Determining the Role of TLR Agonists in T Cell-Based Cancer Therapy

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Determining the Role of TLR agonists in T Cell-Based Cancer Therapy

By

Marshall Andrew Diven

A thesis submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirements for the degree of Masters of Biomedical Science in the College of Graduate Studies.

Department of Microbiology and Immunology, 2015

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List of Figures

**Figure 1.** Increasing the intensity of lymphodepletion with TBI correlates with greater depletion of inhibitory lymphocytes, transient activation of dendritic cells and increased impairment of the gastrointestinal tract.

**Figure 2.** Administration of ultra-pure LPS does not enhance anti-tumor immunity in nonirradiated mice.

**Figure 3.** Administration of ultra-pure LPS significantly enhanced anti-tumor immunity in 5 Gy TBI mice.

**Figure 4.** LPS enhances CD25 expression of adoptively transferred cells and improves their long-term persistence *in vivo.*

**Figure 5.** Bacterial derived TLR agonists enhance ACT tumor treatment in irradiated hosts.

**Figure 6.** *In vitro* priming with LPS enhanced *in vivo* anti-tumor activity of adoptively transferred P-mel-1 CD8+ T cells.

**Figure 7.** CpG ODN increases both IFN-γ and IL-10 secretion by pmel-1 CD8+ T cells in an antigen specific manner.

**Figure 8.** CpG ODN augments the expression of the activation markers CD25 and ICOS on pmel-1 CD8+ T cells.

**Figure 9.** Transient neutralization of IL-10 augments the activation status of CD8+ T cells primed with CpG ODN.

**Figure 10.** CpG ODN promotes the generation of central memory pmel-1 CD8+ T cells.

**Figure 11.** IL-10 blockade impairs the generation of central memory pmel-1 CD8+ T cells.

**Figure 12.** TLR agonists differentially alter the *in vitro* cytotoxicity of pmel-1 CD8+ T cells upon TCR specific re-stimulation.

**Figure 13.** Pmel-1 CD8+ T cells primed with CpG ODN regress melanoma and extend survival in mice.

**Figure 14.** Transiently neutralizing IL-10 during expansion of tumor-specific CD8+ T cells enhanced their *in vivo* anti-tumor capacity and prolonged survival in B16F10 tumor bearing mice.

**Figure 15.** CpG ODN promotes the functional plasticity of TRP-1 Th17 cells *ex vivo.*

**Figure 16.** Simultaneously neutralizing IL-10 while priming Th17 cells with CpG ODN decreases the long-term *in vivo* anti-tumor activity of adoptively transferred Th17 polarized TRP cells.
# Table of Contents

ACKNOWLEDGEMENTS.................................................................................................................. ii

LIST OF FIGURES............................................................................................................................. iii

ABSTRACT.............................................................................................................................................................. v

INTRODUCTION.......................................................................................................................................................... 1

Rationale......................................................................................................................................................................... 3

REVIEW OF LITERATURE............................................................................................................................................ 5

Toll Like Receptor agonists........................................................................................................................................ 5
TLRs 3, 4 & 9............................................................................................................................................................... 5
TLR signaling............................................................................................................................................................... 6
TLR agonists in cancer immunotherapy..................................................................................................................... 7
The paradoxical role of interleukin 10 in immunity.................................................................................................... 9

PRELIMINARY FINDINGS.......................................................................................................................................... 13

EFFECTS OF TLR AGONISTS & IL-10 ON ANTITUMOR T CELLS............................................................. 21

Rationale......................................................................................................................................................................... 21
Results............................................................................................................................................................................ 22

GENERAL DISCUSSION............................................................................................................................................. 39

FUTURE DIRECTIONS & CONCLUSIONS.................................................................................................................. 44

MATERIALS & ADDITIONAL METHODS................................................................................................................ 46

REFERENCES............................................................................................................................................................... 50
Abstract

One of the main mechanisms by which total body irradiation (TBI) enhances adoptive cell transfer (ACT) immunotherapy is by inducing systemic innate immune activation, triggered by microbial LPS released from the radiation-injured gut. Although microbial LPS is a critical mediator of TBI enhancement, it is unknown whether exogenous administration of LPS can augment therapeutic outcome. We report here that administration of LPS to non-irradiated animals did not replicate the ACT effectiveness seen in irradiated animals. In contrast, however, we found that LPS dramatically augmented ACT treatment in irradiated mice. Moreover, bacterial derived TLR agonists other than LPS (such as CpG ODN and Monophospholipid A) but not fungi- or virus-derived signals (such as PolyI:C, Zymosan, Loxoribine, and Imiquimod) improved antitumor immune responses in irradiated animals. Of clinical importance, we found that in vitro priming with LPS, CpG or MPL enhanced the anti-tumor activity of transferred CD8+ T cells. While it would be ideal to simply add TLR agonists to APCs to enhance their immunogenicity and in turn increase CD8+ T cell function, we must consider the potential negative feedback immune-regulatory mechanisms that result from TLR agonist priming, such as elevated IL-10 production. We found that transiently neutralizing IL-10 enhanced anti-tumor activity of CD8+ T-cells. However, priming CD8+ T cells with CpG ODN while neutralizing IL-10 did not augment the anti-tumor response in mice. Furthermore, we found that IL-10 was necessary to maintain IL-17 production and illicit a potent anti-tumor response in Th17 cells primed with CpG ODN.
Chapter 1: Introduction

For a variety of reasons, it is not surprising that the skin accounts for more cases of cancer than any other organ of the body. It is the largest organ and plays a pivotal role in many homeostatic mechanisms. It is divided into 3 layers, the epidermis, dermis and hypodermis. These layers are comprised of specific cell types that aid in the various functions that are critical for host immune-health. Of interest to my thesis, are the melanocytes that are found in the epidermis. Melanocytes are responsible for protecting the deeper layers of skin from harmful ultra violet radiation by secreting the protective pigment melanin.¹

While cancers that arise from melanocytes, or melanomas, account for less than 2% of skin cancer, they cause the majority of skin cancer related deaths. The American Cancer Society estimates that in 2014, 76,100 new melanomas will be diagnosed and roughly 9,710 people will die of the disease in the US alone.² If the disease is diagnosed and treated in stage 1b or sooner, the five and ten year survival rates exceed 90% and 85%, respectively. However, once the melanoma meets stage II criteria, the five and ten year survival rates drop to 81% and 67%, respectively. Unfortunately, patients diagnosed with stage IV melanoma face a poorer prognosis with approximately 20% of patients living longer than five years.³

Current treatments vary according to the stage at which the melanoma is diagnosed. Typically, melanoma treatment utilizes one or a combination of surgery, immunotherapy, targeted therapy, chemotherapy and radiation therapy in an adjuvant, neoadjuvant or concomitant fashion. While surgery often provides effective treatment for early-stage melanomas, later stage disease often require a more sophisticated and combinatorial
treatment approach. A relatively new and burgeoning field for cancer treatment, including melanoma, is immunotherapy and adoptive immunotherapy.\textsuperscript{4}

Immunotherapy, or the use of treatments that bolster and stimulate a patient’s own immune system, aids the host’s ability to distinguish cancer cells and ward them off. This approach provides an exciting and hopeful avenue for the future of melanoma treatment. Within the last decade new and exciting therapies targeting various aspects of the immune system have been developed and applied to the treatment of melanoma. Ipilimumab, approved by the FDA in 2011, is a monoclonal antibody that blocks CTLA-4, an inhibitory T cell receptor, engagement by CD80 (B7-1) and CD86(B7-2) expressed on tumors and host antigen presenting cells.\textsuperscript{5} In a recent clinical trial, this therapy has mediated a 7\% objective response rate in patients with advanced melanoma.\textsuperscript{6}

A similar and more recently developed strategic immunotherapy that is showing success in clinical trials are PD1 and PD-L1 blocking antibodies. These monoclonal antibody therapeutics prevent the engagement of PD1, an inhibitory receptor expressed by activated T cells, by PDL-1 that is expressed by either host antigen presenting cells (APCs) or various types of tumors. In recent clinical trials Nivolumab, a monoclonal antibody against PD1, has achieved objective tumor regressions in 31\% of patients with advanced melanoma.\textsuperscript{5} A deeper understanding of the complexity of melanoma and host immune cell interactions will provide a multitude of avenues for developing therapeutic interventions.

Another therapeutically effective treatment for patients with advanced metastatic melanoma is adoptive T cell therapy (ACT). While there are many variations and competing paradigms related to the best possible approach, the therapy typically involves isolating host-tumor infiltrating lymphocytes (TILs), culturing them to large numbers ex
vivo and then re-infusing these autologous cells back into the patient. Early clinical trials utilizing TILs in addition to IL-2 with cyclophosphamide for the treatment of patients with metastatic melanoma resulted in an overall objective response rate of 52%.

More recently, clinical trials using non-myeloablative chemotherapy pretreatment (consisting of cyclophosphamide and fludarabine) plus total body irradiation (12 Gy TBI, 2Gy BID for three days) prior to TIL infusion yielded objective responses up to 72% among patients with metastatic melanoma. Patients receiving this regimen require CD34+ hematopoietic stem cells (HSCs) the day following TIL infusion to replenish senescent HSCs. Mechanistically, TBI has been shown to enhance the effectiveness of transferred T cells via several mechanisms. Published mechanisms include decreasing CD4+CD25+ regulatory T cells (T-regs); increasing the availability of homeostatic cytokines by removing cytokine “sinks”; activation of APCs via disruption of intestinal homeostasis; and stimulation of lymphocytes with HSCs. Of particular interest are the ways in which Toll Like Receptor agonists, a byproduct of intestinal disruptions from TBI, impact tumor specific CD8+ T cells and CD4+ T cells, particularly Th17 cells.

**Rationale:**

Indigenous gut microbes play a crucial role in health, ranging from nutrient absorption, maintenance of mucosal integrity to the regulation of intestinal immune homeostasis. Many chemotherapeutic regimens compromise intestinal immune homeostasis and can induce microbial translocation. This microbial translocation can initiate a switch in the host-microbe relationship from mutualistic to pathogenic. This phenomenon is implicated in the exacerbation of the pathogenesis of graft versus host
disease (GVHD), inflammatory bowel disease, as well as HIV/AIDS.\textsuperscript{20-22} However, this seemingly deleterious effect has recently been found to be beneficial for cell-based cancer therapies.\textsuperscript{14,23}

Lymphodepletion with a myeloablative chemo-radiotherapy preparative regimen administered prior to adoptive immunotherapy mediates objective immune responses in >70\% of patients with metastatic melanoma.\textsuperscript{24} In addition to removal of inhibitory cells, our lab reported that innate immune activation via TLR4 signaling was a critical mechanism underlying the enhanced effectiveness of TBI in mice.\textsuperscript{25,18,26,14} The purpose of this study is to explore the potential for utilizing LPS and other TLR agonists to enhance the effectiveness of transferred tumor-specific CD8\(^+\) T cells or Th17 cells at eradicating established B16 F10 melanoma. In addition to TLR agonist priming of CD8\(^+\) T cells, we investigate how IL-10, produced in response to microbial challenge, affects these TLR-activated T cells.
**Chapter 2- Review of Literature**

**Toll Like Receptor (TLR) agonists**

One mechanism by which the host innate immune system recognizes pathogen invasion is via detection of pathogen associated molecular patterns (PAMPS) by pathogen recognition receptors (PRRs). TLRs, a type of PRR, are highly expressed on myeloid derived cells of the innate immune system. However, research has also indicated variable TLR expression on numerous T cell subsets. APC engagement by TLR agonists has been reported to enhance antigen presentation and bolster the production of inflammatory cytokines through up-regulation of co-stimulatory molecules. Thus in an indirect fashion, TLR induced maturation and stimulation of dendritic cells has important implications for the adaptive immune response.

Of the 11 known family members, TLRs 3, 4 and 9 will be the focus of this study. The rationale for choosing these TLR agonists is because PolyI:C (TLR3) and CpG (TLR9) have been or are currently in clinical trials. We are using LPS/MPL (TLR4) as a control as it builds on the foundation of our finding that TBI augments adoptively transferred CD8+ T cells via inducing microbial LPS from the injured bowel; which is in our previous manuscript published in the Journal of Clinical investigation.

**TLRs 3, 4 and 9**

TLR3 recognizes polyinosinic:polycytidylic acid (poly I:C), a synthetic double stranded RNA molecule that mimics viral infection. Similarly, TLR9 recognizes unmethylated cytosine-phosphate-guanine (CpG) motifs of DNA which are common to bacterial genomes and viral DNA. TLR4 recognizes the gram negative endotoxin
lipopolysaccharide (LPS). Furthermore, while TLR4 is expressed on the extracellular membrane, TLR3 and TLR9 are located within the endosomal compartment of most professional APCs. Because TLR3 and TLR9 are located in the endosomal compartment, they are mainly activated by endogenous viral nucleic acids. However, studies have shown TLR3 and TLR9 can induce CD8 T cell responses via enhancing APC cross presentation. Conversely, TLR4 signaling does not require phagocytosis of pathogens by APCs and its activation via LPS binding can be initiated extracellularly without the need for cross presentation.

**TLR signaling**

In general, TLR signaling involves ligand binding to a leucine-rich repeat motif with signal transduction initiated through a cytoplasmic Toll/interleukin receptor (TIR) domain. These receptors recruit and homodimerize with TIR domain-containing adaptor proteins. With the exception of TLR3 and TLR4, myeloid differentiation protein 88 (MyD88) is the adaptor protein that is required to link the TLR receptors to down-stream signaling molecules. TLR3 signals through the adaptor molecule Toll/IL-1R domain-containing adaptor-inducing IFN-β (TRIF). TLR4 can signal through either MyD88 or a TRIF dependent fashion. After MyD88 is recruited, it activates IL-1 receptor-associated kinases (IRAKs) and TNF receptor associated factor 6 (TRAF6). Then, this series of signaling events activates multiple transcription factors including NFkB. Furthermore, TLR signaling through MyD88 can activate the PI-3 kinase pathway, induce IFN regulatory factor IRF-1, IRF-5 and IRF-7 and plays a role in Fas and IFNγ-receptor signaling.
**TLR agonists in cancer immunotherapy**

Conceptually, employing bacteria to mediate cancer progression dates back to the late nineteenth century when William B. Coley observed spontaneous tumor regression in patients with erysipelas. In the late twentieth century, the molecular basis behind the effects of bacterial bi-products on the mammalian immune system were uncovered by Charles Janeway and his colleagues. Their discovery of the direct connection between TLR signaling and activation of the innate immune system gave heightened insight into that of William Coley’s famous toxin over a century prior. Currently, research with TLR agonists and their derivatives for cancer treatment is extensive with many ongoing and newly launched clinical trials. In this section, I will briefly discuss current FDA approved TLR agonists for cancer treatment. I will also highlight previous attempts and potential new strategies regarding the utilization of various TLR agonists as anti-oncogenic agents against melanoma.

Currently, only three TLR agonists are licensed by the FDA for use in cancer patients. These include bacillus Calmette Guérin (BCG), an attenuated non-virulent *Mycobacterium bovis* used for the treatment of patients with non-invasive transitional cell carcinoma of the bladder, monophosphoryl lipid A (MPL), an LPS derivative used as an adjuvant in the HPV-16 and -18 vaccine Ceravix®, and Imiquimod, a guanosine analog and imidazquinoline derivative used as a topical therapy for actinic keratosis, superficial basal cell carcinoma and external genitoperitoneal warts. BCG has been shown to work as a heterogeneous TLR2/TLR 4 agonist which is similar to MPL as it also triggers TLR2 and TLR4. The topical imiquimod cream, Aldara®, elicits its immunostimulatory
effects via TLR7 signaling.\textsuperscript{54} Although, these are the only three TLR agonists currently approved for cancer treatment, many other TLR agonists have been used in clinical trials and are currently underway for expanding this anti-oncogenic arsenal.

Previous attempts by Speiser and colleagues were pursued for treating metastatic melanoma in patients by administration of low dose CpG ODN 7909 plus a melanoma antigen A (Melan-A) analog.\textsuperscript{55} They were able to achieve strong antigen-specific CD8\textsuperscript{+} T cell responses in these patients. Interestingly, however the therapy could not effectively promote tumor regression. The investigators of the study suggested that this crucial shortcoming could be attributed to an increase in regulatory CD25\textsuperscript{+}FoxP3\textsuperscript{+}CD4\textsuperscript{+} T cells (i.e. T regs) at the site of the tumor.\textsuperscript{56} It is also plausible that this vaccination strategy generated tumor-specific T cells that were short lived and overall ineffective. Similarly, a clinical trial for treatment of melanoma with Hiltonol\textsuperscript{TM}, PolyI:CLC- TLR3 agonist, began in 2013 in combination with a personalized peptide-based vaccine.\textsuperscript{32} This therapy may encounter obstacles that accompany other vaccine based treatments for melanoma; as they are effective at activating innate and adaptive immune system but they cannot overcome the peripheral tolerance that ultimately abrogates tumor regression.

The efficacy and optimization of future immunotherapeutic regimens involving TLR agonists relies on our ability to combine their distinct immunomodulatory features with current and new treatments. For instance, it is has been shown that combined TLR and CD40 signaling can induce potent CD8\textsuperscript{+} T cell expansion.\textsuperscript{57} Furthermore, selective combinations of TLR agonists can differentially skew immune responses to favor potent Th1 responses.\textsuperscript{58} With this in mind, the next logical step for using TLR agonists for anti-oncogenic therapy in the clinic will likely be in conjunction with the encouraging
checkpoint blockade therapy.\textsuperscript{6,7} For instance, by administering TLR agonists to patients, we are effectively activating the innate and the adoptive immune system. However the inhibitory mechanisms such as Treg induction, CTLA-4 or PD-1 engagement by APCs or cancer cells prevent a prolonged anti-tumor response. By inhibiting the activation of the immune brakes of CTLA-4, PD-1 or Tregs, we could theoretically enhance treatment outcomes using TLR agonist therapy. Accordingly, a recent study demonstrated that blocking CTLA-4 or PD-1 while locally stimulating with a TLR9 agonist effectively broke immune tolerance and enhanced tumor eradication in a murine bladder cancer model.\textsuperscript{59} TLR agonist immunotherapies or use of multiple TLR agonists simultaneously for cancer treatment will not be a highly fortuitous therapeutic venture without addressing the possible immunosuppressive compensatory mechanisms.

In this present study we propose that another incompletely elucidated role for TLR agonists lies in their ability to enhance adoptive T cell therapy regimens. In previous work from our lab, we have reported that TLR4 signaling, induced by microbial translocation, can enhance the function of adoptively transferred CD8\textsuperscript{+} T cells.\textsuperscript{14} In this study we aim to identify other mechanisms by which TLR agonists enhance CD8\textsuperscript{+} adoptive T cell therapies. Furthermore, we seek to suppress the immunomodulatory features induced by TLR agonists that may impair the effectiveness of tumor-specific T cells to potentially replace the need for myeloablative chemotherapy pre-treatments in melanoma patients. Of particular interest to us is to block the immunosuppressive cytokine IL-10, which has been reported to suppress T cell mediated immunity.\textsuperscript{60}

\textbf{The paradoxical role of Interleukin 10 in immunity}
Interleukin-10 (IL-10) is a cytokine that serves an important immunosuppressive role in response to pathogens by inhibiting inflammatory cytokine production by APCs.\textsuperscript{61} Globally, this cytokine protects the host by preventing persistent inflammatory responses which can cause damage to the host.\textsuperscript{62} This damage is evidenced by data in IL-10 deficient mice developing inflammatory bowel disease in response to colonization of certain microorganisms.\textsuperscript{63,64} IL-10 production was first discovered in Th2 cells but has since been reported in many other adaptive and innate cell types including Th1, Tregs, Th17, CD8\textsuperscript{+} T cells, B cells, macrophages and dendritic cells.\textsuperscript{65-68,62} In this section we review the main immune cell types that produce IL-10, some of the immunomodulatory effects of IL-10 and, briefly, how IL-10 might be used in cancer therapies.

Given its role in suppressing immune responses to pathogens, it is no surprise that IL-10 is produced by cells of the innate immune system in response to pattern recognition receptor (PRR) ligation by pathogen-derived products.\textsuperscript{61} Of note, TLR2, TLR3, TLR4, and TLR 9 agonists can stimulate the production of varying amounts IL-10 by macrophages and DCs.\textsuperscript{69,70} It has been reported that macrophages produce the greatest amount of IL-10 followed by myeloid DCs and low amounts by plasmacytoid DCs in response to TLR signaling.\textsuperscript{71} The degree of IL-10 induction by these innate cell types have been shown to correlate with the degree of ERK activation.\textsuperscript{72} As mentioned previously, IL-10 production has also been reported in cells of the adaptive immune system making it an important immunomodulator in diverse inflammatory conditions.

The main source of IL-10 production in cells of the adaptive immune system stem from CD4\textsuperscript{+} T cell subsets including Th1, Th2, Th17 and Treg cells. Moreover, it has been
reported that high TCR signaling along with IL-12 production enhances the differentiation of IL-10 producing Th1 cells in a STAT4 and ERK dependent manner.\textsuperscript{73,74} Briefly, IL-10 production in Th17 cells has been reported to occur in a STAT3 dependent fashion while IL-4, STAT6 and GATA binding protein 3 (GATA3) are important for IL-10 production in Th2 cells.\textsuperscript{75-78} While CD4\textsuperscript{+} lineages are important IL-10 producing cells, CD8\textsuperscript{+} T cells also produce IL-10 following TCR activation by APCs or CD40L interaction on pDCs.\textsuperscript{65-68}

In large part, the role of IL-10 is to suppress the effector adaptive immune responses and minimize tissue damage in response to microbial infections.\textsuperscript{79} IL-10 attains this function by down regulating MHC complex antigens, intercellular adhesion molecule-1(ICAM-1) and costimulatory molecules CD80 and CD86 on APCs.\textsuperscript{80} The role of IL-10 in tumor development and treatment remains incompletely elucidated and often times contradictory.\textsuperscript{81} Studies demonstrate that IL-10 is expressed in a variety of human tumors including melanomas.\textsuperscript{81-83} Furthermore, IL-10 production in some human cancers correlates with poor prognosis possibly due to an increased number of tumor infiltrating DCs and Treg cells which may suppress CD8\textsuperscript{+} T cell function.\textsuperscript{84-87} Thus, neutralizing IL-10 for oncogenic malignancies is an attractive strategy which has shown success in preliminary studies involving DC-based vaccine tumor immunotherapies.\textsuperscript{81} However, there are contradictory studies that suggest high levels of IL-10 administration increase an effective tumor response by enhancing proliferation and function of tumor infiltrating CD8\textsuperscript{+} T cells.\textsuperscript{88-89} Indeed, IL-10 producing CD8\textsuperscript{+} T cells have potent responses to tumor antigen.\textsuperscript{90} Thus, it will be important to neutralize IL-10 to understand how it shapes
immunity to tumors. We will explore if blocking IL-10 is an attractive strategy, which has shown success in preliminary studies involving DC-based vaccine tumor immunotherapies.⁸¹
Chapter 3-Preliminary Findings

Increasing levels of TBI correlate with depletion of inhibitory lymphocytes, transient activation DC activation, and increased impairment of the gastrointestinal tract.

Because intense lymphodepletion with chemo-radiation further enhances ACT treatment in patients, it is important to access how increasing the intensity of irradiation impacts the degree of host cell depletion and innate activation. To address this question, we evaluated the absolute number of splenic lymphocytes and APCs in non-irradiated mice compared with mice given a non-myeloablative regimen (5Gy TBI) or mice given a myeloablative regimen (9 Gy TBI plus HSC). As expected, we found that increasing the intensity of lymphodepletion from 0 to 9 Gy TBI was associated with a greater reduction in the absolute number of splenic CD4 and NK cells (Fig. 1A, D3: 0GyTBI: CD4 and NK

**Figure 1:** Increasing the intensity of lymphodepletion with TBI correlates with greater depletion of inhibitory lymphocytes, transient activation of dendritic cells and increased impairment of the gastrointestinal tract. **A.** TBI depletes endogenous CD4 and NK cells and transiently promotes activation of CD11c+ dendritic cells. Splenocytes were isolated from 0, 5 and 9 Gy irradiated mice 2-5 days after TBI. Mice irradiated with 9 Gy TBI were given HSC. Absolute numbers of CD4, NK and activated CD11c+CD86+ DCs in the spleens of TBI and non-irradiated C57BL/6 mice were enumerated. Data shown are representative of 2 independent experiments. **B-C.** High dose TBI compromises the colon. Colon of mice were analyzed at 3 days post-TBI and scored by a pathologist unaware of the treatment groups. Data shown (n = 5 mice per group) are representative of 1 independent experiment. **D.** High dose TBI promotes translocation of gut derived LPS. Serum from non-irradiated and 5 or 9 Gy irradiated mice were collected and analyzed for the presence of LPS using a LAL assay 6 days after TBI. Data shown (n = 3 mice per group) are representative of 3 independent experiments.
cells –10.6 and 6.45 x 10^6 splenocytes, respectively). Consistent with previous work, the absolute number of activated DCs transiently increased as the intensity of irradiation was increased from 0 to 9 Gy TBI (Fig. 1A, Day 1: 0 Gy TBI: CD11c+CD86high cells: 5e3 splenocytes; 5 Gy TBI: 135e3; and 9Gy TBI+HSC: 211e3 splenocytes).^91,14 Collectively, our data revealed that increasing the intensity of TBI correlated with greater depletion of endogenous lymphocytes and considerable activation of innate immune system.

We next sought to determine if heightened innate immune activation was associated with greater TBI damage to the GI tract. For this, we measured the colon integrity of non-irradiated mice and mice irradiated with 5 or 9 Gy TBI. Mice irradiated with 9 Gy TBI were given HSC support. We found that both 5 and 9 Gy TBI compromised the morphological integrity of the gut by pathological score (Fig. 1B and 1C). When compared with 5 Gy TBI, 9 Gy TBI appeared to more severely impair the colon, as visually indicated by heightened signs of edema as well as by a considerable reduction in crypts and goblet cells. A significant amount of LPS, a key component of gram-negative bacterial cell walls often measured to determine the degree of microbial translocation, was detected in the sera of irradiated mice (Fig. 1D).^20,22,14 As expected, a higher amount of LPS was detected in the blood of mice receiving 9 Gy TBI (plus HSC) than mice given no or 5 Gy TBI. In addition, marked increases in levels of inflammatory cytokines associated with activation of the innate immune system — i.e., IL-1 β, IL-6 and IL-12 — were also detected in the sera of irradiated mice compared with non-irradiated mice (not shown). Collectively, these data revealed that irradiating mice with 9 Gy TBI (plus HSC) further damaged the GI tract than seen in 5 Gy TBI mice. Furthermore, greater impairment of the GI tract resulted in greater translocation of gut-derived LPS in mice receiving the highest level of irradiation.
Addition of ultrapure LPS could not replace the need for TBI.

Because microbial LPS was detected in the serum of irradiated animals (Fig. 1D), we postulated that administering ultrapure LPS to non-irradiated mice would bypass the previous need for TBI. To address this idea, we first determined the highest dose of LPS that could be tolerated in non-irradiated mice given an ACT therapy. To this end, increasing doses of ultrapure LPS, ranging from 0.1 to 10μgs, were administered to non-irradiated animals one day after an ACT therapy and their tolerance to treatment was monitored by their overall appearance and survival. The ACT therapy was administered consisted of the infusion of 5e⁵ transgenic CD8⁺ T cells with a TCR that recognizes the gp100 peptide on B16 tumors, vaccination encoding gp100 peptide and IL-2 cytokine. In contrast to our hypothesis, we found that even the highest tolerable dose of LPS (5μg/mouse) administered to non-irradiated mice could not enhance treatment in non-irradiated mice (Fig. 2).

Figure 2: Administration of ultra-pure LPS does not enhance anti-tumor immunity in nonirradiated mice. Ultra-pure LPS does not augment anti-tumor responses in non-irradiated mice. Mice bearing s.c. B16 tumors were established for 10 days. Mice received an ACT treatment comprised of the adoptive transfer of 5x10⁵ cultured pmel-1 T cells, rFPgp100 vaccination and rhIL-2 or were left untreated. The next day, mice received ultra-pure LPS ranging from 0.1 to 25 μg/mouse or left untreated. Data shown (mean ± SEM of 5-10 mice per group) are representative of 2 independent experiments.
Addition of ultrapure LPS enhances ACT in mice given non-myeloablative TBI.

We next sought to determine what dose of LPS might safely and effectively enhance ACT treatment in animals given a non-myeloablative 5 Gy TBI. Thus, LPS doses ranging from 0.1-50μg were administered to irradiated animals one day after treatment. In contrast to our findings in nonirradiated animals, we found that 1μg of LPS could significantly potentiate CD8+ T cell mediated tumor eradication in irradiated animals.

Likewise, doses of LPS exceeding 1μg of LPS improved ACT treatment (Fig. 3). Of note, we also found that irradiated mice tolerated higher doses of LPS compared to non-irradiated mice (not shown). This is likely because TBI ablates APCs activated by this agonist that secrete inflammatory cytokines that compromise survival.
LPS increases proliferative capacity and persistence of infused CD8+ T cells. LPS increases CD25 but not CD62L expression on infused CD8+ T cells.

How LPS impacts on the phenotypic signature and proliferative capacity of infused tumor-specific CD8+ T cells in vivo remains incompletely elucidated. Thus, we aimed to elucidate how LPS influenced the expression of CD62L, CD44 and CD25 on the transferred cells in irradiated mice 5 days post treatment. Interestingly, we found that LPS greatly increased the expression of CD25, a receptor for IL-2 cytokine, on the transferred cells from irradiated mice (Fig. 4A). These data revealed that CD8+ T cells from irradiated mice given LPS might have an advantage in acquiring homeostatic cytokine IL-2. In contrast to significant differences in CD25 expression due to LPS, there were no differences in the expression of CD62L (Fig. 4A) on the transferred cells, likely due to the differentiation of the infused cells to a full effector phenotype post infusion into the animals.

To investigate how LPS impacts on the in vivo proliferative capacity of the infused CD8+ T cells, we labeled the infused cells with BRDU and determined the percent of these compounds incorporated on day 3 post-transfer. We found that the transferred cells from irradiated mice given LPS incorporated significantly more BRDU than in mice receiving TBI alone (Fig. 4B). These BRDU data suggested that removing suppressive lymphocytes with TBI while concomitantly heightening innate activation by administering a higher concentration LPS to the host, unmasked the proliferative capacity of the transferred cells. Accordingly, the absolute number of CD8+ T cells was considerably greater in the spleen and blood of irradiated mice receiving LPS compared with the absolute number of transferred cells in the spleen and blood of irradiated mice not receiving LPS 30 days after
treatment (Fig. 4C and 4D). Collectively, these data indicated that LPS does not merely drive the proliferative capacity of infused CD8+ T cells but also increases their persistence in irradiated animals. These data might reveal why treatment and survival is superior in irradiated mice given LPS after ACT.

![Figure 4: LPS enhances CD25 expression of adoptively transferred cells and improves their long-term persistence in vivo. A. LPS enhances the expression of CD25 expression on adoptively transferred cells in irradiated mice B. LPS enhances the initial proliferation of adoptive transferred cells as indicated via BRDU incorporation at day 3 post-ACT. C-D. LPS increased the absolute number of transferred pmel-1 T cells in the spleen and blood of irradiated hosts. Absolute numbers of transferred pmel-1 cells (CD8+Thy1.1+) in the spleens and blood were enumerated. Data shown (mean ± SEM of 3-5 mice per group) are representative of 2 independent experiments.]

CpG ODN and MPL augment the antitumor activity of infused CD8+ T cells.

Owing to its inherent toxicity, it is important to find alternate agonist to LPS for tumor therapy in the clinic. Moreover, some patients have TLR4 polymorphisms, rendering their innate immune system resistant to microbial LPS.92 Thus, we sought to determine whether TLR2/TLR4 monophospholipid A (MPL-a detoxified version of LPS) could also augment ACT treatment in irradiated hosts. Similar to ultrapure LPS, we found that MPL was effective in mediating tumor regression by the transferred cells. Importantly, we also found that other bacterial-derived agonist beyond LPS could enhance ACT treatment, such as CpG ODN (Fig. 5F). Interestingly, no enhanced tumor response was observed in mice treated with zymosan, Poly I:C, flagellin, and imiquimod, at least with the doses used in
Collectively, our data revealed other TLR agonists besides LPS that can improve cancer-based cellular therapy, such as clinically available CpG-ODN, an agonist previously used for tumor immunotherapy in patients.\textsuperscript{55,93}

Figure 5: Bacterial derived TLR agonists enhance ACT tumor treatment in irradiated hosts. Detoxified LPS (MPL) and CpG augmented antitumor immunity in irradiated mice. Mice bearing s.c. B16 tumors established for 10 days received 5 Gy TBI. One day after TBI, mice received an ACT treatment comprised of the adoptive transfer of $10^6$ cultured pmel-1 T cells, rFPhgp100 vaccination and rhIL-2 or were left untreated. The next day, mice received either A. zymosan (250μg i.v.), B. PolyI:C (50μg i.v.), C. detoxified LPS – MPL (5μg i.v.), D. Flagellin (200μg i.v.), E. Imiquimod (5% topical cream), F. CpG (10μg i.v.) or were left untreated. Data shown (mean ± SEM of 5-10 mice per group) are representative of 2 independent experiments.

\textbf{In vitro priming of CD8$^+$ T cells with LPS enhanced treatment outcome in mice.}

To further lessen the toxicity of LPS while simultaneously bolstering anti-tumor activity of adoptively transferred CD8$^+$ T cells, we hypothesized that \textit{ex vivo} priming CD8$^+$ T cells prior to their infusion would augment their antitumor activity \textit{in vivo}. Because we wash out LPS from the culture before infusing the cells into mice, we capitalize on the therapeutic potential of LPS without its toxic side effects. To address this idea we added...
1μg of ultrapure LPS to pmel-1 splenocytes during their *in vitro* expansion with hgp10025 peptide. Adding 1μg/mL of ultrapure LPS at the start of the culture and then washing out prior to infusion enhanced their *in vivo* anti-tumor activity compared to cultures not primed with LPS (Fig. 6 A). Similar to what was observed with *in vivo* LPS addition, culturing pmel-1 CD8+ cells *in vitro* with LPS increased CD25, indicating their enhanced ability to competitively uptake IL-2 (Fig. 6B). Furthermore, culturing the CD8+ T cells with LPS prior to infusion enhanced their ability to persist, which is similar to the results when LPS was administered directly to the mice (Figure 5 C-D). Collectively, these results indicate the potential to utilize LPS in a non-toxic way by administering the agonist to the media during the rapid expansion phase of host TILs instead of directly to the host.
Chapter 4- Effects of TLR agonists and IL-10 on antitumor T cells:

Rationale:
Our data indicates the potential use of different TLR agonists, especially LPS, in a non-myeloablative regimen to improve the activity of tumor specific CD8+ T cells. The aim after acquiring the previous data was to elucidate the issues that prevent the practical translation of this work in the clinic. Since LPS is a highly toxic compound, it is necessary to establish the potential of utilizing other clinically relevant and less toxic TLR agonists such as CpG and polyI:C instead of LPS in ex vivo ACT protocols. Even though the TLR agonists are not completely interchangeable at eliciting their immunomodulatory effects, we hope to identify safe alternatives that ultimately improve anti-tumor CD8+ T cell function. Second, although TLR agonists are capable of potentiating a potent immune response, it is often accompanied with an equally strong suppressive response. For instance, one of the reasons a high dose of TBI is required prior to adoptive cell therapy is to rid the body of the immunosuppressive T-reg cells. Thus, while it would be ideal to simply add TLR agonists to APCs to enhance their immunogenicity and in turn increase CD8+ T cell function, we must consider the potential negative feedback mechanisms that result from such priming.

IL-10, a focal interleukin in this study, is an immunosuppressive cytokine that is produced in response to TLR signaling in a variety of immune cells. IL-10 production was first reported in Th2 cells but has recently been reported in Th1, Tregs, Th17, macrophages, DCs and CD8+ T cells following TCR activation or interaction with CD40 ligand by activated pDCs. It has recently been shown that DCs deficient of IL-10 were capable of activating a stronger anti-tumor Th1 and CTL response compared to wild-type
Thus, we posit that neutralizing IL-10 during initial priming will further enhance the anti-tumor effectiveness of our TLR agonist primed tumor specific CD8+ T cells.

**Results:**

**CpG ODN increases both IFN-γ and IL-10 secretion by pmel-1 CD8+ T cells.**

We sought to determine how TLR agonists impact the functional capacity of tumor-specific CD8+ T cells. We hypothesized that culturing CD8+ T cells with different TLR agonists would differentially regulate their capacity to produce IFN-γ. Due to IL-10’s role in limiting inflammation, we suspected that TLR agonists would increase IL-10 production by T cells. After thorough investigation of previous literature TLR agonists concentrations were identified to stimulate pmel-1 CD8+ T cells. We used 1-5μg/mL LPS, 10μg/mL MPL, 20μg/mL PolyI:C and 0.5-5 μg/mL CpG ODN 1668 (CpG ODN or CpG) to prime T cells. Specifically, using these concentrations, we stimulated pmel-1 splenocytes with gp10025-33 peptide plus each of the TLR agonists and rhIL-2 [100IU/mL] for 3d and collected supernatant to analyze IFN-γ and IL-10.

As shown in **Figure 7A**, priming pmel-1 splenocytes in the presence of LPS, MPL or CpG increased their secretion of IFN-γ compared to pmel-1 CD8+ T cells either given no TLR agonists or PolyI:C. However, IL-10 production was increased only by the addition of LPS or CpG (**Fig. 7B**). While these data reveal that TLR4 and TLR 9 agonists
augment CD8+ T cell function, it remains unclear if these agonists augment their function directly or indirectly via antigen presenting cells.

Figure 7

Pmel-1 CD8+ T cells were combined with the splenocytes at 5:1 (pmel-1/BL6). Supernatant was collected 3 days later and an ELISA was performed.

Due to the fact that pmel-1 splenocytes contain a heterogeneous population of various immune cells, including innate immune cells, we sought to modify the culture technique to address if the TLR agonists were acting through the antigen presenting cells (APC) or on the CD8+ T cells directly. Accordingly, C57/BL6 splenocytes were pulsed with TLR agonists overnight, washed three times with PBS, exposed to 10Gy irradiation and loaded with gp10025-33 peptide. In one experimental arm, pmel-1CD8+ T cells were
purified from bulk splenocytes via a negative CD8+ T cell selection kit and combined with the TLR primed, gp10025-33 loaded and irradiated APC-containing C57/BL6 splenocytes. To control for the possibility that TLR priming alone caused an increase in IFN-γ production without activating pmel-1 CD8+ T cells, TLR primed C57/BL6 splenocytes were not loaded with peptide but combined with purified CD8+ pmel-1 T cells. For this experiment, LPS and CpG were the only TLR agonists used primarily because they were the only two shown to increase both IFN-γ and IL-10 production in culture. As expected, pmel-1 cells primed with TLR agonists without activation did not secrete IFN-γ (Fig. 7C). However, pulsing C57/BL6 cells with LPS or CpG and gp10025-33 peptide yielded significant increases in IFN-γ production. This data suggests that TLR agonists act in an indirect fashion through the APC compartment to stimulate the production of IFN-γ by tumor-specific CD8+ T cells.

CpG augments CD25 and ICOS expression on pmel-1 CD8+ T cells.

Given our finding that TLR4 agonists enhance pmel-1 CD8+ T cells, we next determined how TLR agonists impacted their activation status. We hypothesized that TLR agonists would increase the activation of pmel-1 CD8+ T cells, which might account for their heightened secretion IFN-γ. Thus, we activated pmel-1 CD8+ T cells with gp10025-33 peptide with or without TLR agonists. Cells were expanded for 5 days with rhIL-2 [100I.U./mL] and then stained for various activation markers. Analysis via flow cytometry indicated increased MFI of CD25, the alpha chain of IL-2 receptor, for the samples that were stimulated in the presence of LPS, MPL, PolyI:C or CpG (Fig. 8A B). CD25 was significantly increased in samples stimulated with LPS or CpG (Fig. 8B). Furthermore, the inducible T cell costimulatory (ICOS) was upregulated in cells stimulated with LPS or CpG.
during activation and expansion with hgp1025-33 as indicated by an increase in MFI (Fig. 8A and B). These data reveal that TLR4 and TLR9 agonists potentiate the activation status of tumor-specific CD8+ T cells. How the immunosuppressive cytokine IL-10, induced by these agonists, impacts their activation status remains unknown.

Figure 8: CpG augments the expression of the activation markers CD25 and ICOS on pmel-1 CD8+ T cells. A. Representative histograms of flow cytometry samples comparing relative expression of CD25 and ICOS. TLR agonists LPS, MPL, PolyI:C or CpG increase CD25 expression on pmel-1 CD8+ T cells. LPS and CpG but not MPL or PolyI:C enhances ICOS expression on pmel-1 CD8+ T cells. Extracellular staining was performed 5-6 days post after initial stimulation. B. Average MFI of CD25 and ICOS of samples from four and three separate experiments, respectively.
Transient IL-10 blockade augments the activation of CpG-primed CD8+ T cells. Because CpG induces marked IL-10 secretion by pmel-1 splenocytes in vitro, we explored the effects of IL-10 on T cell activation. We used a purified α-mouse CD210R monoclonal antibody (1b1.3a) to neutralize IL-10 in the culture. It has previously been reported that IL-10 regulates macrophage and DC immunostimulatory responses to CpG. Blocking IL-10 also increases ICOS expression on Th17 cells in a colitis model. Thus, we posit that neutralizing IL-10 while priming cells with CpG would increase CD25 and ICOS. We found that transiently neutralizing IL-10 alone increased CD25 compared to control and the simultaneous addition of CpG further increased CD25 compared to CpG alone (Fig. 9 A&B). However, the addition CpG and depletion of IL-10 did not further upregulate CD25.

Figure 9: Transient neutralization of IL-10 augments the activation status of CD8+ T cells primed with CpG ODN. A. Flow cytometry histogram showing relative expression of CD25 and ICOS for CpG ODN stimulated P-mel-1 splenocytes with or without transient neutralization of IL-10. Samples were stained for cytokines and fixed six days post initial stimulation. 10μg/mL of the monoclonal αIL-10(1b1.3a) antibody was added to designated samples at the start of the culture B. Bar graph demonstrating ICOS and CD25 MFI of the same samples represented in panel A. The CD25 and ICOS MFI of CpG stimulated pmel-1 CD8+ T cells increased when those samples were simultaneously IL-10 neutralized.
on pmel-1 T cells. Contrary to our hypothesis, we did not see an increase in ICOS when IL-10 was neutralized compared to control T cells. However, when IL-10 is blocked in the presence of CpG, ICOS increased on pmel-1 CD8⁺ T cells (Fig. 9A & B). This data suggest that IL-10 regulates ICOS expression via TLR stimulation, however, these data are preliminary and thus need to be repeated increase confidence in the results.

**CpG promotes the generation of central memory pmel-1 CD8⁺ T cells.**

Central memory T cells regress tumor to a greater extent than effector memory T cells when infused into mice.⁹⁹ Thus, we sought to determine how TLR agonists impacted the memory profile of pmel-1 CD8⁺ T cells *ex vivo*. To do this, CD44 and CD62L expression was analyzed via flow cytometry to detect central (CD44⁺CD62L⁺)/effector (CD44⁺CD62L⁻) memory cells on day 5. All agonists increased central memory cells in the culture, as shown by flow, pie and bar graphs (Fig. 10 A-C). Interestingly, CpG robustly promoted the generation of central memory CD8⁺ T cells compared to other agonists. This enhanced CD62L expression is characteristic of a more central memory type T cell as well as their capacity to traffic to the lymph node, which has been shown by Klebanoff, Gattinoni and colleagues to correlate with cells that potently regress tumors.⁹⁹

**IL-10 blockade impairs the generation of central memory pmel-1 CD8⁺ T cells.**

Next, we wanted to determine how neutralizing IL10 impacted the memory phenotype of CpG-activated CD8⁺ T cells, given that CpG induces IL-10 secretion by T cells. Interestingly, we found that transient neutralization of IL-10 decreased the proportion of central memory cells compared to control pmel-1 CD8⁺ T cells, as shown by flow, pie chart and bar graph form (Fig. 11A-C). This diminished expression, along with high CD44 expression indicates an effector CD8⁺ T cell phenotype.⁹⁸ Furthermore, when αIL10R was
(combined with CpG, the pmel-1 CD8+ T cells expressed fewer central memory cells compared to those only primed with CpG. This data suggests that CpG stimulation of CD8+ T cells increases their central memory phenotype (CD44+CD62L+), perhaps via IL-10. **TLR agonists differentially regulate the in vitro cytotoxicity of pmel-1 CD8+ T cells.**

Next, we determined how TLR agonists and/or IL-10 neutralization regulated the cytotoxic capacity of tumor-specific CD8+ T cells. We hypothesized that TLR agonists would augment T cell cytotoxicity via stimulating APCs to express MHC and secrete IL-12. As a feedback mechanism, IL-10 is a common cytokine that is released by multiple
cell types in response to TLR signaling. Thus by removing the immunosuppressive effects of IL-10, we also expected that T cells would be more cytotoxicity upon antigen specific re-stimulation. We expected to observe an increase of TNF-α and Granzyme A/B production by pmel-1 CD8+ T cells primed under these conditions. To address this question, pmel-1 splenocytes were activated with gp10025-33 peptide with or without TLR agonist and then expanded with IL-2 for five days. The cells were then re-stimulated with C57/BL6 splenocytes loaded with gp100 peptide. Cells were stained for various cytotoxic cytokines and then analyzed via flow cytometry. We found that blocking of IL-10 or the addition of the TLR agonists LPS or MPL increased the secretion of granzyme A
production by pmel-1 cells (Fig. 12). LPS, MPL, PolyI:C or CpG increased the percentage of granzyme B positive CD8⁺ pmel-1 T cells. TNFα production was increased by the addition of MPL or the transient neutralization of IL-10 but decreased by addition of LPS, PolyI:C, or CpG. Taken together, these results indicate that priming pmel-1 T cells with TLR agonists differentially regulate the in vitro cytotoxic cytokine production of CD8⁺ pmel-1 splenocytes. Furthermore, transiently neutralizing IL-10 also enhances their effector function.

**Figure 12: TLR agonists differentially impact the in vitro cytotoxicity of pmel-1 CD8⁺ T cells.** Pmel-1 splenocytes were cultured for 5ds in the presence of LPS, MPL, PolyI:C, CpG ODN or αIL10 antibody or a combination of CpG ODN and αIL10R. The Vβ13⁺ CD8⁺ T cells were activated with hgp10025-33 peptide pulsed irradiated C57/BL6 splenocytes. Extracellular and intracellular staining of granzyme A/B, and TNFα was performed 6hrs post re-challenge.

**Pmel-1 CD8⁺ T cells primed with CpG regress melanoma and extend survival in mice:**

After discovering a multitude of seemingly beneficial effects that TLR agonists have on pmel-1 CD8⁺ T cells, we determined that they were useful for stimulating potent anti-tumor responses in an in vivo adoptive cell therapy (ACT) model. We postulated that
T cells primed with LPS or CpG would most effectively control tumor growth and prolong survival in melanoma bearing mice, given that cells primed with these agonists generated highly function central memory T cells. As represented in Fig. 13A, C57/BL6 mice were injected subcutaneously on the abdomen with $3 \times 10^5$ B16F10 cells and allowed to develop tumor for 8 days prior to infusion of T cells primed with various TLR agonists. 6 days prior to infusion, pmel-1 mice were sacrificed and T cell cultures were started using splenocytes gathered from these animals. Pmel-1 T cells were expanded with gp100$_{25-33}$ peptide [1μg/mL], IL-2 [100I.U./mL] and effective doses of the TLR agonists LPS, MPL, PolyI:C or CpG. On the day prior to infusion, mice were given 5Gy TBI and pmel-1 T cells were re-stimulated with irradiated hgp100$_{25-33}$ splenocytes. The following day, cells were washed 3X prior to the infusion of $6 \times 10^5$ pmel-1 T cells into tumor-bearing mice. Additionally, mice received rhIL-2 complex (1.5ug rhIL-2 + 7.5ug αhIL-2) on the day of infusion to aid the expansion of the adoptively transferred T cells. We found that tumors grew rapidly in mice treated with only 5Gy TBI and IL-2 complex and met their endpoint for tumor size within 21 days post T cell infusion (Fig. 13B-D). Mice that received pmel-1 T cells had their tumors grow slightly slower than control and approximately 30% were alive 27 days post T cell infusion. The next best treatment group, although not by a large margin, was the PolyI:C primed T cells followed by LPS primed and MPL primed T cells (Fig. 13B-D). Interestingly, pmel-1 CD8$^+$ T cells primed with CpG were most effective at regressing melanoma and extending survival in mice (Fig. 13B-D). In fact, mice that received the CpG primed T cells had 100% survival 27 days post infusion compared to approximately 30% of the mice that received pmel-1 T cells without the priming. This data
closely models the experiments where the TLR agonists were given directly to the mice receiving pmel-1 T cells (Fig. 5). However, this method of utilizing TLR agonists ex vivo to augment adoptively transferred T cells in vivo is more clinically translatable because the TLR agonists are never directly exposed to the patient.

Figure 13: Pmel-1 CD8+ T cells primed with CpG regress melanoma and extend survival in mice. A. B6 mice bearing s.c. B16F10 tumors (established for 8 days) were preconditioned with 5 Gy TBI. One day after TBI, mice were infused with 6e^5 pmel-1 T cells that were cultured with different types of TLR agonists, i.e. LPS [1μg/mL], MPL [10μg/mL], PolyI:C [20 μg/mL] or CpG ODN [5 μg/mL]. All pmel-1 CD8+ T cells were re-stimulated with irradiated and hgp100_25-33 loaded C57/BL6 splenocytes overnight and washed 3X with PBS prior to tail-vein infusion. Mice also received rhIL-2 complex (1.5ug Il-2 + 7.5ug ahIL-2) on the day of infusion via IP injection. B. Individual tumor curves of mice bearing B16F10 tumors (n=6-10). C. Comparison of average tumor burden of mice receiving 5 GY TBI, and pmel-1 T cells or TLR agonist primed pmel-1 T cells (mean ± SEM of 6-10 mice per group). D. Survival curve representing the proportion of tumor bearing mice alive following pmel-1 T cell infusion.
IL-10 blockade augments the antitumor activity of CD8+ T cells in mice.

Next, we determined how neutralizing IL-10 during T cell expansion impacted the ability of transferred CD8+ T cells to control tumor growth in vivo. We hypothesized that neutralizing immunosuppressive IL-10 during T cell expansion would enhance the in vivo anti-tumor response of adoptively transferred T cells. Furthermore, we suspected that neutralizing IL-10 while simultaneously priming cells with CpG would yield the most potent anti-tumor response. As represented in Fig. 14A, B6 mice were injected subcutaneously on the abdomen with 3e5 B16F10 cells and allowed to develop tumor for 8 days prior to receiving adoptive cell therapy. 6 days prior to infusion, pmel-1 mice were sacrificed and T cell cultures were started using splenocytes gathered from these animals. Pmel-1 T cells were expanded with gp10025-33 peptide [1μg/mL], IL-2 [100I.U./mL], CpG ODN [5μg/mL] and/or αIL-10 monoclonal antibody [10μg/mL]. On the day prior to infusion, mice were given 5Gy TBI and pmel-1 T cells were re-stimulated with irradiated hgp10025-33 splenocytes. The following day, cells were washed 3X prior to the infusion of 6e5 pmel-1 T cells into tumor-bearing mice. Additionally, mice received rhIL-2 complex (1.5μg rhIL-2 + 7.5μg αhIL-2) on the day of infusion to aid the expansion of the adoptively transferred T cells. We found that transiently neutralizing IL-10 during the expansion of tumor-specific CD8+ T cells enhanced their in vivo anti-tumor capacity and prolonged survival in B16F10 tumor bearing mice (Fig. 14B-D). Transiently neutralizing IL-10 during in vitro expansion allowed the T cells to thwart melanoma growth comparable to cells primed with CpG alone. In contrast to our hypothesis, however, the neutralization of IL-10 while concomitantly priming pmel-1 T cells with CpG did not generate a synergistic
anti-tumor effect. This data might suggest that extracellular IL-10 does not hinder the ability of CpG primed CD8⁺ T cells to control tumor growth in vivo.

All pmel-1 T cells were re-stimulated with irradiated and hgp10025-33 loaded C57/BL6 splenocytes overnight and then washed 3X with PBS prior to tail-vein infusion. Mice also received IL-2 complex on the day of infusion via IP injection. B. Average tumor burdens comparing the effects that transiently neutralizing IL-10 has on pmel-1 T cells that are primed with CpG or not (mean ± SEM of 6-10 mice per group). C. Individual tumor curves of mice bearing B16F10 tumors and treated with modified P-mel-1 T cells (n=6-10). D. Mice that received T cells that were expanded in the presence of IL-10 neutralizing antibody survived longer compared to those mice that received pmel-1 T cells without further modification. Survival curve represents the proportion of tumor bearing mice alive following pmel-1 T cell infusion.

Figure 14: Transiently neutralizing IL-10 during expansion of tumor-specific CD8⁺ T cells enhanced their in vivo anti-tumor capacity and prolonged survival in B16F10 tumor bearing mice. A. Mice bearing s.c. B16F10 tumors established for 8 days received 5 Gy TBI. One day after TBI, mice received 6e⁵ pmel-1 T cells that were cultured with CpG [5μg/mL], all10 antibody [10μg/mL] or both CpG and all10.
**CpG promotes the functional plasticity of TRP-1 Th17 cells ex vivo.**

After discovering the benefits of priming CD8+ T cells with CpG, we wanted to uncover the effects that this agonist imparted on IL-17 producing CD4+ T cells, called Th17 cells. We were particularly interested in Th17 cells because these cells have shown promise in various preclinical models of cancer. Because CpG ODN causes an increase in IFN-γ production by CD8+ T cells in an APC dependent manner (Fig. 7C) we hypothesized that CpG ODN would promote the functional plasticity, or the conversion of IL-17 producing cells to those that make IFN-γ, of TRP-1 Th17 cells. Also based on experiments outlined previously, we hypothesized that simultaneously neutralizing IL-10 would further the effects that CpG ODN has on Th17 cell function.

To address these ideas, we polarized TRP-1 CD4+ T cells towards a Th17 lineage (described in materials and methods section) in the presence of CpG and/or αIL-10 monoclonal antibody. We then allowed the cells to expand for 7 days before stimulating with PMA plus Ionomycin and analyzing their ability to secrete IL-17 and IFN-γ via flow cytometry. We found that CpG ODN enhanced the functional plasticity of Th17 cells as indicated by an increase in the percentage of IFN-γ secreting cells and decrease in IL-17 producing cells (Fig. 15A&B). Th17 plasticity was further enhanced when IL-10 was simultaneously blocked with CpG activation (Fig. 15A&B).

To determine the cytokine cues that promote Th17 plasticity via TLR activation and IL-10 blocking, supernatant was collected four days post Th17 polarization and various cytokines were assayed with by multi-analyte analysis. After analyzing the data, several cytokines were markedly elevated in samples that received CpG alone or in conjunction with αIL10. These include IL-3, IFN-γ, MCP-1, Rantes (CCL5) and GM-CSF (not shown).
Furthermore, the IL-12 p40 subunit was markedly elevated in samples that received CpG ODN (Fig. 15C). IL-12(p70) was also increased but to a lesser degree than the IL-12 p40 subunit (not shown).

Given that CpG induces IL-12, we hypothesized that this cytokine was responsible for the enhanced ability of Th17 cells to secrete IFN-γ. To test this idea, we neutralized IL-12 with a monoclonal antibody against IL-12(p40) [10μg/mL]. We found that neutralizing IL-12p40 partially prevented the functional plasticity of Th17 cells as indicated by a decrease in the percentage of IFN-γ producing T cells (Fig. 15D vs. 15A). However, IL-17 was not completely maintained compared to control suggesting that other cytokines induced by CpG enhanced the functional plasticity of Th17 cells.

Simultaneously neutralizing IL-10 while priming Th17 cells with CpG decreases the long-term in vivo anti-tumor activity of adoptively transferred Th17 polarized TRP cells.

After discovering that the addition of CpG to Th17 polarizing conditions enhanced the ex vivo functional plasticity of TRP-1 Th17 cells, we determined how this impacted their ability to eradicate melanoma in vivo. Previous research by Muranski and colleagues shows that tumor-specific Th17 cells are superior to Th1 cells at eradicating large established melanoma in a mouse model. Given that CpG caused Th1 polarization in the presence of Th17 cytokines, we hypothesized that these CpG primed Th17 cells would have diminished capacity to eradicate established melanoma. Thus, we injected C57/BL6 mice bearing subcutaneous B16F10 tumors established for 15 days with 1.5e6 CD4+ TRP-1 cells polarized towards a Th17 lineage in the presence of CpG ODN and/or aIL10 monoclonal antibody (Fig. 16A). As part of the adoptive therapy protocol, all tumor
bearing mice received 5Gy TBI 1 day prior to T cell infusion. Additionally, T cells were re-stimulated O/N with TRP peptide-loaded C57/BL6 splenocytes prior to infusion.

As shown in Fig. 16B&C, all mice in the group that received only 5Gy TBI were euthanized due to growth in tumor size within 14 days post infusion. We also found that mice treated with Th17 polarized TRP-1 T cells profoundly regressed tumors. Mice receiving Th17 cells that were polarized in the presence of CpG or αIL-10 had similar effectiveness and prolonged anti-tumor responses compared to mice receiving Th17 control cells. As predicted, TRP-1 CD4+ T cells polarized in the presence of both CpG and αIL-10 were less effective in regressing tumors. These data indicate that IL-10 is an

![Figure 15: CpG ODN promotes the functional plasticity of TRP-1 Th17 cells ex vivo. A. TRP-1 CD4+ T cells were polarized using cytokines towards a Th17 lineage in the presence of CpG [5μg/mL] and/or αIL10 [10μg/mL]. CpG enhances the production of IFN-γ and simultaneously neutralizing IL-10 further enhanced the percentage of IFN-γ producing cells in the samples. B. CpG decreases the relative expression of IL-17 while increasing the relative expression of IFN-γ. Transiently neutralizing IL-10 while stimulating cells with CpG markedly enhanced production of IFN-γ. Relative expression was achieved from 3 separate experiments using flow cytometry as the assay. The percentage of cells producing IL-17 or IFN-γ for the control Th17 sample was used to standardize the relative expression for each experiment. The relative expressions from the three separate experiments were averaged for all samples and are represented as an average plus standard error. C. Data obtained from supernatant collected 4 days post beginning T cell polarization and addition of CpG and/or αIL10. The supernatant was analyzed via multiplex assay and the data indicates increased amount of IL-12 in samples that were given CpG and those that were given CpG and simultaneously neutralized of IL-10. D. Flow cytometry data showing that neutralizing the p40 subunit of IL-12 prevented IFN-γ production of Th17 polarized cells primed with CpG.](image-url)
important cytokine that maintains Th17 polarization in the presence of CpG ODN and that it might be a potential future treatment to enhance adoptively transferred T cells.

Figure 16. Simultaneously neutralizing IL-10 while priming Th17 cells with CpG decreases the long-term in vivo anti-tumor activity of adoptively transferred Th17 polarized TRP cells. A. C57/BL6 mice bearing s.c. B16F10 tumors established for 15 days were given 1.5e6 Th17 polarized TRP CD4+ T cells cultured with CpG and/or aIL-10 monoclonal antibody. 1 day prior to infusion, mice received 5 Gy TBI and T cells were re-stimulated overnight with irradiated C57/BL6 splenocytes loaded with TRP peptide. B. Average tumor area of mice following T cell infusion indicates that Th17 cells polarized in the presence of both CpG and aIL10 antibody did not maintain long-term in vivo anti-tumor activity (N=5-10 mice/group). C. Graphs showing individual tumor area of mice receiving the ACT regimen.
**Chapter 5- General Discussion**

Lymphodepletion with chemotherapy and/or TBI enhances adoptive immunotherapy via several mechanisms. In addition to the removal of myeloid-derived suppressor cells, cytokine sinks and Treg cells, translocation of gut microflora by TBI enhances the outcome of therapy. This intriguing finding that bacterial infection can promote tumor regression are reminiscent of Coley's findings published long ago.

In patients with advanced metastatic melanoma, a non-myelablative regimen prior to infusion of tumor-infiltrating lymphocytes and bolus IL-2 resulted in an objective response rate of 50% in the absence of vaccination. Increasing this regimen to an intense myeloablative chemoradiation preparative regimen further augmented adoptive immunotherapy, resulting in an objective response rate of 72% and lead to more durable responses. While the tolerated doses of lymphodepletion are well established, these systemic approaches are not devoid of toxicities. In mice, we report here that escalating the intensity of lymphodepletion with TBI correlated with greater innate immune activation (Fig. 1A). Heightened innate activation was associated with greater impairment of the gastrointestinal tract, as evidenced by profound destruction of the colon and greater microbial LPS translocation (Fig. 1B-D). We wished to find an effective and safe way to activate the innate immune system without compromising the GI tract with intense 9 Gy TBI. Thus, we administered ultrapure LPS to non-irradiated mice receiving adoptive cell transfer (ACT). We found that LPS could not augment ACT-mediated tumor regression in non-irradiated mice (Fig. 2). However, we found that LPS could improve ACT treatment in mice given a non-myeloablative regimen of irradiation (Fig. 3).
We found that LPS improved ACT treatment by increasing CD25 expression, allowing for transferred cells to better compete for IL-2, and by enhancing initial proliferation post transfer, aiding in engraftment (Fig. 4). However, due to its inherent toxicity, we sought to use other clinically relevant TLR agonists to mimic the beneficial effects seen with LPS. We found that MPL or CpG ODN could also enhance outcomes in mice given nonmyeloablative ACT (Fig. 5). To further explore the potential of using TLR agonists to improve ACT, we hypothesized that we could obtain results similar to that of directly administering LPS to mice by instead priming CD8$^+$ T cells with LPS in vitro during their rapid expansion. By washing LPS from the culture prior to infusion, we thought we could prevent the potential toxicity of this compound that might otherwise prevent the clinical translation of utilizing TLRs to enhance ACT. We found that in vitro priming of CD8$^+$ T cells with LPS enhanced in vivo anti-tumor activity of the cells, increased expression of CD25 and increased their engraftment (Fig. 6).

With this preliminary data we set out to explore some of the potential mechanisms for how TLR agonists augment the anti-tumor activity of CD8$^+$ T cells. We found that LPS, MPL, PolyI:C or CpG could enhance their function, as indicated by their increased ability to secrete IFN-$\gamma$ (Fig. 7A) and that the APC compartment was important for this enhanced function (Fig. 7C). However, along with an increase in IFN-$\gamma$ we found that LPS or CpG also induced an increase in the production of the immunoinhibitory cytokine, IL-10 (Fig. 7). Yet, how IL-10 regulates CD8$^+$ T cell responses to TLR signaling remains incompletely elucidated and is a major focus of this study.
Next, we wanted to determine if other TLR agonists besides LPS could enhance the activation of CD8\(^+\) T cells. We found that CpG significantly increased the expression of CD25 and ICOS on pmel-1 CD8\(^+\) T cells (Fig. 8). We found that neutralizing IL-10 while priming with CpG ODN further enhanced the expression of the activation markers CD25 and ICOS (Fig. 9). Additionally, we wanted to determine how TLR agonists and IL-10 impacted the memory phenotype of CD8\(^+\) T cells. Overall, priming pmel-1 T cells with TLR agonists increased the central memory phenotype of the cells (Fig, 10). Consistent with prior research that suggests IL-10 is responsible for creating optimal T cell memory, we found that neutralizing IL-10 caused cells to obtain a more effector memory-like phenotype (Fig. 10). Furthermore, when IL-10 was transiently neutralized while priming with CpG ODN, the cells were less central memory-like. These data suggest that IL-10 may regulate the maturation of TLR agonist primed CD8\(^+\) T cells from fully activated effector cells into central memory T cells. We also found that priming CD8\(^+\) T cells with TLR agonists augmented their in vitro cytotoxicity and that transiently neutralizing IL-10 also enhanced their function (Fig. 12).

Subsequently, we wanted to test if our TLR-primed T cells in an in vivo model of melanoma to determine if our clinically relevant strategy was successful in regressing tumors. We found that priming CD8\(^+\) T cells with LPS, MPL or CpG ODN during primary expansion hindered tumor growth and enhanced survival when transferred into mice bearing melanoma (Fig. 13). We also found that neutralizing IL-10 during expansion of CD8\(^+\) T cells improved their survival and delayed tumor growth similar to priming cells with CpG (Fig, 14). Furthermore, we found that combining CpG ODN and blocking IL-10
did not enhance the anti-tumor response beyond control. Even though we were able to neutralize external sources of IL-10 with our monoclonal antibody, our methods did not account for the possibility of IL-10 autocrine regulation of CD8+ T cells. In one experiment, we found that the production of IL-10 by pmel-1 CD8+ T cells increased when extracellular IL-10 was neutralized (not shown). Future experiments should account for the potential of IL-10 to be produced by CD8+ T cells and such techniques will be discussed in the future directions section of this thesis.

Following our observation that CpG could be used to improve ACT therapy, we wanted to explore some of its immunomodulatory capabilities on different T cell subsets. We decided to explore Th17 cells due to their ability to mediate a superior melanoma regression in a mouse ACT model. A hallmark of Th17 cells is their plasticity, or the ability to convert from mainly IL-17A producers to IFN-γ producers. This Th17 plasticity has been associated with higher in vivo survival and self-renewal capacity compared to Th1 cells. Using an IL-12 blocking antibody, we found that CpG induced Th17 cell plasticity and their conversion from IL-17 to IFN-γ was driven in part by IL-12 (Fig. 15A-D). We also found that IL-10 was important for preventing full Th1 polarization of CD4+ T cells in the presence of CpG and Th17 polarizing cytokines (Fig. 15A-D). In addition to the production of IL-12, CpG induced the production of other inflammatory cytokines including RANTES, CXCL-1, GM-CSF which could foster trafficking of other immune cells to sites of inflammation (not shown). We found that Th17 cells polarized in the presence of CpG while neutralized of IL-10 did not eradicate B16F10 melanoma as well as control Th17 cells (Fig, 17B & C). However, Th17 cells primed with CpG or neutralized
of IL-10 performed as well as control Th17 cells (Fig. 17B & C). Taken together, our data suggest that IL-10 is important for maintaining the prolonged production of IL-17 for Th17 cells exposed to the TLR agonist CpG.

In conclusion, we found that clinically relevant TLR agonists such as CpG can be used to augment cell based anti-tumor therapies. Utilizing TLR agonists in a variety of ways, either through direct administration to patients or via ex vivo priming of extracted T cells may allow for possibility of avoiding myeloablative preparative regimens in ACT protocols. When employing TLR agonists to augment cell based therapies for melanoma, it is important to consider by-products of this microbial challenge. For this research, we focused on the role of IL-10 in response to TLR agonist stimulation of T cells. We found that IL-10 was important for maintaining central memory characteristics of CD8+ T cells and for preserving the anti-tumor activity of CpG primed Th17 cells. While these findings are interesting, further research needs to be conducted to understand the role of IL-10 in T cell based therapies for cancer.
Chapter 6- Future Directions and Conclusion

In this study, we expand the potential of using TLR agonists for oncogenic malignancies by exploring their impact on tumor specific CD8^+ T cells. We show that the clinically relevant TLR agonists MPL or CpG could potentially replace the need for myeloablative TBI in ACT regimens. While this study focused on administering various TLR agonists independently, it would be interesting to investigate the effects that combinations of different TLR agonists have. For instance it might prove efficacious to trigger both extracellular and intracellular TLRs simultaneously using both MPL and CpG, respectively. Or, using a combination of two intracellular derived TLR agonists such as PolyI:C and CpG could potentiate a robust anti-tumor response of adoptively transferred T cells. Furthermore, it would be interesting, although less clinically relevant and variable-laden, if we exposed CD8^+ T cells to various bacteria or viral lysates. This might better replicate *in vivo* microbial translocation caused by damage to the GI tract as opposed to using a purified TLR agonists. Perhaps microbial by-products other than the classic TLR agonists can induce stronger T cell responses. The possibilities and variability of combining the microbial landscape to augment the immune system are immense. However, the resulting immunoinhibitory consequences such as IL-10 production must be considered.

Our data indicates that IL-10 plays an important role in maintaining a memory like phenotype of CD8^+ T cells. It would be interesting to explore how addition of exogenous IL-10 during various stages of CD8^+ T cell expansion impact the anti-tumor activity of the T cells. Additionally, it may be worthwhile to explore the effects of IL-10 production by
CD8+ T cells in response to combined TCR and TLR stimulation. Our method of neutralizing IL-10 was limited to extracellular sources so it would be intriguing to use TLR agonists in pmel-1 IL-10 knockout mice. In addition to focusing on IL-10, checkpoint blockade therapies might also increase the effectiveness of ACT regimens using TLR primed T cells. With the recent approval of αPD-1 receptor antibodies to treat advanced melanoma and non-small cell lung cancer, the future potential of immunotherapies for cancer treatment is robust.

Furthermore, although we found that the CD2L expression was altered by TLR agonist priming and neutralization of IL-10, our methods were limited to short-term in vitro analysis. T cell memory generation was not tracked in a kinetic fashion which is a major limitation of this present study. In future studies, it would be interesting to track CD2L expression for an extended duration of time. Not only would we initially measure the expression of CD44 and CD2L shortly after T cell activation, we would extract transfer red T cells from mice many weeks post ACT. It would be interesting to perform a biodistribution on the spleen and lymphnodes of the mice to determine if memory T cells were generated. Based on the present data in this study, we would expect for T cells primed with CpG to have a higher percentage of donor T cells in the lymphnodes that co-express CD44 and CD2L. We would also predict that T cells neutralized of IL-10 would have less memory T cells generated. This would be indicated by a decreased number of donor T cells present in the mouse weeks after ACT.

In conclusion, it is important to reiterate that advanced metastatic melanoma is a devastating disease that requires a multilateral treatment approach. When the traditional
treatments such as surgery or chemotherapies have been exhausted, more sophisticated methods of immunotherapy are necessary. The goal is to induce curative responses in patients and adoptive cell therapy has proven efficacious for those left with limited options. By studying the mechanisms underlying TBI’s role in enhancing treatment outcomes in patients receiving ACT regimens, we arrive at a junction where the adoptive and innate immune systems meet. We utilized products from intestinal disruption and were able to show how some TLR agonists could augment T cell based anti-cancer immunity.

Chapter 7-Materials and Additional Methods

Mice and tumor lines. All mice were bred and housed at MUSC facilities. Female pmel-1 TCR Tg mice were crossed with C57BL/6-Thy1.1 Tg mice to derive pmel-1 Thy1.1 double Tg mice (C57BL/6-pmel-1-Thy1.1 mice; Jackson). Male TRP-1 mice used for Th17 ACT experiments were purchased from Jackson. C57BL/6 (Taconic) were used as recipients in ACT experiments. Experiments were conducted with the approval of the MUSC Animal Use and Care Committee. B16-F10 (H-2b), a spontaneous, transplantable gp100+ murine melanoma, was maintained in culture media.

In vitro activation of pmel-1 T cells. Pmel-1 mice are sacrificed via CO₂ asphyxiation and cervical dislocation and spleens were extracted using sterile technique and placed in 2.00 mM EDTA. Spleens were mechanically processed through 70μm filters using the plunger from a plastic syringe. Splenocytes were brought to a concentration 1-2e⁶/mL in T cell media and 1μg/mL hgp10025-33 peptide is added. Recombinant hIL-2 is added daily at 100IU/mL (rhIL-2; Chiron Corp). Assays were completed as indicated anywhere from 4-8 days post activation with hgp10025-33 peptide.
Adoptive cell transfer, vaccination, cytokine administration and TLR agonists. Six-
ten week old mice were injected s.c. with 2-5 x 105 B16-F10 melanoma cells and treated
10 d later with i.v. adoptive transfer of 1-3e6 pmel-1 CD8+ T cells in vitro activated-
splenocytes(to serve as a vaccination). 5 Gy TBI was given to mice on the day of ACT. In
some experiments conducted with our NIH collaborator, mice were vaccinated with 2x10^7
PFU of recombinant fowlpox virus expressing human gp100 (Therion Biologics). 3.6-36
μg/dose of rhIL-2 was administered by i.p. injection twice daily for a total of five doses in
some experiments. Ultra-pure LPS (Invivogen;0.1-50 μg s, i.v.), MPL (5 μg s, i.v. on day
1), CpG ODN 1668 (10 μg s, i.v. daily for 4 consecutive days 5'TCCATGACGTTCCTGATGCT
-3'), Poly I:C (50 μg s, i.v), imiquimod (5% topical cream, everyday for three weeks), flagellin (200 μg s, i.v.), zymosan (250 μg s, i.v.) were
administered 1 day post treatment with ACT. Tumors were measured with calipers and the
perpendicular diameters were recorded. Experiments were performed in a blind,
randomized fashion.

Detection of serum LPS. For experiments in Figure 1, A LAL assay (QCL-1000;
Cambrex) was used to analyze serum LPS on days 1-8.

Enumeration of adoptively transferred cells and host CD11c+CD86+ DCs, and ex
vivo cytokine release assay. At the indicated times, adoptively transferred pmel-1 thy1.1
cells were enumerated. Transferred pmel-1 Thy 1.1 was calculated by multiplying the
percent of Thy1.1/CD8+ T cells in the spleen by the absolute spleen count. Enumeration
of host CD86highCD11c+ DCs was similarly performed. Six days after ACT, pmel-1
thy1.1 cells were used for cytokine release assay via ELISA. Pmel-1 thy1.1 cells were
isolated from splenocytes and were co-cultured at a 1:2 ratio with irradiated splenocytes pulsed with titrated doses of hgp100\textsubscript{25-33} peptide or unpulsed as negative controls.

**Functional and phenotypic assays.** At the days indicated, Pmel-1 T cells cultured with TLR agonists and/or blocked of IL-10 will be assayed by flow cytometry, multi-analyte (Bio-Rad Pro\textsuperscript{TM} Mouse Cytokine 23-Plex assay) and ELISA to determine their capacity to secrete IFN-\(\gamma\), IL-2, TNF-\(\alpha\), IL-10, granzyme A and granzymeB on days 1-7. The activation and memory phenotype of the T cells will also be determined by their expression of CD62L, CD44, CD25, and ICOS.

**Mucosal barrier score.** Colons were removed from mice and placed in 10\% formalin for 48 h, and then embedded in methylacrylate. 4-5-mm sections were taken along papillary-optical axis. Sections were evaluated by a pathologist unaware of the identity of the groups using the scores as follows: normal architecture = 0, some signs of edema = 1, mild cell infiltration and reduction of crypts and goblets = 2, and severe cell infiltration and profound reduction of crypts and goblets = 3, severe cell infiltration and visually undetectable crypt and goblets = 4.

**P-mel Model.** P-mel mice are a transgenic strain of mice that carry a rearranged T cell receptor transgene specific for human premalanosome protein gp100 (hgp100). They also carry the T lymphocyte specific Thy1.1 allele. Greater than 95\% of the CD8\(^+\) T cells express the transgenic TCR based on expression of VB13. These transgenic CD8\(^+\) T cells account for approximately 20\% of the total lymphocyte population\textsuperscript{103,104} Tumor-specific CD8\(^+\) T cells can be expanded by adding hgp100\textsubscript{25-33} peptide single-cell P-mel-1 splenocytes plus IL-2.
**TRP-1 Model.** TRP-1 are a transgenic strain of mice that carry a rearranged T cell receptor transgene specific for the Tyrp-1 protein.\(^{100}\) CD4\(^+\) tumor specific T cells can be expanded from TRP-1 male splenocytes using C57/BL6 splenocytes plus TRP-1 peptide. The rearranged TCR contains V\(\beta\)14 chains which is used as a marker for the tumor-specific CD4\(^+\) T cells.

**Th17 cell culture/ polarization.** Male TRP-1 mice were sacrificed via CO\(_2\) asphyxiation and cervical dislocation. Spleens were extracted under sterile conditions and TRP-1 splenocytes were brought to single-cell suspension using manual dissociation and 70\(\mu\)m filter. TRP-1 splenocytes are then plated in an appropriate culture in CM while C57/BL6 splenocytes are brought to a single cell suspension and loaded with TRP-1 peptide at a concentration of 1\(\mu\)g/mL. C57/BL6 splenocytes + TRP-1 peptide mixture are vortexed intermittently for 30 minutes at 37\(^\circ\)C. After at least 30 minutes the peptide loaded C57/BL6 splenocytes are washed at least 2X and then combined with TRP-1 splenocytes. Th17 polarizing media is then added to the cells so that the final concentrations are \(2\times10^6\)/mL TRP-1 splenocytes, \(4\times10^5\)/mL TRP-1 peptide-loaded C57/BL6 splenocytes (5:1 ratio). Th17 media contains the following final concentrations of cytokines 30ng/mL hTGF-\(\beta\), 100ng/mL hIL-6, 10ng/mL hIL-1\(\beta\), 100ng/mL hIL-21, 10\(\mu\)g/mL \(\alpha\)-mouse IFN-\(\gamma\), 10\(\mu\)g/mL \(\alpha\)-mouse IL-4 and 5\(\mu\)g/mL \(\alpha\)mouse IL-2.

**Negative Selection.** In some experiments, CD8\(^+\) T cells were purified using invitrogen™ Dynabeads\textsuperscript{\textregistered} Untouched\textsuperscript{TM} Mouse CD8 Cells kit. The purity of the selection was measured using flow cytometry and found to be greater than 87\%.
Flow Cytometry Analysis and Cytokine Analysis. Flow cytometry data was analyzed using FlowJo V10 software. Graphs and statistical analysis were performed using Graph Prism 6. ANOVA statistical analysis was performed as indicated and represented as mean +/- standard error. P-values < 0.05 were considered significant.
Chapter 8 - References


78. Chang, H. D., et al. (2007). "Expression of IL-10 in Th memory lymphocytes is conditional on IL-12 or IL-4, unless the IL-10 gene is imprinted by GATA-3." Eur J Immunol 37(3): 807-817.