In Vitro Anti-PD-1/Anti-CTLA-4 Checkpoint Blockade in "Dirty" Mice

Abstract

Cancer immunotherapy research is traditionally conducted with specific pathogen-free (SPF) mice, who most accurately mimic the immune system of a human newborn. This makes translational research challenging, as the mouse model being used in the lab is not an accurate reflection of the adult patients who ultimately receive these newly developed treatments. An opportunity exists to further develop a mouse model that bridges this gap and increases translatability of current cancer immunotherapy research. By cohousing SPF mice with regular pet store mice, we are able to generate a cohoused (CoH) mouse that more accurately reflects the microbial experience of an adult human immune system. We will investigate the differing functions of CD8+ T cells within the tumor microenvironment of both SPF and CoH mice by injecting them with B16-melanoma. We aim to characterize each CD8+ T cell population by flow cytometry, ELISA, and by developing a novel in vitro immune checkpoint blockade assay. In vitro administration of anti-PD-1 and anti-CTLA-4, known melanoma treatments, will allow us to characterize the effector functions of tumor-infiltrating CD8+ T cells in SPF and CoH mice. CD8+ T cell responses to immune checkpoint therapies, in the context of a microbially experienced "dirty mouse model," will provide much needed insight into the impact that previous infection has on the efficacy of immune checkpoint therapy.

Introduction & Background

As humans develop cancer, the immune system provides us the first line of defense. The cells of the immune system, CD8+ T cells in particular, regularly kill cancerous cells that develop in the human body. This ability to recognize and destroy these cancerous cells develops over time as we age, and eventually we possess a whole host of cells that are capable of killing disease-causing organisms (called pathogens) and cancerous cells.

Traditional cancer immunotherapy research is conducted with standard laboratory mice that are raised under specific-pathogen-free (SPF) conditions, and most accurately mimic the immune system of a human newborn (Beura et al., 2016). In stark contrast with the adult immune system, human newborns possess no immune memory, leaving them with little protection against microbial invaders or cancerous cells. While the use of SPF mice provides researchers with a highly reliable and predictable animal model, it offers a poor analogue of the diversely sensitized adult human immune system (Masopust et al., 2017). Therefore, cancer immunotherapy research conducted solely in SPF mice fails to account for a variety of modulating interactions that may occur within a microbially experienced host.

An opportunity exists to further develop an animal model that more accurately recapitulates the human tumor microenvironment, and provides a better understanding of how prior infection and microbial exposure impact responses to cancer (Huggins et al., 2019). Originally developed by researchers at the University of Minnesota, and now well-established in the Renkema lab at Grand Valley State University, the "dirty mouse model" has been shown to accurately mimic the immune system of an adult human (Beura et al., 2016). We believe that this model provides a compelling complement for any cancer immunotherapy research with translational aspirations.

The advent of checkpoint blockade immunotherapies marked the most significant cancer treatment advancement since the early 1900's. Researchers discovered a mechanism by which tumor cells are able to "shut off" the immune response of CD8+ T cells, thus allowing the tumor cells to safely grow and proliferate in the host. In response, checkpoint blockade immunotherapies were developed to interfere with this mechanism. Administration of specific antibodies was shown to inhibit a tumor cells ability to "shut off" the host CD8+ T cell response. As a result, CD8+ T cells remained activated and were able to destroy the cancerous cells (Leach, Krummel, & Allison, 1996). While these treatments have saved countless lives and worked to extend many more, the nuances of their mechanisms remain incompletely characterized, as evidenced by the large populations of cancer

patients who remain unresponsive to immune checkpoint therapies (Padmanee & Allison, 2015). Challenges regarding efficacy may be due to the variability of the tumor microenvironment from one patient to the next. This heterogeneity may be due, in part, to individual variation in microbial exposure and its long-term impact on proliferation and differentiation of CD8+ T cells and their effector functions.

Previous research conducted in the Renkema lab showed that robust microbial experience appears to impact the proliferation of antigen experienced tumor-infiltrating CD8+ T cells in response to B16 melanoma. However, variation in effector function remains largely uncharacterized (Groeber, 2020).

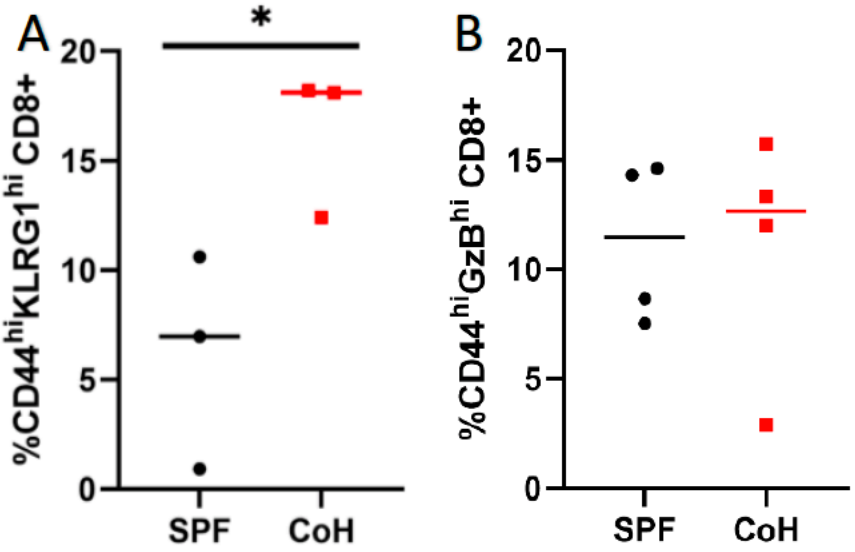

Figure 1: Antigen experienced tumor-infiltrating CD8+ T cells are more prevalent in CoH mice, while increases in effector function appear limited (Groeber, 2020).

Further exploration of the mechanisms underlying this limited difference in effector function are needed. Programmed cell Death-1 (PD-1) and Cytotoxic-T-Lymphocyte Associated protein 4 (CTLA-4) are protein receptors that are expressed on the surface of CD8+ T cells. Both receptors are capable of interacting with ligands or proteins expressed on the surface of tumor cells. Through this interaction, tumor cells are able to "turn off" the CD8+ T cells anti-tumor functionality,

allowing cancerous cells to survive and continue their growth. By administering antibodies that are designed to bind to these protein receptors (anti-PD-1 & anti-CTLA-4), tumor cells are blocked from binding to PD-1 and CTLA-4, thus eliminating their ability to evade the anti-tumor immune response. These treatments allow the CD8+ T cell to attack and kill the cancerous cells (Phan et al., 2003; Blank et al., 2004; Miller & Carson, 2020).

With regard to the previous studies conducted in the Renkema lab (Figure 1), it is our belief that tumor cells may be inhibiting the effector function of *both* SPF and CoH mice by binding PD-1 and CTLA-4 to limit the anti-tumor properties of the CD8+ T cells. Upon treatment with anti-PD-1 or anti-CTLA-4, we believe the cytotoxic potential of these cells may be restored, and variation in anti-tumor effector function between SPF and CoH mice can be better characterized.

Research Question & Hypothesis

Our goal is to further characterize the anti-tumor responses of CD8+ T cells in SPF and CoH mice by developing a novel in vitro anti-PD-1/anti-CTLA-4 checkpoint blockade assay. This will allow us to better understand the impact that microbial experience has on the efficacy of immune checkpoint therapy. We hypothesize that cell cultures from CoH mice treated with anti-PD-1 or anti-CTLA-4 will experience increased anti-tumor effector functions when compared to treated SPF cell cultures.

Methodology & Data Collection

Mice Cohousing. C57BL/6 specific pathogen-free (SPF) mice are purchased from Charles River Laboratories and split into groups. Some groups will be cohoused with pet store mice while others will be housed with only SPF mice to serve as controls. After a cohousing period of approximately 30 days, the SPF mice that were cohoused with the pet store mice can now be referred to as “dirty” or CoH mice, as they have been exposed to various murine pathogens. SPF control mice

are housed in a separate space and proper care is taken to ensure no cross-contamination occurs between the dirty CoH and clean SPF mice.

B16-Melanoma Injection. B16F10-melanoma cells will be injected subcutaneously into the shaved flank of each experimental mouse. The mice will be anesthetized with isoflurane to limit distress experienced during the procedure. As the tumors develop, measurements will be taken with calipers at regular intervals to document the progression of tumor growth in both SPF and CoH mice. In accordance with the lab IACUC protocol (19-08-A), the mice will be euthanized when the tumors reach a size of 1 mm^3.

Cell Culture, Flow Cytometry, & ELISA. Spleen, lymph nodes, and tumors will be harvested for analysis. In vitro checkpoint blockade assays will be run on cell cultures isolated from the tumors of both SPF and CoH mice. After treatment, activation, and incubation, these cells will be analyzed via flow cytometry using a Beckman & Coulter CytoFlex 4 channel flow cytometer. Flow cytometry will allow us to examine the presence of various surface proteins that provide insight into the effector functions that are present post-treatment. Additionally, we will utilize Enzyme-linked immunosorbent assays (ELISA) to analyze the concentrations of various cytokines related to anti-tumor functionality.

Justification of Sample Size

To properly run the in vitro anti-PD-1 and anti-CTLA-4 checkpoint blockade assays, we plan to cohouse one cohort of mice specifically for these experiments. We will cohouse 20 SPF mice with 5 pet store mice. An additional 20 SPF mice will not be cohoused, thus acting as our controls. We will inject all SPF and CoH mice with B16-melanoma cells. 10 SPF and 10 CoH mice will be used for the anti-PD-1 assay, while 10 SPF and 10 CoH mice will also be used for the anti-CTLA-4 assay. 5 SPF mice and 5 CoH mice will comprise the treatment groups in each assay, with the remaining mice acting as controls. Groups of 5 are required to attain statistical significance, as has been determined by a power-calculation and previous experiments.

Plans for Dissemination of Information

I intend to present these findings at a national immunology conference, such as the Autumn Immunology Conference taking place in Chicago, Illinois in November 2021 or the Midwinter Conference of Immunologists, which is being held in Pacific Grove, California in January 2022. I also look forward to participating in the annual 3-Minute-Thesis (3MT) competition where I will present this research in under 3 minutes and with the help of only one slide. Most importantly, my primary goal for disseminating this work is to publish the findings in a peer-reviewed scientific journal.

Project Timeline

We plan to receive the first cohort of mice on April 26th, 2021, with data collection and analysis beginning in earnest on May 31st, 2021. While analyzing the data from the first cohort of mice, we hope to immediately begin cohousing a second cohort, which we will harvest on or around the first week of July 2021. This additional cohort will be used to conduct the in vitro anti-PD-1/anti-CTLA-4 checkpoint blockade assays. Funding from this grant would provide us with the resources needed to pursue this novel experiment. Data analysis will then continue through the fall and into winter 2022, all of which will culminate in a thesis defense in the spring of 2022.

Budget

I am requesting $1,500.00 to fund the majority of the $1,837.95 needed for the in vitro checkpoint blockade assays. My mentor has agreed to pay for the balance from other funding sources.

Dirty Mouse Cohort. As previously described, we intend to cohouse an entire cohort of mice to accomplish both in vitro anti-PD-1 and anti-CTLA-4 checkpoint blockade assays. This will require at least 40 C57BL/6 mice from Charles River Laboratories and an additional 5 mice from a local pet store. These animals will cost approximately $1,333.95 in total.

In vitro Anti-PD-1 & Anti-CTLA-4 Treatment. Antibodies from Tonbo Bio will be purchased to administer the 20 μg/mL dosages of anti-PD-1 to cell cultures taken from 5 SPF and 5 CoH mice. The same dosage of anti-CTLA-4 will be delivered to cell cultures taken from an additional 5 SPF and 5 CoH mice. These experiments will require 200 μg of anti-PD-1 and 200 μg of anti-CTLA-4 which will cost $504.00 in total.

Quantity	Manufacturer	Serial #	Item Description	Price/Unit	Total Cost
40	Charles River Laboratories	C57BL/6NCrl	C57BL/6 Female Mouse	$32.60	$1,304.00
5	PETCO	135143	Female Mouse	$5.99	$29.95
2	Tonbo	50-9985-U100	Anti-Mouse CD279 (PD-1)	$137.00	$274.00
2	Tonbo	50-1522-U100	Anti-Mouse CD152 (CTLA-4)	$115.00	$230.00
					$1,837.95

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