AUGMENTING THE EFFECTOR PHASE OF ADOPTIVE T CELL IMMUNOTHERAPY OF CANCER

By

Kevin M. Friedman

A DISSERTATION

Presented to the Department of Medical and Molecular Genetics
and the Oregon Health & Science University School of Medicine
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

December 2008

School of Medicine
Oregon Health & Science University

CERTIFICATE OF APPROVAL

This is certify that the Ph.D. dissertation of

Kevin M Friedman

has been approved

_____

Mentor
Dr Bernard Fox

_____

Member
Dr Grover Bagby

_____

Member
Dr David Parker

_____

Member
Dr Rosalie Sears

_____

Member
Dr Andrew Weinberg

TABLE OF CONTENTS

LIST OF FIGURES

ABBREVIATIONS USED

AIDS: Acquired immunodeficiency syndrome

BMDC: Bone marrow-derived dendritic cell

CD: Cluster of differentiation

CFA: Complete Freund's Adjuvent

CFSE: Carboxyflourescein succinimidyl ester

CIITA: MHC class II transactivator

eIF: eukaryote initiation factor

ELISA: Enzyme-linked immunosorbent assay

ERK: Extracellular signal-related kinase

FBS: Fetal bovine serum

GM-CSF: granulocyte-macrophage colony-stimulating factor

Gy: Gray

HIV: Human immunodeficiency virus

i.v.: intravenous

IKK: Iκb kinase

IFN: Interferon

IL: Interleukin

IRF: interferon regulatory factor

IU: International unit

ISG: interferon-stimulated gene

IVS: in vitro sensitization

JAK: Janus kinase

KO: Knock-out

LAK: Lymphokine-activated killer cells

LCMV: lymphocytic choriomeningitis virus

MART-1: Melanoma antigen recognized by T cells 1

MCA: 3-methylcholanthrene

MHC: Major histocompatibility complex

NK cells: Natural killer cells

NKT: Natural killer T cells

PBL: Peripheral blood lymphocytes

PKR: Protein kinase R

Poly(I:C): Polyinosinic polycytidylic acid

RAG: Recombinase activating gene

RCC: Renal cell cancer

RIG-I: Retinoic acid-inducible gene

s.c.: subcutaneous

siRNA: short inhibitory RNA

SPF: Specific pathogen free

STAT: Signal transducers and activator of transcription

TAK: TGF- β -activated kinase

TGF-β: Transforming growth factor β

TE: Effector T cells

TIL: Tumor-infiltrating lymphocytes

TLR: Toll-like receptor

TVDLN: Tumor-vaccine draining lymph nodes

TRAF: TNF receptor-associated factor

UBP: Ubiquitin-specific protease

UCRP: Ubiquitin cross-reactive protein

WT: Wild type

ACKNOWLEDGEMENTS

None of this would be possible without the many wonderful people who contributed to this body of work and to my graduate career in general. I could take many pages thanking everyone who helped me reach my degree, but for brevity's sake will highlight the more influential. Thank you to Dr Stefan Lanker who I learned an incredible amount of molecular and cellular biology from. Stefan was responsible for imparting the foundation of careful scientific thinking by demonstrating how to examine a scientific question in its detail instead of performing, as he put it, "phenomenological science." Stefan always made sure that I not only knew what an assay did, he ensured that I knew how the assay worked. His training to focus on the details was invaluable. Similarly, thank you to Dr Rosalie Sears who was instrumental during my graduate career as she was one of the two professors who remained on my thesis advisory committee for the entire trip. Rosie shares a passion for cell signaling and cancer cell biology and was amazingly helpful at sharing her knowledge with me. In many instances, she treated me as a graduate student; even spending an entire day showing me how to produce and purify adenovirus particles. Her constant support was invaluable and incredibly appreciated. Dr Grover Bagby was the other professor who remained on my committee the entire time. I can not thank Grover enough for his constant support throughout my graduate training. When ever I needed help with my project or advice on my scientific career in general, Grover always made time by often spending several hours in discussion with me. His unwavering support was invaluable for helping me get through the more significant hurdles presented to me by graduate school.

Thank you to all the amazing people at the Earle A Chiles Research Institute that made working on the dissertation truly a pleasure. I would like to thank Drs Carl Ruby, Will Redmond, Hong-Ming Hu, Michael Gough and Nick Morris who were always open to scientific discussion and readily shared reagents. Thank you to Dr Walter Urba who spent many hours editing my dissertation and who from I learned an incredible amount about the art of writing scientific works. In particular, I want to thank current and former members of the Fox lab including Marlene Riskey, Lisa Said, Chris Twitty, Michael LaCelle, Dr Ulf Petrausch, Dr Christian Poehlein, and Dr Shawn Jensen. Christian provided unwavering support during my tenure here at the institute and was responsible for initially teaching me how to perform tissue culture. Shawn was absolutely an amazing basic scientist who was always willing to have scientific discussions and provided the backbone for the basic science research done in Dr Bernard Fox's Lab. Finally, I want to give my most heartfelt thanks to Dr Fox for picking up a used graduate student and being an incredibly supportive and amazing mentor for the last half of my graduate school. Bernie was amazing at giving me the freedom to pursue my own ideas and truly wanted the best for me and my career. He did everything in his power to support both. Best exemplifying his supportive nature, Bernie gave a personal tour of the Capital monuments at 3:00 AM just because I have never been there and he didn't want to miss the opportunity. I will never forget the opportunities he created for me and I am deeply indebted to him for all his support.

I want to specifically thank Drs Andrew Weinberg, Rosalie Sears, Grover Bagby, and Bernie Fox for their role and support on my thesis advisory committee.

Lastly, I want to thank all my friends and family who have put up with me for over the last seven years of graduate school. I am fortunate to have such a supportive group of people providing the support necessary to complete graduate school. On many levels, this education tested the limits of what I was able to accomplish and I would not have finished without their love and support. In particular, I want to thank my brother Lorin, my step-father Steve, my dad Mark, and my mom Cinda. I have the greatest family in the world. Thank you to each one of you. Finishing graduate school has as much to do with your love and support as this dissertation.

ABSTRACT

Recent results demonstrated that adoptive T cell immunotherapy of cancer is a potent treatment for patients with advanced melanoma. 52 percent of patients who failed standard treatments achieved objective clinical responses after receipt of adoptively transferred tumor-specific T cells. The marked improvement compared to previous adoptive transfer trials was due to nonmyeloablative lymphopenic conditioning prior to the adoptive transfer. Further studies are necessary to understand the nature of T cell populations which mediate the most effective anti-tumor response in a lymphopenic host. Moreover, novel strategies which enhance the anti-tumor function of the adoptively transferred T cells are also needed. This body of work presents data which address ways to enhance the anti-tumor efficacy of adoptively transferred T cells. We found that the most potent long-term anti-tumor response exerted specifically by the adoptively transferred T cells in a lymphopenic conditioned host required both tumor-specific $CD4^+$ and $CD8^+$ T cells. To elucidate strategies which enhance the T cell's anti-tumor function, we next determined whether a molecular mechanism of enhanced anti-viral potential could in-turn enhance T cell-mediated anti-tumor response. Based on enhanced anti-viral potential previously observed in mice deficient in ubiquitin-specific protease 43 (Ubp43), we found that adoptively transferred tumor-specific $Ubp43^{-/-}$ T cells are significantly more therapeutic then wild type T cells. Taken together these data inform future adoptive T cell transfer clinical trials the necessity of ensuring that tumor-specific $CD4^+$ T cells are contained within the population of transferred tumor-specific $CD8^+$ cells. Further, these data elucidate a strategy to enhance their anti-tumor effect based on the elimination of Ubp43 expression.

CHAPTER ONE: INTRODUCTION

T cell adoptive immunotherapy can be an effective treatment alternative for patients with advanced metastatic melanoma. For example, 50 to 70 percent objective clinical response rates have been reported in patients who were refractory to standard cancer treatment (1). However, even with its success, adoptive immunotherapy is a labor-intensive, patient-specific treatment which restricts its use to only a few institutions world-wide. Better appreciation of strategies that increase the frequency and the anti-tumor function of tumor-specific T cells will facilitate the practicality and more prevalent use of adoptive immunotherapy. This body of work presents strategies that augment the efficacy of adoptive T cell immunotherapy of cancer.

One strategy used to enhance the anti-tumor response is lymphopenic conditioning prior to the adoptive transfer of T cells. The achievement of a 50 to 70 percent response rate is largely attributed to lymphopenic conditioning prior to adoptive transfer. It has been observed that adoptive T cell transfer into a lymphopenic environment enhances the anti-tumor response in part due to the following: the removal of suppressive regulatory T cells (2-4), increased reactivity to self-antigens (5), reduced intraclonal competition for the same peptide-MHC complex (6), and easier access to cytokines like IL-7 and IL-15 (7). A better understanding of the nature of an effective anti-tumor response when adoptively transferring T cells into a lymphopenic environment may elucidate ways to enhance the anti-tumor response. One area requiring further exploration is the contribution of tumor-specific $CD4^+$ T cells to an effective anti-tumor response in a lymphopenic host. Although we know that tumor-specific $CD8^+$ T cells are

required for tumor regression, the nature and necessity of tumor-specific $CD4^+$ T cells in a lymphopenic host has not been addressed. Chapter two describes experiments that explored the nature of $CD4^+$ T cell help in a mouse model that mimics both lymphopenic conditioning and, similar to many cancer patients, the inability to generate de novo T cells. We demonstrate the significance of including tumor-specific $CD4^+$ T cells in the population of adoptively transferred T cells used to treat tumor-bearing mice most effectively.

After we established that tumor-specific $CD4^+$ T cells were important constituents of the adoptively transferred population which mediated the most anti-tumor effects, we examined other ways to enhance their therapeutic potential. Our strategy was to take a lesson from previously described mechanisms which enhanced anti-viral immunity, and determine if they also enhanced anti-tumor immunity. In chapter three, we present experiments that demonstrated that the deficiency of Ubiquitin-specific protease 43 (Ubp43), previously shown to enhance anti-viral immunity, also augmented the efficacy of adoptively transferred T cells. This strategy has the potential to be adapted by clinical trials designed to use patient's T cells as a treatment for advanced cancer. This initial chapter discusses the background of the cancer-immune system relationship, preclinical and clinical adoptive T cell immunotherapy, and the enhanced anti-viral effects observed in $Ubp43^{-/-}$ mice.

Cancer Immunosurveillance

Paul Ehrlich was the first to propose that the incidence of cancer would be higher if we did not have an immune system (8). This 1909 insight spearheaded the hypothesis

of “cancer immunological surveillance,” later coined by Burnet and Thomas to describe the capability of the immune system to detect and destroy nascent malignant cells (9, 10). Well accepted now, the hypothesis of cancer immunosurveillance was not without its growing pains. When initially put to the test thirty-five years ago using the only congenitally immunodeficient mice available at the time, the CBA/H strain of nude mice, investigators found that these mice did not develop more spontaneous or carcinogen-induced tumors, nor was the tumor latency different compared to wild type mice (11). Considered a terminal blow, the cancer immunosurveillance hypothesis did not reemerge for two decades when studies on interferon-gamma (IFN-γ) and tumor formation incited renewed interest. IFN-γ is exclusively expressed by cells of the immune system, particularly T and natural killer (NK) cells (12). So, when it was demonstrated that IFN-γ could protect the host against the formation of primary tumors, induced or spontaneous, and inhibited the growth of transplanted tumors (13-16), the revival of the immunosurveillance hypothesis had begun. The cancer immunosurveillance hypothesis was further supported by the observation that mice lacking perforin, a critical component for T and NK cell cytotoxicity (17), were more susceptible to induced and spontaneous tumor formation than their wild type counterparts (15, 16, 18-20).

Finally, the definitive demonstration of the existence of cancer immunosurveillance came from studies in which mice lacking recombinase activating gene (RAG)-2 were shown to develop sarcomas more rapidly and with greater frequency than genetically matched wild type controls (21). The results with immunodeficient mice were in direct contrast with the earlier experiments in nude mice that derailed the cancer immunosurveillance hypothesis for two decades. The difference can be explained by

caveats to the experiments with nude mice that were not appreciated at the time. Namely, that while the RAG-$2^{-/-}$ mouse was truly deficient in T, B, and NKT cells, the nude mouse was an imperfect model of immunodeficiency because they actually contained a small number of $\alpha\beta$ T cells and normal numbers of NK cells, which could manifest innate immunity and some degree of adaptive immunity (22-24). Because the nude mice used in early studies were not completely devoid of immune cells they may have had some capacity to exert immunosurveillance on their tumors.

Evidence of immunosurveillance in humans is much more circumstantial. An increased incidence of cancer in immunosuppressed transplant recipients (25-30) has been reported suggesting that de novo or occult tumors can form when the pressure of a functional immune system is removed. Tumors examined in these studies had no apparent viral origins so the counterargument of augmented viral susceptibility facilitating nascent tumor formation could not be made. Immunosuppressed patients infected with the human immunodeficiency virus (HIV) also have a higher incidence of non-AIDS-defining cancers with a variety of histological origins (31). A second line of evidence is that the adaptive immune system can spontaneously form a response to tumor-associated antigens. Following the identification of tumor-associated antigens, such as NY-ESO-1, tumor-specific T cells, which can appear in the patient's circulation (32, 33) spontaneously without vaccination or other similar immune system manipulation were detected. A third line of evidence in support of immunosurveillance in humans is the better prognosis for patients whose tumors contain tumor-infiltrating lymphocytes (TIL). This phenomenon has been observed in ovarian, colorectal (34), and melanoma patients (35, 36). Finally, the correlation of graft-versus-host disease severity with

leukemia remission after allogeneic hematopoietic stem cell transplantation highlights the immune system's capability to control tumor growth (37-45). Importantly, this graft versus leukemia effect appeared to be mediated by donor T cells derived from the stem cell products (46-49). The potency of the allogeneic immune response mediated by the transplanted stem cells causes graft versus host disease yet is better capable to eliminate the leukemia.

Taken together, a large amount of circumstantial data has accumulated indicating that cancer patients indeed develop an immune response to their tumors. Compounded with the direct evidence observed in mouse models these findings strongly support a role for the immune system in sculpting the progression of neoplastic disease. Although the immune response may not always be able to prevent cancer development, it may nevertheless function to restrict tumor growth. Clarifying the relationship between the immune system and cancer may facilitate the development of strategies to exploit this relationship to cause tumor regression.

Adoptive Immunotherapy

Adoptive cellular therapy of cancer has long been recognized as a promising strategy to treat metastatic disease. It was initially described by Southam and colleagues who demonstrated that growth of human tumor autografts in patients bearing advanced cancers was inhibited by the transfer of autologous lymphocytes in about half of the patients (50). This early finding suggested that inherent in cancer patient's lymphocytes was the ability to inhibit the implantation and growth of tumor cells. There was,

therefore, a real possibility that these anti-cancer properties could be harnessed and directed against human tumors.

*Lymphokine-activated killer (LAK) cell*s

The foundation of current cancer adoptive immunotherapy procedures was pioneered by the Surgery Branch at the National Cancer Institute over two decades ago. The development of recombinant human interleukin-2 (rIL-2) (51) and the observation that it facilitated the development of LAK cells in culture led to attempts to treat patients with advanced metastatic melanoma. Murine studies demonstrated that LAK cells, generated from IL-2-treated splenocytes, were lytic to a variety of tumors in vitro and reduced the number of experimental pulmonary metastases in adoptive transfer studies (52). Based on these results, the investigators wanted to determine if this effect could be translated into patients. They found that administration of LAK cells with high-dose IL-2 did not show added benefit in comparison to rIL-2 alone in patients with advanced melanoma and renal cell cancer (53). Despite the ineffectiveness of LAK cells, these trials laid the foundation for the design and execution of large-scale human adoptive transfer clinical trials (54).

Tumor-infiltrating lymphocytes (TIL)

The next generation of adoptive transfer studies emerged from the observation that tumor-specific lymphocytes could be obtained from TIL cultures treated with rIL-2. First made from immunogenic mouse tumors (55) and subsequently from resected human tumors of a variety of histologies (56-58), these observations informed many future clinical trials using tumor-specific T cells. The success of adoptively transferred autologous TIL populations co-administered with high-dose rIL-2 was initially

highlighted by 60 percent objective clinical response in a trial treating 15 patients with metastatic melanoma (59). The study expanded to include a final cohort of 86 patients of which 34 percent exhibited objective clinical responses (60). These data demonstrated that tumor-specific T cells from TIL cultures were more effective then rIL-2 alone or with LAK cells and provided the foundation for adoptive T cell immunotherapy as a treatment for patients with advanced melanoma.

Tumor vaccine-draining lymph node (TVDLN) cells

Efforts continued in order to optimize the potential of adoptive T cell immunotherapy. Based on evidence from murine studies demonstrating that therapeutic T cells can be generated from TVDLN following in vitro sensitization (IVS) with the tumor cells and culture in IL-2 (61-63), a clinical trial was initiated using a similar protocol. In this case, patients were vaccinated adjacent to lymph nodes (LN) with irradiated autologous tumor, TVDLN were resected, subjected to IVS, expanded in IL-2, and adoptively transferred back to the patients along with IL-2 administration. One patient exhibited a partial response, one a mixed response, and one patient had stable disease (64). More importantly, patients who received a vaccination and IL-2 did not respond, suggesting that anti-tumor immunity could be passively transferred by the in vitro treated TVDLN cells. However, serious limitations to this strategy were the requirement for surgery to obtain tumor cells and lymph nodes and the large number of autologous tumor cells that were required for the IVS step.

To overcome this hurdle, anti-CD3 stimulation was used in lieu of tumor cells for IVS after mouse models demonstrated that similar results, including tumor regression, could be obtained (65-68). Anti-CD3 activated TVDLN were used for clinical trials

treating patients with renal cell cancer (RCC) (69, 70) and head and neck cancer (71) with a 27 percent overall response rate observed in the RCC trials. The success of these trials laid the foundation for subsequent studies using autologous tumor vaccination and therapeutic TVDLN for treatment of advanced cancer.

The identification of melanoma-rejection antigens, gp100 and MART-1, brought a new tool to select and track tumor-specific $CD8^+$ T cells (72-74). Armed with this new information, phase I clinical trials were initiated to treat advanced melanoma patients. Peripheral blood lymphocytes (PBL) or TIL were cloned by selecting $CD8^+$ T cells reactive to gp100 or MART-1 and then adoptively transferred along with rIL-2. In two trials the transferred cells rapidly declined to undetectable levels and no patient exhibited an objective clinical response (75, 76). The inability of $CD8^+$ T cells to persist in vivo raised the possibility that the transferred cells were competing with endogenous lymphocytes for homeostatic cytokines like IL-7 and IL-15. Subsequent trials attempted to deplete the endogenous lymphocyte repertoire with non-myeloablative chemotherapy prior to adoptive transfer of highly cultured, gp100 or MART-1 –reactive $CD8^+$ T cell clones (77-79) with one trial reporting one objective clinical response and one complete response.

The results of these trials were disappointing compared to those done a decade earlier which transferred bulk TIL populations resulted in a 34 percent objective clinical response rate (60). Because highly cultured tumor-specific $CD8^+$ T cell clones were observed to lack potency and persistence in vivo, the subsequent trials adoptively transferred minimally cultured but highly tumor-reactive oligoclonal or TIL cloid populations, which generally comprised both $CD4^+$ and $CD8^+$ T cells. After non-

myeloablative lymphopenic conditioning, the adoptive transfer of tumor-reactive TIL cloids and the co-administration of high-dose rIL-2 resulted in a 51 percent objective clinical response rate (80). Impressively, these results were obtained treating melanoma patients who all had progressive disease after receiving multiple therapies including surgery and high-dose IL-2. Compounded with the recent update report describing a 72 percent objective clinical response rate in patients receiving high intensity preparative lymphodepleting chemotherapy plus 12 Gy total body irradiation (1), demonstrated the potency and potential of adoptive T cell immunotherapy strategies for the treatment of cancer.

Mouse Model for Adoptive T cell Immunotherapy

Syngeneic mouse models have been invaluable for the identification and optimization of many tumor therapies. It is no surprise that insights into mechanisms of adoptive immunotherapy were first obtained in mouse models and later adapted for clinical studies. Early work on adoptive T cell immunotherapy in murine models focused on the transfer of TVDLN cells after IVS with autologous tumor and expansion in low-dose IL-2 (61-63). IVS treated TVDLN cells were demonstrated to have tumor cytolytic activity in vitro and were highly therapeutic when transferred to tumor-bearing mice. These data demonstrated the following important facets of adoptive T cell immunotherapy: 1) highly therapeutic T cells could be generated from tumor-bearing mice, 2) the IVS protocol was critical for the generation of therapeutic cells, 3) in vivo depletion of Lyt-2^+ $CD8^+$ cytotoxic T cells ablated the therapeutic efficacy, and 4) the co-

administration of IL-2 enhanced the therapeutic efficacy of the adoptively transferred cells.

The next development sought to overcome the necessity of autologous tumor for the IVS step. Based largely on the realization from clinical trials that it would not be possible to obtain the large number of autologous tumor cells required for the IVS step, alternative strategies were explored. Anti-CD3, a polyclonal stimulator of T cells, was demonstrated to mimic the effects of the autologous tumor (65-68). The use of anti-CD3 instead of IVS generated similar numbers of effector cells with tumor-specific cytolytic activity and therapeutic efficacy upon adoptive transfer into tumor-bearing hosts. Importantly the protocols used today for generating tumor-specific effector cells for preclinical studies were optimized in those series of papers. Specifically, TVDLN cells were activated for two days in soluble anti-CD3 followed by a three day in vitro expansion in low-dose IL-2. The resultant effector cells were then used for adoptive transfer into tumor-bearing hosts with the co administration of exogenous IL-2.

In this body of work, the adoptive T cell immunotherapy of D5, a poorly immunogenic sub-clone of the spontaneously arising B16BL6 mouse melanoma, was examined. Because therapeutic T cells could not be generated from lymph nodes draining a D5 tumor vaccination, mice were vaccinated with D5 tumor cells that were transduced to express granulocyte-macrophage colony stimulating factor (GM-CSF) (D5-G6). Vaccination with GM-CSF-producing D5-G6 tumor cells generates TVDLN from which therapeutic tumor-specific T cells could be obtained (81, 82). D5-G6 TVDLN cells after anti-CD3 activation and expansion in IL-2 contained effector T cells with D5-specific activity in vitro and therapeutic activity when adoptively transferred into mice

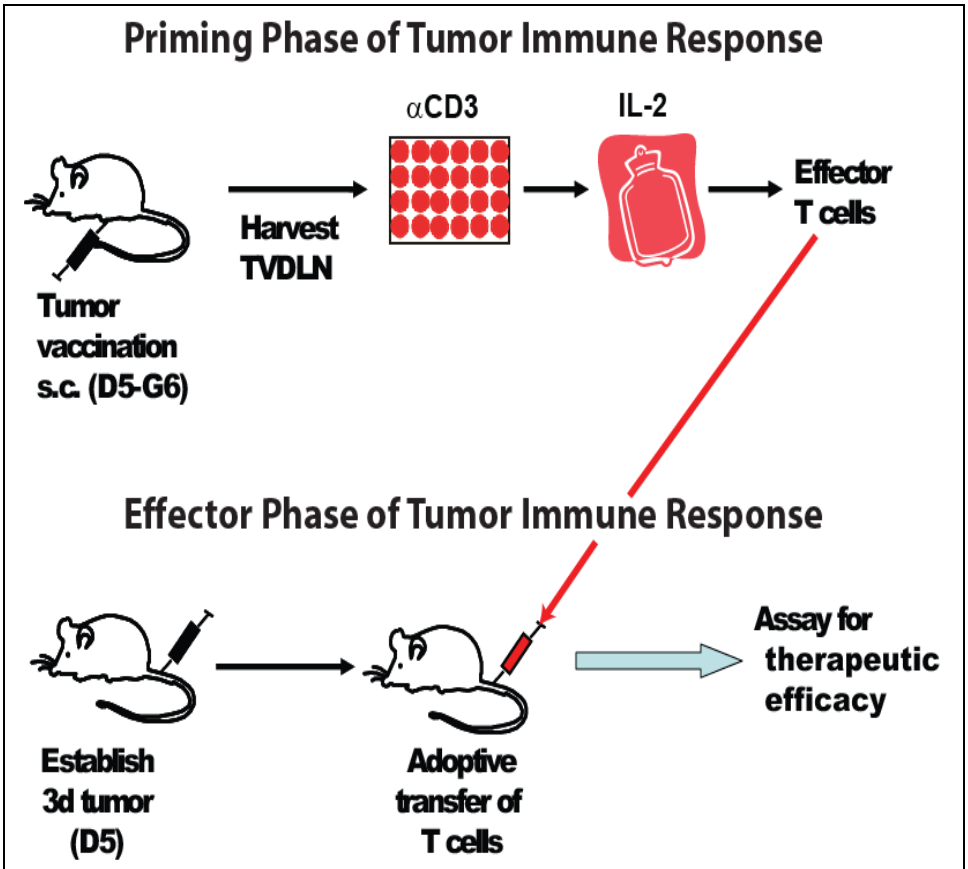

Figure 1: ***Schematic of mouse model of adoptive immunotherapy.***

bearing three-day D5 pulmonary metastases (Figure 1). This mouse model of adoptive immunotherapy presents an ideal opportunity to evaluate mechanisms responsible for effective tumor immunotherapy. Since the tumor-vaccine induced generation of tumor-specific T cells occurs in one animal (priming phase), the TVDLN cells are treated ex vivo, and subsequently adoptively transferred to another syngeneic tumor-bearing animal (effector phase), the contribution of the priming and effector phases to the global anti-tumor immune response can be examined independently. Therefore, not only do mouse models of adoptive immunotherapy have the potential for directing the translation of strategies to the clinic, it also is ideally suited to examine the mechanisms of anti-tumor immunity.

ISG15, UBP43, and enhanced anti-viral potential

One strategy to enhance anti-tumor function might be to harness mechanisms of the anti-viral response to enhance the immune response against neoplasms. This reasoning led us to examine mice deficient in Ubp43 (Usp18) which are hypersensitive to type I IFN exposure due to a hyperactive Jak/Stat signaling axis (83) and exhibit augmented anti-viral and anti-bacterial immunity (84, 85). Ubp43 was initially cloned when analyzing genes differentially expressed between wild type (WT) and leukemia fusion protein AML1-ETO knock-in mice (86, 87). The primary amino acid sequence of Ubp43 contained hallmarks of ubiquitin-specific proteases including the conserved Cys and His domains (88). However, UBP43 showed specific activity toward the ubiquitin-like molecule, interferon-stimulated gene 15 (ISG15). UBP43 specifically cleaved ISG15-peptide fusions instead of ubiquitin-peptide fusions and removed ISG15 from native conjugates (Figure 2). Exemplifying this specificity, cells derived from $Ubp43^{-/-}$ mice exhibit higher than normal amounts of ISG15-modified proteins, but not ubiquitinated proteins (89).

Anti-viral properties of ISG15 protein modification

ISG15 was first described as an interferon-stimulated gene product almost three decades ago (90) and its induction paralleled the acquisition of anti-viral resistance conferred by IFNs (91). Its recognition as an ubiquitin-like molecule came a decade later with the discovery that ubiquitin antibodies cross-reacted to ISG15 and that the two contained significant sequence homology (91). The 15kDa ISG15 was about twice the size of ubiquitin and contained two domains that were each about 30 percent homologous to ubiquitin. Their similarity led to an alternative name for ISG15, ubiquitin-cross

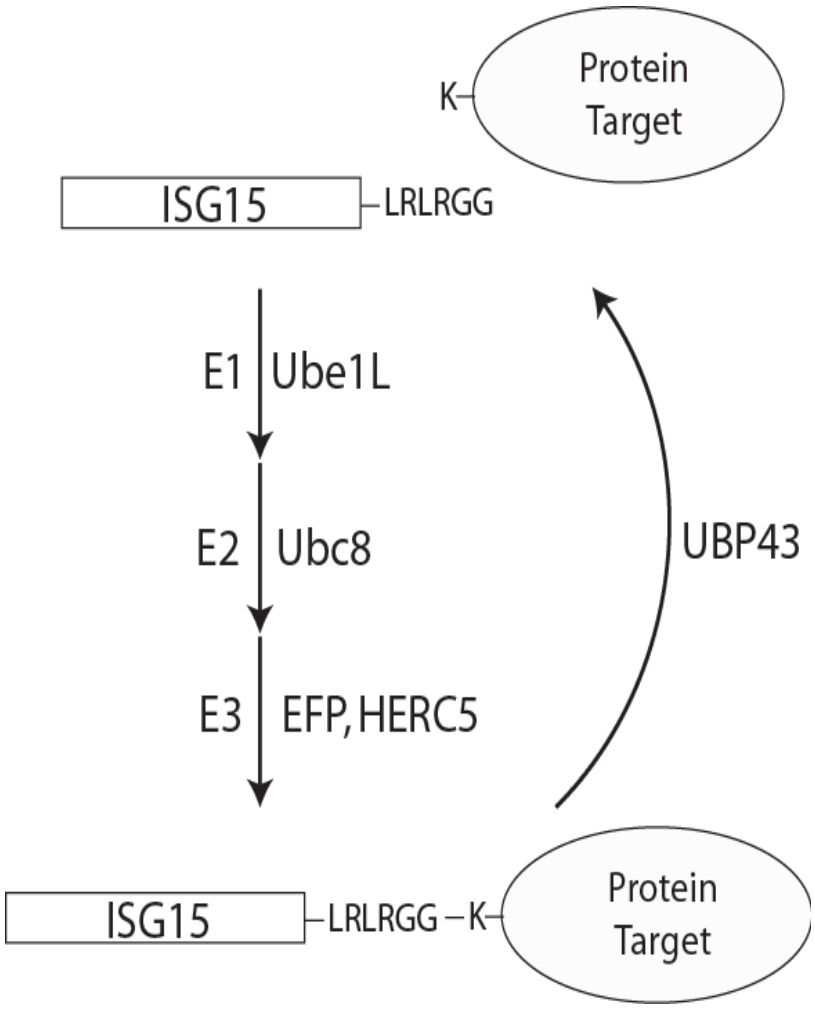

Figure 2: ***Diagram of ISG15 conjugation and UBP43 as its isopeptidase.***

reactive protein (UCRP), and it became the founding member of the molecular family of ubiquitin-like molecules. Solved later, the crystal structure of ISG15 confirmed the similarity to ubiquitin (92). Consistent with the sequence and structural similarities to ubiquitin, ISG15 utilizes a three enzyme protein conjugation pathway similar to but distinct from ubiquitin (93, 94). This pathway involved the coordinated activity of three modification enzymes: An activating (E1), a conjugating (E2), and a ligating (E3) enzyme (Figure 2). The E1 molecule possesses an ATP-binding domain and an active-site cysteine residue which forms a thioester bond with ISG15. The E1 then passes the ubiquitin to an E2 which, along with the E3, mediates the transfer of the ISG15 molecule to a lysine residue on the target substrate whose specificity is established by the E3. The ISG15 E1, UBE1L, was the first

enzyme reported to regulate ISG15 conjugation (95). UBE1L contained high homology to the ubiquitin E1 and, like ISG15, its expression is induced by type I IFN treatment. Because ubiquitin and its other family members each have only one E1, UBE1L will likely be the only E1 for ISG15. Two groups independently identified a ubiquitin E2, UbcH8 (Ubc8) as a major ISG15 E2 (96, 97). UbcH8 can serve as an E2 for both ISG15 and ubiquitin and is induced by IFN treatment. The two known ISG15 E3s, EFP and HERC5, can also serve as a ligase for ubiquitin and are both inducible by IFNs (98-101). Distinguishing itself from ubiquitin, both ISG15 expression and the enzymes required for conjugation are induced by type I IFNs.

In addition to ISG15 induction and conjugation, many ISG15 substrates are induced by or regulate the cellular response to type I IFNs. Initially five ISG15-modified proteins were identified, serpin 2a (102), phospholipase Cγ1, Jak1, STAT1, and ERK1/2 (103). Later, additional substrates were determined by mass spectrometry after ISG15 affinity purification (104, 105). These studies identified ISG15-modified proteins involved in many diverse cellular functions. Several identified targets are type I IFN inducible, including PKR, MxA, and RIG-I. Unexpectedly however, many of the identified proteins are constitutively expressed and not inducible by type I IFNs. These ISG15 conjugates are involved with RNA splicing, chromatin remodeling, cytoskeleton organization, stress response, translation, glycolysis, and motility. Due to their IFN-induced modification by ISG15, the identification of these ISG15 substrates could theoretically expand the repertoire of proteins regulated by IFNs.

Although there is a strong correlation in the development of ISG15-modified proteins with the acquisition of anti-viral immunity, the specific function of ISG15

modification still remains elusive. Sparse examples of the effect of ISG15 conjugation are in the literature and all are associated with regulation of the anti-viral or interferon responses. Discussed in detail below, effects of ISG15 modification have been described for interferon regulatory factor-3 (IRF-3), protein phosphatase 2Cβ (PP2Cβ), and the mRNA cap binding protein 4EHP.

IRF-3 is responsible for inducing type I IFN expression after dsRNA exposure. After ISG15 modification, IRF-3 is resistant to ubiquitin-mediated degradation in virus infected cells and enhances IFN-β transactivation suggesting that virus-mediated proteolysis of IRF-3 is subverted by ISG15 modification (106). IRF-3 is constitutively expressed and is activated by carboxy-terminal phosphorylation after dsRNA is detected by either RIG-I or TLR-3 leading to nuclear translocation and transcription of IFN-β (107). As a mechanism of immune avoidance, some viruses protect themselves from inducing IFN-β by ubiquitinating and subsequently degradation of IRF-3 (108). The cellular response strikes back by modifying IRF-3 with ISG15 resulting in its stabilization and persistence in the face of a viral infection. The ability to prevent its degradation makes ISG15-modified IRF-3 an important molecule responsible for the detection of viral-derived dsRNA.

Along with IRF-3 activation, the concerted activation of NF-κB is critical for the host response against many viruses (109). In addition to its ability to stimulate the production of IFN-β (110, 111), NF-κB plays a critical role in promoting immune and inflammatory cell function (112). Mechanisms which attenuate NF-κB function thus reduce its ability to mediate immunity and inflammatory potentiating function. The negative regulator of NF-κB, PP2Cβ, down-regulates its activation by dephosphorylating

and suppressing the NF-κB activators, TGF-β-activated kinase (TAK1) and IκB kinase (IKK) (113). In turn, when modified by ISG15, PP2Cβ phosphatase activity is suppressed leading to augmented NF-κB activation (114). Therefore, ISG15 modification of PP2Cβ enhances anti-viral immunity by augmenting NF-κB activation.

The above two examples demonstrate that modification by ISG15 mediates diverse effects on the substrate including protein stabilization (IRF-3) and the suppressing phosphatase activity (PP2Cβ). Regardless of the specific effect of ISG15 modification, it globally results in enhanced anti-viral mechanisms. Consistent with its anti-viral role, another ISG15-modified protein is involved with global translation inhibition. This anti-viral process is well characterized and occurs by PKR-dependent phosphorylation and subsequent inhibition of the translation initiation factor, eIF-2. Prior to scanning, cap-dependent translation requires the formation of the 43S pre-initiation scanning complex on the mRNA cap. The binding of the pre-initiation complex, in turn, requires the binding of another translation initiator factor, eIF4E. The levels and thus binding of eIF4E serves as the rate limiting step for translation initiation (115). ISG15 conjugation decreased translation by reducing the cap binding ability of eIF4E (116). In this case, 4EHP, an inhibitor of eIF4E which functions by competing for mRNA cap binding, is modified by ISG15 enhancing cap binding affinity. Thus, ISG15 conjugation further contributes to anti-viral immunity by augmenting the translation inhibitor 4EHP.

Therefore, ISG15 may have diverse effects on its substrate protein but all appear to culminate to confer a state of enhanced anti-viral potential. Even early observations that ISG15 expression was induced by IFNs and that it localized intracellularly with the cytoskeleton led to the suggestion that ISG15 would play an important anti-viral role

(117). Demonstrated directly by observing that ISG15 over-expression prevented the release of HIV-1 virus from infected cells (118), its role as an anti-viral molecule was later formally established by demonstrating that ISG15$^{-/-}$ mice had reduced ability to survive an influenza, herpes, or sinbis virus challenge (119). Importantly, this susceptibility to viral challenges could be overcome by ectopic delivery of WT ISG15, but not by an ISG15 mutant unable to be conjugated. These important data highlight the significance of, not only ISG15 expression, but also its ability to modify proteins. The significance of ISG15 conjugation is made more poignant by the observation that the influenza virus B has developed an immune escape mechanism that prevents ISG15 conjugation by inhibiting UBE1L (95).

Extrapolated from these observations, one could theorize that the maintenance of ISG15 conjugation would enhance the anti-viral response. In this regard, the inability to cleave ISG15 from modified proteins is one phenotype observed in cells derived from Ubp43$^{-/-}$ mice. Thus, we reasoned that, in the Ubp43$^{-/-}$ environment, the ability to cleave ISG15 modifications is ablated resulting in augmented immunity.

Ubp43$^{-/-}$ mice exhibit enhanced viral immunity

Several reports have described an increase in viral and bacterial resistance in Ubp43-deficient mice, including lymphocytic choriomeningitis virus (LCMV) and *Salmonella typhimurium* (84, 85). At doses which a WT animal would succumb, LCMV injected intracranially into Ubp43$^{-/-}$ mice resulted in reduced viral RNA replication and increased survival of the mice. Similarly, increased survival and reduced *S. typhimurium* was observed in Ubp43$^{-/-}$ mice compared to WT littermates. Striking however, the resistance to LCMV was found to be independent of ISG15 (120, 121). Mice deficient in

both ISG15 and UBP43 maintained the resistance against LCMV observed in the Ubp43$^{-/-}$ mice revealing that the UBP43-deficiency may facilitate enhanced anti-viral activity independent of the anti-viral effects of ISG15 conjugation.

The revelation of an ISG15-independent mechanism of enhanced viral resistance in Ubp43$^{-/-}$ mice led to the discovery that UBP43 itself could inhibit type I IFN signaling (122, 123). The presence of UBP43 attenuated proximal type I IFN signaling by sterically preventing the binding of JAK1 to IFNαR2. This steric inhibition prevented JAK1 activation, the first step in the JAK-STAT signaling cascade. Therefore, the absence of Ubp43 ablates this inhibitory mechanism of type I IFN signaling.

It's becoming increasingly clear that the expression of UBP43 serves as a negative regulator of the IFN response and of the acquisition of ISG15-mediated viral resistance in general (Figure 3). Either by inhibiting the anti-viral effects of ISG15 conjugation or by directly attenuating type I IFN signaling, UBP43 has emerged as a central player in regulating the anti-viral response. The genetic ablation of Ubp43, therefore, removes this inhibitor and results in an environment of significantly augmented anti-viral and anti-bacterial immunity.

The third chapter explores whether the enhanced anti-viral potential facilitated by the deficiency of Ubp43 can be harnessed to mediate enhanced anti-tumor efficacy. The enhanced anti-tumor function could be due to either the hypersensitivity to type I IFNs or the inability to cleave ISG15. Importantly, these mechanisms are not mutually exclusive such that both may enhance anti-tumor function. These results are presented after first confirming a role for tumor-specific CD4^{+} T cells to help CD8^{+} T cells treat tumor-bearing lymphopenic mice in chapter two.

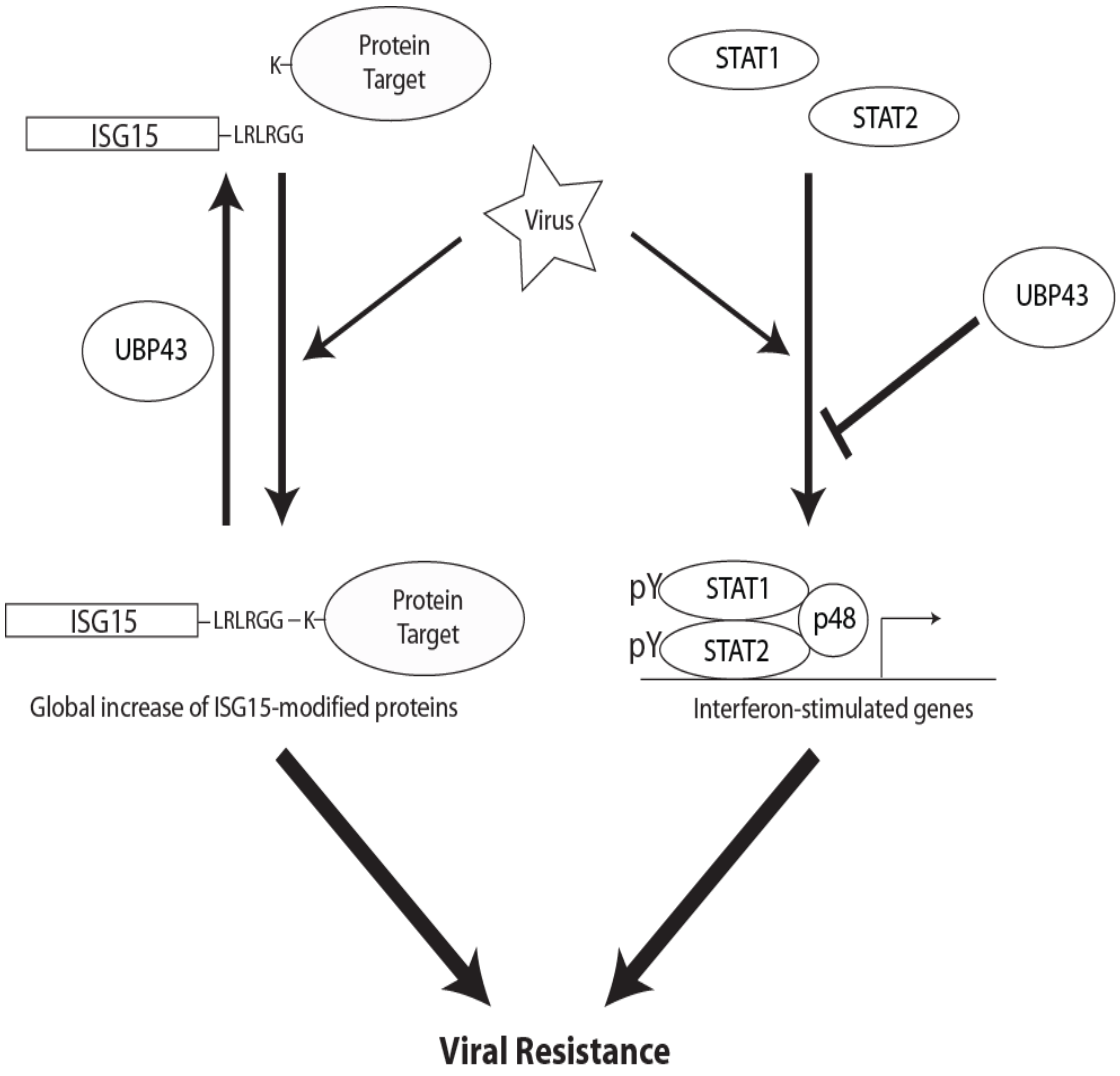

Figure 3*: Schematic diagramming two mechanisms which UBP43 suppresses viral resistance.*

CHAPTER TWO: Tumor-specific $CD4^+$ and $CD8^+$ T cells are both required for the most therapeutic benefit in tumor-bearing reconstituted lymphopenic mice

Abstract

The specific contribution of $CD4^+$ T cells to effective adoptive T cell therapy is not fully understood. Therefore their role in adoptive immunotherapy remains an important focus of research. Previous work has demonstrated that the adoptive transfer of tumor-specific T cells into a lymphopenic host results in superior tumor regression compared to the transfer of the same T cells into normal mice. A clear understanding of the constituents of the population which mediates the most effective therapeutic benefit is necessary to improve their potential. Data obtained from a clinical trial suggest that the direct anti-tumor effect of $CD8^+$ T cells in a lymphopenic host may depend on the presence of tumor-specific $CD4^+$ T cells. To explore this possibility, we determined whether $CD4^+$ T cells were necessary for the enhanced anti-tumor response and, if so, the nature of $CD4^+$ T cells required to support the anti-tumor effects of adoptive immunotherapy in tumor-bearing reconstituted $RAG1^{-/-}$ lymphopenic mice. Importantly, this model allowed us to examine the effect of the adoptively transferred T cells independent of any contribution from endogenous T cells. We confirmed that $CD4^+$ T cells were necessary to achieve the most effective tumor response. We also found that tumor-bearing survival was significantly enhanced after adoptive transfer if the $CD4^+$ T cells were specific to the cognate tumor rather than $CD4^+$ T cells specific to a noncognate tumor or an unrelated antigen. It is known that polyclonal T cells undergo maturation

changes when homeostatically proliferating in a lymphopenic environment which can potentially alter their anti-tumor function. Considering these changes, we identified that, regardless of their ability to proliferate homeostatically, cognate $CD4^+$ T cell help produced a superior tumor-specific $CD8^+$ T cell anti-tumor response. This indicated that $CD4^+$ T cell homeostatic-driven proliferation did not alleviate the requirement of tumor-specificity in order to provide effective help to the tumor-specific $CD8^+$ T cells. The nature of the help provided by cognate $CD4^+$ T cells was to maintain the tumor-specific response longer and thereby delay the progression of metastatic disease. This effect was specific to the maintenance phase as the presence of cognate $CD4^+$ T cells did not enhance the initial anti-tumor response. These results document the importance of tumor-specific $CD4^+$ T cell help in the achievement of an effective anti-tumor immune response and provide insights that will improve the effectiveness of adoptive immunotherapy strategies.

Introduction

The conditioning of the immune system with non-myeloablative lymphodepleting regimens prior to adoptive T cell immunotherapy has emerged as a potent and promising treatment for metastatic melanoma. This was highlighted by a successful clinical trial in which a 51 percent objective clinical response rate was observed in melanoma patients receiving adoptively transferred tumor-specific T cells and systemic IL-2 after non-myeloablative lymphodepletion (80). These results were a marked improvement compared to the authors' previous study in which non-myeloablative lymphodepletion prior to adoptive transfer produced no objective clinical responses (79). Although tumor-specific T cells isolated from melanoma lesions were employed in both studies, only $CD8^+$ T cell clones, which were selected by their reactivity to MART-1 or gp100 melanoma-derived peptides, were transferred in the first trial. In the second trial tumor-reactive tumor-infiltrating lymphocyte (TIL) cloids, which generally contained both $CD8^+$ and $CD4^+$ T cells, were administered. These $CD8^+$ T cells were shown to expand, persist and maintain function possibly leading to the high rate of tumor regression.

Insight into the factors necessary for successful adoptive T cell transfer may be discovered by comparing these two trials. One notable difference between these studies was the inclusion of $CD4^+$ T cells in the successful clinical trial. In this trial, patients were treated with oligoclonal (a.k.a. cloid) populations that, as a group, were tumor-reactive; however, although they seemed to be necessary, the role and specificity of the $CD4^+$ T cells were not determined (80). $CD4^+$ T cell help has been studied extensively in viral models and has been shown to orchestrate and enhance the immune response in both the priming (124, 125) and the subsequent effector and maintenance (126, 127) phases.

While not as well characterized in tumor models, $CD4^+$ T cells were required in the adoptively transferred population in order to maximize the efficacy in MHC $II^{-/-}$ tumor-bearing mice (82, 128). While MHC $II^{-/-}$ mice contain normal amounts of other immune cells, they are devoid of classical $CD4^+$ T cells suggesting that $CD4^+$ T cells played a critical role in this model. This environment was notably different then the lymphopenic environment induced prior to adoptive T cell transfer in the successful clinical trial. Indeed, proliferation in a lymphopenic environment imparts an activated phenotype to both $CD8^+$ (5, 129, 130) and $CD4^+$ (131, 132) T cells. Thus, proliferation in a lymphopenic environment could affect the $CD8^+$ T cell requirement for $CD4^+$ T cells or the nature of the $CD4^+$ T cell help provided, e.g. perhaps the help could be provided in a non-tumor-specific manner.

Wang *et al* demonstrated that tumor-bearing mice treated with both tumor-specific $CD4^+$ and $CD8^+$ T cells after the induction of lymphopenia by sublethal total body irradiation had a synergistic effect compared to the $CD8^+$ T cells alone (128). However, it is possible that the long-term anti-tumor response received contribution from the nascent endogenous T cells that emerge after the total body irradiation. Our previous data demonstrated that $CD4^+$-depleted effector T cells were capable of mediating long-term anti-tumor immunity in tumor-bearing WT mice yet were incapable of effectively treating tumor-bearing $MHCII^{-/-}$ mice (82). This suggested that repopulation of tumor-specific $CD4^+$ T cells from the endogenous T cell pool may contribute to long-term anti-tumor efficacy. Considering the possibility that the lymphopenic environment may alleviate the requirement of endogenous $CD4^+$ T cells, we determined the contribution of tumor-specific $CD4^+$ T cells made to an effective anti-tumor immune response in

lymphopenic mice treated by adoptive transfer of T cells in the absence of endogenous T cells. Specifically, RAG1$^{-/-}$ mice which the therapeutic effect is solely dependent on the adoptively transferred T cells were treated. Precluding any contribution by endogenous T cells to the therapeutic effect, this model mimics what occurs in patients who no longer have thymus activity. We found that tumor-specific CD8^{+} T cells transferred into lymphopenic tumor-bearing mice were more effective long-term if the accompanying CD4^{+} T cells were cognate, in respect to the tumor-specific CD8^{+} T cells, and capable of homeostatic-driven proliferation. Further, the presence of effector CD4^{+} T cells specific for the cognate tumor enhanced the anti-tumor effects by maintaining anti-tumor CD8^{+} T cell responses significantly longer than the noncognate CD4^{+} T cells. However, despite the enhanced tumor immune response and improved survival, using this stringent RAG1$^{-/-}$ model, all mice eventually succumbed to their disease. This was in striking contrast to adoptive immunotherapy in lymphopenic wild type mice capable of maintaining lymphopoeisis and reconstitution with de novo T cells. Therefore, these data describe a significant role for cognate CD4^{+} T cell help in the enhanced anti-tumor response observed in tumor-bearing hosts treated by adoptive T cell transfer after lymphopenic preconditioning. These important principles should inform the design of clinical trials using adoptive tumor immunotherapy to treat patients with cancer.

Materials and Methods

Mice

Syngeneic wild type C57BL/6 mice (H-2^b) were obtained from the National Cancer Institute (Bethesda, MD), Charles River Laboratories, Inc (Wilmington, MA), and The Jackson Laboratory (Bar Harbor, ME). RAG1 (H-2^b) knock-out mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred in our facility. OTII/RAG$^{-/-}$ mice were obtained from Taconic (Hudson, NY) and bred in our facility. Mice were maintained in SPF conditions and recognized principles of laboratory animal care were followed (Guide for the Care and Use of Laboratory Mice, National Research Council, 1996). All animal protocols were approved by the Earle A. Chiles Research Institute Animal Care and Use Committee.

Tumor cell lines

B16BL6-D5 (D5) is a poorly immunogenic subclone of the B16 melanoma cell line B16BL6 (H-2^b). D5-G6 is a clone generated by retroviral transduction of D5 with MFG-mGM-CSF. D5-G6 secretes GM-CSF at 100ng/mL/10^6 cells/24 hour. MCA-304 and MCA-310 are 3-methylcholanthrene-induced fibrosarcomas (H-2^b). In some instances, tumor lines were treated with 2ng/mL IFN-γ for 24 hours in order to increase MHC class I and class II expression. D5 and MCA-304 were transfected with pMTI-GFP-CIITA to express the class II transactivator (D5-CIITA and MCA-304-CIITA); cell lines were enriched to >94% by fluorescent cell sorting. All cells were grown in mouse complete media (CM: RPMI-1640 supplemented with 10 percent FBS, 0.1 mM nonessential amino acids, 1mM sodium pyruvate, 2mM L-glutamine, 50ug/mL

gentamicin sulfate (Biowhittaker Inc, Walkersville, MD), and 50 uM β-mercaptoethanol (Sigma-Aldrich, Milwaukee, WI).

Generation of tumor-specific effector T cells

Lymphopenia was induced in wild type mice by sublethal total-body irradiation (500 cGy) or RAG1$^{-/-}$ mice were used and reconstituted with 20 x 10^6 wild type or OTII/RAG$^{-/-}$ splenocytes, respectively. Mice were vaccinated with 250ug $OVA_{323-339}$ (AnaSpec, San Jose, CA) in CFA, 1 x 10^6 D5-G6, or 1 x 10^6 MCA-304 in all four flanks where indicated. Eight days later, vaccine-draining inguinal and axillary lymph nodes were resected and cells were plated at 4 x 10^6 in 2ml CM in a 24-well plate with 5ug/mL anti-CD3 (2C11) and 2.5ug/mL anti-CD28 (37.51). Two days later, non-adherent cells were harvested, washed, and placed at 0.1 x 10^6/mL CM supplemented with 60 IU/mL rhIL-2 (Chiron Co, Emeryville, CA) in Lifecell tissue culture flasks (Nexell therapeutics Inc, Irvine, CA). Three days later, effector T cells were harvested and subjected to in vitro depletion, tumor restimulation in vitro, and/or adoptive transfer as described below.

T cell depletion and adoptive immunotherapy

Where indicated, mice were depleted of $CD4^+$ or $CD8^+$ T cells 24 and 48 hours prior to lymph node harvest by intraperitoneal injections with 400ug of anti-CD4 (GK1.1) or anti-CD8 (Lyt2.2), respectively. Further enrichment was performed in vitro after IL-2 expansion when effector T cells were depleted of either $CD4^+$ or $CD8^+$ cells using L3T4 or Ly2 MACS beads (Miltenyi Biotec, Auburn, CA), respectively. Purity was verified by flow cytometry. Experimental pulmonary metastases were established by

i.v. injection of 0.2 x 10^6 D5 or 0.75 x 10^6 MCA-304 tumor cells. To assess short-term in vivo anti-tumor activity three days after injection of tumor cells, mice were treated with 20 x 10^6 effector T cells and/or 90 x 10^3 IU rhIL-2 intraperitoneally daily for four days starting the day of adoptive transfer. Mice were sacrificed by CO_2 narcosis ten days later; lungs were resected, and fixed in Fekete's solution. Macroscopic metastases were enumerated. Lungs with metastases too numerous to count were designated as having 250 metastases. For mice with MCA-304 metastases, the lungs were counter-stained by insufflation with India Ink solution prior to resection. To determine effects on survival, 40 x 10^6 effector T cells comprising 36 x 10^6 $CD8^+$ T cells and 4 x 10^6 $CD4^+$ T cells were adoptively transferred three days after metastases were established, IL-2 was administered as above and mice were sacrificed when moribund.

Tumor restimulation and ELISA

Effector T cells were restimulated in vitro at a 10:1 effector T cell to tumor cell ratio in a 24-well plate with the indicated tumors or 5ug plate-bound anti-CD3. 24 hours later, supernatants were harvested and IFN-γ concentration was determined by ELISA following the manufacturer's protocol (Pharmingen, San Jose, CA).

Statistics and nonlinear regression

Student's t-test was used for analysis of ELISA data. A two-tailed p value <0.05 was considered significant. Statistical differences in the number of lung metastases were determined by Mann-Whitney Test. A two-tailed nonparametric p value <0.05 was considered significant. A chi-square test was used for analysis of survival data. A one-

phase exponential nonlinear regression was used for decay analysis. Statistical analysis was performed by GraphPad Prism (Graphpad Software, La Jolla, CA).

Results

$CD4^+$ T cells are required for $CD8^+$ T cells to prolong survival of lymphopenic tumor-bearing hosts

We and others have demonstrated that development of long-term anti-tumor immunity following adoptive immunotherapy in lymphoreplete hosts required $CD4^+$ T cells (82, 128, 133, 134). Recent studies have reported that tumor-specific T cells elicited superior anti-tumor effects when transferred into lymphopenic mice (135). The specific requirement for adoptively transferred effector $CD4^+$ T cells, independent of host T cells, has not been evaluated in lymphopenic mice. Since homeostasis-driven proliferation in a lymphopenic host can induce a memory-like state in $CD8^+$ T cells that increases their sensitivity to stimulation by self-peptide-MHC complexes (5, 129, 130), it is possible that this environment may alleviate the requirement for $CD4^+$ T cells to maintain tumor-specific $CD8^+$ memory T cells and induce tumor regression. To explore this possibility, tumor vaccine-draining lymph node (TVDLN) lymphocytes from mice vaccinated with D5-G6, a poorly immunogenic B16BL6-D5 (D5) melanoma cell line genetically modified to secrete GM-CSF, were stimulated with soluble anti-CD3 and expanded in low-dose (60 IU/mL) IL-2. The resultant tumor-specific effector T cells were used for adoptive transfer before and after they were depleted of $CD4^+$ T cells. The presence of D5-specific T cells was verified by documenting IFN-γ production after in vitro restimulation with D5, but not with the unrelated, but syngeneic MCA-310 sarcoma (Figure 4a). Importantly, the depletion of $CD4^+$ T cells did not significantly affect the quantity of IFN-γ secreted.

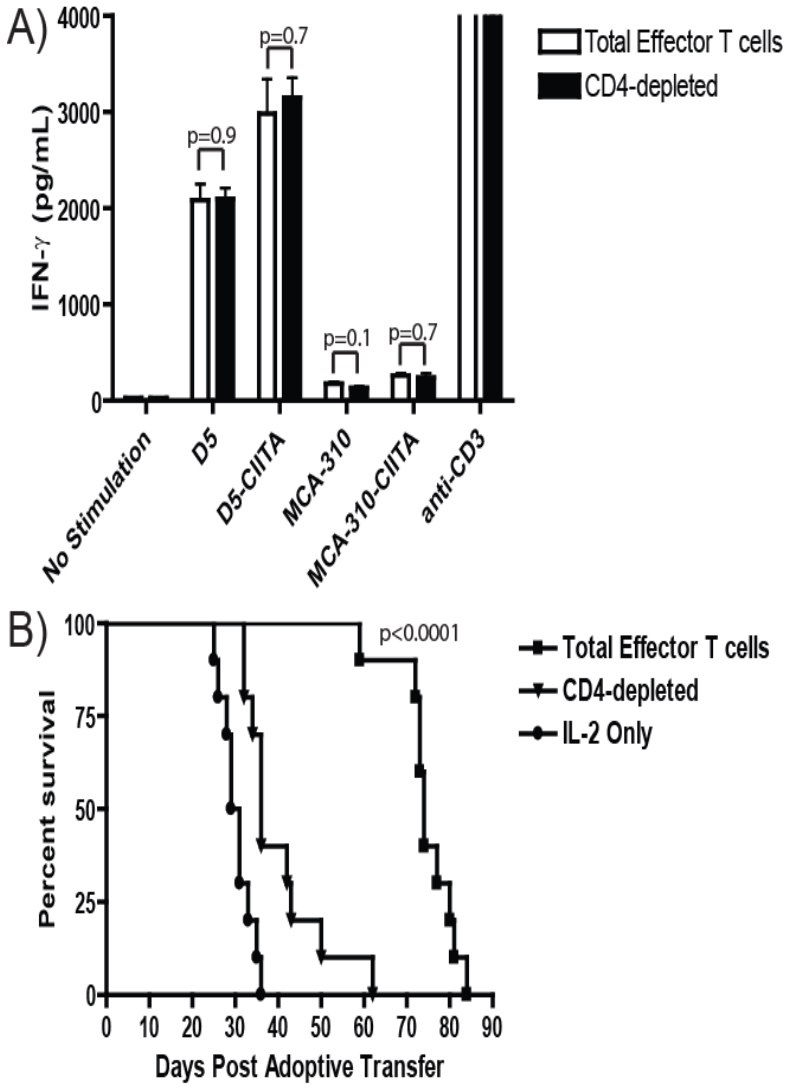

Figure 4: ***Depletion of CD4$^+$ T cells retain tumor-specific IFN-γ release in vitro but reduces long-term therapeutic efficacy in vitro.*** Effector T cells were generated as described in the materials and methods and left untreated or depleted of CD4$^+$ T cells using magnetic beads. A) 4 x 10^6 untreated (open bars) or CD4$^+$-depleted (solid bars) effector T cells were restimulated in vitro with 2 x 10^5 D5, D5-CIITA (D5 engineered to express the MHC class II transactivator) MCA-310, MCA-310-CIITA, or plate-bound anti-CD3. Twenty-four hours later supernatants were harvested and analyzed for IFN-γ by ELISA (n=3). B) RAG1$^{-/-}$ mice were injected with 1 x 10^5 D5 tumor cells i.v. three days prior to the adoptive transfer of 40 x 10^6 untreated (square) or 36 x 10^6 CD4$^+$-depleted (inverted triangle) effector T cells i.v. (effector T cells contained 10 percent CD4$^+$ T cells and the number of CD8$^+$ T cells transferred were matched). Experimental and control mice (circle) were given 90,000 IU IL-2 daily for four days following adoptive transfer. Mice were followed until moribund and then sacrificed (n=10).

To determine if the CD4$^+$ T cell-depleted population was sufficient to mediate effective anti-tumor therapy without endogenous T cells, RAG1-deficient mice bearing three-day established D5 pulmonary metastases were treated with either the non-depleted or CD4$^+$ T cell-depleted population. The number of effector CD8$^+$ T cells transferred was normalized so that all mice received an equivalent number of CD8$^+$ T cells. All mice received 90,000 IU IL-

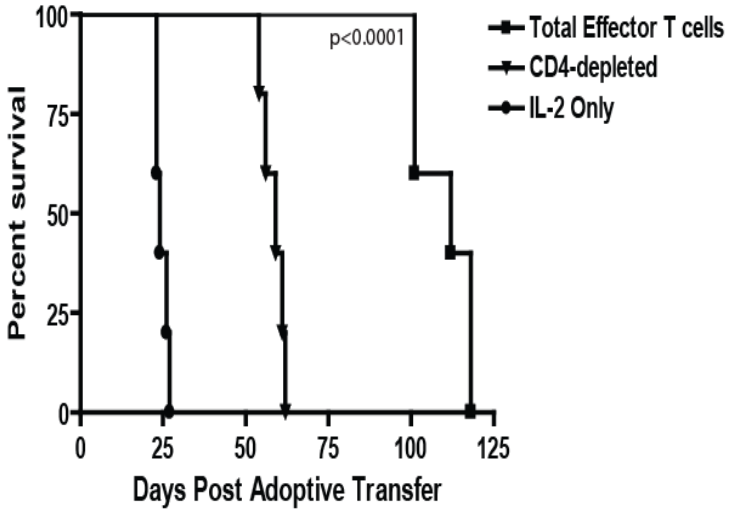

Figure 5: ***Large numbers of adoptively transferred effector T cells do not cure RAG1$^{-/-}$ mice, but efficacy still requires CD4^{+} T cells.*** Effector T cells were generated as described in material and methods and left untreated or depleted of CD4^{+} T cells using magnetic beads. RAG1$^{-/-}$ mice were injected with 1 x 10^{5} D5 tumor cells i.v. three days prior to the adoptive transfer of 70 x 10^{6} untreated (square) or 63 x 10^{6} CD4^{+}-depleted (inverted triangle) effector T cells i.v (effector T cells contained 10 percent CD4^{+} T cells and the number of CD8^{+} T cells transferred were matched). Experimental and control mice (circle) were given 90,000 IU IL-2 once per day for four days following adoptive transfer. Mice were followed until moribund and then sacrificed (n=5).

2 daily for four days following adoptive transfer and were then followed for survival. The survival of mice that received both CD4^{+} and CD8^{+} T cells was significantly ($p<0.0001$) prolonged compared to the survival of mice that received only tumor-specific CD8^{+} T cells (Figure 4b). It was evident that despite an improvement in survival mice treated with non-depleted T cells were not cured. Irrespective of the tumor burden at the time of adoptive transfer or the number of tumor-specific T cells transferred, RAG-deficient mice could not be cured of their disease. Even the transfer of seventy million effector T cells, a dose that cures WT mice (82), merely prolonged the survival of tumor-bearing RAG1$^{-/-}$ mice (Figure 5). We posited that the inability to eradicate tumor was a phenomenon restricted to D5-bearing RAG1$^{-/-}$ mice and possibly due to the lack of endogenous T cells. With this caveat, we concluded that the long-term anti-tumor effect of adoptive immunotherapy using tumor-specific CD8^{+} T cells to treat tumor-bearing lymphopenic mice requires CD4^{+} T cells.

Noncognate CD4$^+$ T cells that are incapable of homeostatic proliferation do not prolong survival of tumor-bearing hosts

Next we explored the ability of noncognate CD4$^+$ T cells to enhance CD8$^+$ T cell-mediated anti-tumor function in RAG1$^{-/-}$ lymphopenic mice. The specificity of CD4$^+$ T cells that help CD8$^+$ T cells prolong survival in tumor-bearing RAG1$^{-/-}$ lymphopenic mice has not been examined. We hypothesized that the presence of activated CD4$^+$ T cells in the lymphopenic environment would be sufficient to help homeostatically proliferating CD8$^+$ T cells, regardless of their specificity or their ability to proliferate in the lymphopenic environment. To determine if activated noncognate, i.e. non D5-specific, CD4$^+$ T cells incapable of homeostasis-driven proliferation could provide sufficient help, we used OTII transgenic CD4$^+$ T cells that were unable to proliferate when transferred to a lymphopenic host (136). These were derived from OTII/RAG$^{-/-}$ transgenic mice which have only I-A^b-restricted CD4$^+$ T cells that recognize the chicken ovalbumin epitope, 323-339. D5-G6-specific effector T cells and those depleted of CD4$^+$ T cells were generated as described earlier. The same number of effector OTII/RAG$^{-/-}$ CD4$^+$ T cells were added back to the CD4$^+$-depleted population as were removed by depletion. Effector OTII CD4$^+$ T cells were generated by subcutaneous vaccination of RAG1$^{-/-}$ mice reconstituted with OTII/RAG$^{-/-}$ splenocytes with $OVA_{323-339}$ in CFA. Vaccine-draining lymph nodes were harvested, activated and expanded as described above. Antigen specificity and tumor-reactivity of T cells was verified by measuring IFN-γ production following restimulation with tumor cells (Figure 6a). Tumors transduced to express the MHC class II transactivator (CIITA) were used as a more sensitive indicator because of their increased expression of MHC class I and II molecules. The addition of

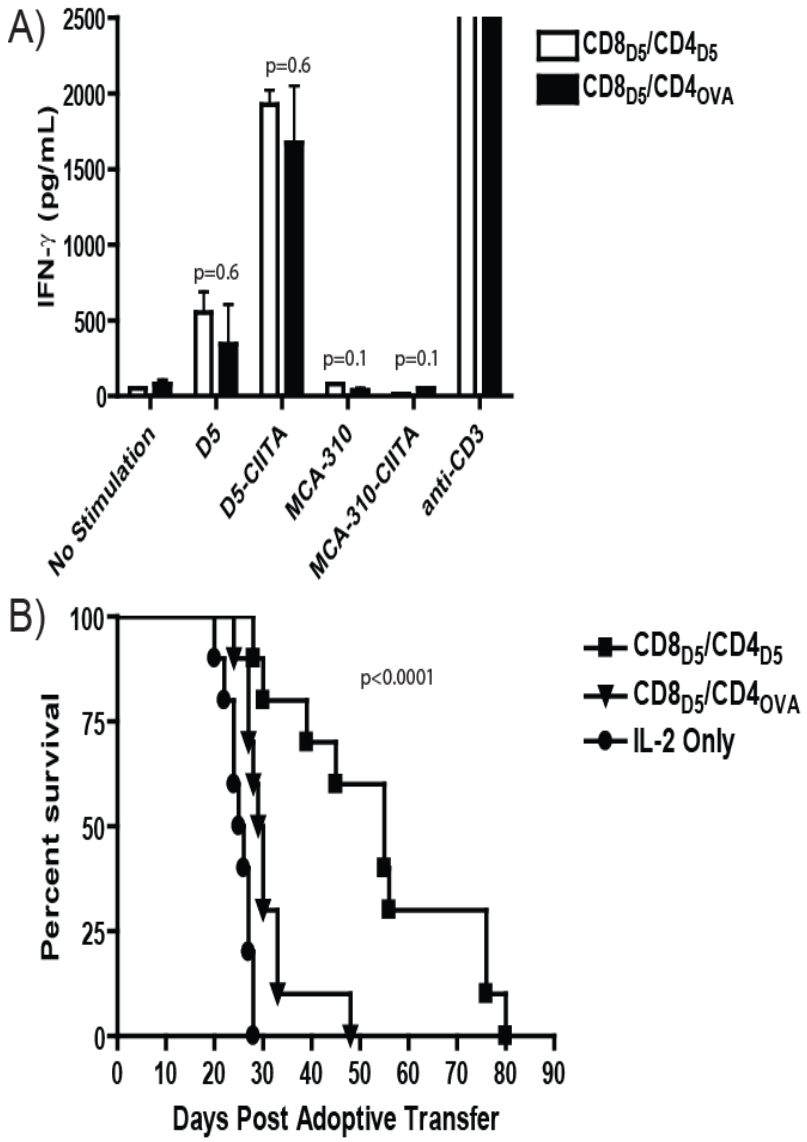

Figure 6: ***CD4$^+$ T cells that are not tumor specific did not reduce IFN-γ secretion in vitro yet significantly reduced ability to support D5-specific CD8+ T cells in vivo.*** D5-specific effector T cells generated as described previously were left untreated ($CD8_{D5}/CD4_{D5}$) or depleted of their $CD4^+$ T cells. Equal number of transgenic OTII $CD4^+$ effector T cells, generated as described in materials and methods, were combined with the depleted D5-specific effector T cells such that the OTII $CD4^+$ effector T cells, like the D5-specific $CD4^+$ effector T cells, comprised 10 percent of the effector T cell population ($CD8_{D5}/CD4_{OVA}$). A) 4 x 10^6 $CD8_{D5}/CD4_{D5}$ (open bars) or $CD8_{D5}/CD4_{OVA}$ (solid bars) were restimulated in vitro with 2 x 10^5 D5, D5-CIITA, MCA-310, MCA-310-CIITA, or plate-bound anti-CD3. Twenty-four hours later supernatants were harvested and analyzed for IFN-γ by ELISA (n=2). B) $RAG^{-/-}$ mice were given 1 x 10^5 D5 i.v. three days prior to the adoptive transfer of 40 x 10^6 of either $CD8_{D5}/CD4_{D5}$ (squares) or $CD8_{D5}/CD4_{OVA}$ (inverted triangles). Experimental and control (circles) mice were given 90,000 IU IL-2 once per day for four days following adoptive transfer. Mice were followed until moribund and then sacrificed (n=10).

OTII effector T cells did not significantly alter the D5-specific IFN-γ secretion by D5-specific $CD8^+$ T cells indicating that they maintained their D5 tumor reactivity regardless of whether the accompanying $CD4^+$ T cells were primed against D5-G6 or $OVA_{323-339}$. To determine if the OTII $CD4^+$ T cells could provide noncognate help in a lymphopenic $RAG1^{-/-}$ host, they were transferred together with $CD8^+$ effector T cells into RAG1-deficient mice bearing three-day D5 pulmonary metastases. In these experiments, the OTII $CD4^+$ T cells were unable to prolong survival significantly when compared to $CD4^+$ T cells specific to the cognate tumor (Figure 6b).

Noncognate $CD4^+$ T cells capable of homeostatic proliferation do not prolong survival of tumor-bearing hosts

The inability of transgenic OTII effector T cells to enhance survival suggested that either the lack of homeostatic proliferation or the absence of cognate help was responsible. To distinguish between these two alternatives, we tested whether noncognate $CD4^+$ T cells that were capable of homeostasis-driven proliferation provided sufficient help to prolong survival of treated lymphopenic mice. Polyclonal $CD4^+$ T cells capable of homeostasis-driven proliferation, but specific for the unrelated but syngeneic sarcoma, MCA-304, were used to provide noncognate $CD4^+$ T cell help. Critical to the interpretation of these experiments was the verification that T cells generated by vaccination with D5-G6 or MCA-304 were tumor-specific and did not cross-react with the other tumor. This was confirmed by restimulation of T cells with untreated tumor cells, including after pre-treatment with IFN-γ to increase MHC class I and class II expression (Figure 7a). Effector T cells induced following immunization with D5-G6

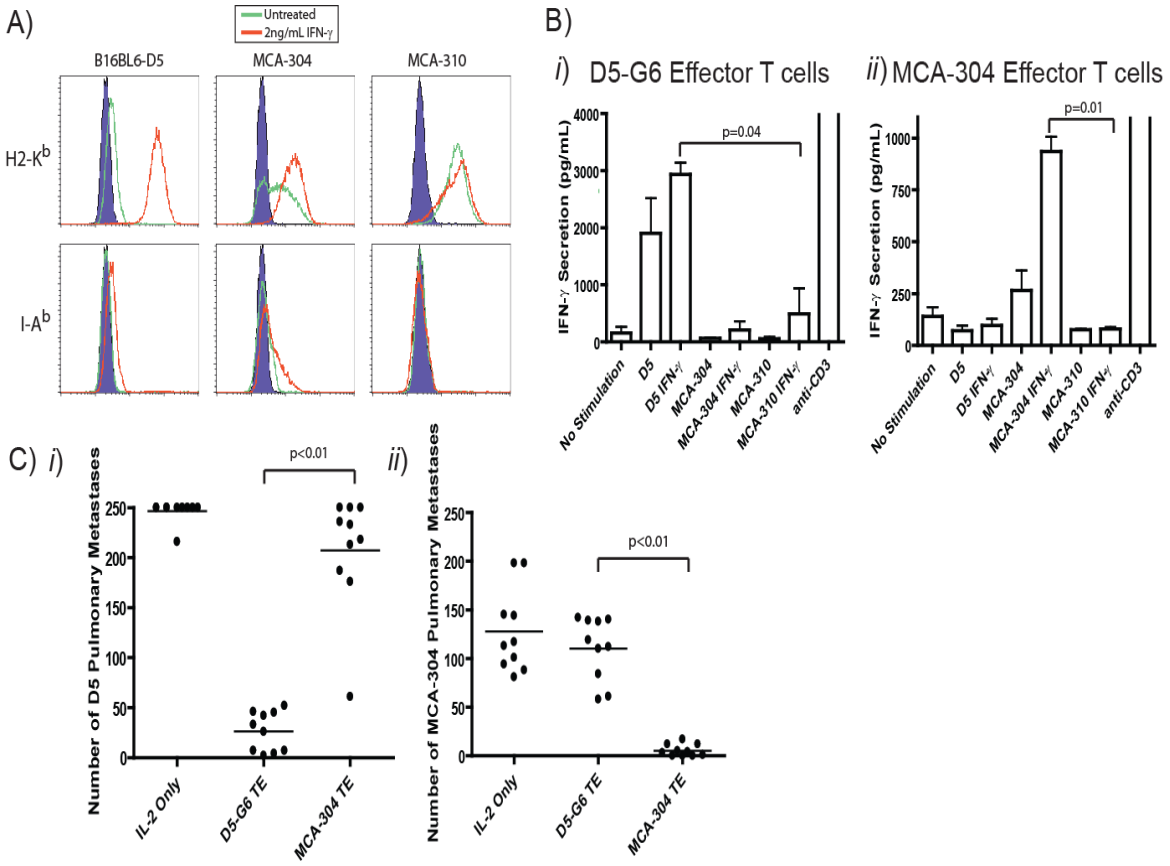

Figure 7: ***TVDLN generated by D5-G6 or MCA-304 vaccination do not exhibit cross reactivity to their noncognate tumor.*** A) The indicated tumor cells were left untreated (green line) or treated (red line) for 24 hours with 2 ng/mL IFN-γ. Each histogram compares the expression levels of H2-K^b or I-A^b with an isotype control (filled line). B) 4 x 10^6 D5-G6-generated (*i*) or MCA-304-generated (*ii*) effector T cells were restimulated in vitro with 2 x 10^5 D5, D5 IFN-γ (pretreated for 24 hours with IFN-γ), MCA-304, MCA-304 IFN-γ, MCA-310, MCA-310 IFN-γ, or plate-bound anti-CD3. Twenty-four hours later supernatants were harvested and analyzed for IFN-γ by ELISA (n=2). C) WT mice were given 2 x 10^5 D5 (*i*) or 7.5 x 10^5 MCA-304 (*ii*) i.v. three days prior to the adoptive transfer of 20 x 10^6 of either D5-G6-specific or MCA-304-specific effector T cells. Experimental and control mice were given 90,000 IU IL-2 daily for four days following adoptive transfer. Mice were sacrificed by CO_2 narcosis ten days later; lungs were resected, fixed in Fekete's solution, and macroscopic metastases enumerated. Results summarize two independent experiments.

reacted strongly against their cognate tumor but not to the noncognate MCA-304 or MCA-310 tumors (Figure 7b). MCA-304-generated effector T cells reacted against MCA-304, but not against D5 or MCA-310. These in vitro results were consistent with in vivo experiments which demonstrated that effector T cells generated after D5-G6 vaccination reduced the number of D5 metastases significantly ($p<0.0001$) compared to

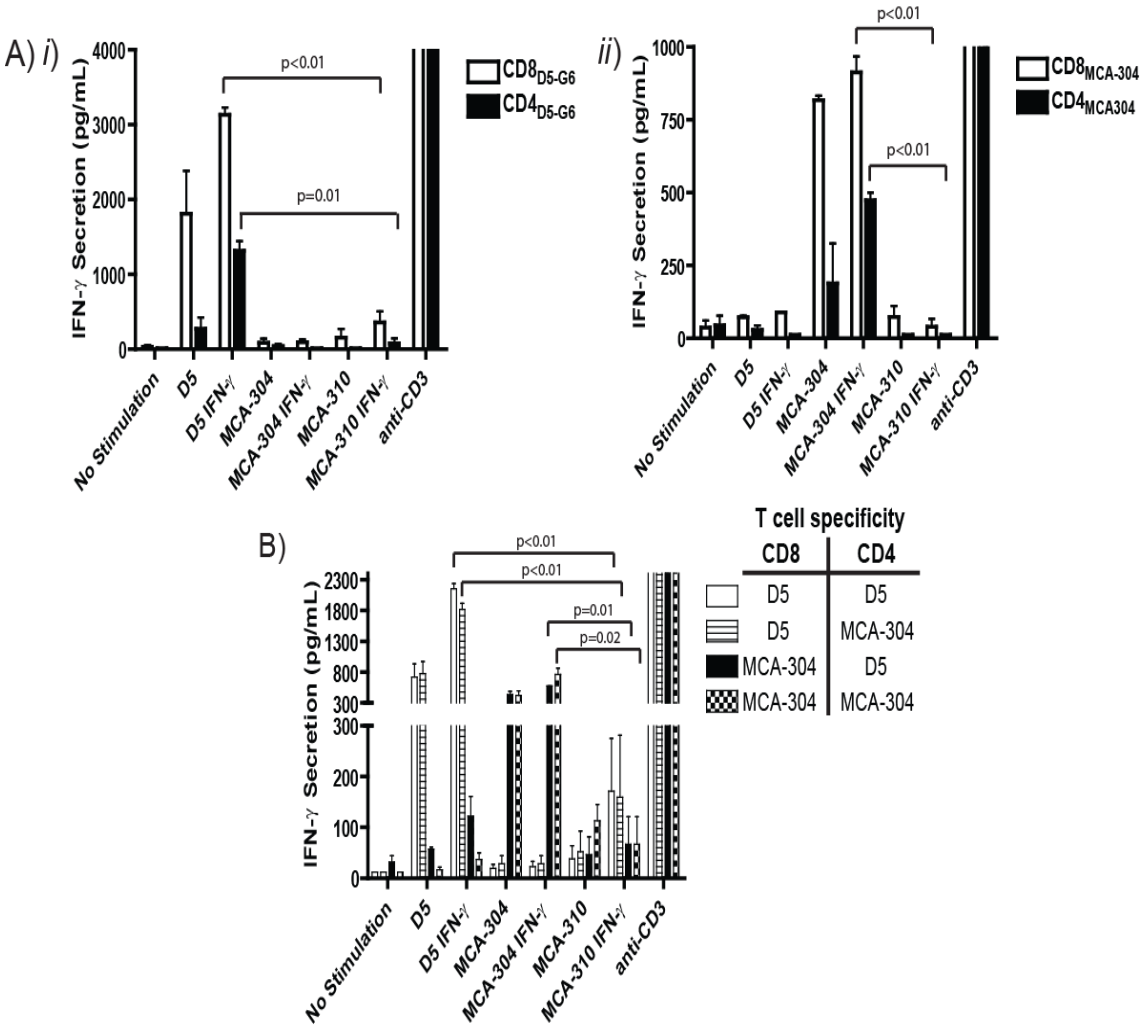

Figure 8: ***Separated and combined CD8$^+$ and CD4$^+$ T cells generated after tumor vaccination have specific tumor reactivity.*** Individual CD8$^+$ and CD4$^+$ effector T cells populations were obtained as described in materials and methods. A) 4 x 10^6 D5-G6-generated CD4$^+$ and CD8$^+$ T cells (*i*) and MCA-304-generated CD8$^+$ and CD4$^+$ T cells (*ii*) were restimulated in vitro with 2 x 10^5 D5, D5 IFN-γ, MCA-304, MCA-304 IFN-γ, MCA-310, MCA-310 IFN-γ, or plate-bound anti-CD3. Twenty-four hours later supernatants were harvested and analyzed for IFN-γ by ELISA (n=3). B) $CD8_{D5}/CD4_{D5}$ (open bars), $CD8_{D5}/CD4_{MCA304}$ (lines bars), $CD8_{MCA304}/CD4_{D5}$ (closed bars), or $CD8_{MCA304}/CD4_{MCA304}$ (boxed bars) T cells were restimulated, supernatants harvested and analyzed as done in A (n=3).

effector T cells generated by MCA-304 vaccination (Figure 7c). Similarly, effector T cells specific to MCA-304 reduced the number of MCA-304 metastases significantly ($p<0.0001$), whereas effector T cells generated by D5-G6 vaccination were ineffective against MCA-304 metastases (Figure 7c). Thus, effector T cells generated against either D5-G6 or MCA-304 recognized their cognate tumor specifically and did not cross-react

with the other tumor cell line. To determine if noncognate help, i.e. $CD4^+$ T cells that recognized a different tumor, would prolong survival in $RAG1^{-/-}$ lymphopenic mice when added to tumor-specific $CD8^+$ T cells, four groups of effector T cells were generated: D5-G6-specific $CD8^+$ effector T cells ($CD8_{D5}$), D5-G6-specific $CD4^+$ effector T cells ($CD4_{D5}$), MCA-304-specific $CD8^+$ effector T cells ($CD8_{MCA304}$), and MCA-304-specific $CD4^+$ effector T cells ($CD4_{MCA304}$). The MCA-304-specific and D5-G6-specific T cells were generated as described previously, and each group was depleted of either the $CD4^+$ or $CD8^+$ effector T cells, both in vivo and in vitro, after IL-2-mediated expansion. Each individual group of $CD4^+$ or $CD8^+$ effector T cells contained greater then 90 percent of the desired T cell population (data not shown), and specifically recognized their cognate tumor targets (Figure 8a). To confirm that the $CD4^+$ effector T cells were tumor specific we showed that more IFN-γ was secreted in response to IFN-γ pretreated tumors that express more MHC class II (Figure 8a). The absence of cross-reactivity verified the tumor specificity of each population. To confirm that the populations used for treatment did not alter the tumor reactivity, each $CD8^+$ effector T cell group were matched with each $CD4^+$ effector T cell group at a 9:1 ratio and their response to tumor cells was verified by restimulation with the appropriate tumor cell lines (Figure 8b). Regardless of the specificity of the accompanying $CD4^+$ T cells, the $CD8^+$ effector T cells released equivalent levels of IFN-γ in this twenty-four hour assay. This demonstrated that there were no suppressive effects after mixing the populations and suggested that the anti-tumor response would be largely unaffected by the specificity of the $CD4^+$ T cells. To assess directly the in vivo anti tumor properties, each group containing different combinations of $CD8^+$ and $CD4^+$ T cells were adoptively transferred into D5 tumor-

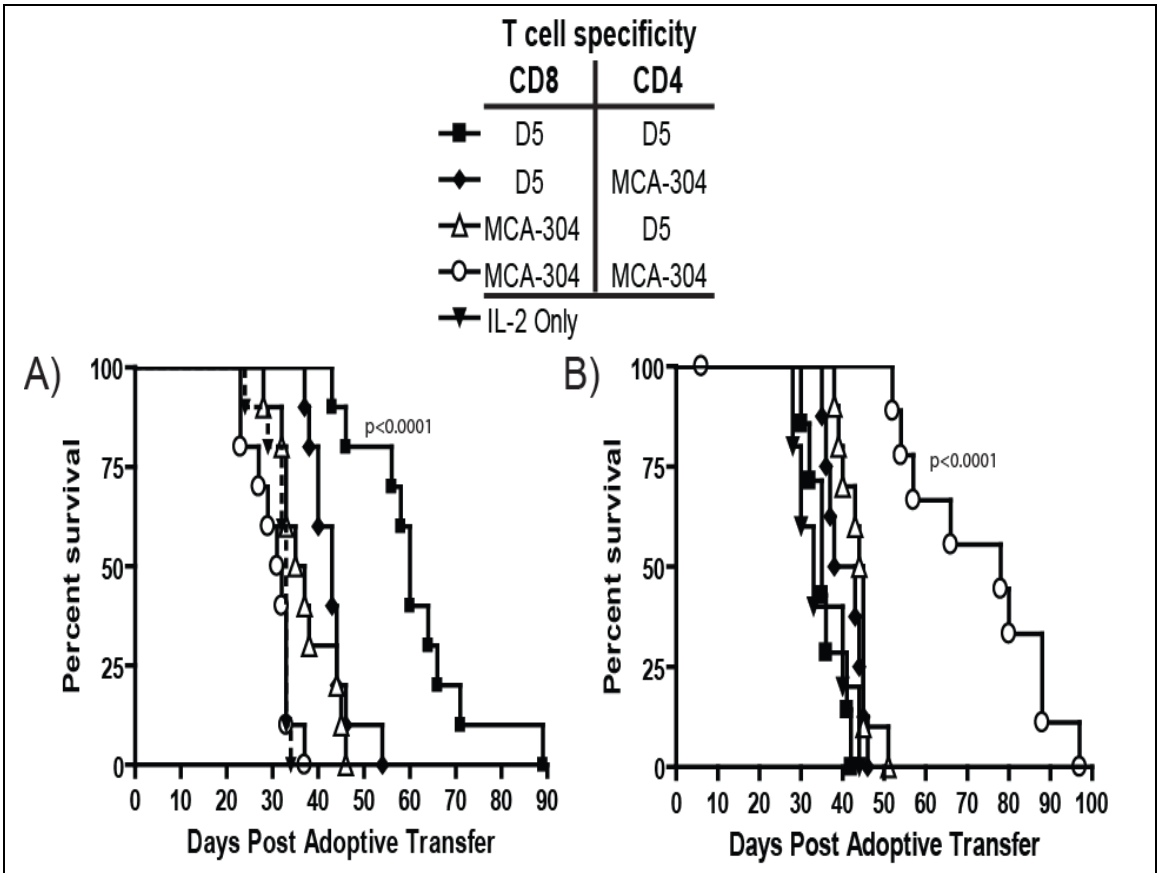

Figure 9: ***Noncognate CD4$^+$ T cells capable of homeostatic proliferation do not prolong survival of lymphopenic tumor-bearing hosts*.** RAG1$^{-/-}$ mice were given 2 x 10^5 D5 (A) or 7.5 x 10^5 MCA-304 tumor cells (B) i.v. three days prior to the adoptive transfer of 40 x 10^6 (9:1 CD8$^+$:CD4$^+$) of either $CD8_{D5}/CD4_{D5}$ (closed squares), $CD8_{D5}/CD4_{MCA304}$ (closed diamonds), $CD8_{MCA304}/CD4_{D5}$ (open triangles), or $CD8_{MCA304}/CD4_{MCA304}$ (open circles). Experimental and control (inverted triangles) mice were given 60,000 U IL-2 once per day for four days following adoptive transfer. Mice were followed until moribund and then sacrificed (n=10, open circles in B had one animal sacrificed due to an infection unrelated to the tumor burden and was excluded from analysis).

bearing RAG1-deficient mice (Figure 9a). Despite the fact that all of the CD4$^+$ T cells were capable of expanding in the lymphopenic host, only cognate $CD4_{D5}$ T cells were able to help CD8$^+$ T cells prolong the survival of D5 tumor-bearing mice significantly (Figure 9a). This suggests that even when both the CD4$^+$ and CD8$^+$ T cells are undergoing homeostasis-driven proliferation, the requirement for cognate CD4$^+$ T cell help was maintained. To verify that these results were not unique to the anti-D5 response, or secondary to the general inability of $CD4_{MCA304}$ T cells to provide help, we

showed that $CD4_{MCA304}$, but not $CD4_{D5}$, could provide sufficient help to $CD8_{MCA304}$ to augment survival of MCA-304 tumor-bearing mice (Figure 9b). Importantly, in one experiment, the D5-specific T cells that were unable to treat MCA-304 lung metastases were able to treat D5 lung metastases and vice-versa. These experiments show for the first time that the requirement for cognate $CD4^+$ T cells in the population of adoptively transferred T cells used to effectively treat tumor-bearing lymphopenic mice is independent of the $CD4^+$ T cell's ability to undergo homeostasis-driven proliferation.

Cognate CD4$^+$ T cell help prolongs survival of tumor-specific effector T cells

It is well established that one function of $CD4^+$ T cell help is to maintain $CD8^+$ T cell memory (126, 127). We hypothesized that cognate $CD4^+$ T cell help prolonged survival of mice by maintaining a functional tumor-specific $CD8^+$ T cell population. An alternative explanation would be that cognate $CD4^+$ T cell help contributed directly to a more complete eradication of the tumor immediately after adoptive transfer, even though the lack of difference of tumor-reactivity in vitro (Figure 8b) would suggest otherwise. To examine these possibilities, D5 tumor-bearing mice treated with adoptively transferred tumor-specific $CD8^+$ effector T cells and cognate or noncognate $CD4^+$ T cells were examined over time. Mice were sacrificed during the course of the experiment and splenocytes were harvested 27, 36, 44, and 50 days after adoptive transfer and the tumor reactivity of $CD8^+$ lymphocytes was monitored by examining IFN-γ release after tumor restimulation. At early time points, e.g. day 27, splenocytes from recipients of cognate or noncognate $CD4^+$ T cells demonstrated a similar response to D5-CIITA, D5 engineered to express higher levels of MHC class I and II (Figure 10a). However, splenocytes from

recipients of noncognate $CD4^+$ T cells lost their D5-CIITA reactivity significantly faster ($p<0.0001$) than the recipients of cognate $CD4^+$ T cells (Figure 10a, 10b). The loss of anti-tumor activity became apparent at day 36 and was complete by day 44, whereas the tumor-specific reactivity of recipients of cognate $CD4^+$ and $CD8^+$ T cells persisted at least until day 50. These data are consistent with the observed anti-tumor effects observed in vivo. At day 27, the tumor burdens were visually similar (Figure 10d) and the number of D5 lung metastases was not significantly different (Figure 10c) between recipients of cognate or noncognate $CD4^+$ T cells. Consistent with the decay in IFN-γ secretion, the tumor burden increased in recipients of noncognate $CD4^+$ T cell help as time progressed, e.g. by day 43 these mice had significantly more tumor metastases (Figure 10c and 10d) than mice receiving cognate $CD4^+$ T cell help. Regardless, the few large metastases observed at day 50 in mice receiving cognate tumor-specific $CD4^+$ and $CD8^+$ T cells would eventually progress to a terminal tumor burden had the mice not been sacrificed early for analysis. Taken together, these data suggest that the mechanism by which cognate help prolongs survival is by the maintenance of tumor-specific $CD8^+$ T cell activity in a lymphopenic host treated with the adoptive transfer of tumor-specific effector T cells.

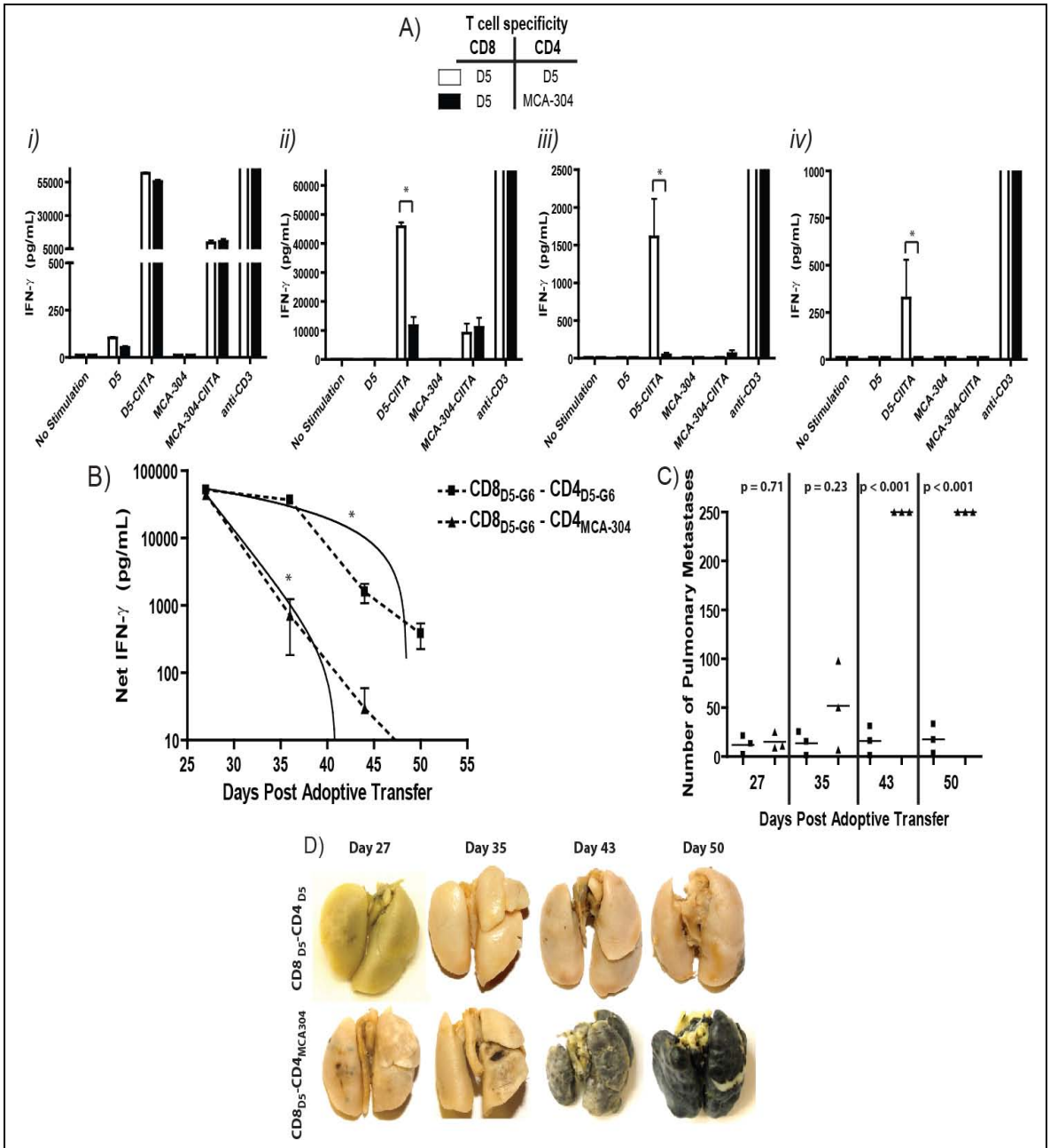

Figure 10: ***Cognate help maintains tumor-specific function of effector T cells longer than noncognate help***. A) 4 x 10^6 splenocytes collected 27 (*i*), 36 (*ii*), 44 (*iii*), and 50 (*iv*) days post adoptive transfer from mice treated with either $CD8_{D5}/CD4_{D5}$ (open bars) or $CD8_{D5}/CD4_{MCA304}$ (closed bars) were restimulated in vitro with $2x10^5$ D5, D5-CIITA, MCA-304, MCA-304-CIITA, or plate-bound anti-CD3. Twenty-four hours later supernatants were harvested and assayed for IFN-γ by ELISA (* indicates that $p<0.05$) (n=3). B) Net D5-specific IFN-γ secretion (D5-CIITA minus MCA-304-CIITA IFN-γ secretion) was plotted as a function of time and results for $CD8_{D5}/CD4_{D5}$ (squares) and $CD8_{D5}/CD4_{MCA304}$ (triangles) were analyzed using nonlinear regression analysis (solid lines) (* indicates that the curves were significantly different ($p<0.0001$)). C) Lungs from $CD8_{D5}/CD4_{D5}$ (squares) and $CD8_{D5}/CD4_{MCA304}$ (triangles) were resected, fixed in Fekete's solution, and macroscopic metastases were enumerated. Examples are shown in (D).

Discussion

Recent clinical data suggest that $CD4^+$ T cells are required to achieve tumor regression following adoptive transfer of tumor-specific $CD8^+$ T cells. We have explored the necessity and nature of the $CD4^+$ T cells that help produce an effective $CD8^+$ T cell anti-tumor immune response in tumor-bearing reconstituted lymphopenic hosts. Since homeostasis-driven proliferation in a lymphopenic host induces phenotypic changes in both $CD8^+$ (5, 129, 130) and $CD4^+$ (131, 132) T cells, we hypothesized that these changes could alter the requirement for $CD4^+$ T cell help or change the nature of the $CD4^+$ T cells that were required for anti-tumor function. To examine these possibilities, we employed a mouse model in which the effector and maintenance phases of effector T cells in a tumor-bearing lymphopenic host could be specifically examined.

We found that, despite the maturational changes that occur in $CD8^+$ T cells during proliferation in a lymphopenic environment, tumor-specific $CD4^+$ T cells were still required to maximize survival in this $RAG1^{-/-}$ model. Not only were $CD4^+$ T cells necessary, but they also needed to be cognate, i.e. specific for the same tumor that the $CD8^+$ T cells recognized. The ability to "help" was independent of the $CD4^+$ T cell's ability to proliferate homeostatically suggesting that, even with the acquired phenotypic changes, $CD4^+$ T cells could not provide help in a non-specific manner. Cognate $CD4^+$ T cells did not enhance the initial anti-tumor immune response, but their presence led to the longer maintenance of the $CD8^+$ T cell anti-tumor response compared to noncognate $CD4^+$ T cells.

These studies are particularly important when considering adoptive T cell transfer into lymphodepleted patients as a treatment for metastatic disease. The paucity of well

characterized tumor-specific $CD4^+$ T cell epitopes has led investigators to include tumor-unrelated $CD4^+$ T cell epitopes in an attempt to boost the $CD8^+$ T cell response in a non-cognate manner (137-140). In this study, attempts to boost the anti-tumor response by providing non-cognate $CD4^+$ T cells were ineffective in maintaining a tumor-specific $CD8^+$ T cell response. Therefore, our studies suggest that clinical trials using adoptive T cell transfer into a lymphopenic conditioned patient to include cognate tumor-specific $CD4^+$ T cells.

While the requirement for cognate $CD4^+$ T cell help during the effector phase in a tumor-bearing lymphopenic conditioned host has not been examined, we have shown that $CD4^+$ T cells were absolutely necessary in the effector phase of the anti-tumor immune response after adoptive transfer of T cells in a $CD8^+$ T cell replete environment (82). Further, others have demonstrated that $CD4^+$ T cells were required to maintain the anti-viral $CD8^+$ T cell response in a lymphocytic choriomeningitis virus model (126). Our current study contributes to this body of literature by highlighting the requirement for cognate $CD4^+$ T cells and by demonstrating that their effect is to prolong the persistence of tumor-specific $CD8^+$ T cells.

Although it is very clear that cognate help in combination with tumor-specific $CD8^+$ T cells prolonged the survival of tumor-bearing mice significantly, it is disappointing that eventually, regardless of the treatment, the tumor-specific activity was eventually lost and all mice succumbed to their disease. This differs from our previous results in which $CD4^+$ T cell help provided in a $CD8^+$ T cell replete host resulted in long-term survival of all mice (>150 days). In this case, tumor-bearing MHC II-deficient mice devoid of endogenous $CD4^+$ T cells were treated with effector T cells. These mice

succumbed to their disease, while wild type mice survived long-term and were immune to subsequent tumor challenge. In our current study, tumor-bearing RAG1-deficient mice, which were devoid of both endogenous $CD4^+$ T cells and $CD8^+$ T cells, were treated. The inability to generate de novo endogenous T cells in the $RAG1^{-/-}$ mice could be the key difference between our earlier and current experiments. Models of chronic viral infection have suggested that repopulation of the effector T cell pool from naïve $CD8^+$ T cells is critical for the maintenance of a heterogeneous and prolonged anti-viral response (141-147). $CD8^+$ T cells generated later in the infection have increased replicative capacity and are critical for maintaining the anti-viral response. One could make the comparison of chronic infection and cancer in terms of the prolonged exposure to antigen. Because $RAG1^{-/-}$ mice lack endogenous T cells, it is possible that endogenous T cell deficiency was responsible for the discrepancy between our former and current reports. Nevertheless, it is apparent that, regardless of the composition of T cells used for treatment, all tumor-specific responses deteriorate over time in $RAG1^{-/-}$ mice. Importantly though, the inevitable deterioration was delayed when cognate $CD4^+$ T cell help was provided in the $RAG1^{-/-}$ mice exemplifying the significant contribution of tumor-specific $CD4^+$ T cells to the long-term anti-tumor immune response.

We hypothesize that one mechanism of cognate help is to facilitate the recruitment of additional tumor-specific T cells from the naïve T cell pool which, in turn, maintains cancer in a state of equilibrium. Indeed, the viral models indicate that $CD4^+$ T cell help is important for this late $CD8^+$ T cell priming (142). In situations where the naïve pool cannot be reconstituted by de novo thymic output, the ability of cognate help to maintain the anti-tumor response is limited by exhaustion of the adoptively transferred

$CD8^+$ T cells. Reduced or eliminated thymic function occurs in people of advanced age or after dramatic treatments such as bone-marrow ablation and reconstitution.

An additional explanation for the eventual demise of the anti-tumor response is that the $CD4^+$ T cells undergo exhaustion or fail to survive. In our model, it is difficult to distinguish a tumor-specific $CD4^+$ T cell response from the overwhelming $CD8^+$ T cell response when examining the tumor-specific reactivity by IFN-γ release even when using tumors transduced with CIITA or pretreated with IFN-γ that result in increased levels of MHC I and MHC II expression. This limitation prevents the appreciation of how long the activated noncognate $CD4^+$ effector T cells can provide help after adoptive transfer into a host with limited amounts of their antigen. Whether exposure of $CD4^+$ T cells to antigen maintains their function (148) or if repeated stimulation can limit their numbers and protective function (149, 150) remains to be established. Further, $CD4^+$ T cells may be supported by homeostatic cytokines like IL-7, which are thought to be in particular abundance in lymphopenic hosts like RAG-deficient mice (151). Thus, although the persistence of tumor-specific "helpful" $CD4^+$ T cells is unclear in our model, it is apparent that inclusion of cognate $CD4^+$ T cells during adoptive transfer resulted in prolonged survival of tumor-bearing hosts compared to noncognate $CD4^+$ T cells.

Taken together these data offer potential insights into the role of $CD4^+$ T cells in the anti-tumor immune response of adoptively transferred effector T cells in melanoma patients treated with non-myeloablative conditioning. Consistent with the results of the recent clinical trial (79), which did not specifically select $CD8^+$ T cell clones for adoptive transfer, our data provide a possible explanation for the beneficial effects of the activated $CD4^+$ T cells transferred with the tumor-specific $CD8^+$ T cells. It further highlights the

therapeutic significance of cognate tumor-specific $CD4^+$ T cells among the population of adoptively transferred T cells used to treat patients with cancer following lymphodepletion.

CHAPTER THREE: Ubp43 ablation enhances the frequency of tumor-specific T cells and therapeutic potential upon adoptive transfer into tumor-bearing mice

Abstract

Ubp43$^{-/-}$ mice exhibit hypersensitivity to type I IFNs (IFN) and elevated levels of ISG15-modified proteins. Both contribute to the enhanced anti-viral potential observed in Ubp43$^{-/-}$ mice. By reducing ISG15 levels by RNAi technology, we demonstrated that the sensitivity to type I IFNs after dsRNA exposure was independent of ISG15, an observation confirmed later by other reports. We next demonstrated that the generation of effector T cells in an Ubp43$^{-/-}$ environment confers enhanced anti-tumor effect after adoptive transfer into tumor-bearing mice. Because we used a mouse model which can distinguish the contribution of T cell priming to the tumor from the subsequent effector function, we were able to decipher that the enhanced tumor therapeutic potential mediated by the Ubp43$^{-/-}$ environment was attributed to both augmented tumor-specific priming and enhanced anti-tumor effector function. While augmented tumor-specific priming was dependent on functional type I IFN signaling, the Ubp43$^{-/-}$ effector T cells mediated enhanced therapeutic efficacy independent of type I IFN sensitivity. Taken together, these data suggest a novel approach to enhance anti-tumor efficacy when employing adoptive T cell immunotherapy for the treatment of metastatic melanoma.

Introduction

Recent clinical trials have demonstrated the plausibility and potential of using T cell-based adoptive immunotherapy in patients with advanced disease (152). However, the labor-intensive nature of this procedure has limited its wide-spread use (80). To facilitate its more frequent use, two general approaches have been taken to increase its success rate: increasing the number tumor-specific T cells and enhancing their anti-tumor potential after adoptive transfer into tumor-bearing hosts. Reasoning that molecular mechanisms of enhanced anti-viral immunity might enhance the immune response against neoplasms, we examined tumor-specific T cells generated in mice deficient in Ubp43 (Usp18), which exhibit augmented anti-viral and anti-bacterial immunity (84, 85), after vaccination with D5-G6. We wanted to determine if Ubp43 deficiency would also facilitate an enhanced anti-tumor immune response.

ISG15 protein modification is not understood, but has been associated with anti-viral mechanisms. UBP43 is an isopeptidase which cleaves ISG15 from modified proteins (89) and, therefore, is a negative regulator of ISG15-mediated anti-viral responses. Independent of its isopeptidase activity, UBP43 also attenuates type I IFN signaling proximal to the IFN receptor (123) (Figure 3). By sterically inhibiting JAK1 binding to IFNαR1, UBP43 prevents JAK1 activation, a necessary step in the cellular response to type I IFNs. UBP43 deficiency therefore ablates several mechanisms which negatively regulate the anti-viral response. One mechanism is dependent on ISG15 and the other dependent on type I IFN signaling.

Enhanced type I IFN signaling capacity could also contribute to an augmented anti-tumor immune response. Type I IFNs have been shown to enhance both innate and

adaptive immunity by increasing cross-priming, clonal expansion, maintenance, and memory formation in T cells (153-157). Ubp43$^{-/-}$ mice could potentially augment these critical aspects of the anti-tumor immune response.

Some have suggested that Ubp43 deficiency enhances toll-like receptor (TLR) signal transduction (85). TLR signaling has long been appreciated to aid in the innate immune response including inflammation induction, dendritic cell maturation, and therapeutic type I cytokine polarization (158-161). Indeed TLR manipulation of the innate immune response has obvious ramifications on molding and enhancing the adaptive response, however it is becoming apparent that TLR signaling has direct effects on T cells themselves. TLR stimulation of T cells can serve as a potent form of costimulation (161), that can lower the signal strength necessary for a T cell response against self antigens (162).

We hypothesized that the mechanisms by which the deficiency of Ubp43 enhances viral and bacterial resistance may also be harnessed to enhance the anti-tumor immune response. Because Ubp43$^{-/-}$ enhances viral resistance in both an ISG15-dependent and IFN-dependent manner, before examining if the Ubp43$^{-/-}$ environment could foster an augmented anti-tumor immune response, we first determined whether these mechanisms were independent of each other. We found that, in terms of the cellular response to viruses, ISG15 regulates the response to the viral dsRNA-mimic, poly(I:C). Importantly, we found no evidence that ISG15 was involved in the direct response to type I IFNs. These results were later corroborated by several other reports (122, 123). Suggesting that ISG15-dependent and IFN-dependent mechanisms of enhanced viral resistance were independent of each other, we next used a model of

adoptive T cell immunotherapy to determine if the Ubp43 deficiency could foster an enhanced anti-tumor immune response. We found that TVDLN cells generated in an Ubp43$^{-/-}$ mouse contained a higher frequency of tumor-specific T cells, a process dependent on functional IFN signaling. Moreover, these tumor-specific T cells were polarized to a therapeutic type I cytokine milieu. This translated to enhanced therapeutic efficacy when Ubp43$^{-/-}$ T cells were adoptively transferred into tumor-bearing mice. Importantly, we demonstrate that the UBP43 deficiency in T cells was intrinsically capable to mediate enhanced anti-tumor efficacy.

Our results outlined in detail below demonstrate that the environment created by the deficiency of Ubp43 does indeed foster a potent anti-tumor immune response by both generating a higher frequency of tumor-specific T cells and by augmenting their anti-tumor potential when adoptively transferred into a tumor-bearing host. These data suggest that disrupting UBP43 expression or by employing small molecules designed to inhibit its activity in patient's T cells may enhance their anti-tumor efficacy.

Materials and Methods

Mice

Syngeneic wild type C57BL/6 mice (H-2^b) were obtained from the National Cancer Institute (Bethesda, MD), Charles River Laboratories, Inc (Wilmington, MA), and The Jackson Laboratory (Bar Harbor, ME). RAG1 (H-2^b) knock-out and FoxP3-GFP mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred in our facility. Ubp43, IFNaR1, and Ubp43/IFNaR1 knock-out mice were kindly provided by Dr DE Zhang (The Scripps Research Institute, La Jolla, CA). $RAG1^{-/-}$/pMel mice were kindly provided by DR HM Hu (Earle A Chiles Research Institute, Portland, OR). Mice were maintained in SPF conditions and recognized principles of laboratory animal care were followed (Guide for the Care and Use of Laboratory Mice, National Research Council, 1996). All animal protocols were approved by the Earle A. Chiles Research Institute Animal Care and Use Committee.

Cell lines

2fTGH is a human fibroblast cell line and is the parental cell line of U3A which was developed by inducing spontaneous IFN-α resistance. Both cell lines were kindly provided by Dr G Stark (Cleveland Clinic, Cleveland, OH). These cell lines were grown in DMEM supplemented with 10 percent FBS and 2mM L-glutamine (Biowhittaker Inc, Walkersville, MD). Polyinosinic-polycytidic acid (Poly(I:C)) (Sigma, St Louis, MO) was added to cultures at 50ug/mL where indicated. B16BL6-D5 (D5) is a poorly immunogenic subclone of the B16 melanoma cell line B16BL6 (H-2^b). D5-G6 is a clone generated by retroviral transduction of D5 with MFG-mGM-CSF. D5-G6 secretes GM-

CSF at 100ng/mL/10^6 cells/24 hour. MCA-304 and MCA-310 are 3-methylcholanthrene-induced fibrosarcomas (H-2^b). D5, MCA-310 and MCA-304 were transfected with pMTI-GFP-CIITA to express the class II transactivator (D5-CIITA, MCA-310-CIITA and MCA-304-CIITA); cell lines were enriched to >94% by fluorescent cell sorting. Mouse tumor cells were grown in mouse complete media (CM: RPMI-1640 supplemented with 10 percent FBS, 0.1 mM nonessential amino acids, 1mM sodium pyruvate, 2mM L-glutamine, and 50ug/mL gentamicin sulfate, 50 uM β-mercaptoethanol (Sigma-Aldrich, Milwaukee, WI).

ISG15 siRNA

ISG15 siRNA targeting hISG15$_{85\text{-}105}$ and a nontargeting (scrambled) control was created using Silencer siRNA Construction Kit (Ambion, Austin, TX), according to manufacture's instructions. The siRNA was delivered by lipofection using Metafectene (Biontex Laboratories, Martinsried/Planegg, Germany).

Luciferase and Apoptotic Assays

Luciferase studies were performed after cloning the hISG15 promoter from the U3A cell line, sequence -100 to -50, into pGL3-basic (Promega, Madison, WI). Luciferase levels were assayed according to manufacturer's instructions. Propidium Iodide (Invitrogen, Carlsbad, CA) treatment followed by flow cytometry was used to determine cellular DNA content according to established protocols.

STAT1 nuclear translocation

A construct expressing a functional STAT1-GFP fusion protein was kindly provided by Dr H Hauser (GBF-National Research Institute for Biotechnology, Braunschweig, Germany). Nuclear STAT1 was determined by fluorescent microscopy according to established protocols (163).

Generation of tumor-specific effector T cells

Mice were vaccinated with 1 x 10^6 D5-G6 in all four flanks where indicated. Eight days later, tumor vaccine-draining inguinal and axillary lymph nodes were resected and cells were plated at $4x10^6$ in 2ml CM in a 24-well plate with 5ug/mL anti-CD3 (2C11) and 2.5ug/mL anti-CD28 (37.51). Two days later, non-adherent cells were harvested, washed, and placed at $0.1x10^6$/mL CM supplemented with 60 IU/mL rhIL-2 (Chiron Co, Emeryville, CA) in Lifecell tissue culture flasks (Nexell therapeutics Inc, Irvine, CA). Three days later, effector T cells were harvested and subjected to tumor restimulation in vitro, flow cytometric analysis and/or adoptive transfer as described below. The frequency of Trp2 tetramer$^+$ (Beckman Coulter, Fullerton, CA) cells were determined by flow cytometry. In some cases, pan-T cells were isolated from splenocytes using bead enrichment (Miltenyi Biotec, Auburn, CA) and used for reconstitution followed by D5-G6 vaccination. Alternatively, splenocytes from RAG1$^{-/-}$ /pMel$^+$ TCR transgenic mice were used for reconstitution followed by D5-G6 vaccination and interrogation of the frequency of pMel$^+$ T cells in the blood using flow cytometric analysis after interrogation with an antibody specific for the pMel TCR, Vβ13 (ebioscience, San Diego, CA).

Adoptive immunotherapy

Experimental pulmonary metastases were established by i.v. injection of 0.25 x 10^6 D5 tumor cells. To assess in vivo anti-tumor activity three days after injection of tumor cells, mice were treated with effector T cells and/or 90 x 10^3 IU rhIL-2 intraperitoneally daily for four days starting the day of adoptive transfer. Mice were sacrificed by CO_2 narcosis ten days later; lungs were resected, and fixed in Fekete's solution. Macroscopic metastases were enumerated. Lungs with metastases too numerous to count were designated as having 250 metastases.

Tumor restimulation, intracellular IFN-γ staining, and ELISA

Effector T cells were restimulated in vitro at a 10:1 effector T cell to tumor cell ratio in a 24-well plate with the indicated tumors or 5ug plate-bound anti-CD3. 24 hours later, supernatants were harvested and IFN-γ concentration was determined by ELISA following the manufacturer's protocol (Pharmingen, San Jose, CA). Alternatively, the frequency of IFN-γ^+ cells were determined by flow cytometry after intracellular IFN-γ staining (ebioscience, San Diego, CA) after 5ug/mL brefeldin A treatment according to manufacturer's protocol.

Treg induction and suppression assays

$CD4^+$ T cells were purified from splenocytes and induced with plate-bound anti-CD3 (2C11) and 600 U/mL IL-2 with and without 5ng/mL TGF-β (ebioscience, San Diego, CA) for two days. The frequency of cells expressing FoxP3 was determined by flow cytometry after intracellular stain for FoxP3 (ebioscience, San Diego, CA). In some

cases, the $CD4^+$ T cells were harvested from transgenic FoxP3-GFP mice, purified to >95% by cell sorting, and mixed with CFSE-stained responder populations. Cellular division was determined by flow cytometry two days later

Statistics

Student's t-test was used for analysis of ELISA and flow cytometry data. A two-tailed p value <0.05 was considered significant. Statistical differences in the number of lung metastases were determined by Mann-Whitney Test. A two-tailed nonparametric p value <0.05 was considered significant. Statistical analysis was performed by GraphPad Prism (Graphpad Software, La Jolla, CA).

Results

IFN insensitivity human fibroblast cells are responsive to dsRNA treatment

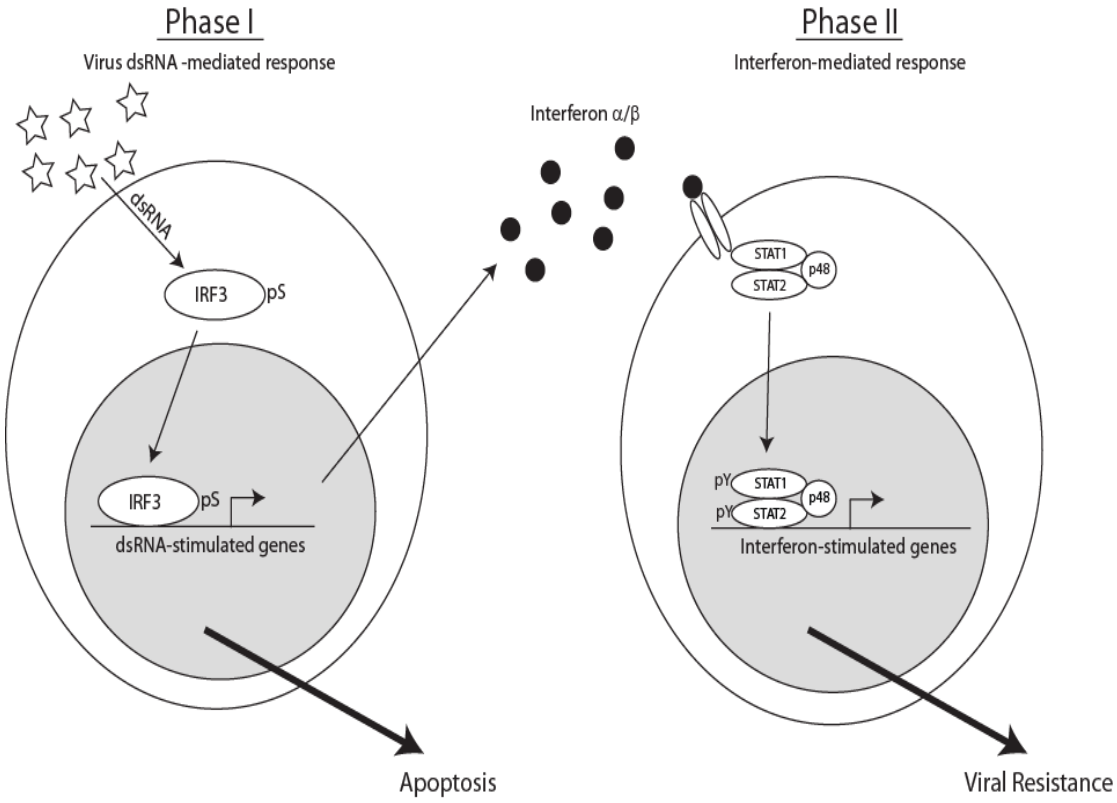

Figure 11: ***Illustration of the two type I IFN-dependent phases of the cellular response to dsRNA.***

To better appreciate the enhanced anti-viral response observed in Ubp43$^{-/-}$ mice, we first examined the role of ISG15 in the dsRNA-induced interferon response. Specifically we wanted to determine if ISG15 could regulate the immediate response to a viral insult, the response to type I IFNs, or both responses. UBP43 negatively regulates proximal type I IFN intracellular signaling, so it was important to determine if ISG15 was also involved in this pathway. The anti-viral response can be divided in two phases: 1) The initial response to dsRNA and 2) the subsequent response to the IFN induced by the dsRNA (Figure 11). The first phase initiates the production of type I IFN which is secreted to inform neighboring cells of the invading virus and is also responsible for the

induction of apoptosis to curb the spread of the virus (164). The second phase of the viral response is dependent on the pleiotropic effects of the IFN produced in the initial phase resulting in cell cycle arrest, apoptosis, and viral resistance (165). In order to dissect in which of these phases ISG15 was involved; we first wanted to establish a model system that could interrogate the two phases independent of each other. Normally when cells are treated with dsRNA, distinguishing the response to the dsRNA from that of the subsequently secreted IFN was difficult due to their redundant effects. Therefore, to dissect the response to dsRNA, a cell line that was unresponsive to IFNs was used. The U3A cell line was established from a human fibroblast cell line, 2fTGH, by inducing mutations leading to IFNα insensitivity (166). The IFN insensitive U3A cell line contained an incapacitating mutation in STAT1, a transcription factor critical for interferon signaling. Because we were not sure of the molecular mechanism of ISG15 action, initially we wanted to use a global readout of dsRNA sensitivity: The ability of dsRNA treatment to reduce cell numbers when treated in culture. This assay could not distinguish whether the treatment increases apoptosis or slowed the growth rates, but, regardless, poly(I:C) affected the accumulation of cell numbers over time in both cell lines (Figure 12a). To determine how IFN sensitivity modulates the effects of poly(I:C), the cell numbers were plotted as a ratio with and without poly(I:C) (Figure 12b). Indicating a more dramatic effect of poly(I:C), the WT 2fTGH cells with functional IFN signaling had fewer cell numbers after treatment then the IFN-insensitive U3A cell line.

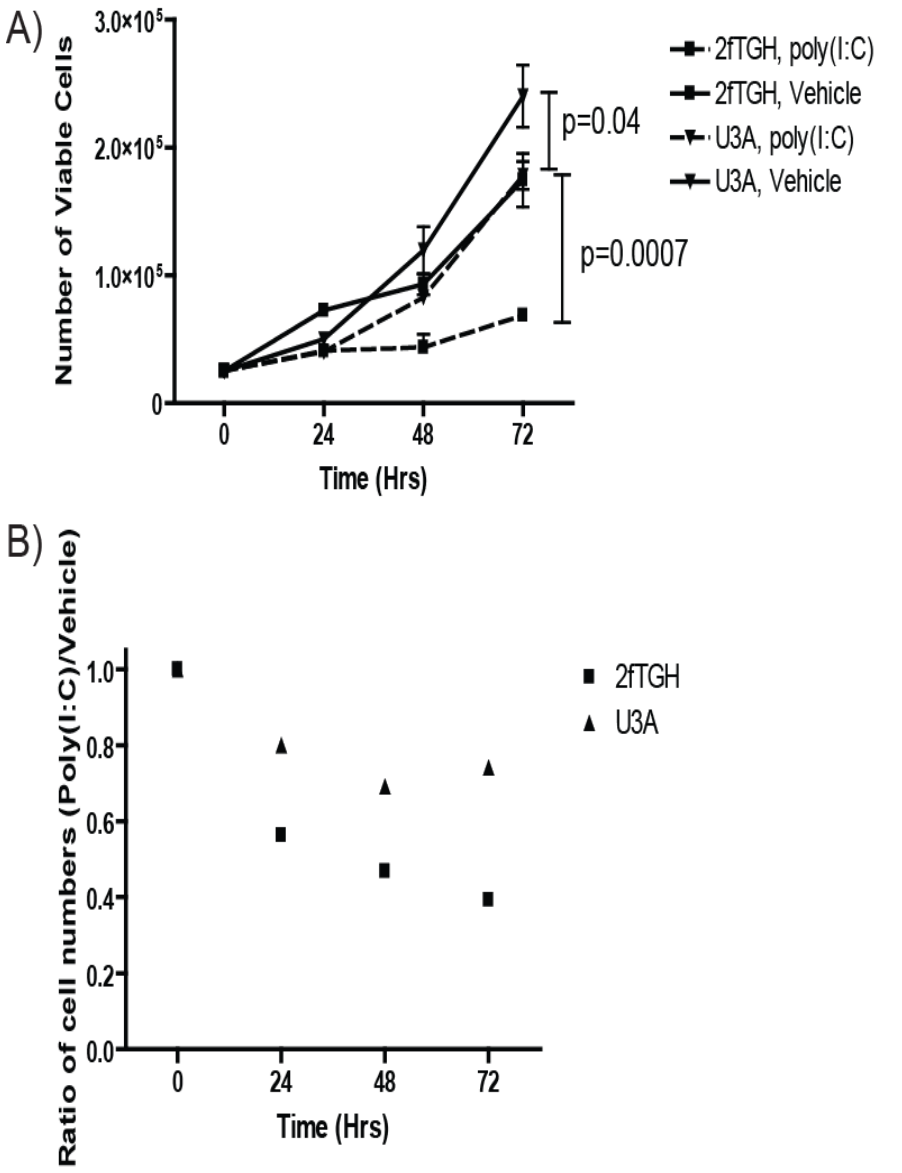

Figure 12: ***IFN insensitive human fibroblast cells are responsive to dsRNA treatment.*** 2fTGH and U3A cell lines with poly(I:C) or vehicle and viable cells were assayed by trypan blue exclusion 24, 48, and 72 hours later. Data was presented as cell numbers (n=3) (A) or as a ratio if poly(I:C) treated to untreated (B).

siRNA-mediated knock-down of ISG15 reduces the sensitivity to dsRNA in a STAT1-independent manner

Because the response of these cell lines to poly(I:C) could be distinguished, the assay could be used to determine if ISG15 regulates the response to poly(I:C) in an IFN-

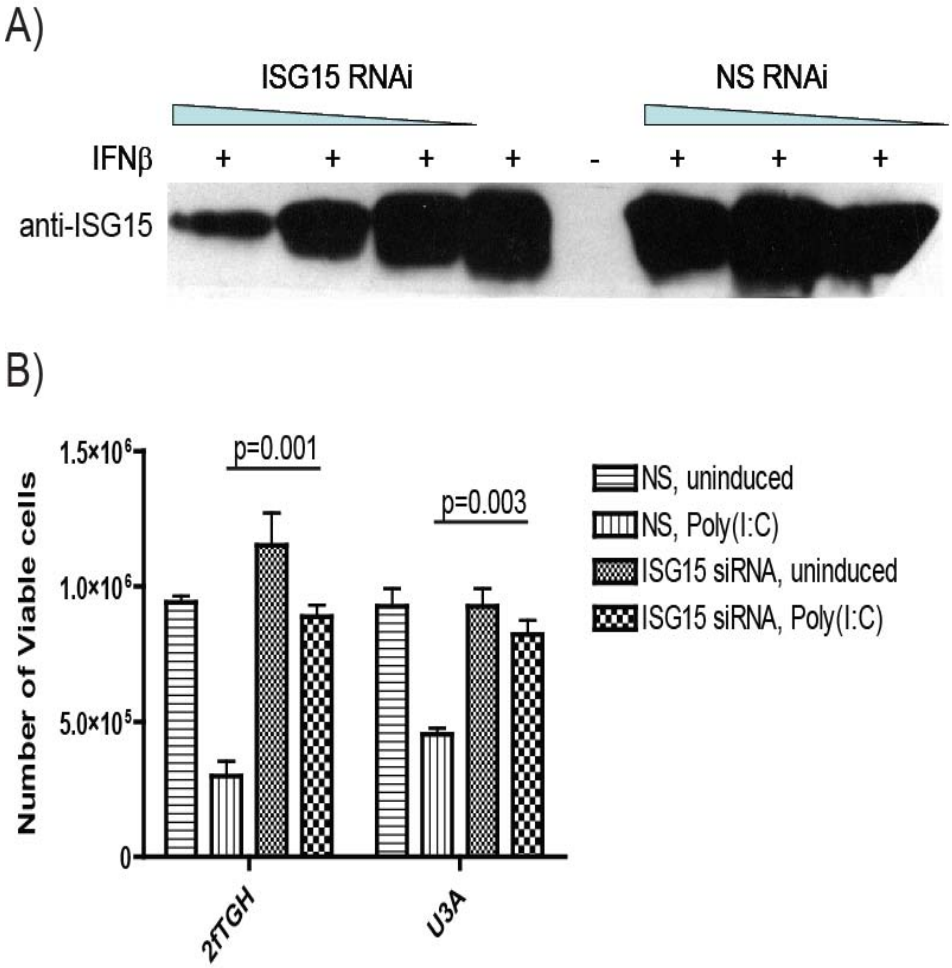

Figure 13: ***siRNA-mediated knock-down of ISG15 reduces the sensitivity to dsRNA in a STAT1-independent manner.*** 2fTGH and U3A cell lines were transfected with a hISG15-siRNA or scrambled control siRNA (NS) and knock-down of ISG15 was assayed by Western blot analysis using anti-hISG15 antibody (kindly provided by Dr E Borden) (A). After 24 hours, the cells were treated with poly(I:C) for 48 hours and the viable cells were assayed by trypan blue exclusion (B) (n=3).

dependent manner. If ISG15 knock-down manipulated the poly(I:C) response in the WT 2fTGH cell line only, it would indicate that ISG15 played a role in modulating the response to IFNs. On the other hand, if the effects were observed in both cell lines, it would indicate that ISG15 modulates the response to poly(I:C) independent of IFN. To this end, 2fTGH and U3A cells were transfected by lipofection with either a hISG15-specific siRNA or a nonsense (NS) siRNA control (Figure 13a). Twenty-four hours after transfection, the cells were treated with poly(I:C) and the number of live cells were

assayed 48-hours later (Figure 13b). As seen before, without ISG15 knock-down, poly(I:C) treatment reduced the cell numbers in both cell lines. Interestingly, ISG15 knock-down significantly dampened the effects of poly(I:C) in both cell lines. This effect occurred regardless of their responsiveness to IFN indicating that ISG15 modulates the direct response to dsRNA.

siRNA-mediated knock-down of ISG15 reduces the ability of poly(I:C) to induce IFN-β transactivation and apoptosis

A direct consequence of dsRNA exposure is the transactivation of the IFN-β promoter and induction of apoptosis. If ISG15 knock-down dampens the response to poly(I:C), it should also attenuate the ability of poly(I:C) to transactivate the IFN-β promoter and induce apoptosis. To test this hypothesis, the human IFN-β promoter was sub-cloned into a luciferase reporter construct and used for transfection of the U3A cell line. Indeed poly(I:C)-induced IFN-β transactivation was attenuated when ISG15 was knocked-down using siRNA (Figure 14a). Further, the number of apoptotic cells, as determined by propidium iodide staining, were decreased in both 2fTGH and U3A cell lines after poly(I:C) treatment and transfection with ISG15-specific siRNA (Figure 14b). Therefore consistent with the notion that ISG15 knock-down directly attenuates the response to poly(I:C), ISG15 knock-down attenuated both IFN-β transactivation and the apoptotic index.

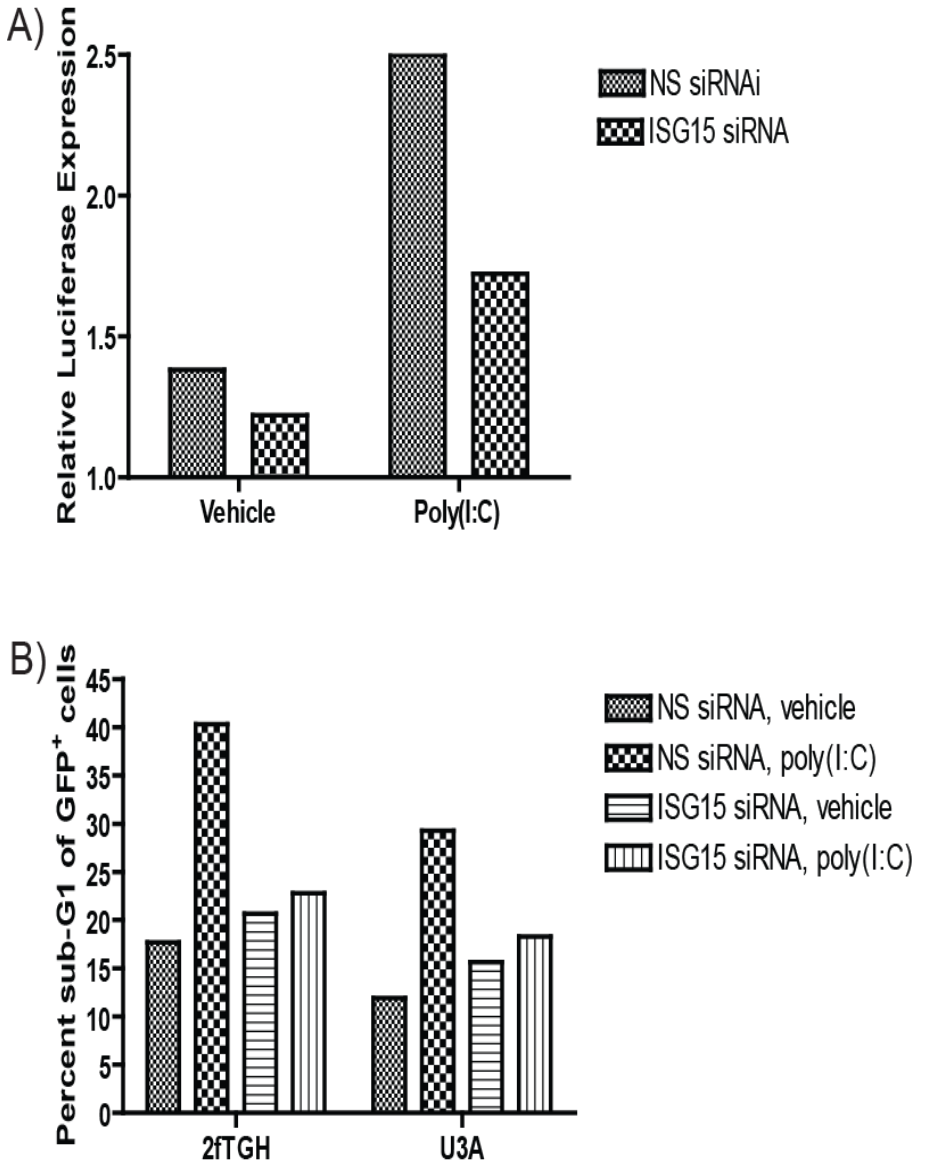

Figure 14: ***siRNA-mediated knock-down of ISG15 reduces the ability of poly(I:C) to induce IFN-β transactivation and apoptosis.*** 2fTGH and U3A cell lines were transfected with a hISG15-siRNA or scrambled control siRNA (NS). Poly(I:C)-induced IFN-β transactivation was assayed by determining IFN-β promoter-driven luciferase expression after co-transfection (A). Poly(I:C)-induced apoptosis was determined by assaying for sub-G1 DNA content after propidium iodide staining and flow cytometric analysis (B). Data are representative of two independent experiments.

ISG15 knock-down does not affect IFN–β– induced STAT1 nuclear translocation

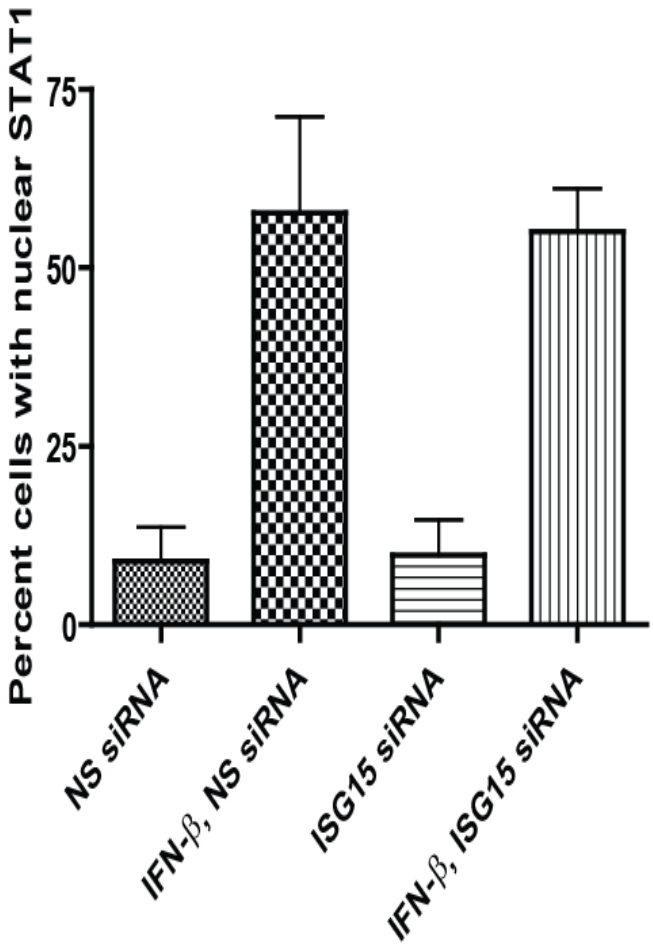

Figure 15: ***ISG15 knock-down does not affect IFN–β– induced STAT1 nuclear translocation.*** U3A cell line was co-transfected with a hISG15-siRNA or scrambled control siRNA (NS) and STAT1-GFP. Performed in a double-blind manner, nuclear STAT1 was determined by GFP localization after treatment with 1000U/mL IFN-β (n=3).

These data strongly suggested that ISG15 sensitizes the cellular response to dsRNA as its knock-down attenuated the poly(I:C)-induced responses independent of IFN signaling. However, early observations in the Ubp43$^{-/-}$ mouse suggested that STAT1 is ISG15 modified facilitating increased DNA binding and augmented type I IFN sensitivity (83). Considering these seemingly contradictory data, we wanted to determine the effect of ISG15 knock-down on STAT1 activity in response to IFN induction. To determine if ISG15 knock-down effected IFN sensitivity, a construct expressing STAT1-GFP fusion protein was co-transfected along with ISG15 siRNA, induced with IFN-β for one hour and percent cells with nuclear STAT1 was enumerated based on GFP localization. We found that ISG15 knock-down had no effect on IFN-induced nuclear STAT1 localization (Figure 15). Since performing these

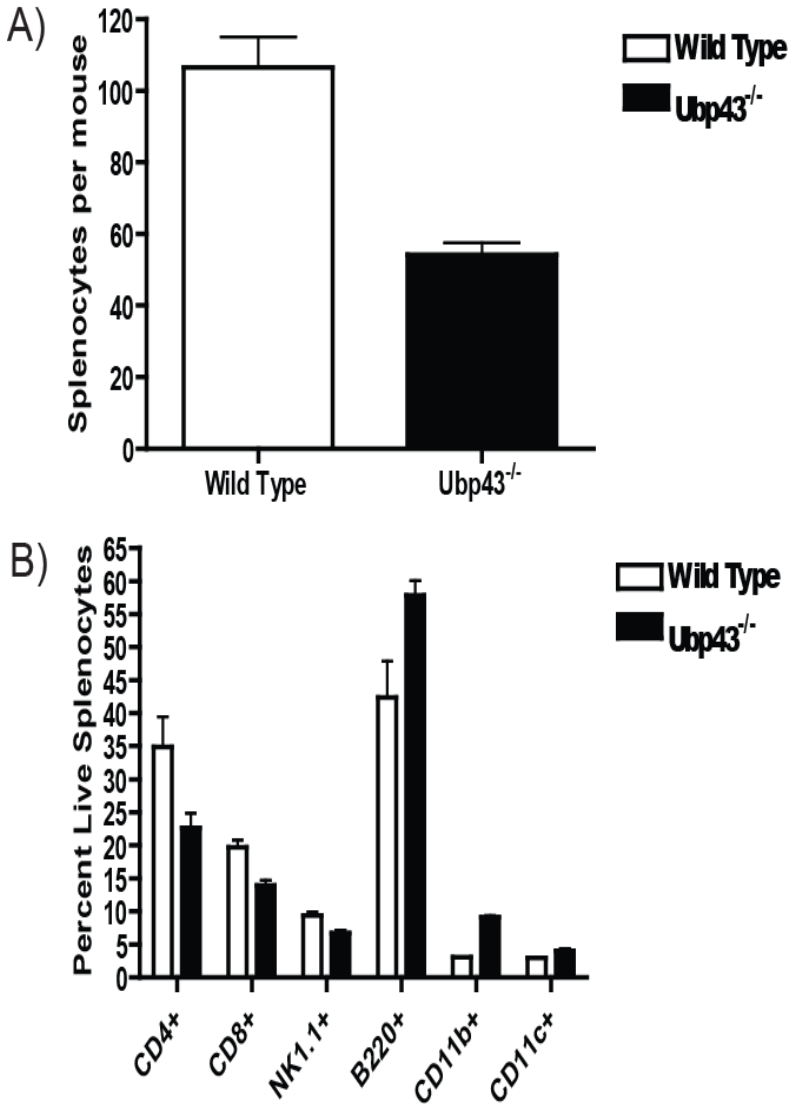

Figure 16: ***Characterization of the immune system in the Ubp43$^{-/-}$ mouse.*** WT and Ubp43$^{-/-}$ splenocytes were interrogated for total cell numbers per spleen (A) (n=3) and by cell type-specific antibodies followed by flow cytometric analysis (B) (n=3).

experiments, the discrepancy in Ubp43$^{-/-}$ mice has been explained by the observation the UBP43 can negatively inhibit type I IFN signaling in an ISG15-independent manner by attenuating IFN receptor proximal signal transduction (123).

Characterization of the immune system in the Ubp43$^{-/-}$ mouse

It is clear that the environment of the Ubp43$^{-/-}$ mouse facilitates a potent anti-viral response in both an ISG15 and IFN -dependent manner. Before addressing whether this environment is conducive to an augmented anti-tumor response, we first wanted to characterize the cellular components of the immune system as compared to WT mice.

Splenocytes from WT and Ubp43$^{-/-}$ mice were harvested and examined for immune system constituents by using cell type-specific antibodies (Figure 16b). Ubp43$^{-/-}$ spleens contained significantly fewer T cells considering they had 50 percent fewer total splenocytes (Figure 16a) and contained a slightly smaller frequency of both CD8^{+} and CD4^{+} cells. WT and Ubp43$^{-/-}$ mice contained equivalent frequencies of NK cells and CD11c^{+} cells while Ubp43$^{-/-}$ mice contained more B220^{+} and CD11b^{+} cells.

Ubp43$^{-/-}$ mice generate a higher frequency of tumor-specific T cells than WT mice after tumor vaccination

To determine whether T cells derived from Ubp43$^{-/-}$ mice resulted in enhanced anti-tumor efficacy in an adoptive transfer model, we initially examined the ability of Ubp43$^{-/-}$ mice to generate tumor-specific T cells. To explore this possibility, TVDLN lymphocytes from WT or Ubp43$^{-/-}$ C57BL/6 mice were stimulated with soluble anti-CD3 and expanded in low-dose (60 IU/mL) IL-2. The frequency of D5-specific T cells was determined as the frequency of IFN-γ^{+} T cells after restimulation with either D5 or the syngeneic sarcoma, MCA-310 (Figure 17a). Effector T cells generated in WT mice contained a small but significant frequency of D5-reactive T cells while those generated in Ubp43$^{-/-}$ mice contained a significantly ($p=0.01$) higher frequency. Consistent with the presence of more tumor-specific T cells, Ubp43$^{-/-}$ effector T cells produced significantly ($p=0.01$) more D5-specific GM-CSF compared to their WT counterparts (Figure 17b).

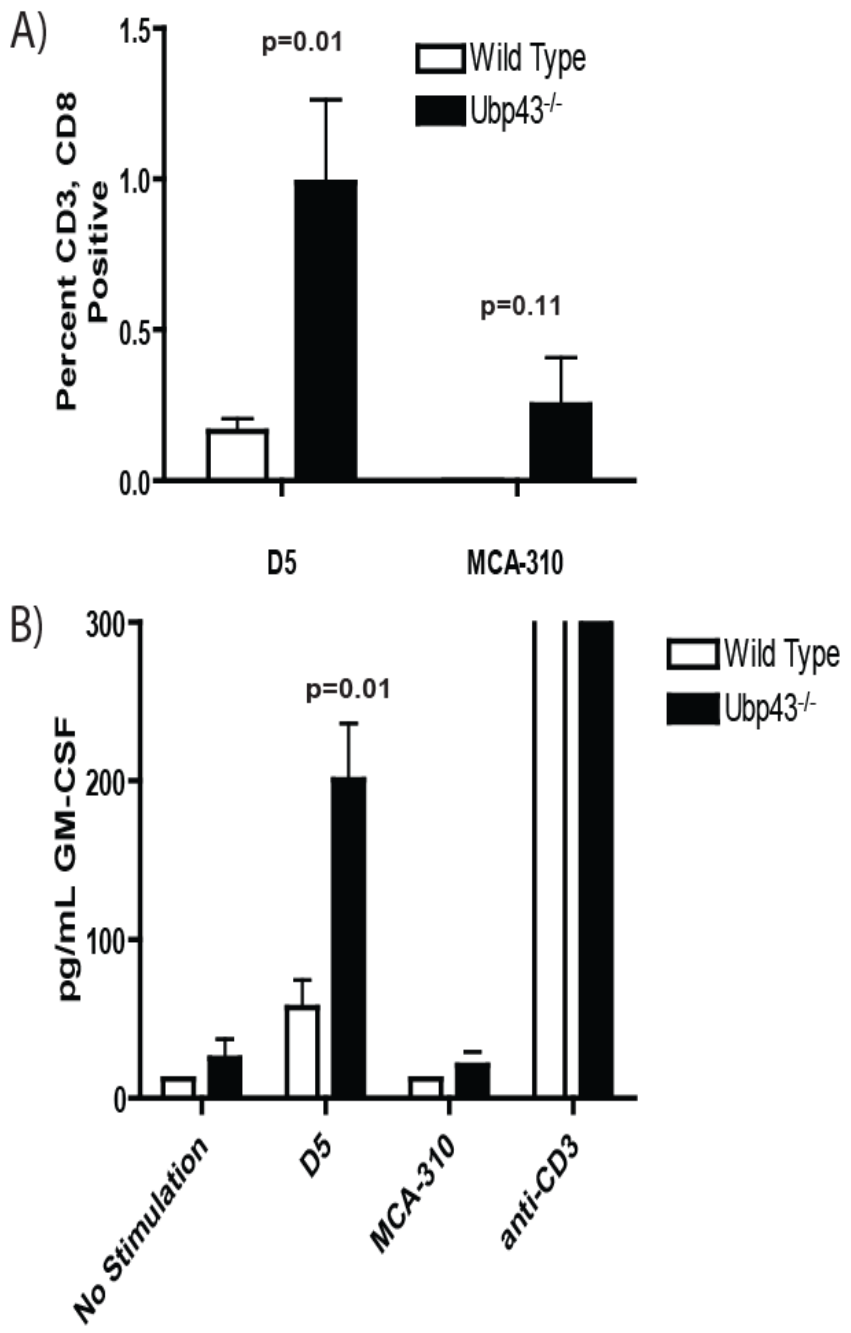

Figure 17: ***After tumor vaccination Ubp43$^{-/-}$ mice generate a higher frequency of tumor-specific T cells then WT mice.*** Effector T cells were generated as described in the materials and methods. 4 x 10^6 WT (open bars) or Ubp43$^{-/-}$ (solid bars) effector T cells were restimulated in vitro with 2 x 10^5 D5, MCA-310, or plate-bound anti-CD3. Frequency of IFN-γ CD8$^+$ effector T cells were assayed by intracellular IFN-γ staining after 5 hours of brefeldin A treatment and presented with the non stimulated background subtracted (A) (n=4). Supernatents were examined after 24 hours of tumor restimulation for GM-CSF secretion by ELISA (B) (n=3).

Tumor-specific Ubp43$^{-/-}$-generated effector T cells are polarized to a therapeutic type I cytokine milieu

The secretion of type I cytokines instead of type II cytokines by tumor-specific T cells is associated with a therapeutic anti-tumor immune response (167-170). Type I cytokines were represented by IFN-γ while type II cytokines were represented by IL-5 and IL-10. We found that Ubp43$^{-/-}$ effector T cells secrete significantly ($p<0.01$) more tumor-specific IFN-γ compared to WT T cells (Figure 18a). Importantly, the large amount of IFN-γ secreted by Ubp43$^{-/-}$ T cells can not be solely ascribed to the higher frequency of tumor-specific T cells observed in the deficient T cells (Ubp43$^{-/-}$ T cells contain <4 times more IFN-γ^{+} T cells yet secrete >10 times more IFN-γ then WT T cells). The higher mean fluorescence intensity (66.81 vs. 54.49) observed in D5-reactive Ubp43$^{-/-}$ is consistent with the observation that Ubp43$^{-/-}$ effector cells secrete more of the type I cytokine, IFN-γ, on a per-cell-basis. To determine whether the increase cytokine secretion also occurred for the type II cytokines IL-5 and IL-10 secretion was interrogated (Figure 18b). WT and Ubp43$^{-/-}$ effector cells produced equivalent amounts of the type II cytokines such that, when the higher frequency of tumor-specific T cells is taken into consideration, the Ubp43$^{-/-}$ T cells secreted less of the type II cytokines on a per-cell-basis. Thus Ubp43$^{-/-}$ effector T cells are polarized to a therapeutic type I cytokine milieu.

Ubp43$^{-/-}$-generated effector cells do not respond to UBP43 peptides

The dramatic difference in both tumor-specific frequency and secretion of IFN-γ between WT and Ubp43$^{-/-}$ led to the question of whether Ubp43$^{-/-}$ effector cells were

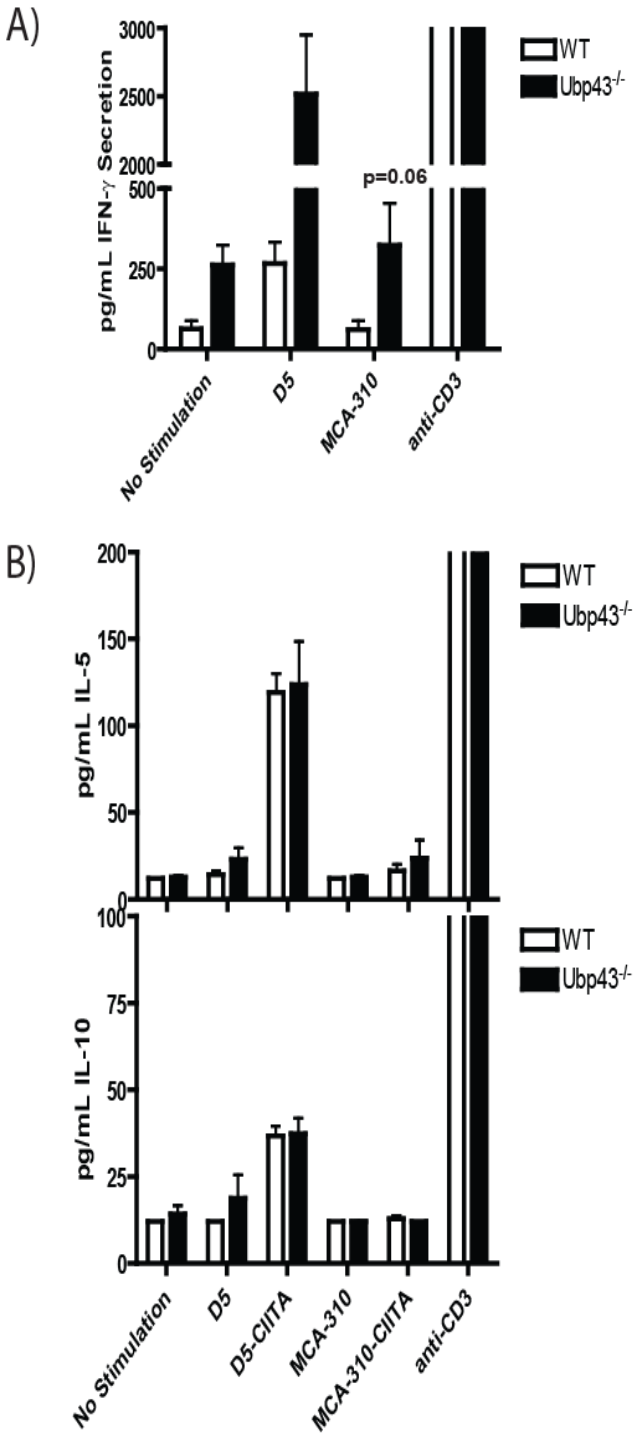

Figure 18: ***Tumor-specific Ubp43$^{-/-}$-generated effector T cells polarize to a therapeutic type I cytokine milieu.*** D5-specific effector T cells generated as described previously. 4 x 10^6 WT (open bars) or Ubp43$^{-/-}$ (solid bars) effector T cells were restimulated in vitro with 2 x 10^5 D5, D5-CIITA (D5 engineered to express the MHC class II transactivator) MCA-310, MCA-310-CIITA, or plate-bound anti-CD3. Twenty-four hours later supernatants were harvested and analyzed for IFN-γ (A) (n=9), IL-5 (top panel), or IL-10 (bottom panel) (B) (n=3) by ELISA.

responding to UBP43 peptides directly presented on the tumors. Thymic emigrants in the Ubp43$^{-/-}$ animal were not centrally tolerized to UBP43 peptides as it was absent in the Ubp43-deficient mice and undetectable in the thymus by both Western blot (86) and RT-PCR (KF, unpublished observations). Thus it was possible that Ubp43$^{-/-}$ effector T cells were simply responding to UBP43 peptides

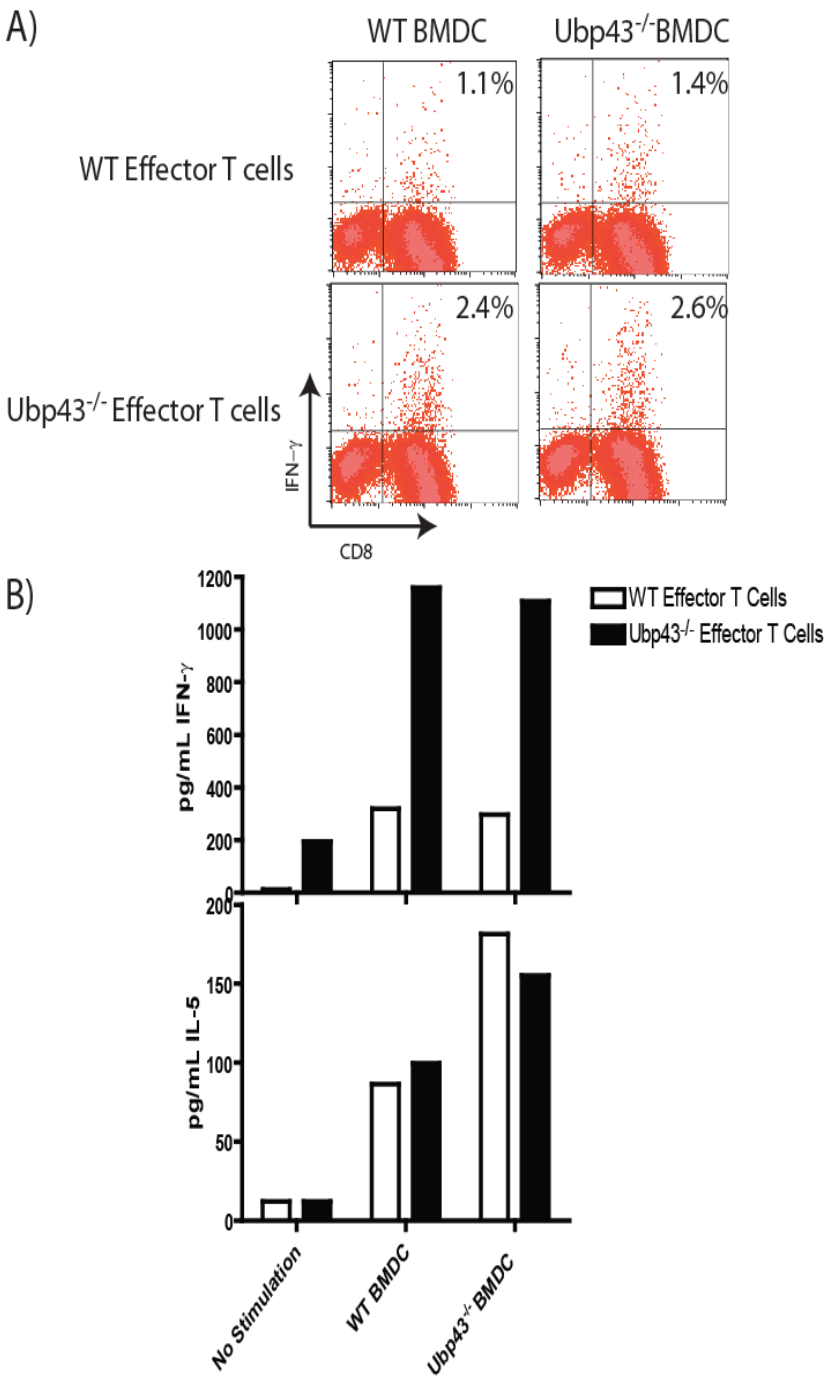

Figure 19: ***Ubp43$^{-/-}$-generated effector cells do not respond to UBP43 peptides.*** D5-specific effector T cells generated as described previously. 4 x 10^6 WT (open bars) or Ubp43$^{-/-}$ (solid bars) effector T cells were restimulated in vitro with 2 x 10^5 BMDCs derived from WT or Ubp43$^{-/-}$ mice. Frequency of IFN-γ CD8$^+$ effector T cells were assayed by intracellular IFN-γ staining after 5 hours of brefeldin A treatment (A). Twenty-four hours later supernatants were harvested and analyzed for IFN-γ (top panel), IL-5 (bottom panel) (B) by ELISA. Data are representative of two independent experiments.

presented as a xenoantigen by the D5 tumor. We considered this possibility unlikely because UBP43 was highly restricted in its tissue expression (86) and the Ubp43$^{-/-}$ effector T cells do not respond well to the unrelated tumor, MCA-310. But it was possible that the D5 melanoma would express UBP43 and not the MCA-310 sarcoma. Performed in a "criss-cross" fashion, WT and Ubp43$^{-/-}$ effector cells were

restimulated with either WT or Ubp43$^{-/-}$ bone-marrow-derived dendritic cells (BMDCs) followed by IFN-γ intracellular staining (Figure 19a). Because UBP43 was highly expressed in monocytes (86), WT BMDCs should present UBP43 peptides:MHC complexes while the Ubp43$^{-/-}$ BMDCs do not. While the Ubp43$^{-/-}$ effector T cells contain a higher frequency of IFN-γ^{+} T cells compared to WT effector T cells, as observed previously, the difference between their response to the WT or Ubp43$^{-/-}$ BMDCs were the same (Δ0.3%). This demonstrated that the Ubp43$^{-/-}$ effector T cells are not being stimulated by UBP43 peptides. These results were confirmed by examining IFN-γ and IL-5 secretion (Figure 19b).

Ubp43 $^{-/-}$-generated effector T cells mediate significantly enhanced therapeutic potential upon adoptive transfer into tumor-bearing mice

To determine if Ubp43$^{-/-}$ effector T cells could mediate enhanced anti-tumor potential, WT and Ubp43$^{-/-}$ effector T cells were adoptive transferred into WT mice bearing 3-day D5 pulmonary metastases (Figure 20). At the highest dose given, fifty million effector T cells, both WT and Ubp43$^{-/-}$ effector T cells could reduce the number of pulmonary metastases to almost zero. This was not surprising because when a high number of effector T cells are transferred, they will eliminate macroscopic metastases in this model. However, at smaller doses, Ubp43$^{-/-}$ effector T cells were significantly ($p < 0.05$) more effective at reducing the number of pulmonary D5 metastases than WT effector T cells. This indicates that effector T cells derived from Ubp43$^{-/-}$ mice are significantly more therapeutic then those derived from WT mice.

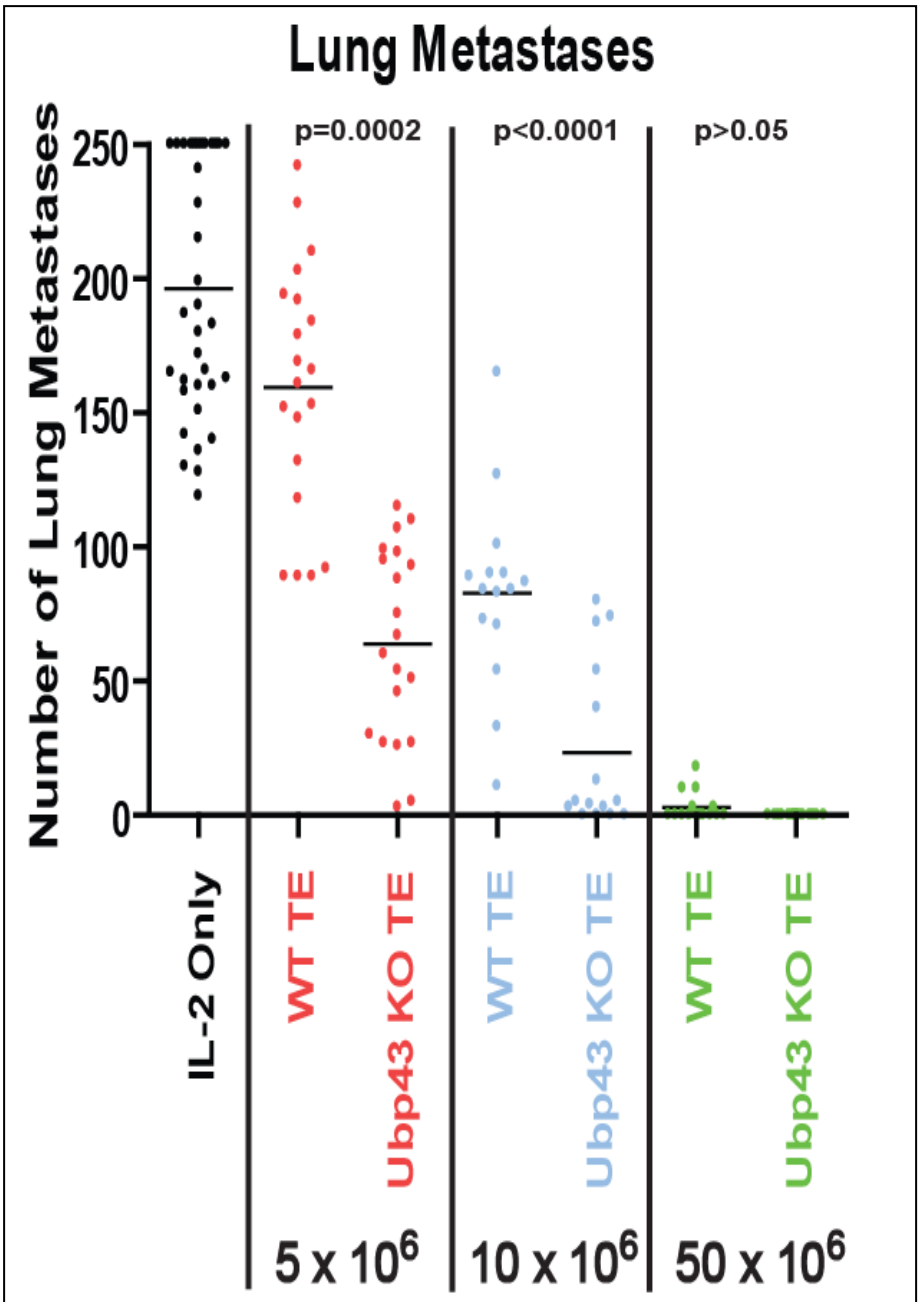

Figure 20: ***Ubp43 $^{-/-}$-generated effector T cells mediate significantly enhanced therapeutic efficacy upon adoptive transfer into tumor-bearing mice.*** WT mice were given 2.5 x 10^5 D5 i.v. three days prior to the adoptive transfer of 50 x 10^6, 20 x 10^6 or 5 x 10^6 of either WT or Ubp43$^{-/-}$ effector T cells. Experimental and control mice were given 90,000 IU IL-2 daily for four days following adoptive transfer. Mice were sacrificed by CO_2 narcosis ten days later; lungs were resected, fixed in Fekete's solution, and macroscopic metastases enumerated. Results summarize five independent experiments.

Enhanced therapeutic potential of Ubp43$^{-/-}$ effector T cells is independent of functional type I IFN signaling

One mechanism of enhanced anti-viral immunity observed in Ubp43$^{-/-}$ mice was enhanced sensitivity to type I IFNs. To determine if the enhanced anti-tumor function is

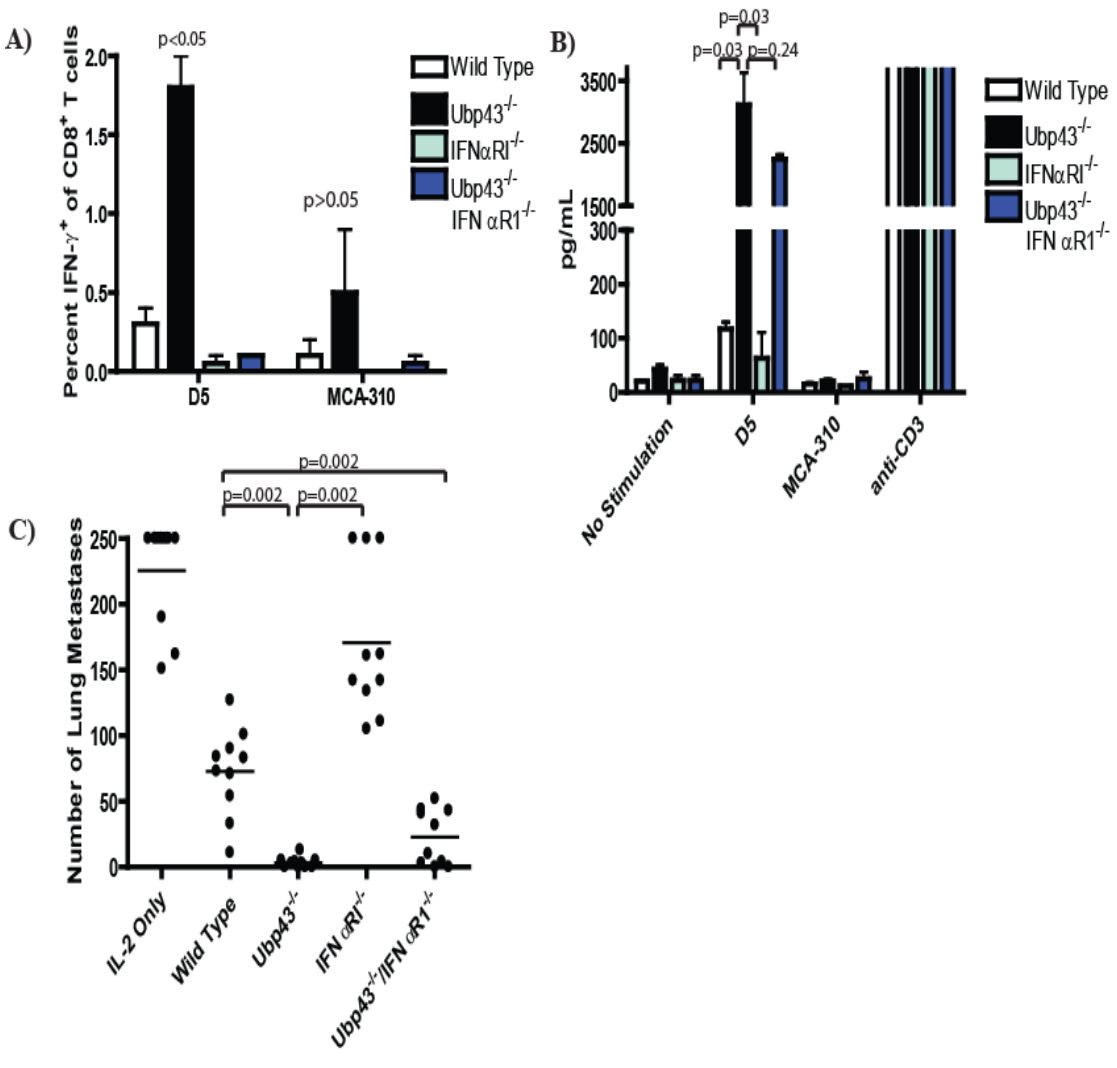

Figure 21: ***Enhanced therapeutic efficacy of Ubp43$^{-/-}$ effector T cells is independent of functional type I IFN signaling.*** Effector T cells were generated as described in the materials and methods. 4 x 10^6 WT (open bars), Ubp43$^{-/-}$ (black bars), IFNαR1$^{-/-}$ (light blue bars) or IFNαR1$^{-/-}$/ Ubp43$^{-/-}$ (dark blue bars) effector T cells were restimulated in vitro with 2 x 10^5 D5, MCA-310, or plate-bound anti-CD3 and supernatants were examined by IFN-γ ELISA (A) (n=3) or cells assayed by intracellular IFN-γ staining after 5 hours of brefeldin A treatment (B) (n=3). WT mice were given 2.5 x 10^5 D5 i.v. three days prior to the adoptive transfer of 5 x 10^6 of either WT, Ubp43$^{-/-}$, IFNαR1$^{-/-}$ or IFNαR1$^{-/-}$/ Ubp43$^{-/-}$ effector T cells. Experimental and control mice were given 90,000 IU IL-2 daily for four days following adoptive transfer. Mice were sacrificed by CO_2 narcosis ten days later; lungs were resected, fixed in Fekete's solution, and macroscopic metastases enumerated (C).

dependent on type I IFN signaling, effector T cells were generated in IFNαR1$^{-/-}$ and Ubp43$^{-/-}$/IFNαR1$^{-/-}$ mice in addition to WT and Ubp43$^{-/-}$ mice. The augmented frequency of tumor-specific T cells observed in Ubp43$^{-/-}$ effector T cells was ablated when type I IFN signaling was also deficient (Figure 21a). Striking however, this was not consistent with the augmented amount of IFN-γ released (Figure 21b) and therapeutic potential (Figure 21c). Ubp43$^{-/-}$ and Ubp43$^{-/-}$/IFNαR1$^{-/-}$ secreted the same amount of IFN-γ and mediated similar therapeutic potential after adoptive transfer into tumor-bearing mice. The discrepancy between these data prompted further investigation into the dependence of Ubp43$^{-/-}$-mediated augmentation of tumor-specific priming on functional IFN signaling. Therefore, we interrogated the frequency of effector T cells which recognize a dominant D5 rejection antigen, TRP-2$_{181-188}$ (171), after D5-G6 vaccination of WT, Ubp43$^{-/-}$, IFNαR1$^{-/-}$ and Ubp43$^{-/-}$/IFNαR1$^{-/-}$ mice (Figure 22a). Ubp43$^{-/-}$-generated effector T cells contained significantly more Trp2^{+} CD8^{+} T cells, an increase not observed in the Ubp43$^{-/-}$/IFNαR1$^{-/-}$ population. Similar results were obtained when pmel-1 TCR transgenic CD8^{+} T cells were used for reconstitution. Pmel^{+} CD8^{+} T cells specifically recognize another D5 rejection antigen, gp100$_{25-33}$ (172). After vaccination with D5-G6, the frequency of pmel-1^{+} CD8^{+} T cells increased dramatically in the Ubp43$^{-/-}$ mice compared to WT, IFNαR1$^{-/-}$ or Ubp43$^{-/-}$/IFNαR1$^{-/-}$ mice (Figure 22b). Taken together, these results clearly demonstrate that, while the augmented frequency of tumor-specific T cells generated in Ubp43$^{-/-}$ mice depends on type I IFN signaling, their therapeutic potential upon adoptive transfer is independent of functional IFN signaling.

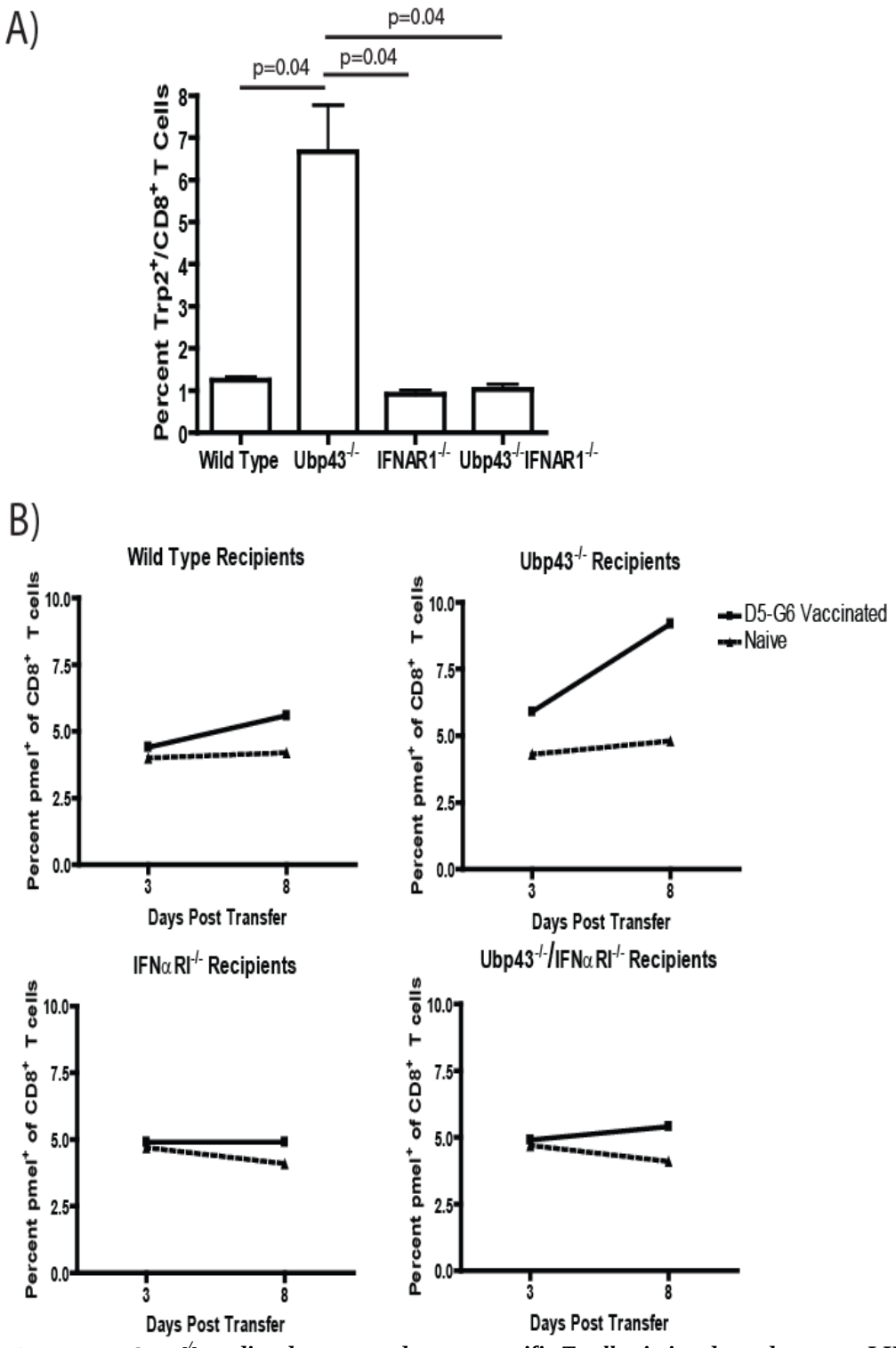

Figure 22: ***Ubp43$^{-/-}$-mediated augmented tumor-specific T cell priming depends on type I IFN signaling*****.** WT, Ubp43$^{-/-}$, IFNαR1$^{-/-}$ or IFNαR1$^{-/-}$/ Ubp43$^{-/-}$ effector T cells were assayed for the frequency of T cells recognizing TRP-2$_{181-188}$ by tetramer analysis (A) (n=3). Pmel^{+} CD8^{+} T cells were used for reconstitution of sublethally irradiated (500 cGy) WT, Ubp43$^{-/-}$, IFNαR1$^{-/-}$ or IFNαR1$^{-/-}$/ Ubp43$^{-/-}$ mice, vaccinated with D5-G6, and percent Pmel^{+} CD8^{+} T cells in the blood was determined three and eight days later (B).

Ubp43$^{-/-}$ T cells are intrinsically more therapeutic

The observation that the tumor-specific T cells generated in Ubp43$^{-/-}$/IFNαR1$^{-/-}$ mice could maintain augmented therapeutic potential without containing a higher frequency of tumor-specific T cells fostered experiments to determine whether the enhanced therapeutic potential was intrinsic to the Ubp43$^{-/-}$ T cells. To this end, pan T cells were purified from WT or Ubp43$^{-/-}$ splenocytes and used for reconstitution of RAG1$^{-/-}$ mice prior to D5-G6 vaccination. RAG1$^{-/-}$ mice contain no lymphocytes but are normal for other immune cells (173) such that priming occurred in essentially a "wild type" environment. The resultant effector T cells were interrogated for their frequency of Trp2^{+} CD8^{+} T cells as a marker for identifying tumor-specific T cells (Figure 23a). Regardless of their T cell composition, an equivalent frequency of tumor-specific cells was obtained from these mice yet secreted significantly ($p=0.001$) more tumor-specific IFN-γ (Figure 23b) and exhibited significantly ($p=0.002$) augmented therapeutic efficacy upon adoptive transfer into WT tumor-bearing mice (Figure 23b). Therefore, the generation of a higher frequency of tumor-specific T cells depends on Ubp43 deficiency in non-T cell compartment yet, even with equivalent numbers of tumor-specific T cells transferred, Ubp43$^{-/-}$ T cells were sufficient to significantly enhance anti-tumor function (Figure 24). These experiments demonstrated that Ubp43 deficiency in T cells is sufficient to impart enhanced anti-tumor potential to tumor-specific T cells.

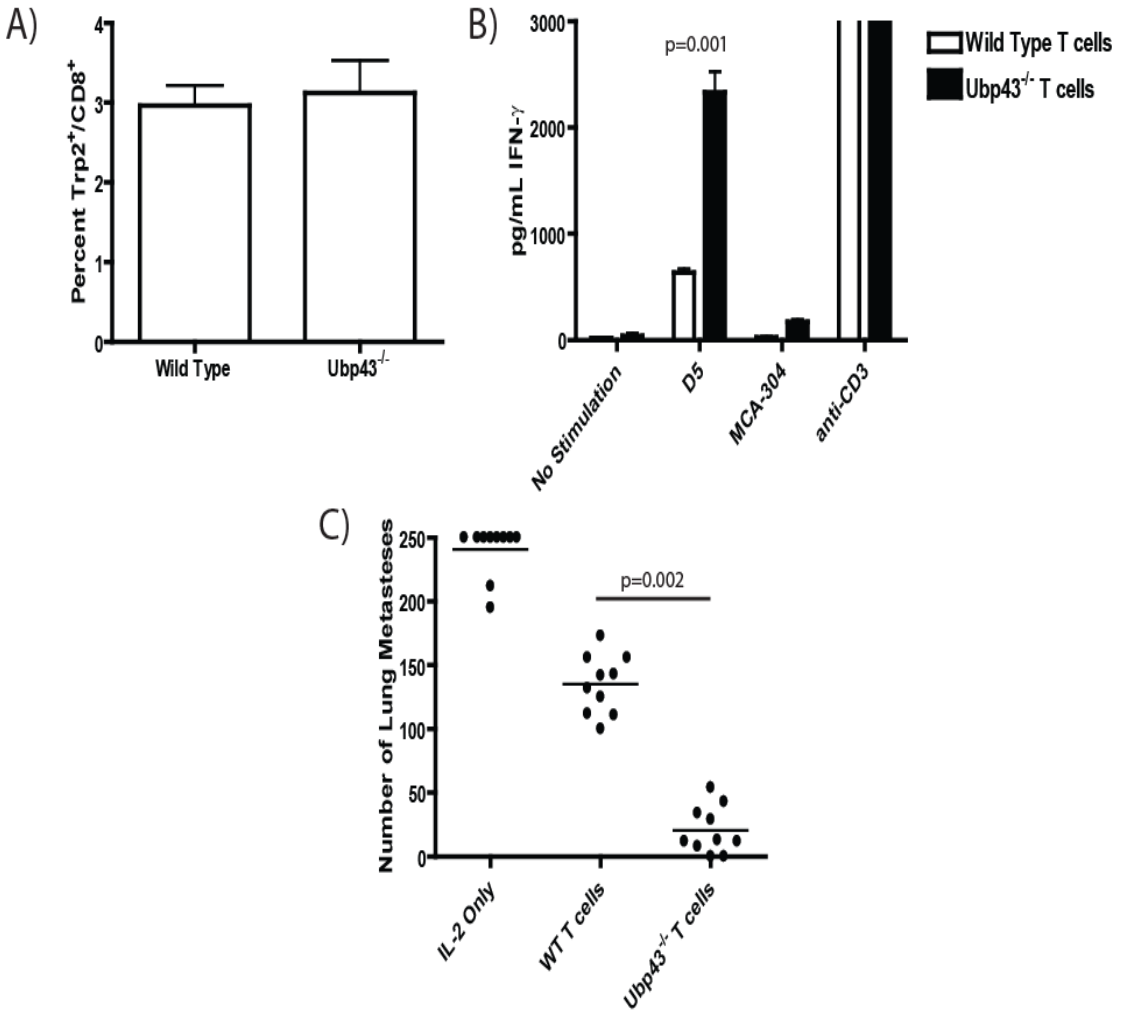

Figure 23: ***Ubp43$^{-/-}$ T cells are intrinsically more therapeutic.*** Purified WT or Ubp43$^{-/-}$ splenic T cells were used for reconstitution of RAG1$^{-/-}$ mice and D5-specific effector T cells generated as described previously. The frequency of T cells recognizing TRP-2$_{181-188}$ by tetramer analysis (A) (n=3). 4 x 10^6 WT (open bars) or Ubp43$^{-/-}$ (black bars) effector T cells primed in RAG1$^{-/-}$ host were restimulated in vitro with 2 x 10^5 D5, MCA-304, or plate-bound anti-CD3 and supernatants were examined by IFN-γ ELISA (B) and adoptively transferred to WT mice bearing 2.5 x 10^5 experimental pulmonary D5 metastases. WT mice were given 2.5 x 10^5 D5 i.v. three days prior to the adoptive transfer of 5 x 10^6 of either WT, Ubp43$^{-/-}$, IFNαR1$^{-/-}$ or IFNαR1$^{-/-}$/ Ubp43$^{-/-}$ effector T cells. Experimental and control mice were given 90,000 IU IL-2 daily for four days following adoptive transfer. Mice were sacrificed by CO_2 narcosis ten days later; lungs were resected, fixed in Fekete's solution, and macroscopic metastases enumerated (C).

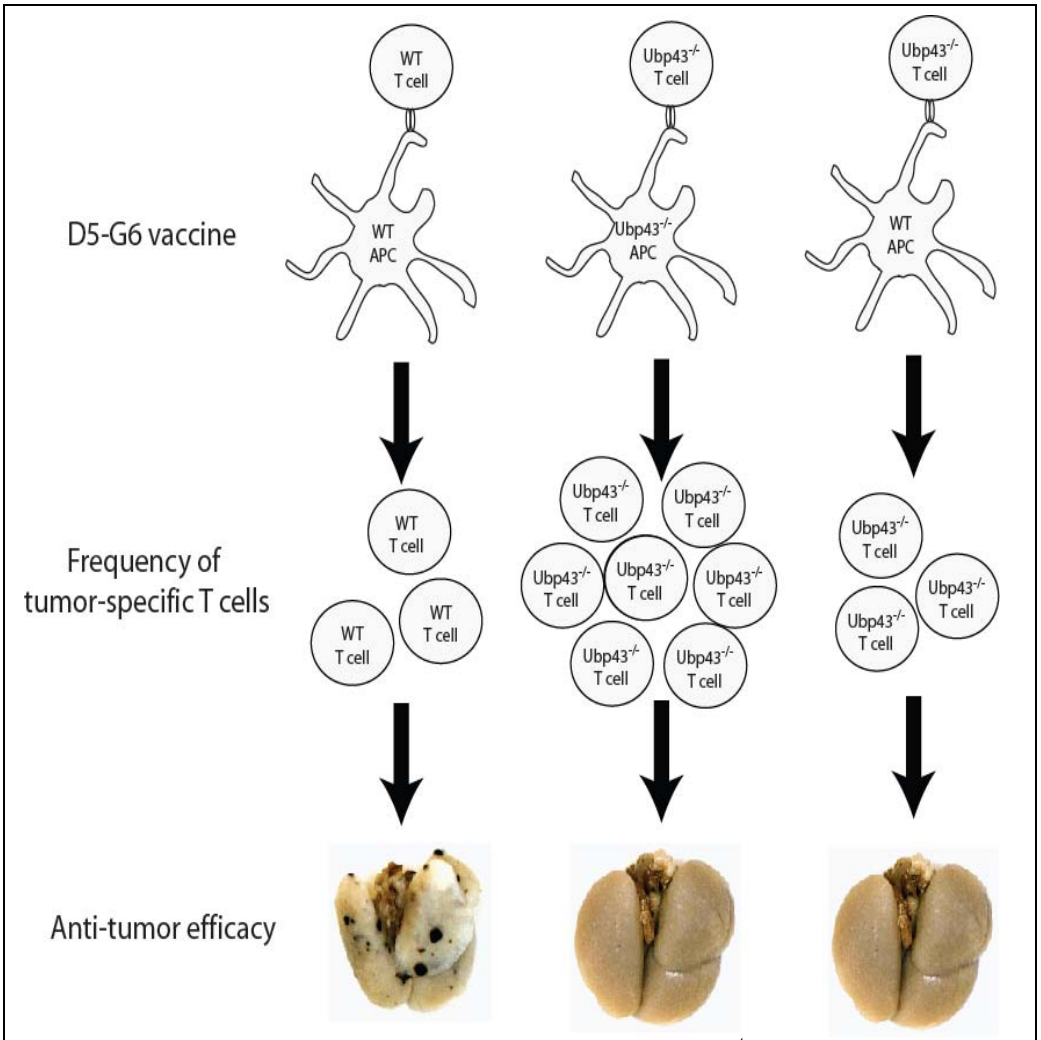

Figure 24: ***Illustration demonstrating intrinsic nature of Ubp43$^{-/-}$ T cells enhanced anti-tumor potential.*** After vaccination, WT or Ubp43$^{-/-}$ pan T cells used for reconstitution of a RAG1$^{-/-}$ host generate a similar frequency of tumor-specific T cells yet mediate enhanced therapeutic potential after adoptive transfer to a tumor-bearing animal.

Ubp43$^{-/-}$ T cells have deregulated FoxP3 induction and insensitivity to Treg-mediated suppression

We next attempted to examine the nature of the Ubp43$^{-/-}$-mediated enhanced anti-tumor function. Regulatory T cells (Treg), characterized by their expression of the FoxP3 transcription factor, are responsible for suppressing the anti-tumor immune response (174). Therefore, we examined whether an altered Treg response could explain

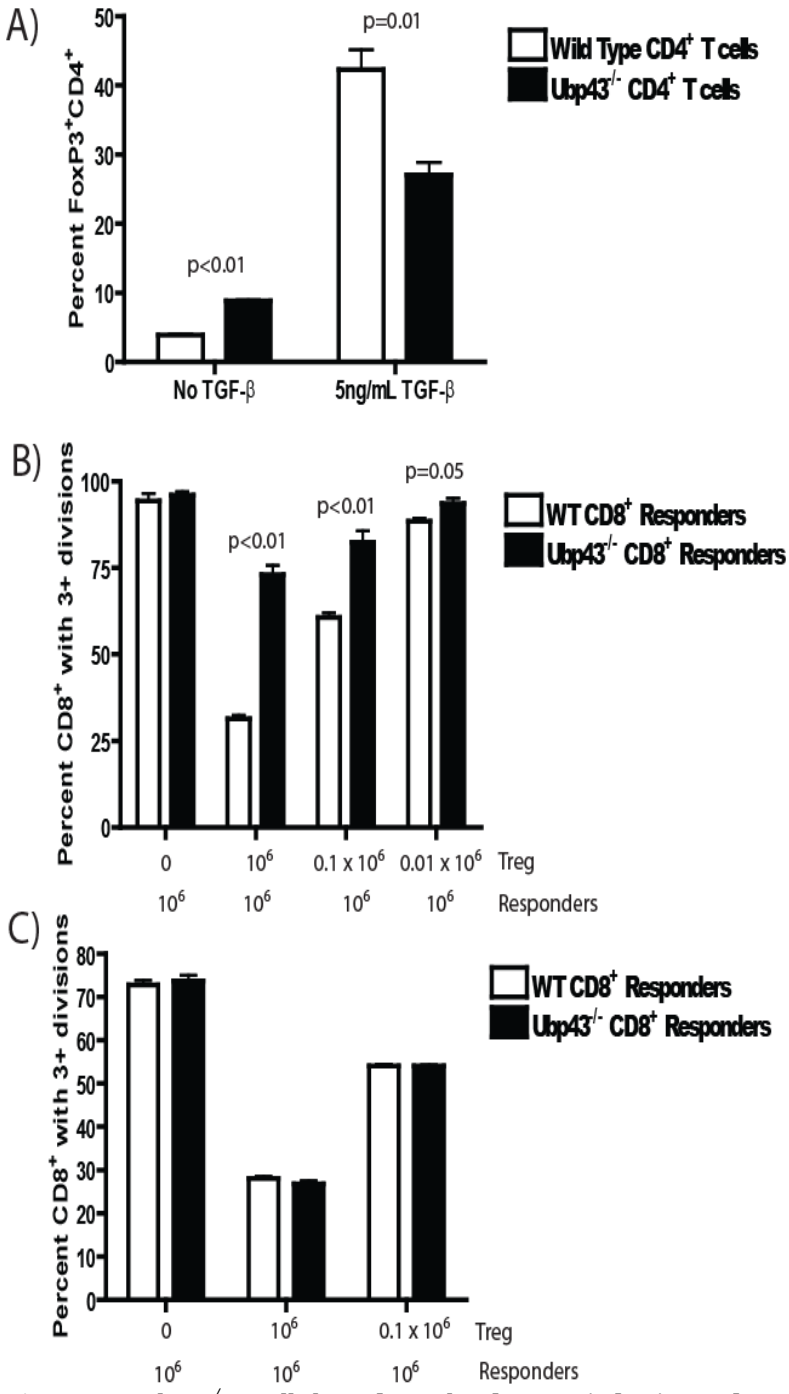

Figure 25: ***Ubp43$^{-/-}$ T cells have deregulated FoxP3 induction and insensitivity Treg-mediated suppression.*** Purified WT or Ubp43$^{-/-}$ splenic CD4^{+} T cells were cultured in vitro with plate-bound anti-CD3 (2C11), 600 U/mL IL-2, and with/without 5ng/mL. Percent FoxP3^{+} CD4^{+} cells were determined by cytometric analysis (A) (n=3). Purified splenic CD4^{+} T cells from the FoxP3-GFP transgenic animal were induced for FoxP3 expression as above and flow sorted to >95% purities. These were co-cultured at the indicated amounts with CFSE labeled WT or Ubp43$^{-/-}$ splenocytes (B) (n=2) or purified CD8^{+} T cells (C) (n=2). CD8^{+} T cell division was determined by cytometric analysis.

the enhanced anti-tumor effect of Ubp43 deficient cells. Because Ubp43$^{-/-}$ effector T cells are polarized to a type I cytokine profile which antagonizes the TGF-β-mediated induction of FoxP3^{+} Tregs (175), we examined the ability of TGF-β to induce FoxP3 expression in WT and Ubp43$^{-/-}$ CD4^{+} T cells (Figure 25a). We found that while the basal levels of FoxP3 expression were significantly ($p<0.01$) higher in Ubp43$^{-/-}$ CD4^{+} T cells, the ability of

TGF-β to induce FoxP3^{+} cells was significantly ($p<0.01$) hampered compared to WT CD4^{+} T cells. We also examined whether Ubp43$^{-/-}$ CD8^{+} T cells were resistant to Treg mediated suppression (Figure 25b,c). Interestingly, Ubp43$^{-/-}$ CD8^{+} T cells were significantly ($p<0.01$) more resistant to Treg-mediate suppression (Figure 25b). However, this only occurred when in the presence of Ubp43$^{-/-}$ splenic antigen presenting cells (APC) (Figure 25c) suggesting that that the Ubp43$^{-/-}$ APC were critical for the resistance against Treg-mediated suppression. Although additional studies are necessary for better dissection, it is clear that the Ubp43$^{-/-}$ environment impacts the ability of TGF-β to induce FoxP3 expression and the sensitivity to Treg-mediated suppression.

Discussion

Based on observations of enhanced anti-viral and bacterial responses in Ubp43$^{-/-}$ mice, we designed experiments that confirmed that this state is also conducive to enhanced anti-tumor efficacy. The presence of UBP43 attenuates anti-viral potential in two ways: By reversing the conjugation of ISG15, a condition associated with viral resistance, and by directly inhibiting the cellular response to type I IFNs. The absence of Ubp43 therefore ablated this negative regulatory response to viral challenges leading to enhanced anti-viral potential. Because the enhanced anti-tumor potential could be due to a multitude of factors (i.e. any number of ISG15-modified proteins and/or the pleiotropic effects of type I IFNs), we used a mouse model which does not necessitate a clear understanding of intracellular molecular mechanisms to determine if the deficiency of Ubp43 facilitates enhanced anti-tumor potential.

After determining these two mechanisms are independent of each other (ISG15 did not directly regulate the response to type I IFNs), an observation also made by others (122, 123), we examined the effect of Ubp43-deficiency on the anti-tumor response. We found that after tumor vaccination, Ubp43$^{-/-}$ TVDLN contained significantly more tumor-specific T cells then TVDLN from WT mice. Moreover, the tumor-specific T cells were polarized to a therapeutic type I cytokine profile. Importantly, after adoptive transfer into tumor-bearing mice, Ubp43$^{-/-}$ effector T cells were significantly more therapeutic then WT effector T cells.

We further demonstrated that Ubp43$^{-/-}$ effector T cells are sufficient to mediate enhanced anti-tumor efficacy. Purified WT or Ubp43$^{-/-}$ T cells used for reconstitution of RAG1$^{-/-}$ hosts generated a similar frequency of tumor-specific T cells after vaccination.

These same Ubp43$^{-/-}$ T cells contained enhanced therapeutic potential compared to WT T cells. We also demonstrated that while the enhanced anti-tumor efficacy was independent of a functional type I IFN signaling, the augmented generation of tumor-specific T cells in Ubp43$^{-/-}$ mice was dependent on type I IFN signaling. Because type I IFNs were shown to increase cross-priming of tumor-specific CD8^{+} T cells (154, 155), the enhanced IFN signaling in Ubp43$^{-/-}$ mice may be responsible for this observation. Further studies are necessary to formally demonstrate whether augmented IFN sensitivity in Ubp43$^{-/-}$ antigen-presenting cells results in increased tumor-specific priming.

Similarly, it is still to be determined whether the enhanced anti-tumor efficacy of Ubp43$^{-/-}$ T cells was dependent on the deregulated ISG15 conjugation. The deficiency of UBP43 enhanced the viral response in either an IFN or ISG15 –dependent manner. We identified that the enhanced anti-tumor efficacy was not dependent on the augmented type I IFN signaling suggesting that it may be dependent on ISG15. This can be tested by using mice doubly deficient in Ubp43 and ISG15. One would hypothesize that the absence of ISG15 would have a minimal effect on the increased frequency of tumor-specific T cells observed after vaccinating Ubp43$^{-/-}$ mice but would ablate the sufficiency of Ubp43$^{-/-}$ T cells to mediate enhanced anti-tumor potential.

Deregulated ISG15 conjugation may also explain the observation that Ubp43$^{-/-}$ CD8^{+} T cells were resistant to Treg-mediated suppression, but only in the presence of Ubp43$^{-/-}$ antigen-presenting cells. Others have reported that TLR agonist-induced inflammatory cytokine and chemokine gene expression is augmented in Ubp43$^{-/-}$ bone-marrow macrophages (85). IL-6 is one of the genes with increased expression. IL-6 can polarize naïve CD4^{+} T cells away from TGF-β-induced Treg differentiation and toward

inflammatory Th17 $CD4^+$ T cells (176, 177). Indeed, $Ubp43^{-/-}$ T cells did demonstrate evidence of aberrant Treg induction. $Ubp43^{-/-}$ $CD4^+$ T cells contained a higher basal frequency of $FoxP3^+$ cells, a marker for identifying Tregs, yet were resistant to FoxP3 induction by TGF-β. Whether this is due to altered cytokine secretion from $Ubp43^{-/-}$ immune cells still remains to be determined.

Similarly, the altered cytokine gene expression in $Ubp43^{-/-}$ immune cells could confer the observed resistance to suppression by purified Tregs. However, it is also possible that the deregulated ISG15 conjugation could play a more direct role. ISG15 conjugation has been demonstrated to modulate TRAF6-mediated signaling (178), an important player in TGF-β sensitivity (179), so that the deregulated ISG15 conjugation observed in Ubp43-deficient cells may be responsible for this observation. Although the Treg compartment is clearly affected by the absence of Ubp43, further experimentation is necessary to better understand the effect of ISG15 conjugation and UBP43 on Treg development and function.

It was not clear how the $Ubp43^{-/-}$ effector T cells mediate enhanced anti-tumor function. Preliminary data suggests that they are hypersensitive to stimulation through the T cell receptor (KF, preliminary observations), but studies using $Ubp43^{-/-}$ TCR transgenic T cells would be necessary to formally demonstrate this. Further, it was not determined whether the $Ubp43^{-/-}$ effector T cells were the terminal cell population mediating the enhanced anti-tumor function. That is, due to the large amounts of tumor-specific IFN-γ secreted, it was possible that these cells exert their enhanced function by recruiting natural killer cells to the tumor which, in turn, serve as the terminal effector population for tumor regression.

A better understanding of the mechanism which mediates the enhanced anti-tumor function observed in tumor-specific Ubp43$^{-/-}$ effector T cells will facilitate the translation of this treatment strategy to the clinic. In particular, if the enhanced therapeutic potential is dependent on ISG15 and, by extension ISG15 conjugation, one could find small molecules that specifically inhibit UBP43 isopeptidase activity and determine if it enhances the anti-tumor efficacy of adoptively transferred T cells. Taken together, these data demonstrate a novel strategy to enhance the anti-tumor potential of adoptively transferred T cells.

CONCLUDING REMARKS

Recent clinical trials have demonstrated the potential of using adoptive T cell transfer for the treatment of progressive metastatic disease. At the heart of successful adoptive T cell immunotherapy is the need for a better understanding of the relationship between the patient's immune system and the progressing disease. By the time cancer has presented itself in the clinic there likely has been a significant amount of interaction between the immune system and the neoplastic disease. The interaction has culminated to a point which the immune system is no longer able to properly restrict its growth. Multiple reasons have been proposed for this malfunction including tumor antigen-loss variants, reduced expression of MHC molecules, and the induction of immune tolerance. Regardless, with the selective pressure of the immune system abated, the disease can then progress. Adoptive T cell immunotherapy attempts to "reprogram" the patient's immune system so that it can properly recognize and eliminate the disease. Because there is an opportunity to manipulate the tumor-specific T cells ex vivo, in order to reinvigorate their anti-tumor function, adoptive transfer has a strategic advantage due to accessibility to the T cells.

Mouse models of adoptive immunotherapy attempt to recapitulate the tumor-immune system relationship in order develop strategies which can be employed in the clinic. Due to the ability to independently interrogate the priming phase from the effector phase of the anti-tumor immune response, adoptive transfer models are especially informative. Even though the efficiency of priming (i.e. the frequency of tumor-specific

T cells) has direct ramifications in the effector phase, the fact that these two phases occur in separate mice allows for their independent interrogation.

The demonstration of a 72 percent objective clinical response rate in patients with advanced melanoma highlights the recent success of adoptive T cell immunotherapy. Presented in this thesis are mouse studies addressing how to further enhance the efficacy of adoptive T cell immunotherapy. However, because the treatment is patient-specific, research directed to improve upon these results is limited by the cost. Nevertheless, finding strategies to improve its efficacy may encourage more prevalent research and use. Preclinical mouse models provide a more cost effective tool to examine strategies to be potentially examined using the more costly clinical trials. The work presented in this body of work used a mouse model of adoptive transfer to elucidate strategies to potentially enhance adoptive immunotherapy.

Based on observations made in prior successful clinical trials, we found that tumor-specific $CD4^+$ T cell help was required for an effective anti-tumor response when tumor-bearing lymphopenic hosts were treated with adoptively transferred T cells. In this model, the recipient $RAG1^{-/-}$ mice were incapable of generating de novo T cells such that the anti-tumor effect was completely mediated by the adoptively transferred population. The presence of tumor-specific $CD4^+$ T cells prolonged both the maintenance of tumor-specific $CD8^+$ T cells and the accumulation of systemic tumor burden in the mice. These data highlighted the necessity of ensuring that tumor-specific $CD4^+$ T cells are included within the population of the adoptively transferred T cells used for treatment of advanced metastatic disease. Although the mechanism for this effect was not identified, the contribution of $CD4^+$ T cells to the anti-tumor response was appreciated. Further, the

specific contribution and requirement of tumor-specific $CD4^+$ T cells have not been examined in a tumor-bearing lymphopenic host that lacks endogenous T cells. Suspecting a significant contribution from activated $CD4^+$ T cells, we examined the necessity and nature of $CD4^+$ T cell help in this setting. Researchers have long suspected a significant contribution from $CD4^+$ T cells but the nature of the $CD4^+$ T cell help remained elusive. Nevertheless, prior clinical trials have induced activation of $CD4^+$ T cells in a tumor nonspecific manner. Activation of $CD4^+$ T cells by vaccination with the pan-DR-epitope (PADRE) (180) was one such strategy. While PADRE vaccinations have enhanced the $CD8^+$ T cell anti-tumor response in some reports (181, 182), the data presented here suggests that, when capitalizing on lymphopenic conditioning to treat tumor-bearing hosts, the most effective response will be mediated by tumor-specific $CD4^+$ T cells. Even activated $CD4^+$ T cells specific to a different tumor did not provide effective $CD4^+$ T cell help compared to the cognate tumor-specific $CD4^+$ T cells. This suggested that the tumor-specific $CD4^+$ T cells facilitated an enhanced response in a tumor-specific manner, such as dendritic cell licensing, cytokine support, or tumor trafficking.

A recent case report described a melanoma patient that was treated with NY-ESO-specific $CD4^+$ T cells without prior conditioning regimens and who experienced a long term complete remission (183). The authors found evidence of nascent $CD8^+$ T cells specific to tumor antigens unrelated to NY-ESO that did not exist prior to adoptive transfer of the $CD4^+$ T cells. Because the patient was treated with only $CD4^+$ T cells, these data supported the hypothesis that tumor-specific $CD4^+$ T cells are capable of initiating occult anti-tumor responses from the endogenous $CD8^+$ T cell pool. In our

RAG1$^{-/-}$ model, long-term anti-tumor immunity could not be permanently established in the absence of endogenous T cells suggesting that the ability to generate de novo tumor-specific T cells is critical for a long-term anti-tumor response. These data further suggested that lymphopenic conditioning of the tumor-bearing host does not significantly alter the requirement of tumor-specific CD4^{+} T cells for the most effective treatment.

After establishing that the most anti-tumor effect occurs when both tumor-specific CD4^{+} and CD8^{+} T cells are adoptively transferred, we explored other strategies which, like lymphopenic conditioning, enhanced the efficacy of adoptively transferred T cells. The final chapter demonstrated that T cells deficient in Ubp43$^{-/-}$ were sufficient to mediate enhanced therapeutic efficacy compared to WT T cells. We determined that this was independent of the augmented type I IFN signaling previously observed in Ubp43$^{-/-}$ mice. We did, however, demonstrate that the enhanced priming of tumor-specific T cells in D5-G6 vaccinated Ubp43$^{-/-}$ mice was dependent on functional type I IFN signaling. Importantly, we found that the enhanced anti-tumor effect was intrinsic to the Ubp43$^{-/-}$ T cells creating the possibility that reducing Ubp43 expression in patient's T cells prior to adoptive transfer will enhance their anti-tumor function.

Whether the inhibition of UBP43 expression in WT T cells will mimic the enhanced anti-tumor function in genetically ablated T cells still remains to be determined. If so, to enhance the efficacy of currently established adoptive transfer protocols, knock-down of Ubp43 with delivery of short-hairpins expressing Ubp43-specific siRNA may be utilized. One potential caveat with this strategy is the technical challenge to decrease Ubp43 expression in a significant number of the tumor-specific T cells, which only constitute a fraction of the T cells used for treatment (2 – 5 percent MART1$_{26-35}$ reactive

$CD8^+$ T cells (80)). To circumvent this potential pit-fall, recent research has focused on the ectopic delivery of T cell receptors which recognize tumor-associated antigens (184, 185). To ensure tumor-specific T cells have reduced Ubp43 expression, T cells could be co-transduced with constructs expressing these T cells receptors and Ubp43-specific short-hairpins. The possibility that Ubp43 knock-down strategies can be used in conjunction with other ways to enhance the anti-tumor immune response adds to the potential clinical ramifications of these observations.

Taken together, these data presented in this body of work demonstrates several strategies to enhance the efficacy of adoptive T cell immunotherapy of cancer. Both strategies have ramifications on the design of clinical trial protocols using adoptive immunotherapy to treat advanced metastatic melanoma.

REFERENCES

1. Dudley, M. E., Yang, J. C., Sherry, R., Hughes, M. S., Royal, R., Kammula, U., Robbins, P. F., Huang, J., Citrin, D. E., Leitman, S. F., Wunderlich, J., Restifo, N. P., Thomasian, A., Downey, S. G., Smith, F. O., Klapper, J., Morton, K., Laurencot, C., White, D. E., and Rosenberg, S. A. Adoptive Cell Therapy for Patients With Metastatic Melanoma: Evaluation of Intensive Myeloablative Chemoradiation Preparative Regimens. J Clin Oncol, 2008.

2. Ghiringhelli, F., Larmonier, N., Schmitt, E., Parcellier, A., Cathelin, D., Garrido, C., Chauffert, B., Solary, E., Bonnotte, B., and Martin, F. CD4+CD25+ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. Eur J Immunol, *34:* 336-344, 2004.

3. Ikezawa, Y., Nakazawa, M., Tamura, C., Takahashi, K., Minami, M., and Ikezawa, Z. Cyclophosphamide decreases the number, percentage and the function of CD25+ CD4+ regulatory T cells, which suppress induction of contact hypersensitivity. J Dermatol Sci, *39:* 105-112, 2005.

4. Brode, S., Raine, T., Zaccone, P., and Cooke, A. Cyclophosphamide-induced type-1 diabetes in the NOD mouse is associated with a reduction of CD4+CD25+Foxp3+ regulatory T cells. J Immunol, *177:* 6603-6612, 2006.

5. Park, J. H., Adoro, S., Lucas, P. J., Sarafova, S. D., Alag, A. S., Doan, L. L., Erman, B., Liu, X., Ellmeier, W., Bosselut, R., Feigenbaum, L., and Singer, A. 'Coreceptor tuning': cytokine signals transcriptionally tailor CD8 coreceptor expression to the self-specificity of the TCR. Nat Immunol, *8:* 1049-1059, 2007.

6. Sojka, D. K., Bruniquel, D., Schwartz, R. H., and Singh, N. J. IL-2 secretion by CD4+ T cells in vivo is rapid, transient, and influenced by TCR-specific competition. J Immunol, *172:* 6136-6143, 2004.

7. Gattinoni, L., Finkelstein, S. E., Klebanoff, C. A., Antony, P. A., Palmer, D. C., Spiess, P. J., Hwang, L. N., Yu, Z., Wrzesinski, C., Heimann, D. M., Surh, C. D., Rosenberg, S. A., and Restifo, N. P. Removal of homeostatic cytokine sinks by lymphodepletion enhances the efficacy of adoptively transferred tumor-specific CD8+ T cells. J Exp Med, *202:* 907-912, 2005.

8. Ehrlich, P. [About the current state of carcinoma research]. Ned Tijdschr Geneeskd, *5:* 273-290, 1909.

9. Burnet, F. M. The concept of immunological surveillance. Prog Exp Tumor Res, *13:* 1-27, 1970.

10. Thomas, L. Cellular and Humoral Aspects of the Hypersensitive State. *In:* E. b. H. Lawrence (ed.), Reactions to homologous tissue antigens in relation to hypersensitivity, pp. 529–532. New York: Hoebner-Harper, 1959.

11. Stutman, O. Tumor development after 3-methylcholanthrene in immunologically deficient athymic-nude mice. Science, *183:* 534-536, 1974.

12. Schroder, K., Hertzog, P. J., Ravasi, T., and Hume, D. A. Interferon-gamma: an overview of signals, mechanisms and functions. J Leukoc Biol, *75:* 163-189, 2004.

13. Dighe, A. S., Richards, E., Old, L. J., and Schreiber, R. D. Enhanced in vivo growth and resistance to rejection of tumor cells expressing dominant negative IFN gamma receptors. Immunity, *1:* 447-456, 1994.

14. Kaplan, D. H., Shankaran, V., Dighe, A. S., Stockert, E., Aguet, M., Old, L. J., and Schreiber, R. D. Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. Proc Natl Acad Sci U S A, *95:* 7556-7561, 1998.

15. Street, S. E., Cretney, E., and Smyth, M. J. Perforin and interferon-gamma activities independently control tumor initiation, growth, and metastasis. Blood, *97:* 192-197, 2001.

16. Street, S. E., Trapani, J. A., MacGregor, D., and Smyth, M. J. Suppression of lymphoma and epithelial malignancies effected by interferon gamma. J Exp Med, *196:* 129-134, 2002.

17. Russell, J. H. and Ley, T. J. Lymphocyte-mediated cytotoxicity. Annu Rev Immunol, *20:* 323-370, 2002.

18. van den Broek, M. E., Kagi, D., Ossendorp, F., Toes, R., Vamvakas, S., Lutz, W. K., Melief, C. J., Zinkernagel, R. M., and Hengartner, H. Decreased tumor surveillance in perforin-deficient mice. J Exp Med, *184:* 1781-1790, 1996.

19. Smyth, M. J., Thia, K. Y., Street, S. E., Cretney, E., Trapani, J. A., Taniguchi, M., Kawano, T., Pelikan, S. B., Crowe, N. Y., and Godfrey, D. I. Differential tumor surveillance by natural killer (NK) and NKT cells. J Exp Med, *191:* 661-668, 2000.

20. Smyth, M. J., Thia, K. Y., Street, S. E., MacGregor, D., Godfrey, D. I., and Trapani, J. A. Perforin-mediated cytotoxicity is critical for surveillance of spontaneous lymphoma. J Exp Med, *192:* 755-760, 2000.

21. Shankaran, V., Ikeda, H., Bruce, A. T., White, J. M., Swanson, P. E., Old, L. J., and Schreiber, R. D. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature, *410:* 1107-1111, 2001.

22. Maleckar, J. R. and Sherman, L. A. The composition of the T cell receptor repertoire in nude mice. J Immunol, *138:* 3873-3876, 1987.

23. Ikehara, S., Pahwa, R. N., Fernandes, G., Hansen, C. T., and Good, R. A. Functional T cells in athymic nude mice. Proc Natl Acad Sci U S A, *81:* 886-888, 1984.

24. Hunig, T. and Bevan, M. J. Specificity of cytotoxic T cells from athymic mice. J Exp Med, *152:* 688-702, 1980.

25. Birkeland, S. A., Storm, H. H., Lamm, L. U., Barlow, L., Blohme, I., Forsberg, B., Eklund, B., Fjeldborg, O., Friedberg, M., Frodin, L., and et al. Cancer risk after renal transplantation in the Nordic countries, 1964-1986. Int J Cancer, *60:* 183-189, 1995.

26. Sheil, A. G., Flavel, S., Disney, A. P., and Mathew, T. H. Cancer development in patients progressing to dialysis and renal transplantation. Transplant Proc, *17:* 1685-1688, 1985.

27. Pham, S. M., Kormos, R. L., Landreneau, R. J., Kawai, A., Gonzalez-Cancel, I., Hardesty, R. L., Hattler, B. G., and Griffith, B. P. Solid tumors after heart transplantation: lethality of lung cancer. Ann Thorac Surg, *60:* 1623-1626, 1995.

28. Penn, I. Malignant melanoma in organ allograft recipients. Transplantation, *61:* 274-278, 1996.

29. Sheil, A. G. Cancer after transplantation. World J Surg, *10:* 389-396, 1986.

30. Penn, I. Sarcomas in organ allograft recipients. Transplantation, *60:* 1485-1491, 1995.

31. Patel, P., Hanson, D. L., Sullivan, P. S., Novak, R. M., Moorman, A. C., Tong, T. C., Holmberg, S. D., and Brooks, J. T. Incidence of types of cancer among HIV-infected persons compared with the general population in the United States, 1992-2003. Ann Intern Med, *148:* 728-736, 2008.

32. Jager, E., Chen, Y. T., Drijfhout, J. W., Karbach, J., Ringhoffer, M., Jager, D., Arand, M., Wada, H., Noguchi, Y., Stockert, E., Old, L. J., and Knuth, A. Simultaneous humoral and cellular immune response against cancer-testis antigen NY-ESO-1: definition of human histocompatibility leukocyte antigen (HLA)-A2-binding peptide epitopes. J Exp Med, *187:* 265-270, 1998.

33. Jager, E., Nagata, Y., Gnjatic, S., Wada, H., Stockert, E., Karbach, J., Dunbar, P. R., Lee, S. Y., Jungbluth, A., Jager, D., Arand, M., Ritter, G., Cerundolo, V., Dupont, B., Chen, Y. T., Old, L. J., and Knuth, A. Monitoring CD8 T cell responses to NY-ESO-1: correlation of humoral and cellular immune responses. Proc Natl Acad Sci U S A, *97:* 4760-4765, 2000.

34. Galon, J., Costes, A., Sanchez-Cabo, F., Kirilovsky, A., Mlecnik, B., Lagorce-Pages, C., Tosolini, M., Camus, M., Berger, A., Wind, P., Zinzindohoue, F., Bruneval, P., Cugnenc, P. H., Trajanoski, Z., Fridman, W. H., and Pages, F. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science, *313:* 1960-1964, 2006.

35. Reiman, J. M., Kmieciak, M., Manjili, M. H., and Knutson, K. L. Tumor immunoediting and immunosculpting pathways to cancer progression. Semin Cancer Biol, *17:* 275-287, 2007.

36. Swann, J. B. and Smyth, M. J. Immune surveillance of tumors. J Clin Invest, *117:* 1137-1146, 2007.

37. Fefer, A., Sullivan, K. M., Weiden, P., Buckner, C. D., Schoch, G., Storb, R., and Thomas, E. D. Graft versus leukemia effect in man: the relapse rate of acute leukemia is lower after allogeneic than after syngeneic marrow transplantation. Prog Clin Biol Res, *244:* 401-408, 1987.

38. Sullivan, K. M., Weiden, P. L., Storb, R., Witherspoon, R. P., Fefer, A., Fisher, L., Buckner, C. D., Anasetti, C., Appelbaum, F. R., Badger, C., and et al. Influence of acute and chronic graft-versus-host disease on relapse and survival after bone marrow transplantation from HLA-identical siblings as treatment of acute and chronic leukemia. Blood, *73:* 1720-1728, 1989.

39. Weisdorf, D. J., Nesbit, M. E., Ramsay, N. K., Woods, W. G., Goldman, A. I., Kim, T. H., Hurd, D. D., McGlave, P. B., and Kersey, J. H. Allogeneic bone marrow transplantation for acute lymphoblastic leukemia in remission: prolonged survival associated with acute graft-versus-host disease. J Clin Oncol, *5:* 1348-1355, 1987.

40. Weiden, P. L., Sullivan, K. M., Flournoy, N., Storb, R., and Thomas, E. D. Antileukemic effect of chronic graft-versus-host disease: contribution to improved survival after allogeneic marrow transplantation. N Engl J Med, *304:* 1529-1533, 1981.

41. Butturini, A., Bortin, M. M., and Gale, R. P. Graft-versus-leukemia following bone marrow transplantation. Bone Marrow Transplant, *2:* 233-242, 1987.

42. Gale, R. P., Horowitz, M. M., Ash, R. C., Champlin, R. E., Goldman, J. M., Rimm, A. A., Ringden, O., Stone, J. A., and Bortin, M. M. Identical-twin bone marrow transplants for leukemia. Ann Intern Med, *120:* 646-652, 1994.

43. Jones, R. J., Ambinder, R. F., Piantadosi, S., and Santos, G. W. Evidence of a graft-versus-lymphoma effect associated with allogeneic bone marrow transplantation. Blood, *77:* 649-653, 1991.

44. Odom, L. F., August, C. S., Githens, J. H., Humbert, J. R., Morse, H., Peakman, D., Sharma, B., Rusnak, S. L., and Johnson, F. B. Remission of relapsed leukaemia during a graft-versus-host reaction. A "graft-versus-leukaemia reaction" in man? Lancet, *2:* 537-540, 1978.

45. Tricot, G., Vesole, D. H., Jagannath, S., Hilton, J., Munshi, N., and Barlogie, B. Graft-versus-myeloma effect: proof of principle. Blood, *87:* 1196-1198, 1996.

46. Champlin, R., Ho, W., Gajewski, J., Feig, S., Burnison, M., Holley, G., Greenberg, P., Lee, K., Schmid, I., Giorgi, J., and et al. Selective depletion of CD8+ T lymphocytes for prevention of graft-versus-host disease after allogeneic bone marrow transplantation. Blood, *76:* 418-423, 1990.

47. Goldman, J. M., Gale, R. P., Horowitz, M. M., Biggs, J. C., Champlin, R. E., Gluckman, E., Hoffmann, R. G., Jacobsen, S. J., Marmont, A. M., McGlave, P. B., and et al. Bone marrow transplantation for chronic myelogenous leukemia in chronic phase. Increased risk for relapse associated with T-cell depletion. Ann Intern Med, *108:* 806-814, 1988.

48. Horowitz, M. M., Gale, R. P., Sondel, P. M., Goldman, J. M., Kersey, J., Kolb, H. J., Rimm, A. A., Ringden, O., Rozman, C., Speck, B., and et al. Graft-versus-leukemia reactions after bone marrow transplantation. Blood, *75:* 555-562, 1990.

49. Martin, P. J., Clift, R. A., Fisher, L. D., Buckner, C. D., Hansen, J. A., Appelbaum, F. R., Doney, K. C., Sullivan, K. M., Witherspoon, R. P., Storb, R., and et al. HLA-identical marrow transplantation during accelerated-phase chronic myelogenous leukemia: analysis of survival and remission duration. Blood, *72:* 1978-1984, 1988.

50. Southam, C. M., Brunschwig, A., Levin, A. G., and Dizon, Q. S. Effect of leukocytes on transplantability of human cancer. Cancer, *19:* 1743-1753, 1966.

51. Rosenberg, S. A., Grimm, E. A., McGrogan, M., Doyle, M., Kawasaki, E., Koths, K., and Mark, D. F. Biological activity of recombinant human interleukin-2 produced in Escherichia coli. Science, *223:* 1412-1414, 1984.

52. Mule, J. J., Shu, S., Schwarz, S. L., and Rosenberg, S. A. Adoptive immunotherapy of established pulmonary metastases with LAK cells and recombinant interleukin-2. Science, *225:* 1487-1489, 1984.

53. Rosenberg, S. A., Lotze, M. T., Yang, J. C., Topalian, S. L., Chang, A. E., Schwartzentruber, D. J., Aebersold, P., Leitman, S., Linehan, W. M., Seipp, C. A., and et al. Prospective randomized trial of high-dose interleukin-2 alone or in conjunction with lymphokine-activated killer cells for the treatment of patients with advanced cancer. J Natl Cancer Inst, *85:* 622-632, 1993.

54. Aebersold, P., Carter, C. S., Hyatt, C., Johnson, S., Ottaway, K., Leitman, S. F., and Rosenberg, S. A. A simplified automated procedure for generation of human lymphokine-activated killer cells for use in clinical trials. J Immunol Methods, *112:* 1-7, 1988.

55. Rosenberg, S. A., Spiess, P., and Lafreniere, R. A new approach to the adoptive immunotherapy of cancer with tumor-infiltrating lymphocytes. Science, *233:* 1318-1321, 1986.

56. Schwartzentruber, D. J., Topalian, S. L., Mancini, M., and Rosenberg, S. A. Specific release of granulocyte-macrophage colony-stimulating factor, tumor necrosis factor-alpha, and IFN-gamma by human tumor-infiltrating lymphocytes after autologous tumor stimulation. J Immunol, *146:* 3674-3681, 1991.

57. Topalian, S. L., Solomon, D., and Rosenberg, S. A. Tumor-specific cytolysis by lymphocytes infiltrating human melanomas. J Immunol, *142:* 3714-3725, 1989.

58. Yannelli, J. R., Hyatt, C., McConnell, S., Hines, K., Jacknin, L., Parker, L., Sanders, M., and Rosenberg, S. A. Growth of tumor-infiltrating lymphocytes from human solid cancers: summary of a 5-year experience. Int J Cancer, *65:* 413-421, 1996.

59. Rosenberg, S. A., Packard, B. S., Aebersold, P. M., Solomon, D., Topalian, S. L., Toy, S. T., Simon, P., Lotze, M. T., Yang, J. C., Seipp, C. A., and et al. Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. A preliminary report. N Engl J Med, *319:* 1676-1680, 1988.

60. Rosenberg, S. A., Yannelli, J. R., Yang, J. C., Topalian, S. L., Schwartzentruber, D. J., Weber, J. S., Parkinson, D. R., Seipp, C. A., Einhorn, J. H., and White, D. E. Treatment of patients with metastatic melanoma with autologous tumor-infiltrating lymphocytes and interleukin 2. J Natl Cancer Inst, *86:* 1159-1166, 1994.

61. Chou, T., Chang, A. E., and Shu, S. Y. Generation of therapeutic T lymphocytes from tumor-bearing mice by in vitro sensitization. Culture requirements and characterization of immunologic specificity. J Immunol, *140:* 2453-2461, 1988.

62. Shu, S. Y., Chou, T., and Rosenberg, S. A. Generation from tumor-bearing mice of lymphocytes with in vivo therapeutic efficacy. J Immunol, *139:* 295-304, 1987.

63. Shu, S., Chou, T., and Rosenberg, S. A. In vitro sensitization and expansion with viable tumor cells and interleukin 2 in the generation of specific therapeutic effector cells. J Immunol, *136:* 3891-3898, 1986.

64. Chang, A. E., Yoshizawa, H., Sakai, K., Cameron, M. J., Sondak, V. K., and Shu, S. Clinical observations on adoptive immunotherapy with vaccine-primed T-lymphocytes secondarily sensitized to tumor in vitro. Cancer Res, *53:* 1043-1050, 1993.

65. Wahl, W. L., Sussman, J. J., Shu, S., and Chang, A. E. Adoptive immunotherapy of murine intracerebral tumors with anti-CD3/interleukin-2-activated tumor-draining lymph node cells. J Immunother Emphasis Tumor Immunol, *15:* 242-250, 1994.

66. Peng, L., Shu, S., and Krauss, J. C. Treatment of subcutaneous tumor with adoptively transferred T cells. Cell Immunol, *178:* 24-32, 1997.

67. Yoshizawa, H., Chang, A. E., and Shu, S. Specific adoptive immunotherapy mediated by tumor-draining lymph node cells sequentially activated with anti-CD3 and IL-2. J Immunol, *147:* 729-737, 1991.

68. Yoshizawa, H., Sakai, K., Chang, A. E., and Shu, S. Y. Activation by anti-CD3 of tumor-draining lymph node cells for specific adoptive immunotherapy. Cell Immunol, *134:* 473-479, 1991.

69. Chang, A. E., Aruga, A., Cameron, M. J., Sondak, V. K., Normolle, D. P., Fox, B. A., and Shu, S. Adoptive immunotherapy with vaccine-primed lymph node cells secondarily activated with anti-CD3 and interleukin-2. J Clin Oncol, *15:* 796-807, 1997.

70. Chang, A. E., Li, Q., Jiang, G., Sayre, D. M., Braun, T. M., and Redman, B. G. Phase II trial of autologous tumor vaccination, anti-CD3-activated vaccine-primed lymphocytes, and interleukin-2 in stage IV renal cell cancer. J Clin Oncol, *21:* 884-890, 2003.

71. Chang, A. E., Li, Q., Jiang, G., Teknos, T. N., Chepeha, D. B., and Bradford, C. R. Generation of vaccine-primed lymphocytes for the treatment of head and neck cancer. Head Neck, *25:* 198-209, 2003.

72. Kawakami, Y., Eliyahu, S., Delgado, C. H., Robbins, P. F., Rivoltini, L., Topalian, S. L., Miki, T., and Rosenberg, S. A. Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor. Proc Natl Acad Sci U S A, *91:* 3515-3519, 1994.

73. Salgaller, M. L., Afshar, A., Marincola, F. M., Rivoltini, L., Kawakami, Y., and Rosenberg, S. A. Recognition of multiple epitopes in the human melanoma antigen gp100 by peripheral blood lymphocytes stimulated in vitro with synthetic peptides. Cancer Res, *55:* 4972-4979, 1995.

74. Kawakami, Y., Eliyahu, S., Jennings, C., Sakaguchi, K., Kang, X., Southwood, S., Robbins, P. F., Sette, A., Appella, E., and Rosenberg, S. A. Recognition of multiple epitopes in the human melanoma antigen gp100 by tumor-infiltrating T lymphocytes associated with in vivo tumor regression. J Immunol, *154:* 3961-3968, 1995.

75. Yee, C., Thompson, J. A., Byrd, D., Riddell, S. R., Roche, P., Celis, E., and Greenberg, P. D. Adoptive T cell therapy using antigen-specific CD8+ T cell clones for the treatment of patients with metastatic melanoma: in vivo persistence, migration, and antitumor effect of transferred T cells. Proc Natl Acad Sci U S A, *99:* 16168-16173, 2002.

76. Dudley, M. E., Wunderlich, J., Nishimura, M. I., Yu, D., Yang, J. C., Topalian, S. L., Schwartzentruber, D. J., Hwu, P., Marincola, F. M., Sherry, R., Leitman, S. F., and Rosenberg, S. A. Adoptive transfer of cloned melanoma-reactive T lymphocytes for the treatment of patients with metastatic melanoma. J Immunother, *24:* 363-373, 2001.

77. Powell, D. J., Jr., Dudley, M. E., Hogan, K. A., Wunderlich, J. R., and Rosenberg, S. A. Adoptive transfer of vaccine-induced peripheral blood mononuclear cells to patients with metastatic melanoma following lymphodepletion. J Immunol, *177:* 6527-6539, 2006.

78. Mackensen, A., Meidenbauer, N., Vogl, S., Laumer, M., Berger, J., and Andreesen, R. Phase I study of adoptive T-cell therapy using antigen-specific CD8+ T cells for the treatment of patients with metastatic melanoma. J Clin Oncol, *24:* 5060-5069, 2006.

79. Dudley, M. E., Wunderlich, J. R., Yang, J. C., Hwu, P., Schwartzentruber, D. J., Topalian, S. L., Sherry, R. M., Marincola, F. M., Leitman, S. F., Seipp, C. A., Rogers-Freezer, L., Morton, K. E., Nahvi, A., Mavroukakis, S. A., White, D. E., and Rosenberg, S. A. A phase I study of nonmyeloablative chemotherapy and adoptive transfer of autologous tumor antigen-specific T lymphocytes in patients with metastatic melanoma. J Immunother, Vol. 25, pp. 243-251, 2002.

80. Dudley, M. E., Wunderlich, J. R., Yang, J. C., Sherry, R. M., Topalian, S. L., Restifo, N. P., Royal, R. E., Kammula, U., White, D. E., Mavroukakis, S. A., Rogers, L. J., Gracia, G. J., Jones, S. A., Mangiameli, D. P., Pelletier, M. M., Gea-Banacloche, J., Robinson, M. R., Berman, D. M., Filie, A. C., Abati, A., and Rosenberg, S. A. Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. J Clin Oncol, *23:* 2346-2357, 2005.

81. Arca, M. J., Krauss, J. C., Aruga, A., Cameron, M. J., Shu, S., and Chang, A. E. Therapeutic efficacy of T cells derived from lymph nodes draining a poorly immunogenic tumor transduced to secrete granulocyte-macrophage colony-stimulating factor. Cancer Gene Ther, *3:* 39-47, 1996.

82. Hu, H. M., Winter, H., Urba, W. J., and Fox, B. A. Divergent roles for CD4+ T cells in the priming and effector/memory phases of adoptive immunotherapy. J Immunol, *165:* 4246-4253, 2000.

83. Malakhova, O. A., Yan, M., Malakhov, M. P., Yuan, Y., Ritchie, K. J., Kim, K. I., Peterson, L. F., Shuai, K., and Zhang, D. E. Protein ISGylation modulates the JAK-STAT signaling pathway. Genes Dev, *17:* 455-460, 2003.

84. Ritchie, K. J., Hahn, C. S., Kim, K. I., Yan, M., Rosario, D., Li, L., de la Torre, J. C., and Zhang, D. E. Role of ISG15 protease UBP43 (USP18) in innate immunity to viral infection. Nat Med, *10:* 1374-1378, 2004.

85. Kim, K. I., Malakhova, O. A., Hoebe, K., Yan, M., Beutler, B., and Zhang, D. E. Enhanced antibacterial potential in UBP43-deficient mice against Salmonella typhimurium infection by up-regulating type I IFN signaling. J Immunol, *175:* 847-854, 2005.

86. Liu, L. Q., Ilaria, R., Jr., Kingsley, P. D., Iwama, A., van Etten, R. A., Palis, J., and Zhang, D. E. A novel ubiquitin-specific protease, UBP43, cloned from leukemia fusion protein AML1-ETO-expressing mice, functions in hematopoietic cell differentiation. Mol Cell Biol, *19:* 3029-3038, 1999.

87. Yergeau, D. A., Hetherington, C. J., Wang, Q., Zhang, P., Sharpe, A. H., Binder, M., Marin-Padilla, M., Tenen, D. G., Speck, N. A., and Zhang, D. E. Embryonic lethality and impairment of haematopoiesis in mice heterozygous for an AML1-ETO fusion gene. Nat Genet, *15:* 303-306, 1997.

88. Chung, C. H. and Baek, S. H. Deubiquitinating enzymes: their diversity and emerging roles. Biochem Biophys Res Commun, *266:* 633-640, 1999.

89. Malakhov, M. P., Malakhova, O. A., Kim, K. I., Ritchie, K. J., and Zhang, D. E. UBP43 (USP18) specifically removes ISG15 from conjugated proteins. J Biol Chem, *277:* 9976-9981, 2002.

90. Farrell, P. J., Broeze, R. J., and Lengyel, P. Accumulation of an mRNA and protein in interferon-treated Ehrlich ascites tumour cells. Nature, *279:* 523-525, 1979.

91. Haas, A. L., Ahrens, P., Bright, P. M., and Ankel, H. Interferon induces a 15-kilodalton protein exhibiting marked homology to ubiquitin. J Biol Chem, *262:* 11315-11323, 1987.

92. Narasimhan, J., Wang, M., Fu, Z., Klein, J. M., Haas, A. L., and Kim, J. J. Crystal structure of the interferon-induced ubiquitin-like protein ISG15. J Biol Chem, *280:* 27356-27365, 2005.

93. Loeb, K. R. and Haas, A. L. The interferon-inducible 15-kDa ubiquitin homolog conjugates to intracellular proteins. J Biol Chem, *267:* 7806-7813, 1992.

94. Narasimhan, J., Potter, J. L., and Haas, A. L. Conjugation of the 15-kDa interferon-induced ubiquitin homolog is distinct from that of ubiquitin. J Biol Chem, *271:* 324-330, 1996.

95. Yuan, W. and Krug, R. M. Influenza B virus NS1 protein inhibits conjugation of the interferon (IFN)-induced ubiquitin-like ISG15 protein. Embo J, *20:* 362-371, 2001.

96. Kim, K. I., Giannakopoulos, N. V., Virgin, H. W., and Zhang, D. E. Interferon-inducible ubiquitin E2, Ubc8, is a conjugating enzyme for protein ISGylation. Mol Cell Biol, *24:* 9592-9600, 2004.

97. Zhao, C., Beaudenon, S. L., Kelley, M. L., Waddell, M. B., Yuan, W., Schulman, B. A., Huibregtse, J. M., and Krug, R. M. The UbcH8 ubiquitin E2 enzyme is also the E2 enzyme for ISG15, an IFN-alpha/beta-induced ubiquitin-like protein. Proc Natl Acad Sci U S A, *101:* 7578-7582, 2004.

98. Zou, W. and Zhang, D. E. The interferon-inducible ubiquitin-protein isopeptide ligase (E3) EFP also functions as an ISG15 E3 ligase. J Biol Chem, *281:* 3989-3994, 2006.

99. Dastur, A., Beaudenon, S., Kelley, M., Krug, R. M., and Huibregtse, J. M. Herc5, an interferon-induced HECT E3 enzyme, is required for conjugation of ISG15 in human cells. J Biol Chem, *281:* 4334-4338, 2006.

100. Nakasato, N., Ikeda, K., Urano, T., Horie-Inoue, K., Takeda, S., and Inoue, S. A ubiquitin E3 ligase Efp is up-regulated by interferons and conjugated with ISG15. Biochem Biophys Res Commun, *351:* 540-546, 2006.

101. Wong, J. J., Pung, Y. F., Sze, N. S., and Chin, K. C. HERC5 is an IFN-induced HECT-type E3 protein ligase that mediates type I IFN-induced ISGylation of protein targets. Proc Natl Acad Sci U S A, *103:* 10735-10740, 2006.

102. Hamerman, J. A., Hayashi, F., Schroeder, L. A., Gygi, S. P., Haas, A. L., Hampson, L., Coughlin, P., Aebersold, R., and Aderem, A. Serpin 2a is induced in activated macrophages and conjugates to a ubiquitin homolog. J Immunol, *168:* 2415-2423, 2002.

103. Malakhov, M. P., Kim, K. I., Malakhova, O. A., Jacobs, B. S., Borden, E. C., and Zhang, D. E. High-throughput immunoblotting. Ubiquitiin-like protein ISG15 modifies key regulators of signal transduction. J Biol Chem, *278:* 16608-16613, 2003.

104. Giannakopoulos, N. V., Luo, J. K., Papov, V., Zou, W., Lenschow, D. J., Jacobs, B. S., Borden, E. C., Li, J., Virgin, H. W., and Zhang, D. E. Proteomic identification of proteins conjugated to ISG15 in mouse and human cells. Biochem Biophys Res Commun, *336:* 496-506, 2005.

105. Zhao, C., Denison, C., Huibregtse, J. M., Gygi, S., and Krug, R. M. Human ISG15 conjugation targets both IFN-induced and constitutively expressed proteins functioning in diverse cellular pathways. Proc Natl Acad Sci U S A, *102:* 10200-10205, 2005.

106. Lu, G., Reinert, J. T., Pitha-Rowe, I., Okumura, A., Kellum, M., Knobeloch, K. P., Hassel, B., and Pitha, P. M. ISG15 enhances the innate antiviral response by inhibition of IRF-3 degradation. Cell Mol Biol (Noisy-le-grand), *52:* 29-41, 2006.

107. Hiscott, J. Triggering the innate antiviral response through IRF-3 activation. J Biol Chem, *282:* 15325-15329, 2007.

108. Lin, R., Heylbroeck, C., Pitha, P. M., and Hiscott, J. Virus-dependent phosphorylation of the IRF-3 transcription factor regulates nuclear translocation, transactivation potential, and proteasome-mediated degradation. Mol Cell Biol, *18:* 2986-2996, 1998.

109. Ishii, K. J., Coban, C., Kato, H., Takahashi, K., Torii, Y., Takeshita, F., Ludwig, H., Sutter, G., Suzuki, K., Hemmi, H., Sato, S., Yamamoto, M., Uematsu, S., Kawai, T., Takeuchi, O., and Akira, S. A Toll-like receptor-independent antiviral response induced by double-stranded B-form DNA. Nat Immunol, *7:* 40-48, 2006.

110. Merika, M., Williams, A. J., Chen, G., Collins, T., and Thanos, D. Recruitment of CBP/p300 by the IFN beta enhanceosome is required for synergistic activation of transcription. Mol Cell, *1:* 277-287, 1998.

111. Wathelet, M. G., Lin, C. H., Parekh, B. S., Ronco, L. V., Howley, P. M., and Maniatis, T. Virus infection induces the assembly of coordinately activated transcription factors on the IFN-beta enhancer in vivo. Mol Cell, *1:* 507-518, 1998.

112. Baldwin, A. S., Jr. Series introduction: the transcription factor NF-kappaB and human disease. J Clin Invest, *107:* 3-6, 2001.

113. Prajapati, S., Verma, U., Yamamoto, Y., Kwak, Y. T., and Gaynor, R. B. Protein phosphatase 2Cbeta association with the IkappaB kinase complex is involved in regulating NF-kappaB activity. J Biol Chem, *279:* 1739-1746, 2004.

114. Takeuchi, T., Kobayashi, T., Tamura, S., and Yokosawa, H. Negative regulation of protein phosphatase 2Cbeta by ISG15 conjugation. FEBS Lett, *580:* 4521-4526, 2006.

115. Gebauer, F. and Hentze, M. W. Molecular mechanisms of translational control. Nat Rev Mol Cell Biol, *5:* 827-835, 2004.

116. Okumura, F., Zou, W., and Zhang, D. E. ISG15 modification of the eIF4E cognate 4EHP enhances cap structure-binding activity of 4EHP. Genes Dev, *21:* 255-260, 2007.

117. Loeb, K. R. and Haas, A. L. Conjugates of ubiquitin cross-reactive protein distribute in a cytoskeletal pattern. Mol Cell Biol, *14:* 8408-8419, 1994.

118. Okumura, A., Lu, G., Pitha-Rowe, I., and Pitha, P. M. Innate antiviral response targets HIV-1 release by the induction of ubiquitin-like protein ISG15. Proc Natl Acad Sci U S A, *103:* 1440-1445, 2006.

119. Lenschow, D. J., Lai, C., Frias-Staheli, N., Giannakopoulos, N. V., Lutz, A., Wolff, T., Osiak, A., Levine, B., Schmidt, R. E., Garcia-Sastre, A., Leib, D. A., Pekosz, A., Knobeloch, K. P., Horak, I., and Virgin, H. W. t. From the cover: IFN-stimulated gene 15 functions as a critical antiviral molecule against influenza, herpes, and Sindbis viruses. Proc Natl Acad Sci U S A, *104:* 1371-1376, 2007.

120. Osiak, A., Utermohlen, O., Niendorf, S., Horak, I., and Knobeloch, K. P. ISG15, an interferon-stimulated ubiquitin-like protein, is not essential for STAT1 signaling and responses against vesicular stomatitis and lymphocytic choriomeningitis virus. Mol Cell Biol, *25:* 6338-6345, 2005.

121. Knobeloch, K. P., Utermohlen, O., Kisser, A., Prinz, M., and Horak, I. Reexamination of the role of ubiquitin-like modifier ISG15 in the phenotype of UBP43-deficient mice. Mol Cell Biol, *25:* 11030-11034, 2005.

122. Kim, K. I., Yan, M., Malakhova, O., Luo, J. K., Shen, M. F., Zou, W., de la Torre, J. C., and Zhang, D. E. Ube1L and protein ISGylation are not essential for alpha/beta interferon signaling. Mol Cell Biol, *26:* 472-479, 2006.

123. Malakhova, O. A., Kim, K. I., Luo, J. K., Zou, W., Kumar, K. G., Fuchs, S. Y., Shuai, K., and Zhang, D. E. UBP43 is a novel regulator of interferon signaling independent of its ISG15 isopeptidase activity. Embo J, *25:* 2358-2367, 2006.

124. Janssen, E. M., Lemmens, E. E., Wolfe, T., Christen, U., von Herrath, M. G., and Schoenberger, S. P. CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. Nature, *421:* 852-856, 2003.

125. Janssen, E. M., Droin, N. M., Lemmens, E. E., Pinkoski, M. J., Bensinger, S. J., Ehst, B. D., Griffith, T. S., Green, D. R., and Schoenberger, S. P. CD4+ T-cell help controls CD8+ T-cell memory via TRAIL-mediated activation-induced cell death. Nature, *434:* 88-93, 2005.

126. Sun, J. C., Williams, M. A., and Bevan, M. J. CD4+ T cells are required for the maintenance, not programming, of memory CD8+ T cells after acute infection. Nat Immunol, *5:* 927-933, 2004.

127. Sun, J. C. and Bevan, M. J. Defective CD8 T cell memory following acute infection without CD4 T cell help. Science, *300:* 339-342, 2003.

128. Wang, L. X., Shu, S., Disis, M. L., and Plautz, G. E. Adoptive transfer of tumor-primed, in vitro-activated, CD4+ T effector cells (TEs) combined with CD8+ TEs provides intratumoral TE proliferation and synergistic antitumor response. Blood, *109:* 4865-4876, 2007.

129. Cho, B. K., Rao, V. P., Ge, Q., Eisen, H. N., and Chen, J. Homeostasis-stimulated proliferation drives naive T cells to differentiate directly into memory T cells. J Exp Med, *192:* 549-556, 2000.

130. Goldrath, A. W., Bogatzki, L. Y., and Bevan, M. J. Naive T cells transiently acquire a memory-like phenotype during homeostasis-driven proliferation. J Exp Med, *192:* 557-564, 2000.

131. Min, B., McHugh, R., Sempowski, G. D., Mackall, C., Foucras, G., and Paul, W. E. Neonates support lymphopenia-induced proliferation. Immunity, *18:* 131-140, 2003.

132. Min, B. and Paul, W. E. Endogenous proliferation: burst-like CD4 T cell proliferation in lymphopenic settings. Semin Immunol, *17:* 201-207, 2005.

133. Klarnet, J. P., Matis, L. A., Kern, D. E., Mizuno, M. T., Peace, D. J., Thompson, J. A., Greenberg, P. D., and Cheever, M. A. Antigen-driven T cell clones can

proliferate in vivo, eradicate disseminated leukemia, and provide specific immunologic memory. J Immunol, *138:* 4012-4017, 1987.

134. Klarnet, J. P., Kern, D. E., Dower, S. K., Matis, L. A., Cheever, M. A., and Greenberg, P. D. Helper-independent CD8+ cytotoxic T lymphocytes express IL-1 receptors and require IL-1 for secretion of IL-2. J Immunol, *142:* 2187-2191, 1989.

135. Wang, L. X., Shu, S., and Plautz, G. E. Host lymphodepletion augments T cell adoptive immunotherapy through enhanced intratumoral proliferation of effector cells. Cancer Res, *65:* 9547-9554, 2005.

136. Ernst, B., Lee, D. S., Chang, J. M., Sprent, J., and Surh, C. D. The peptide ligands mediating positive selection in the thymus control T cell survival and homeostatic proliferation in the periphery. Immunity, *11:* 173-181, 1999.

137. Rojas, J. M., Knight, K., Wang, L., and Clark, R. E. Clinical evaluation of BCR-ABL peptide immunisation in chronic myeloid leukaemia: results of the EPIC study. Leukemia, *21:* 2287-2295, 2007.

138. Simon, R. M., Steinberg, S. M., Hamilton, M., Hildesheim, A., Khleif, S., Kwak, L. W., Mackall, C. L., Schlom, J., Topalian, S. L., and Berzofsky, J. A. Clinical trial designs for the early clinical development of therapeutic cancer vaccines. J Clin Oncol, *19:* 1848-1854, 2001.

139. Holmberg, L. A., Oparin, D. V., Gooley, T., Lilleby, K., Bensinger, W., Reddish, M. A., MacLean, G. D., Longenecker, B. M., and Sandmaier, B. M. Clinical outcome of breast and ovarian cancer patients treated with high-dose chemotherapy, autologous stem cell rescue and THERATOPE STn-KLH cancer vaccine. Bone Marrow Transplant, *25:* 1233-1241, 2000.

140. Slingluff, C. L., Jr., Yamshchikov, G., Neese, P., Galavotti, H., Eastham, S., Engelhard, V. H., Kittlesen, D., Deacon, D., Hibbitts, S., Grosh, W. W., Petroni, G., Cohen, R., Wiernasz, C., Patterson, J. W., Conway, B. P., and Ross, W. G. Phase I trial of a melanoma vaccine with gp100(280-288) peptide and tetanus helper peptide in adjuvant: immunologic and clinical outcomes. Clin Cancer Res, *7:* 3012-3024, 2001.

141. Miller, N. E., Bonczyk, J. R., Nakayama, Y., and Suresh, M. Role of thymic output in regulating CD8 T-cell homeostasis during acute and chronic viral infection. J Virol, *79:* 9419-9429, 2005.

142. Kemball, C. C., Pack, C. D., Guay, H. M., Li, Z. N., Steinhauer, D. A., Szomolanyi-Tsuda, E., and Lukacher, A. E. The antiviral CD8+ T cell response is differentially dependent on CD4+ T cell help over the course of persistent infection. J Immunol, *179:* 1113-1121, 2007.

143. Wherry, E. J., Barber, D. L., Kaech, S. M., Blattman, J. N., and Ahmed, R. Antigen-independent memory CD8 T cells do not develop during chronic viral infection. Proc Natl Acad Sci U S A, *101:* 16004-16009, 2004.

144. Shin, H., Blackburn, S. D., Blattman, J. N., and Wherry, E. J. Viral antigen and extensive division maintain virus-specific CD8 T cells during chronic infection. J Exp Med, *204:* 941-949, 2007.

145. Kemball, C. C., Lee, E. D., Vezys, V., Pearson, T. C., Larsen, C. P., and Lukacher, A. E. Late priming and variability of epitope-specific CD8+ T cell responses during a persistent virus infection. J Immunol, *174:* 7950-7960, 2005.

146. Vezys, V., Masopust, D., Kemball, C. C., Barber, D. L., O'Mara, L. A., Larsen, C. P., Pearson, T. C., Ahmed, R., and Lukacher, A. E. Continuous recruitment of naive T cells contributes to heterogeneity of antiviral CD8 T cells during persistent infection. J Exp Med, *203:* 2263-2269, 2006.

147. Turner, D. L., Cauley, L. S., Khanna, K. M., and Lefrancois, L. Persistent antigen presentation after acute vesicular stomatitis virus infection. J Virol, *81:* 2039-2046, 2007.

148. Obst, R., van Santen, H. M., Mathis, D., and Benoist, C. Antigen persistence is required throughout the expansion phase of a CD4+ T cell response. J Exp Med, *201:* 1555-1565, 2005.

149. Jelley-Gibbs, D. M., Lepak, N. M., Yen, M., and Swain, S. L. Two distinct stages in the transition from naive CD4 T cells to effectors, early antigen-dependent and late cytokine-driven expansion and differentiation. J Immunol, *165:* 5017-5026, 2000.

150. Jelley-Gibbs, D. M., Dibble, J. P., Filipson, S., Haynes, L., Kemp, R. A., and Swain, S. L. Repeated stimulation of CD4 effector T cells can limit their protective function. J Exp Med, *201:* 1101-1112, 2005.

151. Calzascia, T., Pellegrini, M., Lin, A., Garza, K. M., Elford, A. R., Shahinian, A., Ohashi, P. S., and Mak, T. W. CD4 T cells, lymphopenia, and IL-7 in a multistep pathway to autoimmunity. Proc Natl Acad Sci U S A, *105:* 2999-3004, 2008.

152. Dudley, M. E. and Rosenberg, S. A. Adoptive-cell-transfer therapy for the treatment of patients with cancer. Nat Rev Cancer, *3:* 666-675, 2003.

153. Kolumam, G. A., Thomas, S., Thompson, L. J., Sprent, J., and Murali-Krishna, K. Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection. J Exp Med, *202:* 637-650, 2005.

154. Le Bon, A., Durand, V., Kamphuis, E., Thompson, C., Bulfone-Paus, S., Rossmann, C., Kalinke, U., and Tough, D. F. Direct stimulation of T cells by type I IFN enhances the CD8+ T cell response during cross-priming. J Immunol, *176:* 4682-4689, 2006.

155. Le Bon, A., Etchart, N., Rossmann, C., Ashton, M., Hou, S., Gewert, D., Borrow, P., and Tough, D. F. Cross-priming of CD8+ T cells stimulated by virus-induced type I interferon. Nat Immunol, *4:* 1009-1015, 2003.

156. Durand, V., Wong, S. Y., Tough, D. F., and Le Bon, A. IFN-alpha/beta-dependent cross-priming induced by specific toll-like receptor agonists. Vaccine, *24 Suppl 2:* S2-22-23, 2006.

157. Havenar-Daughton, C., Kolumam, G. A., and Murali-Krishna, K. Cutting Edge: The direct action of type I IFN on CD4 T cells is critical for sustaining clonal expansion in response to a viral but not a bacterial infection. J Immunol, *176:* 3315-3319, 2006.

158. Medzhitov, R. Toll-like receptors and innate immunity. Nat Rev Immunol, *1:* 135-145, 2001.

159. Iwasaki, A. and Medzhitov, R. Toll-like receptor control of the adaptive immune responses. Nat Immunol, *5:* 987-995, 2004.

160. Schnare, M., Barton, G. M., Holt, A. C., Takeda, K., Akira, S., and Medzhitov, R. Toll-like receptors control activation of adaptive immune responses. Nat Immunol, *2:* 947-950, 2001.

161. Nembrini, C., Abel, B., Kopf, M., and Marsland, B. J. Strong TCR signaling, TLR ligands, and cytokine redundancies ensure robust development of type 1 effector T cells. J Immunol, *176:* 7180-7188, 2006.

162. Marsland, B. J. and Kopf, M. Toll-like receptors: paving the path to T cell-driven autoimmunity? Curr Opin Immunol, 2007.

163. Koster, M. and Hauser, H. Dynamic redistribution of STAT1 protein in IFN signaling visualized by GFP fusion proteins. Eur J Biochem, *260:* 137-144, 1999.

164. Gantier, M. P. and Williams, B. R. The response of mammalian cells to double-stranded RNA. Cytokine Growth Factor Rev, *18:* 363-371, 2007.

165. Malmgaard, L. Induction and regulation of IFNs during viral infections. J Interferon Cytokine Res, *24:* 439-454, 2004.

166. McKendry, R., John, J., Flavell, D., Muller, M., Kerr, I. M., and Stark, G. R. High-frequency mutagenesis of human cells and characterization of a mutant

unresponsive to both alpha and gamma interferons. Proc Natl Acad Sci U S A, *88:* 11455-11459, 1991.

167. Oldford, S. A., Robb, J. D., Codner, D., Gadag, V., Watson, P. H., and Drover, S. Tumor cell expression of HLA-DM associates with a Th1 profile and predicts improved survival in breast carcinoma patients. Int Immunol, *18:* 1591-1602, 2006.

168. Kondo, T., Nakazawa, H., Ito, F., Hashimoto, Y., Osaka, Y., Futatsuyama, K., Toma, H., and Tanabe, K. Favorable prognosis of renal cell carcinoma with increased expression of chemokines associated with a Th1-type immune response. Cancer Sci, *97:* 780-786, 2006.

169. Mortara, L., Castellani, P., Meazza, R., Tosi, G., De Lerma Barbaro, A., Procopio, F. A., Comes, A., Zardi, L., Ferrini, S., and Accolla, R. S. CIITA-induced MHC class II expression in mammary adenocarcinoma leads to a Th1 polarization of the tumor microenvironment, tumor rejection, and specific antitumor memory. Clin Cancer Res, *12:* 3435-3443, 2006.

170. Nishimura, T., Nakui, M., Sato, M., Iwakabe, K., Kitamura, H., Sekimoto, M., Ohta, A., Koda, T., and Nishimura, S. The critical role of Th1-dominant immunity in tumor immunology. Cancer Chemother Pharmacol, *46 Suppl:* S52-61, 2000.

171. Bloom, M. B., Perry-Lalley, D., Robbins, P. F., Li, Y., el-Gamil, M., Rosenberg, S. A., and Yang, J. C. Identification of tyrosinase-related protein 2 as a tumor rejection antigen for the B16 melanoma. J Exp Med, *185:* 453-459, 1997.

172. Overwijk, W. W., Theoret, M. R., Finkelstein, S. E., Surman, D. R., de Jong, L. A., Vyth-Dreese, F. A., Dellemijn, T. A., Antony, P. A., Spiess, P. J., Palmer, D. C., Heimann, D. M., Klebanoff, C. A., Yu, Z., Hwang, L. N., Feigenbaum, L., Kruisbeek, A. M., Rosenberg, S. A., and Restifo, N. P. Tumor regression and autoimmunity after reversal of a functionally tolerant state of self-reactive CD8+ T cells. J Exp Med, *198:* 569-580, 2003.

173. Mombaerts, P., Iacomini, J., Johnson, R. S., Herrup, K., Tonegawa, S., and Papaioannou, V. E. RAG-1-deficient mice have no mature B and T lymphocytes. Cell, *68:* 869-877, 1992.

174. Wang, H. Y. and Wang, R. F. Regulatory T cells and cancer. Curr Opin Immunol, *19:* 217-223, 2007.

175. Wei, J., Duramad, O., Perng, O. A., Reiner, S. L., Liu, Y. J., and Qin, F. X. Antagonistic nature of T helper 1/2 developmental programs in opposing peripheral induction of Foxp3+ regulatory T cells. Proc Natl Acad Sci U S A, *104:* 18169-18174, 2007.

176. Bettelli, E., Carrier, Y., Gao, W., Korn, T., Strom, T. B., Oukka, M., Weiner, H. L., and Kuchroo, V. K. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature, *441:* 235-238, 2006.

177. Veldhoen, M., Hocking, R. J., Atkins, C. J., Locksley, R. M., and Stockinger, B. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity, *24:* 179-189, 2006.

178. Takeuchi, T. and Yokosawa, H. ISG15 modification of Ubc13 suppresses its ubiquitin-conjugating activity. Biochem Biophys Res Commun, *336:* 9-13, 2005.

179. Sorrentino, A., Thakur, N., Grimsby, S., Marcusson, A., von Bulow, V., Schuster, N., Zhang, S., Heldin, C. H., and Landstrom, M. The type I TGF-beta receptor engages TRAF6 to activate TAK1 in a receptor kinase-independent manner. Nat Cell Biol, *10:* 1199-1207, 2008.

180. Alexander, J., Sidney, J., Southwood, S., Ruppert, J., Oseroff, C., Maewal, A., Snoke, K., Serra, H. M., Kubo, R. T., Sette, A., and et al. Development of high potency universal DR-restricted helper epitopes by modification of high affinity DR-blocking peptides. Immunity, *1:* 751-761, 1994.

181. Kim, D., Monie, A., He, L., Tsai, Y. C., Hung, C. F., and Wu, T. C. Role of IL-2 secreted by PADRE-specific CD4+ T cells in enhancing E7-specific CD8+ T-cell immune responses. Gene Ther, *15:* 677-687, 2008.

182. Brossart, P., Heinrich, K. S., Stuhler, G., Behnke, L., Reichardt, V. L., Stevanovic, S., Muhm, A., Rammensee, H. G., Kanz, L., and Brugger, W. Identification of HLA-A2-restricted T-cell epitopes derived from the MUC1 tumor antigen for broadly applicable vaccine therapies. Blood, *93:* 4309-4317, 1999.

183. Hunder, N. N., Wallen, H., Cao, J., Hendricks, D. W., Reilly, J. Z., Rodmyre, R., Jungbluth, A., Gnjatic, S., Thompson, J. A., and Yee, C. Treatment of metastatic melanoma with autologous CD4+ T cells against NY-ESO-1. N Engl J Med, *358:* 2698-2703, 2008.

184. Rosenberg, S. A., Restifo, N. P., Yang, J. C., Morgan, R. A., and Dudley, M. E. Adoptive cell transfer: a clinical path to effective cancer immunotherapy. Nat Rev Cancer, *8:* 299-308, 2008.

185. Morgan, R. A., Dudley, M. E., Wunderlich, J. R., Hughes, M. S., Yang, J. C., Sherry, R. M., Royal, R. E., Topalian, S. L., Kammula, U. S., Restifo, N. P., Zheng, Z., Nahvi, A., de Vries, C. R., Rogers-Freezer, L. J., Mavroukakis, S. A., and Rosenberg, S. A. Cancer regression in patients after transfer of genetically engineered lymphocytes. Science, *314:* 126-129, 2006.