge. At the late stage, probably caused by CTL depletion or $T_{\text{REG}}$ prevalence [308], the tumor sizes increased sharply and three mice succumbed between day 42 and day 45. Only 3 mice that received OT-I CTLs survived 50 days to the end of the observation.

Not surprisingly, as observed previously that iPS/OT-I derived CTLs persisted longer than OT-I CTLs, iPS/OT-I transferred mice all survived the tumor challenge to the end of this experiment. This mouse survival study substantially confirmed our previous findings about iPS/OT-I cells in all aspects. The mouse survival curve as depicted by Kaplan-Meier plot was summarized in Figure 35.

So far, with all information we have obtained, it was very clear that iPS/OT-I cells were able to differentiate into antigen-specific CTLs in the host with the stimulations from both TCR signaling and a complete set of *in vivo* growth and differentiate factors. The iPS/OT-I derived CTLs could kill tumor cells and promote the survival of recipients.

**B.9 No significant number of Treg cells were developed from iPS/OT-I cells**

In many studies, $T_{\text{REG}}$ cells have been shown that play a negative effect in suppressing the CTL mediated anti-tumor responses [309-312]. The presence of $T_{\text{REG}}$ cells in any cancer patients receiving CTL-based or relevant ACT therapy could significantly lower their therapeutical effects or cause the failure of the approach. Excluding $T_{\text{REG}}$ cells and other immunosuppressive cells has been suggested in carrying out any cancer immunotherapy [313, 314]. In our scenario, one interesting question we
Figure 35: Animal survival chart. Different groups of mice survival on day 50 post-tumor challenges. Kaplan–Meier survival curves were shown (n=6). (*, P < 0.05; **, P < 0.001), 1-way ANOVA with Newman–Keuls multiple comparison test was used to perform the statistic analysis. Data were representative of 3 independent experiments.
Figure 35
wanted to look into was whether iPS/OT-I cells could also develop into T\textsubscript{REG} cells \textit{in vivo}. Our hypothesis is due to the nature of the MHC-I restricted OT-I TCR, it is unlikely that OT-I TCR would direct iPS cells to develop into CD4\textsuperscript{+} T cells, especially the CD4\textsuperscript{+} FoxP3\textsuperscript{+} T\textsubscript{REG} cells \textit{in vivo}.

To check the T\textsubscript{REG} profile in cell transferred mice, on week 8 after post-cell transfer, mice were sacrificed and lymphocytes were pooled from their lymphoid tissues. Single lymphocyte suspensions were stained with various fluorochrome-conjugated antibodies and flowcytometric analyses were performed thereafter.

It was observed that statistically there were no significant differences in T\textsubscript{REG} population between mice receiving iPS/OT-I (6.65% of total CD4\textsuperscript{+}CD25\textsuperscript{+} cells) and mice receiving no cells (6.69% of total CD4\textsuperscript{+}CD25\textsuperscript{+} cells). However, the T\textsubscript{REG} population slightly increased in the mice receiving iPS/MiDR control cells (8.71% of total CD4\textsuperscript{+}CD25\textsuperscript{+} cells) but within the normal range of naturally occurring T\textsubscript{REG} cells [315]. Figure 36 showed the dot-plots of the CD4\textsuperscript{+}FoxP3\textsuperscript{+} cells gated on the CD4\textsuperscript{+}CD25\textsuperscript{+} T cells.

This study demonstrated that our approach of using iPS cells that overexpressed MHC-I restricted TCR did not increase the development of T\textsubscript{REG} cells.

\textbf{B.10 Summary and conclusion}

When finished this second part of my thesis research, it was quite clear our central hypothesis is strongly supported by solid data we have obtained that iPS cells are able to differentiate into T lymphocytes.
Figure 36: Development of T<sub>REG</sub> cells. As described previously, iPS cells were adoptively transferred into C57BL/6J mice. After eight weeks, CD4<sup>+</sup> Foxp3<sup>+</sup> cells from pooled lymph nodes and spleen were analyzed by flow cytometry, after gating on CD4<sup>+</sup>CD25<sup>+</sup> cell populations. Pooled lymph nodes and spleen from C57BL/6J mice that did not receive iPS cell transfer were used as a control. Meanwhile, the CD4<sup>+</sup>CD25<sup>+</sup> T<sub>REG</sub> cells are TCR Vβ5 negative (data not shown). Data were representative of two independent experiments.
Figure 36
In the first section, we investigated the capability of iPS cells in generalized T lineage differentiation. And a conclusion that Notch signaling stimulation could induce iPS cells to progenitor T lymphocytes has been drawn. However, in designing a personalized cancer immunotherapy by using iPS cells, the most important feature of iPS-derived T cells should be their reactivity to tumor antigen. According to previous studies, a successful cancer regression could only be achieved by using tumor infiltrating T cells or genetically modified T cells that express either tumor antigen-specific TCR or chimeric antigen receptor (CAR) [15, 63, 316]. Therefore iPS cell-derived, antigen-specific T cells were greatly favored in terms of conducting a successfully ACT-based anti-cancer regimen. Thanks to other group of scientists who have already performed some pioneering work in understanding of the important role TCR has played in directing T lymphocyte development from stem cells [123, 124]. We decided to understand in what extent that TCR could determine the T lineage differentiation in iPS cells. To simply the condition, we used the in vivo developing system in immunocompetent C57BL/6J mice other than the previously used in vitro OP9-DL1 co-culture system to study this question. Because the immunocompetent C57BL/6J mice have a full spectrum of factors that playing role in T cell development, by subtracting the differences between negative and positive controls, we can exclusively focus on the sole function of TCR in determining T cell development and differentiation. In doing this, TCR overexpressed iPS cell line (iPS/OT-I) were generated.

First, we observed that iPS/OT-I cells were able to differentiate in vivo in C57/BL6J mice to T cells as detected by flow cytometric analysis. In contrast, the control iPS/MiDR cells could not differentiate into T cells in vivo. This result suggested that TCR played
function as the determinant of stem cell differentiation in T lineage. Further analysis of these \textit{in vivo} generated T cells showed that MHC-I restricted TCR directed iPS to differentiate into CD8$^+$ T cells and the corresponding TCR was expressed on the surface of developed iPS cell. Second, iPS/OT-I derived CD8$^+$ CTLs were able to persist in the lymphoid organs of the recipient mice possibly due to the mechanism of memory T cell formation. Third, iPS/OT-I derived CTLs were able to respond to antigen peptide stimulation as secreting cytokines. Also, cytotoxicity of iPS/OT-I derived CTLs could be observed as antigen peptide-pulsed target cells were lysed. These studies showed functional, antigen-specific CTLs could be programmed by introducing a corresponding TCR to iPS cells.

In the hope of using iPS cells for cancer immunotherapy, we decided to evaluate their interactions with tumor cells such as tumor infiltration and tumor killing. A xenograft tumor model was used to test the tumor-reactivity of \textit{in vivo} developed iPS/OT-I cells. E.G7-OVA, a murine thymoma cell line overexpress OVA, was used as the tumor model to challenge cell transferred mice intraperitoneally. It was found that iPS/OT-I derived CTLs were able to infiltrate into the tumor tissue, kill tumor cell and control the tumor growth by different assays. Last but not least, iPS/OT-I derived CTLs could significantly prolong the survival of the recipient mice compared to other groups, which substantially supported the previous studies of tumor interaction.

Based on these findings, it was obvious that engineered iPS cells could be a potential source of getting an unlimited numbers of tumor antigen-specific T cells required for a successful, personalized cancer immunotherapy.
To further explore this possibility, on top of our current work, a lot of more efforts were still pending. Two major one I have looked into in my thesis study were deciphering the synergistic roles of TCR and Notch signals in guiding the TCR-restricted T lineage differentiation of iPS cells and generating large numbers of melanoma-specific CTLs from iPS cells \textit{in vitro}.

\textbf{C. Notch signal and TCR signal synergistically promote T lineage development from iPS cells}

We have tested both \textit{in vitro} and \textit{in vivo} approaches of inducing T lymphocytes from iPS cells. The take home messages from our previous studies as well as other publications were both Notch signaling and TCR signaling pathway played critical roles in the T lineage differentiation process from stem cells, especially the iPS cells. In the first part of our study, Notch signal alone could support the generation of T lymphocytes from iPS cells. From the second part of my research project we learned that TCR played major roles in iPS-derived T cell development based on two facts: first, immunocompetent mice express a full spectrum of required factors for T cell development including Notch ligands, and second, TCR overexpressed iPS cells were able to develop into corresponding T cells. These clearly suggested Notch and TCR mutually promoted T cell development from iPS cells. However, no study has been done to explain the relative roles of these two signals.

To better understand the mechanisms of T cell development from iPS cells, therefore we could generate large numbers of different subtypes of T cells for
immunotherapy to either cancer or autoimmune disorders. We have performed several preliminary studies trying to illustrate the relationship between Notch and TCR.

In doing this, the *in vitro* differentiation system used in **Part A** and the OT-I TCR-transduced iPS cells used in **Part B** were combined together. In short, iPS/OT-I cells or control iPS/MiDR cells were cocultured with confluent OP9-DL1 cell monolayer. The general coculture protocol has been introduced in Materials and Methods. At different time points of coculture, iPS/OT-I or control cells were harvested from culture to perform different experiments. The simplified experimental scheme is summarized in Figure 7.

**C.1 the Notch and TCR dual signals promoted iPS/OT-I cells towards CD8 differentiation *in vitro***

In one of the initial experiments, iPS/OT-I cells were cocultured with OP9-DL1 cells, after 15 days of differentiation, cells were harvested from culture and stained with various types of fluorescent-conjugated antibodies prior to flow cytometric analysis. The flow-cytometric data showed that iPS/OT-I cells at day 15 of differentiation have a higher percentage of CD8$^+$ T cells (3.84% of DsRED$^+$ cells) compared to the control iPS/MiDR cells (1.23% of DsRED$^+$ cells). However the TCR V$\beta$5 chain could not be detected on the surface of iPS/OT-I cells (0.029% of DsRED$^+$ cells) at this time of the differentiation. As we proposed and observed previously, MHC-I restricted OT-I TCR determined CD8$^+$ direction differentiation, in this study we also observed that the percentage of CD4 expressing DsRED+ cells were slightly lower in iPS/OT-I cells (1.44% of DsRED$^+$ cells) compared to iPS/MiDR cells (1.78% of DsRED$^+$ cells). Figure 37 shows the expression levels of different surface markers in each group.
Figure 37: Surface marker study on day 15 of differentiated iPS cells. On day 15 of coculture, iPS/OT-I cells were harvested and flowcytometric analysis on the surface expression of CD4, CD8 and TCRVβ5 were performed.
To further characterize these differentiated cells, CD3⁺ pan-T cells were gated to evaluate the expression levels of either CD4 or CD8 molecules. It was found that in iPS/OT-I groups, more than half of the CD3⁺ cells were CD8⁺ SP cells (50.4% of total CD3⁺ cells). However, only a small fraction of SP cells could be spotted in iPS/MiDR group (26.8% of total CD3⁺ cells). Figure 38 showed the dot-plots representing the expression levels of CD4 and CD8 molecules in each group.

Taken together, the readout of this experiment was, under the way of in vitro T lineage differentiation, Notch signal provided initial stimulation to turn on the differential process; later on, the TCR signaling pathway got activated to further direct early stage T-determined precursor cells to undergo a TCR or MHC-restricted sub-lineage development. This work supported our previous findings that Notch signal promoted iPS cells’ T lineage determination and TCR directed the MHC-restricted T lymphocyte development of the iPS cells. However, this was the very preliminary study we have done so far. More repeats are still needed. Additional experiments at different time points of in vitro differentiation should also be performed to delineate a clear picture of the synergistic role Notch and TCR played in directing T lineage differentiation.

C.2 the Notch and TCR signals might crosstalk via the TGF-b signaling pathway

In our previous work, we have already observed the phenotypic and morphological changes of iPS cells undergoing T lineage differentiation. And, we understood that dual Notch and TCR signals played significant function in determining T lineage differentiation. However, no detailed exploration about the intrinsic mechanism how
Figure 38: Study on the day 15 of the iPS cells differentiated CD3\(^+\) population. On day 15 of coculture, iPS/OT-I cells were harvested and flowcytometric analysis on the surface expression of CD3, the CD4 and CD8 expression levels were gated on the CD3\(^+\) population.
Figure 38

[iPS/MiDR graph]

[iPS/OT-1 graph]
Notch and TCR modulate T cell development has been performed yet. We were very interested to look into this intriguing question. Therefore, following the previous ones, after consulting with Drs. Xin Liu and Thomas Loughran at the Penn State Cancer Institute, we tried to evaluate the gene expression and propose a possible relationship between Notch and TCR signals. In conducting this study, qRT-PCR assays were used to check the gene expression profiles of iPS/OT-I cells under T lineage differentiation.

The scheme is described in Figure 7 as described above. In summarized of the experimental protocol, iPS/OT-I cell or control iPS/MiDR cell was cocultured with OP9-DL1 cells for 13 days. At the end of coculture, cells were harvested and RNAs were exacted for qRT-PCR analysis. In all qRT-PCR experiments, the expression level of a house keeping gene Glyceraldehyde 3-phosphate dehydrogenase (GADPH) RNA was used as the internal control and all experimental readings were normalized to the GADPH value.

First we understood that the Nanog gene was the house keeping gene in both ESCs and iPS cells. Its expression was maintained in the pluripotent stage but when stem cells started to differentiate, the Nanog gene would be ultimately shut down. There is no Nanog expression in differentiated tissue [268]. To validate the qRT-PCR setting and understand the status of differentiation of iPS cell under in vitro T lineage differentiation, the Nanog expression levels were recorded. Being consistent with published data, Nanog expression levels were relatively high in undifferentiated cells but significantly reduced in differentiated iPS/OT-I cells (4 folds decrease) or iPS/MiDR cells (25 folds decrease). Figure 39 showed the relative expression levels of Nanog to GADPH in different group of samples. This finding agreed with our previous observation that TCR signal alone
Figure 39: Expression level of Nanog gene. On day 13 of coculture, different groups of cells were harvested to isolate the total RNA for the qRT-PCR reaction were performed thereafter. * indicates P<0.05; ** indicates P<0.001. Error bars were determined from triplicates of each sample. Data were representative of two independent experiments.
would not automatically turn on the T lineage differentiation of iPS cells because the majority of TCR-overexpressed cells kept the stem cell morphology and expressed GFP in the culture. In order to activate the differentiation process, additional stimulation such as Notch ligand binding was required. The qRT-PCR results of Nanog expression supported that the binding of Notch ligand to its receptor on iPS cells was the determining event to turn on their T lymphocyte differentiation since the stemness marker disappeared and T cell markers emerged.

The Nanog expression profiles in undifferentiated and differentiated iPS/OT-I cells indicated that Notch signal was the initiating event in programming T lineage differentiation. Therefore, we were very interested to look into the molecular pathways in regulating this process. It has been shown that Notch signaling pathway has a very close relationship with the transforming growth factor-beta (TGF-β) mediated signal transduction pathway in both synergistic and antagonistic patterns [120-122, 317]. In this study, based on the insightful suggestion from our collaborators, we investigated the possible cross-talk between the Notch signaling pathway and TGF-β signaling pathway in terms of regulating the T cell development from stem cells especially the iPS cells.

RNAs were extracted from the different cells were prepared as described before in Figure 7. In short, after 13 days of coculturing with OP9-DL1 cells, either iPS/OT-I cells or control iPS/MiDR cells were harvested. Day 0 iPS/OT-I cells and iPS/MiDR cells were used as controls to extract RNAs. qRT-PCR was performed as previously described and readings were normalized to GADPH values in each setting. It was observed that before the start of differentiation that stimulated by Notch ligand, both the TGF-β1 and TGF-β2 expression levels were low in either iPS/OT-I cells or iPS/MiDR cells. However,
after 13 days of Notch ligand stimulation, their relevant expression levels have dramatically increased. Figure 40 showed the expression differences in both TGF-β1 and TGF-β2 mRNAs in different groups of cells.

After finishing this experiment, it was indicated that Notch and TCR signals stimulated T lineage differentiation from iPS cells might be a TGF-β signaling mediated cross-talk. However, this was another very preliminary experiment that trying to understand the mechanism of T cell differentiation from stem cells. More experiments would be lined up to further explore how Notch and TCR determined T cell development from stem cell, especially iPS cells. Unveiling the complete mechanism would significantly improve our understanding about the T cell development and outline a better approach to generate antigen-specific T cells for ACT-based cancer immunotherapy.

C.3 Summary and Conclusion

As described in previous section, our initial findings indicated that Notch signaling stimulation could promote T lineage differentiation from iPS cells in vitro. Later on, a second study showed that TCR signals could further drive iPS-derived cell into antigen-specific T cell in vivo in which a full spectrum of differentiation-determinant factors including Notch ligands were present. By combining two pieces of information together, it was highly suggested that dual Notch and TCR played important roles in directing iPS cell differentiation into T lymphocyte. Hence, it would be very interesting to investigate in what extent Notch and TCR signaling pathways have played to determine T lineage development from iPS cells. To study this, both in vitro Notch signaling stimulation system (OP9-DL1 cells) and TCR-bearing iPS cells (iPS/OT-I cells) were used. After a
Figure 40: TGF-β1 and TGF-β2 expression levels in iPS/OT-I cells. On day 13 of coculture, different groups of cells were harvested to isolate the total RNA for the qRT-PCR reaction were performed thereafter. * indicates P<0.05; ** indicates P<0.001. Error bars were determined from triplicates of each sample. Data were representative of two independent experiments.
brief coculture of iPS/OT-I with OP9-DL1 cells, it was found that although OT-I specific TCR (Vβ5 chain) was not detected from partially differentiated iPS/OT-I cells, these cells has skewed their differentiation towards the CTL subtype in terms of expressing CD8 over CD4 molecules. Meanwhile, no significant CD8 population increase was observed in control cells. This observation indicated Notch and TCR played a synergistic role in directing T cell development and this was consistent with the current model of T cell development. As documented, mature TCR molecules could only be detected at the late DP stage which was later than CD4 and CD8 [318, 319]. Similarly, CD3 molecules could be detected in partially developed iPS/OT-I or control derived T cells; however, iPS/OT-I derived CD3+ cells have a higher CD8+ population than control. This finding supported our hypothesis that TCR direct a subtype differentiation. The CD3 expression profile was also consistent with current model of T cell development that CD3 molecule could be constitutively detected on T cells from the very early stage of the intrathymic T cell commitment [320].

Subsequent study by analyzing the gene expression profile further showed that after coculture, gene expression profile has changed. Stem cell marker such as Nanog was down-regulated but other molecules were up-regulated. In this study, we showed that TGF-β signaling pathway was highly involved in the T cell differentiation process. Previous studies have clearly shown that TGF-β signaling, especially TGF-β1 was critical in T cell development, our findings was generally consistent with previous reports [118, 119, 321-323]. However, we also found that besides TGF-β1, TGF-β2 and other signaling pathway components were upregulated after Notch and TCR signals turned on the T lineage commitment of iPS cells.
Although this part of my thesis research was largely exploratory and more solid works needed to be finished to further confirm my observation. From current picture, it was quite clear that both Notch and TCR signaling pathway have determinant roles in T cell commitment from iPS cells. By analyzing the data, it was suggested that the role of TCR in T cell development was later than Notch. Generally, binding of the Notch ligand to iPS cells was the initiating event in T lineage commitment as supported by our data that TCR transduction alone would not down-regulate the stem cell marker Nanog gene (Figure 39). However, under Notch induced T lymphocyte differentiation, TCR signal promoted this process as the qRT-PCR data pointed out that compared to control group, the expression levels of genes-of-interest in iPS/OT-I group were always higher.

In summary, a broad picture of the signaling pathways getting involved in T lineage commitment from iPS cells could be initially drawn in Figure 41. Generally, Notch signal would turn the switch of T cell differentiation on, and then TCR signal would join in with the Notch later on. Both of them would play certain significant roles in regard to T cell commitment, especially a TCR-restricted commitment, in a synergistic fashion. To fill more information into this picture, extensive works are still pending.

At the same time of trying to understand the mechanisms of iPS-derived T cell development, we were also very interested to perform some cutting-edge translational study by using iPS cells. As mentioned previously, our long-term goal of studying T cell differentiation from iPS cells was to develop a new generation of personalized, ACT-based cancer immunotherapy. Although OVA system has been widely used in the study of immunology for many years, some researchers argued that OVA protein was not naturally expressed in our current murine and human model. Compared to the over-
**Figure 41: Schematic illustration about the cross-talk between Notch and TCR signals.** The possible cross-talk between the Notch pathway and the TCR pathway via the TGF-β mediators were proposed based on the data. First, Notch ligand stimulation activates the T lineage differentiation of iPS cell, later on, TCR signal joins this event and further promotes iPS differentiation to T cell. Other evidence shows TGF-β might be the intermediate that connects these two signaling pathways.
expressed OVA protein, the expression levels of some endogenous protein, especially those tumor markers were significantly low. To further test the possibility of using iPS cells in the personalized cancer immunotherapy, we have to show that real tumor could be tackled by iPS cell derived CTLs. In answering this question, melanoma and melanoma-antigen specific TCR were picked up.

**D. Generating cell lines for melanoma immunotherapy**

Melanoma is the most lethal type of skin cancer in human especially when it gets distal metastasis. Although many new drugs in treating melanoma have been developed, the long-term prognosis of the patients with metastatic melanoma has not changed a lot. For instance, Vemurafenib, a B-RAF\(^{V600E}\) kinase inhibitor that has been approved by FDA as the most promising drugs in treating metastatic melanoma, could only achieve a median progression-free survival of 7 months [224]. Compared to chemotherapeutical management such as Vemurafenib, adoptive T cell transfer-based melanoma therapy has achieved some substantial improvements, especially a complete remission, in certain late stage patients by using either tumor TILs or lymphocytes genetically engineered to express tumor antigen-specific TCRs [63, 324-326]. Although it was still far from perfect, the initial success of this novel approach could give us a new hope to fight cancer, especially by harnessing our own immune system. In comparison to TILs, the genetically engineered T lymphocytes by unanimously overexpressing a tumor antigen-specific TCR have several important properties including the high affinity and specificity of their TCR that could direct a precise targeting and killing of the tumor cells [63, 327]. The keys to conduct an effective ACT-based cancer immunotherapy by using these genetically modified T cells include two major steps: first, the identification of tumor-specific
antigen and its corresponding TCR, and second the preparation of a large number of T cells. By collaborating with other researchers who endeavored in discovering tumor antigens and relevant TCRs, our focus in modulating this ACT-based cancer immunotherapy was mainly on the second part, *i.e.* the acquisition of adequate T cells for this anti-cancer regimen.

In the majority of current clinical trials for ACT-based cancer immunotherapy, the T cells used for *ex vivo* expansion and genetic modification came from the patient’s own peripheral blood. As the nature of human immune system that it was continuously exposed to various antigens, the major population of the peripheral T cell pool was consisted by effector (T\textsubscript{EFF}) and effector memory (T\textsubscript{EM}) T cells. It was found that several developmental and/or functional defects have been associated with these T\textsubscript{EFF} and T\textsubscript{EM} cells such as anergy, low *ex vivo* proliferation and most important short-life span due to different factors [325, 328-330]. Meanwhile other studies suggested that using naïve phenotyped T (T\textsubscript{N}) cells were more efficient than T\textsubscript{EFF} or T\textsubscript{EM} cells in ACT-based cancer immunotherapy [328, 331, 332]. Moreover, it has been observed that less differentiated T cells used in adoptive transfer could gain a better persistence compared to other types of T cells, including T\textsubscript{N} cells [324, 330, 333]. This suggested that central memory T (T\textsubscript{CM}) cells would be a promising candidate in adoptive transfer thanks to their robust proliferation, persistence and differential capabilities into all subsets of T cells [112, 334].

In summary, it was obvious that using T\textsubscript{N} cells or T\textsubscript{CM} cells would be better for conducting an ACT-based cancer immunotherapy. But how could we get enough these cells from the cancer patients, especially adult patients was still in vague. As mentioned above, HSCs and ESCs have been shown being able to develop into T lymphocytes even
antigen-specific CTLs by different approaches. Here in our studies, we showed that iPS cells were similar to both HSCs and ESCs in the T lineage differentiation capabilities. Therefore, our approach by using iPS cells in generating large numbers of CTLs for ACT-based cancer immunotherapy could bypass several ethical and technical problems regarding to the use of human stem cells.

With our previous results, we aimed at generating large numbers of melanoma antigen-specific CTLs from iPS cells for the adoptive transfer to treat the melanoma patients. By collaborating with other groups, we obtained both human and murine TCRs that target different antigens in either human or murine melanoma such as human tyrosinase and murine tyrosinase related protein 2 (TRP-2). These two melanoma-related antigens have been shown involved in the melanin synthesis and were unanimously expressed in the majority of melanoma tissues [335-338]. Both human anti-tyrosinase TCR (ATT) and mouse anti-TRP-2 TCR (TRP-2) have been shown effective in control melanoma growth in different models [242, 262]. Based on these preliminary studies, we would like to investigate whether or not giving iPS cell these TCRs could generate melanoma-specific CTLs for melanoma management in murine models.

Although our previous work showed iPS/OT-I cells could develop into CTLs in vivo, several studies suggested the in vivo tumorigenesis of directly introduced undifferentiated ESCs or iPS cells [339, 340]. Because it was focused on the characterization of the T lineage differentiation, especially antigen-specific CTL differentiation from iPS cells, this tumorigenic property of iPS cell has not been taken into consideration. However, for designing a melanoma-specific immunotherapy by using iPS cells for potential clinical utilizations, this was a serious problem. It was
suggested that some approaches could be applied to reduce the possibility of tumorigenesis from stem tissue, such as *in vitro* selection and apoptosis-induction [341, 342]. The purposes of these approaches were to remove undifferentiated cells. In the Part C of this study, it was shown that Notch ligand DL1 stimulation could promote the CTL development from iPS/OT-I cell, therefore, the in vitro T cell differentiation system could be incorporated into our protocol of generating melanoma-specific CTLs for two reasons: avoid potential tumorigenesis and promote early maturation.

In short, our general approach of generating melanoma-specific CTLs from iPS cells for an ACT-based immunotherapy consisted by two portions, *in vitro* differentiation induction and *in vivo* maturation as well as melanoma management.

**D.1 Generation of OP9-DL1/DL4 cell line**

There are two major families of Notch ligands, *Delta-like* (DL) family and *Jagged* family [343]. In *Delta-like* family, there are three members DL-1, DL-3 and DL-4 [344, 345]; in *Jagged* family, two members have been discovered are *Jagged-1* and *Jagged-2* [346, 347]. In the T lymphocyte development area, Notch ligand DL-1 and DL-4 have been shown play key roles in T lineage lymphogenesis in the thymus [95, 96, 265, 348, 349]. Moreover, a study showed that DL-1 knockout mice have an intact T cell development however T cell development was disrupted in DL-4 conditionally knockout mice[96, 350]. In summary, above data indicated that both DL-1 and DL-4 were important for T lymphocyte development however DL-4 was indispensable. For this reason and to better modulate the *in vitro* T lineage differentiation induction system, we
would like to introduce both DL-1 and DL-4 molecules into the OP9 bone marrow stromal cell to generate the OP9-DL1/DL4 cells.

The constructs containing either DL-1 or DL-4 genes were kind gifts from one of our collaborators. PCR primers were designed to amplify those genes (Please refer to Table 2 for sequence information). To obtain an equal expression of both DL-1 and DL-4 gene in OP9 cells, a picornavirus-2A self-cleaving sequence was placed in between of two genes by PCR. Figure 42 showed the schematic representation of the construct. Amplified PCR fragments were subcloned into pMiG-DL1/DL4 retroviral vector.

Retroviral transduction was performed as described above. GFP+ OP9 cells were identified as genes transduced cells. Figure 43 showed the fluorescent microscopic representation of transduced GFP+ OP9-DL1/DL4 cells. Fluorescent activated cell sorting was performed collect the GFP+ cells. Flow-cytometric analysis of DL-1 and DL-4 expression on sorted OP9-DL1/DL4 cells is summarized in Figure 44. In this work, OP9-DL1/DL4 cell line was established for future use.

D.2 Generation of iPS/TRP-2 and iPS/ATT cells

To test the tumor-antigen reactivity of iPS cell for designing an ACT-based, iPS-derived melanoma immunotherapy, the first work to be done was the construction of iPS cell lines that expressing tumor antigen reactive TCRs. Therefore, at the same time of generating the OP9-DL1/DL4 cell line, iPS cell lines expressing melanoma antigen-specific TCRs were also undertaken. Through collaboration, we obtained two sets of melanoma-specific TCRs: one was a human TCR that recognized human tyrosinase and the other was a murine TCR that targeted TRP-2 protein.
Figure 42: Schematic representation of the DL1/DL4 MiG construct. The simplified vector information was provided on the schematic description.
Figure 42
Figure 43: Fluorescent microscopic profile of OP9-DL1/DL4 cells. After the retroviral-mediated gene transduction, cells were checked by fluorescent microscope. Data represented two sets of independent experiments.
Figure 43

Merge

GFP Channel

Bright field
**Figure 44: Flow-cytometric profiles of OP9-DL1/DL4 cells.** After sorting, cells were evaluated for the surface expression of DL1 and DL4 ligand by flowcytometry. OP9 cells were used as negative control. Date were representative of three sets of independent experiments.
Figure 44

OP9 cells

OP9–DL1/DL4 cells
Previous work has demonstrated a transgenic mouse whose CD8⁺ T cells universal express an MHC-I restricted anti-TRP-2 TCR is established and its CD8⁺ T cells are able to control melanoma growth [262]. Therefore, we decided to test this anti-TRP-2 TCR in iPS cell. In brief, the system is based on the genetic background of C67BL/6J mouse strain, which includes the murine iPS cell line we used previously, murine anti-TRP-2 TCR, murine B16-F10 melanoma cell line and C57BL/6J mouse strain. This section will describe the generation of iPS cells expressing anti-TRP-2 TCR (or the iPS/TRP-2 cells).

Meanwhile, in collaboration with Dr. Richard Morgan in NIH, we obtained the human anti-tyrosinase TCR (ATT) for the generation of human iPS cell line that could specifically target tyrosinase (hiPS/ATT) for controlling the melanoma. For the initial evaluation, we would use mouse iPS cell line and a humanized mouse model to investigate their anti-melanoma efficacies in animals (it is designated as iPS/ATT cell line). Human study would only be considered until all safety and efficacy issues have been completely addressed. Because of the time limit, only cell line construction has been done and following story would be finished by coming students or post-doctoral fellows in the laboratory. Therefore, I would only briefly summarize the cell line preparation in this section.

For the generation of miPS/ATT cells, it was very straightforward. Morgan group has already linked the TCR-α chain and TCR-β chain with a P2A self-cleaving sequence. According to the genetic map, ATT gene was cloned by PCR with specific endonuclease cutting sites added in both flanks. And then, PCR fragments were recovered by shuttle vector and subsequently introduced into DsRed tagged retroviral vector MiDR to make the ATT-MiDR construct. Retroviral transduction was performed as previously described.
After transduction, recovered GFP\(^+\) DsRED\(^+\) double-positive cells were sorted at the Flowcytometry Core Facility by FACSARia cell sorter. Sorted GFP\(^+\) DsRED\(^+\) iPS cells were placed on irSNL76/7 feeder cell for coming characterization. Figure 45 showed the fluorescent microscopic pictures of transduced GFP\(^+\) DsRED\(^+\) miPS/ATT colony on the feeder cells. PCR analysis of the full length ATT gene by using the genomic DNA extracted from sorted miPS/ATT cell showed the presence of ATT gene. Figure 46 showed the detection of ATT gene in the genome of miPS/ATT cells. Based on these data, it was indicated that iPS/ATT cells were generated by retroviral-mediated transduction. Sorted cells were preserved in 10% DMSO FBS freezing buffer and frozen in liquid nitrogen for future use.

Compared to the relatively easy generation of ATT-MiDR construct, the generation of TRP-2-MiDR construct was quite similar to the generation of DL1/DL4-MiG construct. Figure 47 showed the schematic representation of the construct. Individual TCR-\(\alpha\) chain and TCR-\(\beta\) chain genes were received from Hurwitz group at National Cancer Institute of the National Institutes of Health. The genes were cloned and linked by a P2A sequence by using PCR. Shuttle vector recovered PCR fragments were ligated into the MiDR vector to make TRP-2-MiDR retroviral vector. After initial characterization of the construct, retroviral-mediated transduction was performed and GFP\(^+\) DsRED\(^+\) double-positive iPS cells were sorted as described in previous paragraph. FACSARia separated GFP\(^+\) DsRED\(^+\) iPS cells were also evaluated as by the approached described previously. Figure 48 showed the fluorescent microscopic presentations of transduced iPS/TRP-2 cells. Figure 49 displayed the presence of TRP-2 gene in the genome of transduced and sorted iPS/TRP-2 cells.
D.3 Summary and Conclusion

In this part of the work, three different types of cell lines have been successfully constructed and characterized, due to the limit of time, this work will be continued after my thesis defense and it will be further assisted by coming lab personnel.

The simplified scheme for this study is summarized in Figure 50.
Figure 45: Fluorescent microscopic profile of miPS/ATT cells. After the retroviral-mediated gene transduction and fluorescent-activated cell sorting, GFP$^{+}$DsRED$^{+}$ double positive cells were checked by fluorescent microscope. Figure shows an iPS colony expresses both GFP and DsRED. Data were representative of two independent experiments.
Figure 45

ATT iPS Bright field  ATT iPS GFP

ATT iPS DsRED  ATT iPS Overlay
Figure 46: PCR profile of the gene integration in miPS/ATT cells. After cell sorting, the total cell DNA was harvested and a PCR assay was performed to check the gene integration. Date were representative of two sets of independent experiments.
Figure 46

PCR

Marker  Vector Ctrl  iPS/MiDR  miPS/ATT
Figure 47: Schematic representation of the TRP-2 MiDR construct. The simplified vector information was provided on the schematic description.
Figure 48: Fluorescent microscopic profile of iPS/TRP-2 cells. After the retroviral-mediated gene transduction and fluorescent-activated cell sorting, GFP$^+$DsRED$^+$ double positive cells were checked by fluorescent microscope. Figure shows an iPS colony expresses both GFP and DsRED. Data were representative of two independent experiments.
Figure 48

TRP2 iPS Bright field

TRP2 iPS GFP

TRP2 iPS DsRED

TRP2 iPS Overlay
Figure 49: PCR profile of the gene integration in iPS/TRP-2 cells. After cell sorting, the total cell DNA was harvested and a PCR assay was performed to check the gene integration. Data were representative of two sets of independent experiments.
Figure 49

PCR

Marker  Vector Ctrl  iPS/MiDR  iPS/TRP-2
Figure 50: The schematic illustration of the experiment approaches to investigate the iPS-derived, melanoma-specific CTLs. The general approach will be divided into *in vitro* evaluation and *in vivo* evaluation.
Figure 50

**In vitro** characterization:
Surface marker
T cell function
Antigen reactivity

**In vivo** Characterization:
T cell development
T cell persistence
Tumor management

Adoptive transfer

IPS/TRP2 cells

GF9-DL1/DL4 monolayer
Chapter V: Discussion and Future Directions

A. Discussion

Although there are numerous papers coming out every day in the study of the iPS cells from many aspects, before the start of my thesis research, there was little information available about the differential capabilities about iPS cells. There were studies reporting the successful programming of iPS cells into functional cardiomyocytes [274, 275]. However, no study has been documented to investigate the ability iPS cells in hematopoietic lineage, especially T lymphocyte lineage differentiation. The Notch signaling pathway has already been demonstrated to play an important role in the commitment of T lymphocytes fate, and HSCs or ESCs cocultured with OP9-DL1 cells have showed a robust T lymphopoiesis [17, 136]. Based on these data as well as the resemblance of iPS cells to ESCs in both morphology and function, we proposed that iPS cells are able to differentiate into T lymphocytes as their naturally occurring counterparts do by using the OP9-DL1 cells. Studying the T lineage differential capability has two major impacts. First, it would help us fully characterize iPS cells; second, it could help provide a new source of getting large numbers of naïve-phenotyped T lymphocytes for the application in the personalized medicine. In this first part of my thesis research, it was clearly shown that by using the simple in vitro, OP9-DL1 cells-based, Notch signaling pathway mediated differentiation induction system, iPS cells could differentiate towards hematopoietic lineage and further into a more specific T lineage. In my observation, following the Notch ligand DL1 stimulation, first, the morphologies of iPS cells changed drastically. Then, the expression of certain stem cell markers such as CD117 and CD45
has discontinued but T lymphocytes markers such as CD4, CD8, CD3 and TCR-β have been detected. Along with the morphological changes, these differentiated cells also acquired the T lymphocyte function in the context of cytokine secretion upon costimulatory signals mediated T cell activation. These in vitro observations that iPS cells were able to differentiate towards T lymphoid tissue was further backed by the in vivo study that in vitro differentiated cells could reach a full maturation in the notion of CD4+CD8+ double positive populations disappeared and only either CD4+ or CD8+ single positive populations could be identified. Moreover, these iPS cell-derived T lymphocytes reconstituted the lymphoid organs in the endogenously lymphopenic Rag1−/− mice.

This first published study suggested that in terms of T lineage differentiation, iPS cells are similar to ESCs and HSCs. This report significantly improved our understanding of using stem tissue for clinical studies, because it helped overcome the ethical and technical obstacles related to the usage of human embryonic and hematopoietic tissues. However, generating a subgroup of specialized T lymphocytes would be more beneficial for designing a personalized medicine. For example, T lymphocytes either isolated from tumor tissues or genetically modified to express tumor antigen-specific T cell receptor have been shown to reduce tumor burden and substantially increase the life quality of the patients. This gave us a new direction of using iPS for the personalized medicine that was to generate antigen-specific T lymphocytes from iPS cells. On the other hand, this study also showed that, by using iPS cells, generating regulatory T cells for the treatment of autoimmune disorders were also possible.

The major shortage of this study was the T lineage differentiation of iPS cells was only investigated by using the simplified in vitro OP9-DL1 co-culture system. As noticed,
the OP9-DL1 co-culture system does not support the development of CD4\(^+\) SP cells for
the reason that the parent OP9 cell only expresses MHC-I but not MHC-II molecule [136].
In one of our studies, I have generated a new OP9 cell line overexpressing MHC-II
molecule (OP9-DL1-MIAb) and co-culture of FoxP3-expressing iPS cells with OP9-
DL1-MIAb favors the generation of progenitor CD4\(^+\) T\(_{REG}\) cells *in vitro* [351]. Therefore,
for generating both functional CD4 and CD8 SP cells in culture, we could adopt this
OP9-DL1-MIAb cell that expresses both MHC-I and MHC-II molecules. Meanwhile,
previous study suggested that fetal thymus organ culture (FTOC) could provide a better
environment in the awareness that the FTOC has a full range of molecules that necessary
for T cell development. Second, although morphologically and functionally, the *in vitro*
differentiated iPS cells were evaluated in our study, the intracellular molecular events
such as gene expression patterns and some epigenetic changes after differentiation were
not characterized. Additional studies to obtain this information could better understand T
lineage differentiation from stem cells. Third, in this proof-of-concept study trying to
understand T lineage differentiation capability of iPS cell, we only performed evaluations
at several time points such as Day 12, Day 18 and Day 22 during the course of T cell
development. We understand that T cell development is a continuous process therefore a
complete evaluation of surface marker and function changes in a daily basis is important
to fully understand T cell differentiation from iPS cell.

After finishing this first part of my thesis research, we put our focus on the
understanding of the capability of iPS cells to develop into antigen-specific T cells. At
the conclusion of the second study, it was quite clear our central hypothesis that iPS cells
can develop into antigen-specific T cells was strongly supported by our solid data.
In the first part, it was shown iPS cells are able to develop into a mixture of both CD4$^+$ and CD8$^+$ cells after \textit{in vivo} maturation. However, in designing personalized cancer immunotherapy by using iPS cells, in the context of mediating a direct tumor killing, the most important goal is to obtain iPS-derived CTLs that are reactive to tumor antigen. According to previous studies, a partial cancer regression could be achieved by using TILs or genetically modified T cells that express either tumor antigen-specific TCR or CAR [15, 63, 316]. Therefore, iPS cell-derived, antigen-specific CTLs would be greatly favored in conducting a successful ACT-based anti-cancer regimen. Due to other pioneering work toward understanding the important role TCR has played in directing T lymphocyte development from stem cells [123, 124], we decided to explore to what extent TCR could determine the T lineage differentiation in iPS cells. To bypass the \textit{in vitro} two-step developmental system described in \textbf{Part A}, we used the \textit{in vivo} differentiation system in immunocompetent C57BL/6J mice instead of the OP9-DL1 system for the reason immunocompetent C57BL/6J mice have a full spectrum of factors that playing role in the T cell development. By excluding differences between different controls, we can exclusively focus on the sole function of TCR in the determination of T cell development and differentiation. To do so, TCR bearing iPS cell line (iPS/OT-I) was generated by retroviral transduction.

We first observed that iPS/OT-I cells were able to differentiate in C57/BL6J mice to T cells \textit{in vivo} as detected by flow cytometric analysis. In contrast, the control iPS/MiDR cells could not differentiate into T cells \textit{in vivo}. This result suggests that TCR plays a function as T lineage differentiation determinant in stem cells. Further analysis of these \textit{in vivo} generated T cells shows that MHC-I restricted TCR directs iPS to differentiate
into CD8+ T cells and the corresponding TCR is expressed on the surface of the
developed iPS cells. Second, iPS/OT-I derived CD8+ CTLs are able to persist in the
lymphoid organs and tissues of the recipient mice possibly due to the mechanism of
memory T cell formation. Third, iPS/OT-I derived CTLs are able to respond to antigen
stimulation by secreting cytokines. Also, cytotoxicity of iPS/OT-I derived CTLs can be
observed as antigen peptide-pulsed target cells are lysed. These studies showed functional,
antigen-specific CTLs could be programmed by introducing a corresponding TCR to iPS
cells.

In the hope of using iPS cells for cancer immunotherapy, we decided to investigate
the interactions between iPS-derived T cells and tumor cells such as tumor infiltration,
tumor killing and animal survival. A xenograft tumor model was picked up to test the
tumor-reactivity of in vivo developed iPS/OT-I cells. E.G7-OVA, a murine thymoma cell
line that overexpresses OVA, was used as the tumor model to intraperitoneally challenge
the mice receiving different types of cells. It is found that iPS/OT-I derived CTLs are
able to infiltrate into the tumor tissue, kill tumor cell and control the tumor growth by
different assays. Meanwhile, iPS/OT-I derived CTLs can significantly enhance the
survival rate of the mice receiving iPS/OT-I cells compared with other groups, which
further supports the previous studies of tumor interaction.

Based on these findings, it is suggested that engineered iPS cells could be a
potential source of large number of tumor antigen-specific T cells that required for a
successful, personalized cancer immunotherapy.
In conducting this study, several problems are identified and needed to be further addressed. Starting from stem cells, it took up to 6 to 8 weeks for TCR-bearing iPS cells to develop into fully differentiated T cells. There were possible methods to shorten the time frame and enhance the development. For example, first, researchers have evaluated the efficacy of ACT-based therapy by transferring tumor-specific CD8+ CTLs at various stages of differentiation into tumor bearing mice. These studies indicated that administration of naïve and early effector T cells, in combination with a lymphodepletion regimen, γc cytokine administration, and also vaccination, resulted in the eradication of established tumors [242, 352, 353]. Second, a conditioning treatment of mice such as sublethal irradiation prior to iPS cells transfer or IL-2 or IL-15 based cytokine treatment may also benefit iPS cell-based therapies. Third, in vitro priming of iPS cells by using Notch ligands DL1 and DL4 might shorten the time needed for the following in vivo differentiation. Addressing these questions will be helpful for the translation of the studies for the treatment of cancer patients.

Despite the observed control of tumor growth, we also identified some limitations of ACT-based therapy with TCR gene-transduced iPS cells. First, at least 6 weeks of in vivo development were required for T cell differentiation to occur from the transferred iPS cells. Although there were Ag-specific CD8+ T cells presenting in lymph nodes and spleen 4 weeks after cell transfer, these cells are less than 3.55% of the total CD3+Vβ5+ population, which was not sufficient to generate an efficient antitumor immunity. From weeks 6 to 10 after cell transfer, approximately 24% of the CD3+Vβ5+ population were in lymph nodes and spleen, and more than 80% of these cells were CD8+ CD4-. Second, we noted hair loss and bone softening in mice receiving TCR-transduced iPS cells. These
effects might be caused by the generation of other immune cells from the transferred iPS cells. And how did such cells get generated \textit{in vivo} remained unknown currently. Nevertheless, we did not observe that immunosuppressive T cell subsets such as CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells were developed from genetically engineered iPS cells.

The other issue about iPS-derived ACT therapy, which is also found in other trials using T cell-mediated ACT therapy is it is difficult to reach a tumor free status. Although iPS-derived CTLs are able to control tumor growth and benefit animal survival, no tumor-free status has been observed. There are many possible reasons to address this issue, for example, we only performed a single transfer with 3 million cells and second, the immunosuppressive factors such as T$_{REG}$ and MDSC in tumor microenvironment. To solve this problem, we could either give multiple transfers or pre-condition the recipient by irradiation or cytokines to eliminate those immunosuppressive cells.

With the observation of the phenotypical changes of T lineage differentiation from the iPS cells, to better design a directed T lineage differentiation from any stem cells, an important question to answer is the mechanism involved in this process. To further explore this, in my thesis study, I have partly worked on deciphering the synergistic roles of TCR and Notch signals engaged in guiding the TCR-restricted T lineage commitment differentiation of iPS cells.

As described above, our initial findings indicated that Notch signaling stimulation could promote T lineage differentiation from iPS cells \textit{in vitro}. Later on, a second study showed that TCR signals could further drive iPS-derived cell into antigen-specific T cell
in vivo in a MHC-restricted manner. The advantage of using in vivo system was a full spectrum of differentiation-determinant factors including Notch ligands was present. By combining two pieces of information together, it was highly suggested that a dual Notch and TCR signal played some important roles in directing iPS cell differentiation into T lymphocyte. Hence, it would be very interesting to investigate that in what extent Notch and TCR signaling pathways have played to determine T lineage commitment from the iPS cells. To study this, both in vitro Notch signaling stimulation system (OP9-DL1 cells) and TCR-bearing iPS cells (iPS/OT-I cells) were used. After a brief coculture of iPS/OT-I with OP9-DL1 cells, it was found that although OT-I specific TCR (Vβ5 chain) was not detected from partially differentiated iPS/OT-I cells, these cells has skewed their differentiation towards the CTL subtype in terms of expressing CD8 over CD4 molecules. Meanwhile, no significant CD8 population increase was observed in control cells. This observation indicated that Notch and TCR played a synergistic role in directing T cell development and this was consistent with the current model of T cell development. As documented, mature TCR molecules could only be detected at the late DP stage which was presented later than CD4 and CD8 molecules [318, 319]. Similarly, CD3 molecules could be detected in both partially developed iPS/OT-I and T cells derived from vector control; however, iPS/OT-I derived CD3⁺ cells had a higher percentage of CD8⁺ cells than the control. This finding supported our hypothesis that TCR directs a subtype-specific, MHC-restricted T cell differentiation. The CD3 expression profile was also consistent with the current model of T cell development that it could be constitutively detected on T cells from the very early stage of the intrathymic T cell commitment [320].
Subsequent study by analyzing the gene expression profile further showed that after coculture, gene expression profile has changed. Stem cell marker such as *Nanog* was down-regulated but other molecules were up-regulated. In this study, we showed that TGF-β signaling pathway was highly involved in the T cell differentiation process. Previous studies have clearly shown that TGF-β signaling, especially TGF-β1 was critical in T cell development, our findings was generally consistent with previous reports [118, 119, 321-323]. However, we also found that besides TGF-β1, TGF-β2 and other signaling pathway components were upregulated after Notch and TCR signals turned on the T lineage commitment of iPS cells.

Although this part of my thesis research was largely exploratory and more systemic and solid works are needed to further confirm this observation. From current picture, it was clear that both Notch and TCR signaling pathway have determinant roles in T lineage commitment development from iPS cells. By analyzing the data, it was suggested that the role of TCR in deciding T cell development was activated later than the Notch signaling activation. Generally, binding of the Notch ligand to iPS cells was the initiation event in governing T lineage commitment as supported by our data. TCR transduction alone would not down-regulate the stem cell marker *Nanog* gene. However, under the path of Notch induced T lymphocyte differentiation, TCR signal further promoted the proceedings of this process as the qRT-PCR data pointed out that compared to control group, the expression levels of genes-of-interest in iPS/OT-I group were always higher.

Taken together, Notch signal would turn the switch of T cell differentiation on, and then TCR signal work with the Notch signaling together. Both of them would play certain significant roles in regards to T lineage, especially a TCR-restricted commitment
development, in a synergistic fashion. To fill more information into this picture, extensive works are still demanding.

To thoroughly understand the signaling transduction mechanisms involved in the T cell development. More molecules and more pathways should be investigated. In this study, to make a simplified system, only TCR and Notch pathways were evaluated. However, based on the results from previous studies, other signals were also engaged in the T cell developmental process. Also, the T lineage differentiation process from stem cells, especially the ESCs, was a multi-stage event, therefore different signals and pathways might get involved at different time points and different locations. It was too general and still early to claim the exact roles of TCR and Notch have played. Extensive and intensive works could help finally address the real roles these signaling pathway components have played in the stem cell-derived T lineage differentiation.

At the same time of trying to understand the developmental mechanisms of iPS-derived T cell, we were also very interested to perform some cutting-edge translational study by using iPS cells. As mentioned previously, our long-term goal of studying T cell differentiation from iPS cells was to develop a new generation of personalized, ACT-based cancer immunotherapy. Although OVA system has been widely used in the study of immunology for decades, some researchers argued that OVA protein was not a natural protein that expressed in our current murine and human model. Compared to the over-expressed OVA protein, the expression levels of some endogenous protein, especially those tumor-associated or specific antigens were significantly low. To further test the possibility of using iPS cells in the personalized cancer immunotherapy, we have to test that real tumor reactivity of the iPS cell derived CTLs. In answering this question,
melanoma and melanoma-antigen specific TCR were picked up. However, due to the limited time of graduate study, only preliminary work has been finished, further work will be done in the near future.

**B. Future directions**

To clarify the relationship of TCR and Notch signaling played in the T cell development. The second is the generation of melanoma-specific CTLs from iPS cells. The third is the modulation of the current *in vitro* T cell differentiation system to shorten the required time for the *in vivo* maturation of the iPS-derived T cells.

In performing the first project, different time points and more intracellular signaling pathway components should be investigated to facilitate the interpretation of the actual role that Notch and TCR signaling have played in T lineage differentiation. To continue the second project, the anti-TRP-2 TCR-bearing iPS cell line has been established, it is suggested to start the T lineage differentiation by taking either *in vivo* or *in vitro* systems. Following the differentiation, characterizations of the iPS-derived T cells such as CTLs’ phenotype, function and persistence should be conducted. Meanwhile, iPS-derived CTLs-mediated tumor management should also be evaluated. The third one could be done in combining with the other projects. Currently, for the T cell differentiation purpose, the OP9 cell lines express different Notch ligand(s) such as OP9, OP9-DL1, OP9-DL4 and OP9-DL1/DL4 cell lines are already on hand. By using different types of iPS cells such as TCR-bearing or plain iPS cells, the T lineage differentiation efficiencies of different OP9 cell lines could be evaluated. With the information generated from this project, an improved iPS-based ACT-therapy to melanoma could be proposed accordingly. For
instances, the *in vitro* priming of iPS cells with different Notch ligand(s) could possibly help shorten, in some extend, the time needed for the final maturation of iPS-derived T cells, which could helpfully determine an optimal time point to administrate the primed iPS cells to achieve an ideal therapeutical efficacy.

Other than these three ongoing projects, there are still many related studies could be done. Using human iPS cell other than mouse iPS cell to perform the study is the best way to translate the basic bench work to bedside patient-care.

Other types of TCR that target either viral-related or tumor-related antigens could also be tested under this approach. In future, when the T cell development determinant on the TCR is identified, it is possible to make a unique CAR that containing this domain to direct the generation of novel antigen-specific, iPS-derived CAR-T.

In terms of modulating and understanding the T cell developmental process, when new molecules or new systems have been characterized, it could also be applied in our iPS system to further evaluate their roles as well as to improve our iPS-based T lymphocyte differentiation approaches in the aim of designing ACT-based immunotherapy.

In addition to those T cells related works. Under the concept of designing a personalized immunotherapy by using iPS cells, we can broad our scope of study by investigating the iPS-derived differentiation of other types of immune cells such as B cells and DCs. By doing these researches, it is likely that a comprehensive treatment regimen by using several inter-related mechanisms to manage certain types of disease
could be proposed. In this combined regimen, pharmaceutical agents and physical therapy are also warmly welcomed.

In summary, my thesis research was the first proof-of-concept study of exploring the T lineage differential capability of the iPS cells. From this point, it is suggested that a personalized, T cell-based cancer immunotherapy by using iPS technique is theoretically feasible. Although the road will be winding, with the continuing comprehension of the cancer, the immune system and the stem cell biology, it is believed that through the mutual endeavors from both basic and clinical scientists, this concept will finally become a novel cancer management approaches.

Figure 51 shows the designed scheme of the iPS cell based personalized immunotherapy for cancer. The general approach to design an iPSC-based cancer/patient-specific ACT therapy could be divided into two major parts: one is the identification of tumor antigen-reactive TCR and the other is the preparation of patient-derived iPS cells. In the first part, TCR will either be isolated as well as characterized from TILs obtained from surgical procedures or directly constructed from tumor antigen library. Through general protocol of antigen-specific TCR cloning, tumor-antigen reactive T cells could be identified and clonally expanded upon tumor antigen stimulation. After finishing this, tumor antigen-specific TCR will be selected from these clonally amplified T cells which have the strongest response towards antigen stimulation. The characterized TCR will be genetically processed and subcloned into a lentiviral vector for upcoming gene transduction. For the second part of this approach, generation of iPS cells is relatively easy thanks to the great improvement in iPS technologies. With the help of commercial iPS induction kit, iPS cells could be easily generated and characterized from a tube of
blood or a small chunk of tissue. At this point, TCR will be introduced into iPS cells via well-documented lentiviral transduction approach. To increase the T lineage differentiation efficacy and reduce the spontaneous full spectrum differentiation of stem cells, TCR bearing iPS cells could be briefly stimulated by Notch ligand in vitro. After finishing all in vitro steps, TCR-bearing iPS cells will be re-infused back to patients via their peripheral veins.
Figure 51: Scheme of the future personalized cancer immunotherapy. The general approach to design an iPSC-based cancer/patient-specific ACT therapy.
References


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2011  The Class of 1971 Alumni Endowed Scholarship, PSCOM
2011  American Association of Immunologists (AAI) Trainee Abstract Award, AAI, Bethesda, MD
2010  Dean’s Travel Award, PSCOM
2010  Finkelstein Memorial Student Research Fellowship Award, PSCOM
2002-2005 University Scholarships (Dean’s List), CMU

Selected Publications:

Peer Reviewed Journals
1*. Lei F et al. PLOS ONE. 2013; Accepted

Book Chapters

Selected Oral Presentations:
1. “Notch and TCR signals synergistically promote in vitro T cell development from stem cell”. 15th TRCCC Annual Meeting, 2012, Seven Springs, PA
3. “Lymphopoiesis of Antigen-specific CTLs from induced Pluripotent Stem Cells”. The 23rd Annual Graduate Student Research Forum, 2011, Hershey, PA