

Proposal for a Thesis
in the Field of Biology
in Partial Fulfillment of Requirements for
the Master of Liberal Arts Degree

Harvard University

Extension School

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I.

Tentative Title

The Role of Chemokines CCL19b and CCL25b in T cell Recruitment into Melanoma Tumors

II.

The Research Problem

Melanoma is a type of skin cancer that once becomes metastatic has very poor prognosis. Although the survival rate of patients diagnosed with melanoma has improved over the past decades, the lifetime risk and overall mortality because of melanoma rise yearly (Schatton and Frank 2008). Melanoma prevalence has increased an average of 4 percent per year in the United States (Berwick et al., 2009; WHO, 2014). When diagnosed early melanomas are largely curable (>95%) using surgical excision, however when the cancer has spread to the regional lymph node the 5-year survival of patients decreases to 50%. Additionally, patients with visceral metastases have a median survival rate of only a few months. The minimal therapeutic benefit of current treatment options demonstrates the importance for novel strategies to overcome chemoresistance (Schatton and Frank, 2008).

New therapies for melanoma have recently been introduced including specific inhibitor for the enzyme *BRAF*, which is prevalently mutated in melanoma, and immunotherapy aiming at directing the adaptive immune system towards the tumor (Ackerman et al., 2014). *BRAF* inhibitors are efficient in eradicating the tumor, but many patients acquire resistance to the treatment and relapse without the ability to provide the

treatment again. Immunotherapies include adoptive T cell therapy and immune checkpoint blockade. In adoptive T cell therapy, T cells are isolated from the patient tumor, activated *in vitro* and then transplanted back to the patient. Immune checkpoint blockade involved antibodies administration against two major receptors that deactivate T cells. While both therapies show promising results, a large fraction of the treated patients does not respond to this treatment.

One of the major problems in immunotherapy accounting for the lack of response is the inadequate arrival of T cells to the tumor and tumor recognition. For example, a mechanism by which melanoma is able to evade immune response is by losing expression of major histocompatibility complex (MHC) molecules leading to a failure to recognize antigen result in a lack of T cell activity against the tumor. This lack of T cell recruitment to the tumor poses a major drawback in cancer immunotherapy, since it renders T cell activation ineffective when they are not at the tumor site. Thus, improving the ability to recruit T cells to tumor sites will increase the effectiveness cancer immunotherapy.

A working model needs to be established in order to improve our understanding of the mechanisms governing T cell recruitment to tumors. This model could be established by transplanting a melanoma cell line, which is altered to express T cell recruiting factors, into an animal and monitoring T cell content in the tumor. We plan to use this model to evaluate the upregulation of chemokines CCL19 and CCL25.

We will evaluate chemokines CCL19 and CCL25 due their roles in T cell recruitment. CCL19 is part of the ligand pair (with CCL21) for the chemokine receptor CCR7, and is important for migration of antigen-presenting cells (APC) and lymphocytes

into the lymph node, where antigen education and immune surveillance occur.

Meanwhile, CCL25 is the ligand for the chemokine receptor CCR9, which mediates the localization of recently activated CD8+ lymphocytes (Esche et al., 2005). We hypothesize that inducing expression of chemokines CCL19 and CCL25 we can increase T cell recruitment in melanoma tumors, thus reducing tumor size.

If results are as expected then there will T cell recruitment to that melanoma tumors, which will result reduced tumor burden. There is a possibility that our hypothesis will be wrong because this study has not been attempted before. If we observe no T cell recruitment and no reduction in the tumor, when no technical limitations are present, we would reject our hypothesis that increased expression of chemokines CCL19 and CCL25 will increase T cell recruitment to melanoma tumors. This could be because the function of chemokines is not well conserved in zebrafish. Additionally there is a possibility that the melanoma inhibit T cell recruitment via other mechanisms that masks the recruiting effect of the chemokine.

Finally, this research is important because it represents a significant therapeutic option for patients with melanoma. It can suggest the ability to augment immune response to tumors by elevating the recruitment of T cells in to the tumor. Combined with drugs that activate the immune system, like immune checkpoint blockade drugs, this can improve treatment outcome. This research could represent a new treatment option, or a possible compliment to other treatments, which would increase the survival rate of many patients.

III.

Definition of Terms

“Adoptive T cell transfer”: Adoptive T cell transfer involves the isolation and reinfusion of T lymphocytes into patients to treat disease (Kalos and June, 2013).

“Antigen-presenting cells (APCs)”: a cell that displays antigen complexed with major histocompatibility complexes (MHCs) on their surfaces

“Benign nevi”: an abnormal, noncancerous patch of skin caused by an overgrowth of skin cells.

“Chemokines”: A subfamily of the cell signalling molecules or cytokines, which are secreted by cells to induce chemotaxis in nearby cells.

“CD4 cells (T-helper cells)”: A type of T cell that provides help to other cells in the immune response by recognizing foreign antigens and secreting substances called cytokines that activate T and B cells.

“CD8 cells (cytotoxic T cells)”: a type of T cell that bind to other cells through MHCs and induce the target cells to undergo programmed cell death.

“Cytokines”: small, secreted proteins released by cells have a specific effect on the interactions and communications between cells.

“Dendritic cells”: a type of antigen-presenting cell (APC) that induce a primary immune response in the inactive or resting naïve T lymphocytes.

“Dysplastic nevi”: a type of mole that may be bigger than a common mole, and its color, surface, and border may be different. Though it may resemble melanoma in shape, it is benign.

“Epidermis”: the outermost and nonvascular layer of the skin.

“*ex vivo*” an experimentation or measurements done in or on tissue from an organism in an external environment.

“Major histocompatibility complex”: A group of genes that code for proteins found on the surfaces of cells that help the immune system recognize foreign substances. Class I MHC molecules span the membrane of almost every cell in an organism, while class II molecules are restricted to cells of the immune system called macrophages and lymphocytes.

“Melanin”: broad term for a group of natural pigments. It is produced in a specialized group of cells known as melanocytes.

“Melanoblasts”: The precursor cell of a melanocyte.

“Melanocytes”: the melanin-producing cells located in the bottom layer of the skin's epidermis

“Melanosomes”: an organelle found in animal cells and is the site for synthesis, storage and transport of melanin.

“Neural crest”: a temporary group of cells unique to vertebrates that arise from the embryonic ectoderm cell layer.

“Papillary dermis” the uppermost layer of the dermis

“PD-1 (programmed death-1)”: an immune checkpoint that plays an important role in down regulating the immune system by preventing the activation of T-cells. PD-1 binds to two ligands, PD-L1 and PD-L2.

“Tumor infiltrating lymphocytes (TIL)”: are immune cells that have left the bloodstream and migrated into a tumor.

IV.

Background of the Problem

Melanoma

Malignant melanoma is an extremely metastatic cancer that is highly resistant to conventional therapy. Although the survival rate of patients diagnosed with melanoma has improved over the past decades, the lifetime risk and overall mortality because of melanoma rise yearly (Schatton and Frank 2008). Melanoma prevalence has increased an average of 4 percent per year in the United States (Berwick et al., 2009; WHO, 2014). When diagnosed early melanomas are largely curable (>95%) using surgical excision, however when the cancer has spread to the regional lymph node the 5-year survival of patients decreases to 50%. Additionally, patients with visceral metastases have a median survival rate of only a few months. The minimal therapeutic benefit of current treatment options demonstrates the importance for novel strategies to overcome chemoresistance (Schatton and Frank, 2008).

The melanocytes in the skin are the site of formation for melanoma. Melanocytes are located in the epidermis and hair follicles and their primary function is melanin production. The life cycle of melanocytes consists of several steps including lineage specification from embryonic neural crest cells (melanoblasts), migration and proliferation of melanoblasts, differentiation of melanoblasts into melanocytes, and then maturation of melanocytes. Melanin production occurs in special organelles called melanosomes, which are transport to keratinocytes to protect them from UV radiation (Cichorek et al., 2013). Preceding the formation of a melanoma, mutations to melanocytes lead to the formation of benign melanocytic formations, known as nevi. This

is often caused by mutations in the *NRAS*, *HRAS*, and *BRAF* oncogenes (Ceol et al. 2011). Following the formation of nevi, secondary oncogenic mutations lead to the progression of nevi to melanoma. These common secondary mutations occur in *TP53*, *PTEN*, and *BAP1* (Bastian, 2014).

Melanomas pass through a five-stage process. It begins as a benign nevi with an increased number of nested melanocytes. Next, it becomes a dysplastic nevi with irregular borders, multiple colors, increased diameter, and random and discontinuous cytological atypia. During the radial growth phase melanoma with malignant cells proliferating within the epidermis (Pacheco et al., 2012). The next stage of melanoma development, the tumor begins to grow vertically into the epidermis and the papillary dermis. This is referred to as the invasive radial growth phase. Once it has reached the vertical growth phase it becomes the invasive melanoma. The tumor becomes able to grow into the surrounding tissue and can spread around the body through the blood. At this point blood vessels have developed within the tumor and the cells of the immune system begin to interact with the tumor (Hsu et al., 1998) (Krochmann et al., 2012).

The Immune System

The immune system is a collection of cells, tissue, and molecules that mediate resistance to infection. The coordinated response of these cells and molecules to antigens is called an immune response. The host defense mechanisms consist of innate immunity, which mediates the initial protection against infections, and the adaptive immunity, which develops slower and mediates the later and even more effective, defense against infection.

The innate immune system, made up of macrophages and neutrophils, provide the first line of defense against many common microorganisms. There are two types of adaptive immunity, humoral immunity and cell-mediated immunity. Humoral immunity is mediated by the macromolecules found in the extracellular fluids such as secreted antibodies, complement proteins, and antimicrobial peptides. Cell-mediated immunity is an immune response that involves the activation of phagocytes, antigen-specific cytotoxic T-lymphocytes, and the release of various cytokines in response to an antigen (Abbas and Lichtman, 2012). Tumors are attacked through mechanisms of the cell-mediated immunity.

Malignant tumors express various molecules that are detected by the immune system as foreign antigens. The immune system is able to react against a tumor when it is expressing antigens that are recognized as non-self. These can be mutations of normal proteins, mutated or translocated oncogenes, or tumor suppressor genes (Abbas and Lichtman, 2012).

The principle immune mechanism used for tumor eradication is the killing of tumor cells by cytotoxic T cells (CD8⁺ T cells) specific for tumor antigens. The majority of tumor antigens that elicit immune responses are displayed as class I major histocompatibility complex (MHC)- associated proteins. CTL responses against tumors are often induced by recognition of tumor antigens on host antigen presenting cells (APCs), which ingest tumor cells or their antigens and present the antigens to T cells. These antigens are recognized by class I MHC-restricted CD8⁺ CTLs whose function is to kill cells producing the tumor antigen. The APCs also stimulate Class II MHC-

restricted CD4+ helper T cells, which may provide signals for more CTL development (Abbas and Lichtman, 2012).

Typically, T cell specific for the tumor antigen will recognize the antigen and the T cell will activate, however tumors have evolved mechanisms to evade detection of antitumor immune responses. One method that tumors have developed to evade attack is through the lack of T cell recruitment because a failure to recognize tumor antigen because of low MHC expression Another method tumor utilize is the production of immunosuppressive proteins which can cause an inhibitory effect. Melanoma is an example of a tumor that is able to evade immune response. Although the mechanism is not fully understood, it is believed that the MHC expression is downregulated. Recent immunotherapy strategies aim to counter act these characteristics by inducing T cell recruitment.

Immunotherapy

Immunotherapy involves harnessing the immune system to fight cancer (Couzin-Frankel, 2013). The main strategies for cancer immunotherapy aim to provide antitumor effectors (antibodies and T cells) to patients, actively immunize patients against their tumors, and stimulate the patients' own antitumor immune response (Abbas and Lichtman, 2012). Additionally, identification of cancer cell T cell inhibitory signals have prompted the development of a new class of cancer immunotherapy that specifically hinders immune effector inhibition (Chen and Mellman, 2013).

Recently, several immunotherapies for melanomas have been introducing drugs that act as immune checkpoint inhibitors to inactivate effector T cells from attacking the

tumor (Sharma & Allison, 2015). Many of the patients treated with these drugs showed significant tumor shrinkage that was correlated with enhanced effector T cells activity. However, the efficiency of these treatments relies on T cell recruitment to the tumor site and its infiltration, which differs between patients and affect treatment outcome (Tumeh et al., 2014). These reports highlight the T cell population as a pivotal player in cancer progression. Moreover, they demonstrate the power of endogenous T cells activation against the developing tumor as a potent therapeutic approach.

Adoptive T cell transfer is one of the method that been used to enhance T cell immunization of melanoma. Adoptive T cell transfer involves the isolation and reinfusion of T lymphocytes into patients to treat disease. A number of strategies have been evaluated, initially using T cells isolated from tumor infiltrating lymphocytes (TIL). Adoptive transfer of bulk T lymphocytes, obtained from the periphery and expanded *ex vivo* to generate large numbers prior to re-infusion into patients is an alternative strategy for adoptive T cell therapy. Unfortunately few attempts have succeeded at creating complete and long-lasting durable clinical responses (Kalos and June, 2013). It was not until T cell receptor recombination that adoptive T cell therapy became a viable option because it allowed for more accurate targeting of antigens expressed by tumors (Restifo et al, 2012).

Since immune checkpoints are initiated by ligand-receptor interactions, antibodies can be used as blocked by or modulated by recombinant forms of ligands or receptors. Cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) is a protein receptor that functions as an immune checkpoint to downregulate the immune system. It is expressed by activated T cells and transmits an inhibitory signal to other T cells. CTLA4 antibodies

are used to downregulate the protein receptor and increase T cell response. These antibodies were the first of this class of immunotherapeutics to achieve US Food and Drug Administration (FDA) approval (Pardoll, 2012).

Recently, PD-1 (programmed death-1) has gained attention in melanoma immunotherapy. PD-L1 seems to be upregulated in the microenvironment of many tumors. Upregulation of PD-L1 inhibits the last stages of the cancer immunity cycle by deactivating cytotoxic T cells in the tumor microenvironment. The activated T cells in the tumor microenvironment release interferon gamma (Chen et al., 2012). As a result, the tumor cells and tumor-infiltrating immune cells overexpress PD-L1. PD-L1 binds to T-cell receptors B7.1 and PD-1, deactivating cytotoxic T cells. Once deactivated, T cells remain inhibited in the tumor microenvironment (Chen and Mellman, 2013)(Chen et al., 2012). PD-L1 overexpression can also inhibit propagation of the cancer immunity cycle by preventing the priming and activation of T cells in the lymph nodes (Chen and Mellman, 2013) (Chen et al., 2012) (Keir et al., 2008). PD-L1 expression is upregulated on dendritic cells within the tumor microenvironment (Chen et al., 2012)(Keir et al., 2008). PD-L1–expressing dendritic cells travel from the tumor site to the lymph node (Motz and Coukos, 2013). The PD-L1 binds to B7.1 and PD-1 receptors on cytotoxic T cells, leading to their deactivation (Keir et al., 2008). The administration of anti-PD-1 antibodies has become considered as a way to reduce T cell inhibition.

The aim of this study is to improve T cell recruitment to melanoma. Our approach is to induce expression of the zebrafish chemokines CCL19 and CCL25 in order to establish T cell recruitment in zebrafish melanoma tumors. CCL19 is part of the ligand pair (with CCL21) for the chemokine receptor CCR7, and is important for migration of

antigen-presenting cells (APC) and lymphocytes into the lymph node, where antigen education and immune surveillance occur. Meanwhile, CCL25 is the ligand for the chemokine receptor CCR9, which mediates the localization of recently activated CD8+ lymphocytes (Esche et al., 2005). We hypothesize that inducing expression of chemokines CCL19 and CCL25 can increase T cell recruitment in melanoma tumors, thus reducing tumor size.

We will establish our model by transplanting the melanoma cell line *MelB*, which is altered to express T cell recruiting factors, into a zebrafish and monitoring T cell content in the tumor. We plan to use this model to evaluate the effect of upregulation of chemokines (in this case, CCL19 and CCL25) on T cell recruitment. Tumors will be excised 2-3 weeks after cell line transplantation. T cell recruitment to the melanoma tumors will be evaluated using flow cytometry.

If results are as expected then there will be T cell recruitment to melanoma tumors, which will result in reduced tumor burden. If we observe no T cell recruitment and no reduction in the tumor, we would reject our hypothesis. This could be because the function of chemokines is not well conserved in zebrafish. Additionally, there is a possibility that melanoma inhibit T cell recruitment via other mechanisms.

Our justification for using zebrafish is that they are cheaper to maintain than mice, zebrafish produce hundreds of offspring at weekly intervals providing an ample supply of embryos, their larval stages is transparent, zebrafish have a similar genetic structure to humans and their genome is fully sequenced, and it is easy enough to create mutant lines using embryo injection. The adaptive immune system is well conserved in zebrafish, containing CD8 and CD4 cells as well as FOXP3 regulatory T cells. The T cell receptor

and MHC molecules are conserved and T cell receptor rearrangement and signaling is similar to the mammal processes. The melanoma model developed in the Zon lab, together with T cell reporter fish lines, such as *lck:GFP* available in the lab and *CD8:GFP*, enable us to study the mechanisms underlying T cell recruitment and activity in the tumor settings

V.

Research Methods

The purpose of this study is to determine if upregulation of zebrafish chemokines CCL19 and CCL25 will induce T cell recruitment to melanoma tumors and reduced tumor burden. Zebrafish are maintained as specified by the guidelines of the Harvard Institutional Animal Care and Use Committee. The zebrafish lines that will be used are the *CD8:GFP* and *Lck:GFP*, which report T cells.

We will establish our model creating a melanoma cell line, which is altered to express T cell recruiting factors and then injecting it into T cell reporting fish, to assess their recruitment and possible effect on tumor growth. First we will generate a plasmids that upregulates chemokines CCL19 or CCL25. Next, these plasmids will be inserted into melanoma cell line *MelB* using lipofectamin transfection to create constitutive expression of the cytokine in the melanoma cells. In order to implant the cell line into the fish they will be radiated with 30 Gy, split between two doses. On day 1 the fish will be radiated with the first dose of 15 Gy, 24 hours later the fish will be radiated with a second dose of 15 Gy, 24 hours after that the fish will be injected sub cutaneously with the *MelB* cell line. In the first experiment we will use the *Lck* fish to observe T cell recruitment. If *Lck*-

GFP cell recruitment will be evident, we will repeat the experiment using CD8-GFP fish to evaluate the recruitment of the subpopulation of cytotoxic T cells. The experiment will be performed as follows:

The experimental groups will be comprised of fish injected with: 1.) *MelB* 2.) *MelB* with GFP plasmid 3.) *MelB* with CCL19-GFP or 4.) *MelB* with CCL25-GFP. Each group will contain 10 zebrafish. The experiment will be repeated 3 times in order to avoid any potential technical problems and assess statistical significance.

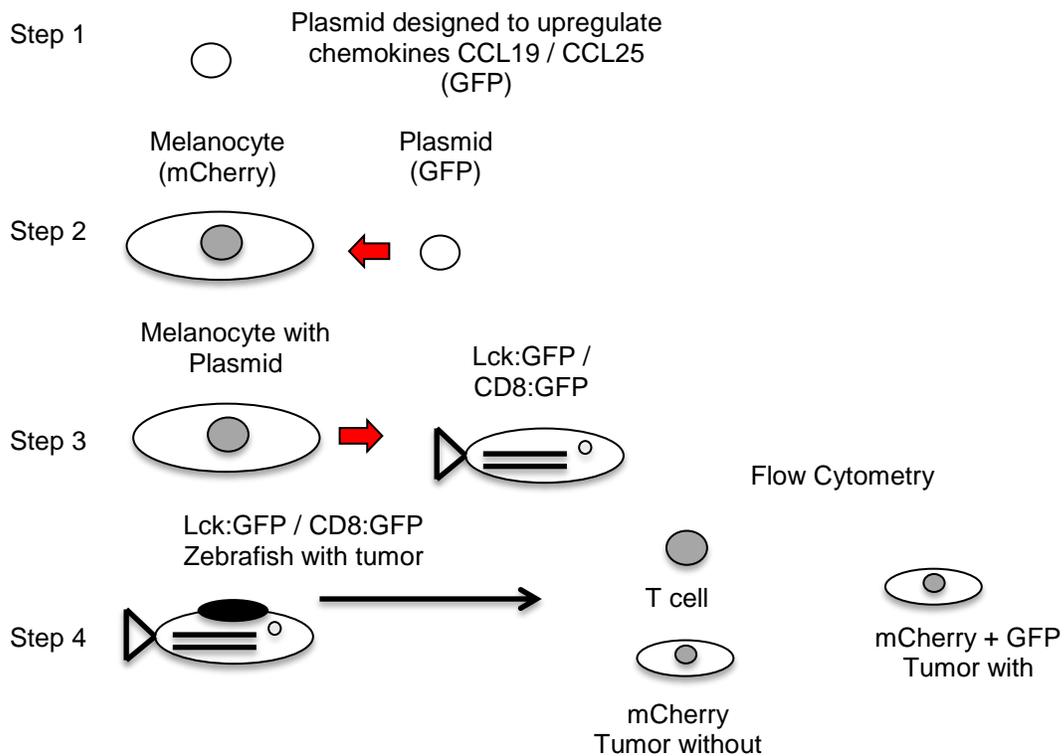
Possible problems include cell line contamination, unhealthy fish, or technical issues with injections. Tumors will be excised 2-3 weeks following cell line transplantation.

Table 1
Layout of experimental groups

<u>Lck/CD8 fish will be injected with:</u>	<u>Group 1</u>	<u>Group 2</u>	<u>Group 3</u>
1) <i>MelB</i>	10 fish	10 fish	10 fish
2) <i>MelB</i> + GFP plasmid	10 fish	10 fish	10 fish
3) <i>MelB</i> + CCL19-GFP	10 fish	10 fish	10 fish
4) <i>MelB</i> + CCL25-GFP	10 fish	10 fish	10 fish

The transplanted fish will be examined for T cell recruitment to the tumor and for tumor size, to evaluate whether T cell recruitment reduces the number or size of emerging melanoma tumors. T cell recruitment to the melanoma tumors will be evaluated quantitatively using flow cytometry. *MelB* will be tagged with mCherry. The plasmid will be tagged with GFP. In the CD8:GFP fish, CD8 (cytotoxic T cells) will be tagged with GFP. In the Lck:GFP fish, CD8 (cytotoxic T cells) and CD4 (helper T cells)

cells will be tagged with GFP. The control will be constitutive expression of EGFP. The reason for using both fish lines is to gain insight into the specific T cells that are being recruited to the tumor. When the tumor is evaluated using flow cytometry red cells will represent melanoma cells without the plasmid, green and red cell will represent melanoma cells with the plasmid, and green cells will represent T cells that have been recruited into the tumor (Figure 1). We will excise the tumor, then using flow cytometry we will measure the T cell count per million cells in order to determine the percentage of T cells within the tumor.



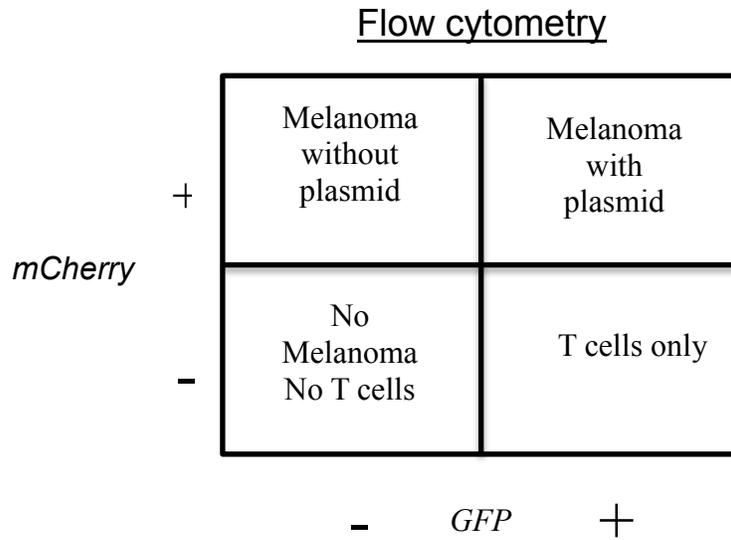


Figure 1
Visual layout of experiment and demonstration of flow cytometry results

VI.

Research Limitations

Melanoma inhibits T cell recruitment via multiple mechanisms including the downregulation of MHC expression and immunosuppressive proteins that have an inhibitory effect. There is a chance that chemokine expression is upregulated but the melanoma inhibits T cell recruitment using other methods.

Despite the fact that 70 per cent of protein-coding human genes are related to genes found in the zebrafish, there may be a chance that the function of chemokines is not well conserved in zebrafish.

VII.

Tentative Schedule

Submission of proposal to research advisor	August 1, 2016
Generate plasmid	August 1, 2016
Insert plasmid into cell line	August 19, 2016
Injecting CCL19 cells and CCL25 cells into fish (sub-cutaneous)	
Round one	August 26, 2016
Round two	September 2, 2016
Round three	September 9, 2016
Draft 1 Proposal returned for revision	September 1, 2016
Collect melanoma from fish and analyze using flow cytometry	
Round one	September 16, 2016
Round two	September 23, 2016
Round three	September 30, 2016
Draft 2 Proposal returned for revision	October 1, 2016
Submission of final proposal	November 1, 2016
Proposal accepted by research advisor	December 30, 2016
Data collection complete	November 18, 2016
First draft completed	January 1, 2016
First draft returned by thesis director	January 16, 2016
Revised draft completed	January 30, 2016
Revised draft returned by thesis director	February 17, 2016
Final text submitted to thesis director and research advisor	March 1, 2016

Final text approved

March 15, 2016

Bound copies delivered to Extension

March 30, 2016

Graduation

May 25, 2017

VIII.

Working Bibliography

Abbas, A. K., Lichtman, A. H., & Pillai, S. (2012). *Basic immunology: functions and disorders of the immune system*. Elsevier Health Sciences.

- The immune system is divided into the innate immune system, made up of macrophages and neutrophils and the adaptive immune system.
- The adaptive immune system consists of humoral immunity and cell-mediated immunity. Humoral immunity is mediated by the macromolecules found in the extracellular fluids such as secreted antibodies, complement proteins, and antimicrobial peptides. Cell-mediated immunity is an immune response that involves the activation of phagocytes, antigen-specific cytotoxic T-lymphocytes, and the release of various cytokines in response to an antigen.
- Malignant tumors are detected by the immune system as foreign antigens. Tumor antigens can come in the form of mutations of normal proteins, mutated or translocated oncogenes, or tumor suppressor genes. Tumor antigens that are displayed as class I major histocompatibility complex (MHC)- associated proteins.
- Cytotoxic T cells (CD8+ T cells) target tumor cells. CTL responses are induced by recognition of tumor antigens on host antigen presenting cells (APCs). The APCs also stimulate Class II MHC- restricted CD4+ helper T cells, which may provide signals for more CTL development.
- The main strategies for cancer immunotherapy aim to provide antitumor effectors (antibodies and T cells) to stimulate the patients' own antitumor immune response

Ackerman, A., Klein, O., McDermott, D. F., Wang, W., Ibrahim, N., Lawrence, D. P., Gunturi, A., Flaherty, K., Hodi, F. S., Kefford, F., Menzies, A. M., Atkins, M., Long, G. V. & Sullivan, R.J. (2014). Outcomes of patients with metastatic melanoma treated with immunotherapy prior to or after BRAF inhibitors. *Cancer*, 120(11), 1695-1701.

- New therapies for melanoma include specific inhibitor for the enzyme *BRAF*, which is prevalently mutated in melanoma, and immunotherapy aiming at directing the adaptive immune system.

Chen, D. S., & Mellman, I. (2013). Oncology meets immunology: the cancer-immunity cycle. *Immunity*, 39(1), 1-10.

- Identification of cancer cell T cell inhibitory signals have prompted the development of a new class of cancer immunotherapy that specifically hinders immune effector inhibition

- Tumor cells and tumor-infiltrating immune cells overexpress PD-L1. PD-L1 binds to T-cell receptors B7.1 and PD-1, deactivating cytotoxic T cells.
- PD-L1 overexpression inhibits propagation of the cancer immunity cycle by preventing the priming and activation of T cells in the lymph nodes
- PD-L1 overexpression prevents the priming and activation of T cells in the lymph nodes

Chen, D. S., Irving, B. A., & Hodi, F. S. (2012). Molecular pathways: next-generation immunotherapy—inhibiting programmed death-ligand 1 and programmed death-1. *Clinical Cancer Research*, 18(24), 6580-6587.

- PD-L1 is upregulated in the many tumors. Upregulation of PD-L1 deactivates cytotoxic T cells in the tumor microenvironment. The activated T cells release interferon gamma, which causes the tumor cells and tumor-infiltrating immune cells to overexpress PD-L1.
- PD-L1 overexpression prevents the priming and activation of T cells in the lymph nodes
- PD-L1 expression is upregulated on dendritic cells within the tumor microenvironment

Cichorek, M., Wachulska, M., Stasiewicz, A., & Tyminska, A. (2013). Skin melanocytes: biology and development. *Postepy Dermatol Alergol*, 30(1), 30-41.

- The melanocytes are the site of formation for melanoma. They are located in the epidermis and hair follicles and their primary function is melanin production.
- The life cycle of melanocytes includes specification from embryonic neural crest cells (melanoblasts), migration and proliferation of melanoblasts, differentiation of melanoblasts into melanocytes, and then maturation of melanocytes.
- Melanin production occurs in special organelles called melanosomes, which are transport to keratinocytes to protect them from UV radiation.

Couzin-Frankel, J. (2013). Cancer immunotherapy. *Science*, 342(6165), 1432-1433.

- Immunotherapy involves harnessing the immune system to fight cancer

Esche, C., Stellato, C., & Beck, L. A. (2005). Chemokines: key players in innate and adaptive immunity. *Journal of Investigative Dermatology*, 125(4), 615-628.

- CCL19 is part of the ligand pair (with CCL21) for the chemokine receptor CCR7, and is important for migration of antigen-presenting cells (APC) and lymphocytes into the lymph node, where antigen education and immune surveillance occur.
- CCL25 is the ligand for the chemokine receptor CCR9, which mediates the localization of recently activated CD8+ lymphocytes.

Hsu, M. Y., Shih, D. T., Meier, F. E., Van Belle, P., Hsu, J. Y., Elder, D. E., Buck, C. A., & Herlyn, M. (1998). Adenoviral gene transfer of $\beta 3$ integrin subunit induces conversion from radial to vertical growth phase in primary human melanoma. *The American journal of pathology*, 153(5), 1435-1442.

- During the invasive radial growth phase the tumor begins to grow vertically into the epidermis and the papillary dermis.
- Once it has reached the vertical growth phase the tumor becomes able to grow into the surrounding tissue and can spread around the body through the blood. At this point blood vessels have developed within the tumor and the cells of the immune system begin to interact with the tumor.

Iwai, Y., Ishida, M., Tanaka, Y., Okazaki, T., Honjo, T., & Minato, N. (2002). Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proceedings of the National Academy of Sciences*, 99(19), 12293-12297.

- The administration of anti-PD-1 antibodies has become considered as a way to reduce T cell inhibition.

Kalos, M., & June, C. H. (2013). Adoptive T cell transfer for cancer immunotherapy in the era of synthetic biology. *Immunity*, 39(1), 49-60.

- Adoptive T cell transfer involves the isolation and reinfusion of T lymphocytes into patients to treat disease.
- One strategy includes the use of T cells isolated from tumor infiltrating lymphocytes (TIL).
- An alternative strategy for adoptive T cell therapy is the adoptive transfer of bulk T lymphocytes, obtained from the periphery and expanded *ex vivo* to generate large numbers prior to re-infusion into patients.

Keir, M. E., Butte, M. J., Freeman, G. J., & Sharpe, A. H. (2008). PD-1 and its ligands in tolerance and immunity. *Annu. Rev. Immunol.*, 26, 677-704.

- PD-L1 overexpression can inhibit propagation of the cancer immunity cycle by preventing the priming and activation of T cells in the lymph nodes.

- PD-L1 expression is upregulated on dendritic cells within the tumor microenvironment.
- The PD-L1 binds to B7.1 and PD-1 receptors on cytotoxic T cells, leading to their deactivation.

Krochmann, J., Sinnberg, T., Meier, F., Garbe, C., & Busch, C. (2012). Melanoma cells in distinct growth phases retain specific invasive qualities during brain metastasis in vivo. *Pigment cell & melanoma research*, 25(1), 113-114.

- Once a tumor has reached the vertical growth phase it becomes the invasive melanoma. The tumor becomes able to grow into the surrounding tissue and can spread around the body through the blood. At this point blood vessels have developed within the tumor and the cells of the immune system begin to interact with the tumor.

Markovic, S. N., Erickson, L. A., Rao, R. D., McWilliams, R. R., Kottschade, L. A., Creagan, E. T., Weenig, R.H., Hand, J. L., Pittelkow, M. R., Pockaj, B. A. & Bardia, A. (2007, March). Malignant melanoma in the 21st century, part 1: epidemiology, risk factors, screening, prevention, and diagnosis. In *Mayo Clinic Proceedings* (Vol. 82, No. 3, pp. 364-380). Elsevier.

- Malignant melanoma is an aggressive, therapy-resistant tumor. The prevalence of melanoma has been steadily increasing worldwide

Motz, G. T., & Coukos, G. (2013). Deciphering and reversing tumor immune suppression. *Immunity*, 39(1), 61-73.

- PD-L1-expressing dendritic cells travel from the tumor site to the lymph node

Pardoll, D. M. (2012). The blockade of immune checkpoints in cancer immunotherapy. *Nature Reviews Cancer*, 12(4), 252-264.

- CTLA4 is a protein receptor that functions as an immune checkpoint to downregulate the immune system.
- It is expressed by activated T cells and transmits an inhibitory signal to other T cells.
- CTLA4 antibodies are used to downregulate the protein receptor and increase T cell response.

Restifo, N. P., Dudley, M. E., & Rosenberg, S. A. (2012). Adoptive immunotherapy for cancer: harnessing the T cell response. *Nature Reviews Immunology*, 12(4), 269-281.

- T cell receptor recombination allows for more accurate targeting of antigens expressed by tumors when using adoptive T cell therapy.

Schatton, T., & Frank, M. H. (2008). Cancer stem cells and human malignant melanoma. *Pigment Cell & Melanoma Research*, 21(1), 39–55.

- Melanoma has very poor prognosis once it has become metastatic. The lifetime risk and overall mortality because of melanoma rise yearly.
- When diagnosed early melanomas are largely curable (>95%) using surgical excision, however when the cancer has spread to the regional lymph node the 5-year survival of patients decreases to 50%.
- Patients with visceral metastases have a median survival rate of only a few months.

Silva, C. O., Martinho, N., Aniceto, N., & Reis, C. P. (2016). Melanoma Prevention: Challenges and Progresses in Nanotechnology for Melanoma Prevention and Treatment.

- Melanoma begins as benign nevi with increased number of nested melanocytes. Next, it becomes dysplastic nevi with irregular borders, multiple colors, increased diameter, and random and discontinuous cytological atypia. During the radial growth phase melanoma with malignant cells proliferating within the epidermis

Tsatmali, M., Ancans, J., & Thody, A. J. (2002). Melanocyte function and its control by melanocortin peptides. *Journal of Histochemistry & Cytochemistry*,

- Melanocytes are cells of neural crest origin.
- Melanocytes produce and distribute melanin to keratinocytes to protect them from UV radiation.

Zhang, J. M., & An, J. (2007). Cytokines, inflammation and pain. *International anesthesiology clinics*, 45(2), 27.

- Cytokines are small secreted proteins released by cells have a specific effect on the interactions and communications between cells.
- Chemokine are cytokines with chemotactic functions.