January 2012

Lenalidomide targets the T-cell co-stimulatory pathway to mediate immune modulation

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Lenalidomide targets the T-cell co-stimulatory pathway to mediate immune modulation

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Cell Biology, Microbiology, and Molecular Biology
College of Arts and Sciences
University of South Florida

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Date of Approval:
August 24, 2012

Keywords: CD28, myelodysplastic syndrome, IMiDs, PP2A, anti-tumor immunity

DEDICATION

I dedicate this dissertation to…

…my husband, Chad, who has been my rock and my support, and who never stopped believing in me, even when I doubted myself. Four years ago, it was my acceptance to USF that brought us 1,000 miles away from our families and friends, and you never once complained. You've always had faith in my abilities; you have been there to wipe away the tears and encourage me when things got tough, and to smile and laugh with me in celebration of my successes. Truly, I would not be where I am today if it weren't for you in my life. I love you.

…my parents, Scott and Cindy, and my brother Benjamin. You have been my biggest cheerleaders throughout my entire life, and always told me I could achieve anything I put my mind to. Mom and dad, you were the first ones to introduce me to science, and it is because of your hard work and support that I have been afforded the opportunities to get where I am today. Thank you for listening to me vent about failed experiments on my way home from late nights in the lab and encouraging me not to quit. Ben, I could always count on you to make me laugh after a crappy day and to encourage me to "keep on, keeping on." I love you all.

…my father-in-law Chris, and my sister-in-laws Erica and Gwen. You all have become my family, and your support means the world to me. From television shows to family vacations, we have many experiences that I cherish

and have kept me sane throughout school, and I'm looking forward to all that lay ahead. I'm thankful that "we're sisters…do do do doo".

…my friends. Near and far, from Florida to France to Chicago and everywhere in between, you have encouraged me in my educational ventures and enrich my life. I guess I'll always be "the nerdy one", and even though you never really understood "what it is I actually do", you never hesitate to let me know how proud you are of me and to reassure me I could do this. Thank you from the bottom of my heart.

ACKNOWLEDGEMENTS

I would first and foremost like to thank my mentor, Dr. PK Epling-Burnette, for all of her encouragement, support, and teaching over these past four years. Your guidance has allowed me to become the scientist and independent-thinker that I am today. The supportive learning environment and inviting/collegial atmosphere of your lab made it feel like “home” from day one, and I couldn’t have asked for anything better. Special thanks to the Burnette lab members Adam Mailloux, Jeff Painter, Eric Padron, and Lili Yang for all of your support and help. Also to past lab members Fanqi, Zou, Mayenha, and Amber I could not have done this if it weren’t for all your expertise and insight into protocols and experimental design. I feel lucky to have had such wonderful labmates/friends to work with on a daily basis, and I truly will miss all of your playful banter.

I would also like to thank my committee members, Dr. Djeu, Dr. Wei, Dr. Pinilla, and Dr. Yu. Thank you so much for your insight, time, support, and camaraderie. You have all been instrumental to my graduate experience and I thank you for everything. I feel especially honored to have Dr. Neal Young, the head of the hematology branch at the National Heart Lung and Blood Institute (NHLBI), serve as the outside chairperson of my dissertation committee.

I cannot forget to thank all the members of the Sotomayor, Pinilla, and Villagra labs (Winston, Jason, John, Eva, Jennifer, Allejandro, etc.) for sharing reagents, cell lines, ideas, and jokes. Lab meetings and conferences were

definitely more fun with us all together, and your input on my experiments was invaluable.

A special thanks to my undergraduate student in the lab, Danielle Grams, for all your help with my experiments and for sharing in my frustrations on PP2A. Thank you for giving me the opportunity to share my love of science with you, and to teach you the ways of the lab. I could not be luckier to have worked with such a bright, motivated, kind, and hard-working young woman, and am so proud of how far you have come since your freshman year. I cannot wait to hear about all you will accomplish in your life.

Last, but certainly not least, I must thank my best friend and classmate, Melissa Buckley. Whether it was training for and running marathons or venting about frustrating experiments, you were always someone I could lean on for guidance, knowledge, and support. I know for certain I never would have finished even one marathon, or maybe even made it this far in graduate school, without your understanding of my failures and encouragement to keep going. I'm truly blessed and thankful to have had such a wonderful person to get to know and share this experience with.

TABLE OF CONTENTS

List of Tables iv

List of Figures v

Abstract vii

Chapter 1: Background 1
T-cells 1
T-cell development 1
T-helper and cytotoxic T-cells 2
Memory T-cells 5
Aging in the immune system 7
T-cell activation 11
Co-stimulatory receptors 12
Co-inhibitory receptors 12
CD28 15
CD28 structure 15
CD28 signaling cascade 16
CD28 is necessary for interleukin-2 production 18
Negative regulation of CD28 signaling 20
Cbl-b 20
PP2A 21
Tumor Immunology 23
Tumor immune surveillance (elimination) 23
Tumor immune equilibrium 24
Tumor immune escape 25
Mechanisms of escape 25
Immunotherapeutics 27
Lenalidomide and the immunomodulatory drugs (IMiDs®) 28
Thalidomide and the generation of IMiDs® 28
Lenalidomide in Myelodysplastic Syndrome 31
Effect on hematopoiesis 31
RPS14 and MDM2 32
PP2cα and CDC25c 33
Cereblon 34
Immune-modulating effects 35
Immunomodulatory capacity of lenalidomide in other hematologic malignancies 37
Enhanced T-cell co-stimulation and signaling 37

Effects on Tregs 39
Increased Natural Killer cell recognition and cytotoxicity of leukemia cells 40
Enhanced NK-cell ADCC by lenalidomide with combination antibody therapy 43

Chapter 2: Reversal of T-cell tolerance in myelodysplastic syndrome through lenalidomide immune modulation 46
Introduction 46
Results 48
Characteristics and hematologic response of lenalidomide-treated MDS cohort 48
MDS patient T-cells were inherently tolerant to *in vitro* stimulation 49
Lenalidomide recovers T-cell proliferation and augments Th1 cytokine production *in vitro* 51
Improved homeostatic regulation after lenalidomide treatment *in vivo* 54
Reversal of functional defects in lenalidomide-responsive patients *in vivo* 56
Improved T-cell homeostasis is associated with functional improvement in lenalidomide-responsive patients 59
The mechanism for increased naïve T-cell distribution after lenalidomide treatment differs in CD4+ and CD8+ T-cells 61
Discussion 62
Materials and Methods 66
Patients and healthy controls 66
T-cell activation 67
Proliferation 67
Intracellular cytokine staining 68
Detection of naïve and memory T-cell populations 69
Preparation of lenalidomide for *in vitro* studies 69
Statistical analysis 69

Chapter 3: CD28 expression is required for lenalidomide immune modulation: identification of a potential mechanism of drug resistance 71
Introduction 71
Results 73
Lenalidomide induces robust interleukin-2 (IL-2) production in the absence of CD28 co-stimulation 73
Surface expression of CD28 is increased after LEN treatment upon TCR activation 76
CD28 surface expression is essential for LEN-induced IL-2 production 78

CD28^{null} cells are resistant to LEN 79
Accumulation of CD28- T-cells in MDS patients is associated with LEN failure 81
Discussion 85
Materials and Methods 88
Healthy Donor T-cell isolation and activation 88
Preparation of lenalidomide for *in vitro* studies 89
T-cell proliferation 89
Cell Sorting 90
siRNA Transfection and ELISA 90
RT-qPCR 91
Chromatin Immunoprecipitation (ChIP) 91
MDS Patient Samples 92
Analysis of T-cell naïve and memory populations 93

Chapter 4: Lenalidomide and the IMiDs inhibit the negative regulatory activity of protein phosphatase 2A in T-cells to mediate T-cell co-stimulation 94
Introduction 94
Results 97
PP2A activity in T-cell lines and primary T-cells is inhibited with lenalidomide treatment 97
PP2A catalytic activity is inhibited after *in vitro* treatment with the IMiDs 99
Virtual computer modeling reveals a potential direct interaction of the IMiDs within the active site of the PP2A catalytic subunit 101
Mutations in the lenalidomide-binding sites of the PP2A catalytic subunit render PP2A enzymatically inactive 103
Discussion 105
Materials and Methods 109
Cell culture and creation of stably transfected cell lines 109
Drug treatment and cell lysate preparation 110
Phosphatase activity assay 111
T-cell isolation and stimulation 112
Virtual Modeling 112
Site-directed mutagenesis 113

Chapter 5: Discussion 115

References Cited 122

About the Author ..... END PAGE

LIST OF TABLES

Table 1. Characteristics and hematologic responses in MDS patients treated with lenalidomide. 49

LIST OF FIGURES

Figure 1. T-cell co-receptors that shape responsiveness to activation 14

Figure 2. Schematic of CD28 receptor signaling motifs 16

Figure 3. Schematic of T-cell signaling 17

Figure 4. Mechanisms of tumor immune escape 27

Figure 5. Structures of the immunomodulatory drugs 30

Figure 6. Schematic of various T-cell signaling pathways up-regulated after lenalidomide treatment 39

Figure 7. Lenalidomide alone, or in combination with a variety of therapeutic monoclonal antibodies, increases NK-cell mediated killing of multiple myeloma cells 42

Figure 8. T-cells from MDS patients are inherently anergic 51

Figure 9. Lenalidomide augments Th1 type cytokine production and proliferation in MDS patient T-cells *in vitro* 53

Figure 10. Naïve T-cells and immune reconstitution after lenalidomide treatment in hematologic responders 55

Figure 11. Absolute lymphocyte count remains unchanged during lenalidomide treatment 57

Figure 12. Lenalidomide reverses T-cell tolerance in MDS patients with hematologic response through increased proliferation and cytokine production *in vivo* 58

Figure 13. Increase in naïve cell production after lenalidomide treatment correlates with increased proliferation, IL-2 production, and IFN-γ production 60

Figure 14. Naïve T-cell proliferation after lenalidomide treatment correlates with younger age in hematologic responders 61

Figure 15. Lenalidomide increases IL-2 production in the absence of CD28 external ligation 74

Figure 16. CD28 downstream transcriptional element is activated after LEN treatment 76

Figure 17. Surface expression of CD28 is increased after LEN treatment upon TCR activation 77

Figure 18. Knockdown of CD28 expression abrogates LEN activity in T-cells 80

Figure 19. $CD28^{null}$ T-cells are associated with LEN Response 82

Figure 20. Non-Responder MDS patients have higher levels of Terminal Effector Memory (TEM) CD8+ T-cells and lower levels of CD28+ T-cells 84

Figure 21. Lenalidomide increases IL-2 production and inhibits phosphatase activity of PP2A isolated from Jurkat and primary T cells 98

Figure 22. PP2A catalytic activity is inhibited after *in vitro* treatment with all of the IMiDs 100

Figure 23. Virtual modeling of the IMiDs reveals potential interacting sites within the catalytic pocket of the PP2Ac subunit 103

Figure 24. Mutations of theorized IMiD-interacting sites alters PP2A enzymatic activity 105

ABSTRACT

T-cells are lymphocytes that make up part of the adaptive arm of the immune system, and are essential for efficient protection from and eradication of viruses and pathogens. T-cells not only play an important role in protection from external agents, but also regulate and prevent activation towards self-peptides and detect and remove erratically growing cells. Alterations in T-cell activation and suppression contribute to auto-immunity, immunocompromised disorders, and cancer progression.

The immune system, and T-cells in particular, provides daily surveillance, recognition and destruction of aberrant cells. Although the immune system is proficient at suppressing malignant progression, tumor cells acquire various methods of immune evasion. Myelodysplastic Syndrome (MDS) is a pre-malignant dysplastic disorder of the bone marrow characterized by ineffective hematopoiesis and clonality in the myeloid lineage, where lack of immune response has been implicated in the propensity for progression to acute myeloid leukemia (AML). Leukemia progression is associated with the acquisition of complex genetic abnormalities. Alterations in immune system regulation have been implicated in various stages of the disease process, although the role of the immune system in response to several therapies in MDS has not been fully discovered.

Lenalidomide is a small molecule therapeutic preferentially effective in MDS patients with an interstitial chromosome 5q deletion (del(5q)). Improved erythropoiesis has also been reported to occur in 20-30% of low-risk, non-del(5q) patients. Although lenalidomide is a potent immunomodulatory drug that potentiates T-cell and NK-cell responses, the T-cell compartment in MDS is highly deregulated by aberrant repertoire skewing, decreased function and abnormal naïve and memory cell homeostasis. The presence of lymphoid infiltrates in the bone marrow of lenalidomide-responsive patients suggests that T-cells may participate in the hematopoietic response, but it is unclear if lenalidomide is capable of reversing these functional T-cell defects. We therefore assessed immunological changes in low-risk MDS patients before and after 16-weeks of lenalidomide therapy, and assessed the relationship to erythroid response. Although MDS T-cells were anergic prior to treatment, we have shown that T-cells in responders have a significant increase in antigen-induced proliferative response and T helper type-1 (Th1) cytokine production (IL-2, IFN-γ, TNF-α) compared to non-responders. The change in function positively correlated with an increase in naïve T-cells and a decrease in memory cells, indicating that lenalidomide has immunomodulatory activity to reverse anergy in MDS.

Although it is known that lenalidomide may increase T-cell activation and proliferation in the absence of co-stimulatory signals, a direct mechanism of action has yet to be elucidated. Since CD28 is one of the most important co-stimulatory molecules deregulated in cancer, we therefore set out to determine if

the expression of CD28 was essential for lenalidomide's mechanism in T-cells. We knocked out CD28 expression in healthy donor T-cells, and sorted on inherently deficient, $CD28^{null}$, T-cells that accumulate in older healthy donors and found that lenalidomide-induced proliferation and function were completely ablated within the $CD28^{null}$ subset. These data indicate the immunotyrosine-based activation motifs (ITAMs) on the intracellular domain of the CD28 receptor are necessary for lenalidomide action.

Interestingly, during the natural aging process, repeated exposure to antigens results in the accumulation of $CD28^{null}$ T-cells that are phenotypically distinct and functionally deficient due to excessive proliferative history *in vivo*. We therefore examined whether CD28 expression on MDS patient T-cells affected responses to lenalidomide, and if this could be used as a predictive biomarker of responsiveness. We found that patients who fail lenalidomide therapy had higher $CD8^{+}$ Terminal Effector Memory (TEM), which are inherently $CD28^{null}$, and that non-responders had an overall increase in CD4+ and CD8+$CD28^{null}$ T-cells, as well as an increase in $CD28^{null}$ cells within the TEM compartment compared to hematologic responders.

We then sought to determine the particular protein target of lenalidomide responsible for increased CD28 receptor signaling in T-cells. Several targets in a variety of cell types have been postulated, although the direct mechanism in T-cells is unclear. Our group has previously shown that lenalidomide inhibits the activity of two haplodeficient phosphatases located within the commonly deleted region (CDR) on chromosome 5q in the MDS myeloid clone, Protein

Phosphatase 2A (PP2A) and Cdc25c. PP2Ac is known to bind CD28 and is hypothesized to inhibit T-cell co-stimulation. Therefore, it is plausible that lenalidomide and other IMiDs inhibit the phosphatase activity of PP2A which leads to increased activation of T-cell proximal signals dependent on CD28 expression. We examined this hypothesis using molecular modeling and virtual screening and found that all of the IMiDs (lenalidomide, pomalidomide, and thalidomide) can theoretically interact with the catalytic pocket of the PP2A heterotrimer, potentially inhibiting PP2Ac activity. *In vitro* phosphatase activity assays supported these findings as lenalidomide-inhibition of PP2Ac activity was seen in both ad293 and Jurkat cell lines, and in primary T-cells. Mutations of theorized lenalidomide hydrogen-bond sites within the catalytic pocket of PP2A rendered the enzyme catalytically dead, indicating that these are important residues for enzymatic activity, but unfortunately could not be used to determine if lenalidomide activity was disrupted by mutation of those sites.

These data together suggest that the ability of lenalidomide to augment immune activation *in vivo* in MDS patients, and potentially other diseases, is extremely important to patient response. Also, that CD28 expression on T-cells is essential for lenalidomide immune-mediated tumor eradication through CD28 downstream signaling, and potentially through inhibition of PP2A. These results are useful in designing future lenalidomide-combination therapy trials in other hematologic and solid malignancies, and could be used to help stratify patients for future therapeutic decisions in MDS and other malignancies.

CHAPTER 1

Background

A note to the reader: Portions of this chapter have been previously published in a review article in the journal *Advances in Hematology*, McDaniel et al. 2012. (1) and has been reproduced here with permission from the publisher.

T-cells

T-cells, along with B-cells and Natural Killer (NK) cells make up the lymphoid compartment, with T- and B-cells comprising the adaptive arm of the immune system. The immune system has both innate and adaptive mechanisms to seek out, recognize, and destroy foreign pathogens such as bacteria, viruses, helminthes, etc., and even erratically-growing autologous cells. T-cells express antigen-specific receptors (T-cell Receptors, TCRs) that allow them to specifically recognize over a million different protein epitopes, both self- and non-self, to stave off attack from the thousands of potential pathogens we encounter on a daily basis.

T-cell development. The creation of a lymphocyte compartment occurs in the thymus where cells are selected for their ability to recognize and destroy foreign pathogens while displaying tolerance towards self-antigens. Immature “T-cell precursors” are produced from hematopoietic stem cells during hematopoiesis within the bone marrow, and home to the thymus through a variety of chemo-attractants, including CCL25 and CCL21, where they develop

and mature (2, 3). The thymus is a primary lymphoid organ with several different structural features, including a cortex and medulla that separate and organize the various stages of lymphocyte development. When an immature lymphocyte enters the thymus, it goes through several CD4/CD8 “double negative” stages (DN1 to DN4) marked by lack of surface receptor expression or down-regulation of CD44 and CD25 (4). During the DN3 stage, the TCR-β chain undergoes irreversible V(D)J recombination via the recombination activating gene (RAG), generating a unique sequence (5). T-cells then continue to the DN4 stage where they undergo VJ TCR-α chain gene rearrangement. T-cells are then either CD4 or CD8 positive, and undergo positive thymic selection. Positive selection involves expression of relatively rare, low-affinity self-peptides that are necessary to ensure the creation of a functional αβTCR. Full maturation of the TCR is not complete until self- MHC-recognition within the thymus (6). Interestingly, the TCR-α chain can undergo multiple VJ recombination events to allow for the creation of a functional positively-selected TCR (7, 8). T-cells surviving positive selection then undergo negative selection, where high-affinity binding of the TCR and secondary signal induction promotes apoptosis of self-reactive T-cells, preventing them from being released into the periphery where they may induce auto-immune reactions (9, 10).

T-helper and cytotoxic T-cells. There are two distinct subsets of T-cells involved in adaptive immunity, namely CD4+ T-helper cells (Th) and CD8+ cytotoxic T-cells (Tc). CD4+ cells are helper T-cells that recognize antigen peptides presented on MHC-II expressed on antigen presenting cells. Activation

of Th cells induces proliferation and robust cytokine secretion to provide support to other helper T-cells and Tc cells in a variety of pathogenic settings. CD4+ T-cells can differentiate in the periphery into a variety of different T-helper subtypes based upon their cytokine secretion profile, including Th1, Th2, Th17, T follicular helper (Tfh) and induced regulatory T-cells (iTreg) (11). The differentiation decision is actually governed by cytokine exposure within the microenvironment of the non-differentiated CD4+ T-cells, and to some extent on the strength of the TCR-antigen interaction (12). Exposure of naïve CD4+ T-cells to various cytokines in the presence of TCR-stimulation induces epigenetic changes at DNA-loci that encode for subset-specific cytokines (13-15). It was originally thought that commitment to one T-helper cell lineage was a permanent epigenetic imprint, but recent evidence suggests that inter-conversion between the different lineages can be induced using epigenetic modifying drugs (16), and may occur between Th1 and Th2 cells under appropriate inflammatory conditions *in vivo* (17).

Each T-helper subset has been defined based upon unique gene expression profiles, and their roles in providing T-cell cytokine help in different pathological settings. Interferon-γ (IFN-γ) and interleukin-12 (IL-12) polarize CD4+ T-cells towards the Th1 lineage, characterized by production of IFN-γ and IL-2 and activation of the T-bet transcription factor (18). Th1 are involved in cellular immunity and are extremely important in providing help to CD8+T-cells during microbe infection, as well as enhancing anti-tumor activity of CD8+ tumor-antigen specific T-cells (19, 20). IL-4 polarizes CD4+ T-cells to become Th-2

through induction of GATA3 transcription factor expression. These cells secrete IL-4, IL-5, and IL-13 cytokines important for humoral B-cell support and they are essential for protection against helminthes and other multicellular pathogens (21). Th17 or “T-inflammatory” cells are a more recently defined population of T-helper cells that require (ROR)γt transcription factor expression and are induced through exposure to IL-6, IL-23, IL-21, and TGF-β (22). Th17 cells secrete IL-17 and IL-22, and play a role in the clearance of extracellular bacteria and fungi and are involved in exacerbating GVHD after bone marrow transplantation (22, 23). Aberrant Th1 and Th17 cell activation is involved in organ-specific auto-immunity, whereas Th2 erratic signaling is involved in allergy and asthma. Another recently defined subset of T-helper cells are the T-follicular helper cells (Tfh) which migrate to follicles to provide help in B-cell maturation and antibody production and are induced through IL-21 exposure (24-26) and activation of Bcl-6 (27).

Another unique subset of CD4+ T-cells are the regulatory T-cells defined by forkhead box P3 (denoted Foxp3 in mice and FOXP3 in humans) expression, which help to maintain immune system homeostasis and regulate effector T-cell responses to prevent auto-immunity. Mice lacking T-regs have overwhelming Th1 and Th2-induced autoimmunity (28-30). There are two types of Tregs, including natural Tregs (nTregs) that are produced and develop in the thymus and are characterized through CD4+CD25+ expression, and TGF-β-induced Tregs (iTregs) that are induced to express Foxp3 and become regulatory T-cells in the periphery (31, 32). Tregs are also involved in promoting immune evasion in the setting of tumors by suppressing tumor-specific cytotoxic T-cells.

CD8+ T-cells are cytotoxic T-cells (Tc) that recognize peptides presented through MHC-I not only on antigen presenting cells, but all cells within the body. CD8+ T-cells are also called “T-killer” cells because activation of these cells against a target cell causes them to release perforin and granzymes directly into the target cell, activating the caspase cascade and inducing cell death (33-35). Like CD4+ T-cells, there are different Tc subsets that have different cytokine profiles similar to their corresponding CD4 counterparts (i.e. Tc1, Tc2, Tc17), although the function of the separate subsets in different pathological settings has not been as extensively defined as in CD4 T-cells (36, 37).

Memory T-cells. Once naïve T-cells, both CD4+ and CD8+ are stimulated by their cognate antigen, they expand and eradicate the pathogen, and then during the contraction phase either become memory T-cells or die. The T-cell fate is usually governed by the strength of signal through the TCR, as well as concurrent cytokine activation (38). A majority of the activated population will undergo activation induced cell death (AICD) and will be eliminated, while others will die due to neglect, and the remainder of the population will become memory cells.

There are three types of memory T-cells: Central Memory (T_{CM}), Effector Memory (T_{EM}), and Terminal Effector Memory (T_{TEM}). Central Memory T-cells are $CD45RA^{-}CCR7^{+}CD62L^{+}$, with expression of the latter two receptors necessary for cell extravasation and migration to the secondary lymphoid organs where they have limited effector function until secondary stimulation (39). T_{CM} provide reactive memory and upon secondary activation will proliferate and differentiate

into effector cells (40). Effector Memory T-cells are CD45RA$^-$CCR7$^-$CD62L$^-$, express high amounts of perforin, and home to peripheral tissues where they are activated rapidly to produce effector cytokines and provide immediate protection against pathogens (41, 42). The third memory population is CD45RA+CCR7-CD62L- Terminal Effector Memory cells, characterized as “end-stage” differentiation. These cells are thought to accumulate following intense cytokine-driven proliferation (43). Interestingly, CD45RA+ T_{TEM} cells are generally senescent with a majority of the population lacking the CD28 co-stimulatory molecule, and increase through aging and in autoimmune diseases (44, 45). This particular memory population is found only in humans, as murine T-cells do not down-regulate the CD28 receptor. Recently it has been determined that the senescence of these cells is due to decreased p38-MAPK pathway signaling, and since they have normal telomerase activity, their senescence is potentially reversible (46). There is some controversy though about the stages of memory cell development. It is unclear if T-cells undergo linear transformation from naïve to T_{CM} and then progress to T_{EM}, and then T_{TEM} after multiple rounds of stimulation (47, 48), or if there is asymmetric memory development. Interestingly, it was recently discovered that there is asymmetric memory T-cell division in response to antigen re-challenge, indicating that there may be a subset of T-cells that remain in the T_{CM} subset after activation (49).

Memory T-cells are an extremely important component of the adaptive immune system, as they persist throughout a person’s lifetime through homeostatic proliferation to provide antigenic memory and swift reaction time to

antigen re-exposure. Memory T-cells turnover at a very slow rate in the absence of their cognate antigen through exposure to the common-γ chain cytokines Interleukin-7 (IL-7) and IL-15, allowing for continued maintenance of the memory compartment (50-53). IL-7 and IL-15 are necessary for memory maintenance of both CD4+ and CD8+ T-cells, but not memory induction (52, 54, 55). CD4+ T-cell help, likely through IL-2 secretion, is an absolute requirement for the generation of functional CD8+ T-cell memory (56). Factors driving the generation of CD8+ effector vs. memory cells are not completely understood, but it seems to require the expression of the transcription factor eomesodermin (eomes, a member of the T-bet family of transcription factors) and exposure to IL-2 (57, 58). Alterations within homeostatic regulation of the memory cell compartment are found in aging individuals, cancer, and several autoimmune disorders, alluding to the importance of memory T-cell generation and maintenance. The strength of antigen stimulation, exposure to homeostatic cytokines, and the phenotype of the memory compartment are all extremely important in shaping the immune reaction to pathogens and cancer.

Aging in the immune system. As with many other systems in the body, aging induces profound alterations within both the innate and adaptive arms of the immune system, and in turn affecting the immune system's ability to respond to and eradicate pathogens. Unlike T and B-cells, NK cell numbers actually increase with age, and normal healthy aging is associated with only a slight decrease in proliferation and response to IL-2 (59). Overall, NK-cell cytotoxicity and IFN-γ production decrease on a "per cell basis" as one ages, but only

significant decreases in cytotoxicity are associated with increased morbidity and mortality associated with infections and poor influenza vaccination response (60-62). The humoral immune response is also dramatically altered through aging. The phenotype of the B-cell compartment is altered through a decrease in overall naïve B-cell numbers and an increase in memory B-cells, leading to overall decreased BCR diversity (63, 64). The B-cells, like T-cells, also have a decrease in co-stimulatory molecule expression, antibody affinity, and a decreased ability for antibody isotype switching which contributes to an overall reduction in infection-fighting capacity (63, 65) . Included with these changes are deficiencies within the T-cell compartment leading to inefficient T-cell help and contributing greatly to overall decreased humoral immune response (66) .

Aging also has a particularly pronounced effect on the phenotype and function of the T-cell compartment, namely alterations in memory T-cell development and differentiation, as well as cellular senescence (67). Thymic T-cell production essentially stops once a child has finished growing and has sufficiently seeded the peripheral T-cell compartment with naïve T-cells (68). The thymus begins to involute around the age of 20, replacing the functional thymic cortex and medulla with adipose tissue, and causing a steady decline in the release of new naïve T-cells into the periphery (69, 70). Therefore, naïve T-cells produced in young humans must persist and survive throughout one's lifetime. Steady-state naive T-cell numbers are maintained in the periphery via homeostatic, mostly TCR-independent, mechanisms through cytokines like IL-7 and IL-15 (71, 72). But, the ability for naïve T-cells to continually divide and keep

populating the T-cell compartment diminishes with age because of the accumulated numbers of memory T-cells that proliferate and crowd the T-cell niche in both the CD4 and CD8 subsets.

Not only does the production of naïve T-cells decline with age, but also the function of memory T-cells that have undergone many rounds of homeostatic proliferation, and newly-generated memory T-cells as well. Poor memory T-cell response and the loss of cognate T-helper cell function among CD4+ T-cells are one of the causes of poor vaccine efficacy in the elderly (73, 74). Aged T-cells have decreased calcium flux after TCR stimulation, as well as decreased ability to produce IL-2 and other cytokines, although some pro-inflammatory functions are enhanced (75, 76).

One of the most profound and consistent phenomenon observed with aging and the immune system is the accumulation of terminally differentiated $CD45RA^{+}CD28^{-}$ T-cells (77, 78), which can also be accelerated through chronic CMV infection (79, 80). Humans over the age of 80 have approximately 10-15% $CD4^{+}CD28^{-}$ T-cells and approximately 60% $CD8^{+}CD28^{-}$ T-cells (44) that have decreased T-cell receptor diversity (81) and decreased ability to proliferate in response to cognate antigen (82). $CD28^{-}$ terminally differentiated memory T-cells are hypo-responsive in their inability to fully support stimulation with the lack of the co-stimulatory molecule, demonstrating a decreased ability to secrete IL-2 or induce telomerase expression, and are more susceptible to activation induced cell death (AICD) due to their long proliferative history *in vivo* (83-85). The accumulation of $CD8+CD28^{-}$ T-cells negatively impacts immune responses to

foreign antigens in the elderly because these cells proliferate and expand, constraining the adaptive immune response through competition for available space within the T-cell niche, restricting naïve T-cell homeostatic proliferation.

There are several documented molecular mechanisms for the perpetual loss of CD28 surface expression on terminally differentiated T-cells. Enhanced TNF-α exposure has been experimentally shown to decrease CD28 surface receptor expression on T-cells, suggesting that these cells are produced in inflammatory conditions, and persistent infection, like CMV, has been demonstrated to drive their accumulation and induce pre-mature aging of the immune system (86, 87). Interestingly, CD4+CD28- T-cells are readily triggered by inflammation and thereafter perpetuate and amplify the inflammatory process (88). CD28 surface expression is transiently down-regulated after ligation with B7-1/2 or with anti-CD28 antibody ligation, but is usually re-expressed on the cell surface within 48 hours. Under persistent T-cell stimulation, CD28 expression is gradually reduced and eventually lost without the ability to re-induce expression, suggesting that transcriptional silencing controls receptor down-regulation. Utilizing a CD28-reporter gene construct method, it was discovered that T-cells lacking CD28 are not able to induce expression of CD28 due to a lack of protein complex formation that binds to the CD28 initiator (89-91). Two proteins within the complex, nucleolin and A isoform of heterogeneous nuclear riboprotein-D0 (hnRNP-D0A), have been shown to be essential for the induction of CD28 expression, and a coordinate loss of alpha-beta INR bound-complexes contributes to decreased CD28 expression in senescent T-cells (89).

Overall, there are significant changes in all the immune system compartments associated with the deterioration of the immune system during aging, but the accumulation of CD28- T-cells is a significant player contributing to the reduced overall responsiveness to pathogens and viruses, and likely to the decreased ability to eradicate tumor cells. Exhausted T-cells usually develop in the presence of highly replicated viruses and tumors through increased inhibitory molecule interaction (like PD-1) and along with the increased expression of TNF-α in the tumor microenvironment, contributing to increased immune senescence (92, 93). The impact of the accumulation of CD28- T-cells on the immune system's ability to eradicate tumors has not been fully elucidated, but is important to understand in relation to the use of immunotherapeutic vaccines for cancer.

T-cell activation. TCR binding to cognate ligand presented on antigen presenting cells induces receptor clustering, phosphorylation of downstream signaling molecules, and activation of transcription factors leading to proliferation, cytokine production and effector/regulatory functions. There are multiple important co-receptors in the B7-CD28 and TNF-Receptor superfamilies involved in this process, such as CD28, cytotoxic T lymphocyte antigen 4 (CTLA-4), inducible co-stimulatory molecule (ICOS), programmed cell death protein-1 (PD-1) and 4-1BB and OX40, respectively. These receptors have both activating and inhibitory properties involved in shaping and altering the T-cell response upon association with cognate ligand (94, 95), as seen in **Figure 1.**

Co-stimulatory receptors. Co-stimulatory receptors, including CD28, ICOS, 4-1BB, and CD40L, expressed on T-cells are necessary for a fully competent T-cell response. CD28 appears to be the most important co-stimulatory molecule involved in activation and proliferation, and will be described in great detail later in this chapter. ICOS is a more recently discovered co-stimulatory molecule that is not natively expressed on naïve T-cells, but is up-regulated quickly on effector T-cells upon activation, indicating it may provide co-stimulatory signals to activated T-cells (96-98). Ligation of ICOS by ICOS-L (B7h) on APCs is involved in co-stimulation of both Th1 and Th2-type effector responses (99, 100), and is necessary in regulating immunoglobulin-isotype class switching and germinal center formation (101). 4-1BB and OX40 are members of the tumor necrosis factor receptor (TNFR) family that bind to 4-1BBL and OX40L respectively on antigen presenting cells, and are also involved in co-stimulation. The expression of both molecules is triggered by TCR/CD28 ligation, as well as IL-2 binding, and the ligation of these receptors increases T-cell proliferation and up-regulation of anti-apoptotic molecules such as Bim, Bcl-xl, and Bcl-2 (102-104). Their role appears to depend on T-cell activation, with 4-1BB and OX40 ligands important in regulating the frequency of effector memory cells generated during recall responses while enhancing T-cell function directly through induction of IL-4 and IFN-γ production (105).

Co-inhibitory receptors. CTLA-4 is the best characterized T-cell co-inhibitory receptor that is essential in regulating T-cell activation and tolerance. CTLA-4 expression on naïve T-cells is low and becomes up-regulated

after activation to intrinsically regulate CD28 signaling through competition for B7-1/2 (CD80/CD86) binding (106, 107) (**Figure 1**). In naïve T-cells, CTLA-4 accumulates in the golgi and is released to the external membrane fraction upon activation (108), where it binds to B7-1 or B7-2 to disrupt CD28 stabilization at the immune synapse, effectively shutting off T-cell co-stimulation and signaling (109). The importance of CTLA-4 in regulating T-cell homeostasis not only lies in its ability to down-regulate activation after stimulation, but in inducing tolerance and suppression mechanisms of regulatory T-cells as well. CTLA-4 knockout mice die within 3-4 weeks of age due to lethal polyclonal CD4+ T-cell dependent lymphoproliferation and autoimmunity, leading to multi-organ failure (110-112). Interestingly, the intracellular cytoplasmic tail of CTLA-4 is not always necessary to induce T-cell inhibition, as mouse models with mutant intracellular motifs were still able to regulate T-cell signaling. This data suggests that CTLA-4 attenuates T-cell activation by physical competition of the extracellular domain binding to B7-1/2 ligands and displacement of CD28 (113, 114).

PD-1 is another co-inhibitory molecule from the B7-CD28 family that is highly expressed on activated CD4+ and CD8+ T-cells, B-cells, and myeloid cells (115). PD-1 ligation by either PD-L1 or PD-L2 has been shown to abrogate TCR-mediated proliferation and cytokine production in activated T-cells, and cannot be overcome through strong CD28 co-stimulatory signals (116, 117). PD-1 only exerts inhibitory activities in the presence of TCR signaling, and does so through activation of SHP-2, which de-phosphorylates signaling molecules within the proximal-TCR signaling cascade, halting TCR-induced activation (118). PD-L1

expression on tumor cells inhibits tumor-specific killing by CD8+ cytotoxic T-cells, and up-regulation of PD-1 expression on functionally exhausted antigen-specific T-cells indicates the negative role PD-1 plays in T-cell activation and proliferation. On the reverse, PD-1 plays an extremely important role in regulating normal T-cell homeostasis and prevention of auto-immunity, as PD-1 knockout mice of the C57BL/6 strain develop progressive arthritis and a lupus-like syndrome (119), or early onset cardiomyopathy in the Balb/c strain (120). These data all demonstrate the importance of co-inhibitory and co-stimulatory molecules in shaping T-cell responses under normal conditions, and how erratic expression or activation of either can lead to severe pathologic conditions.

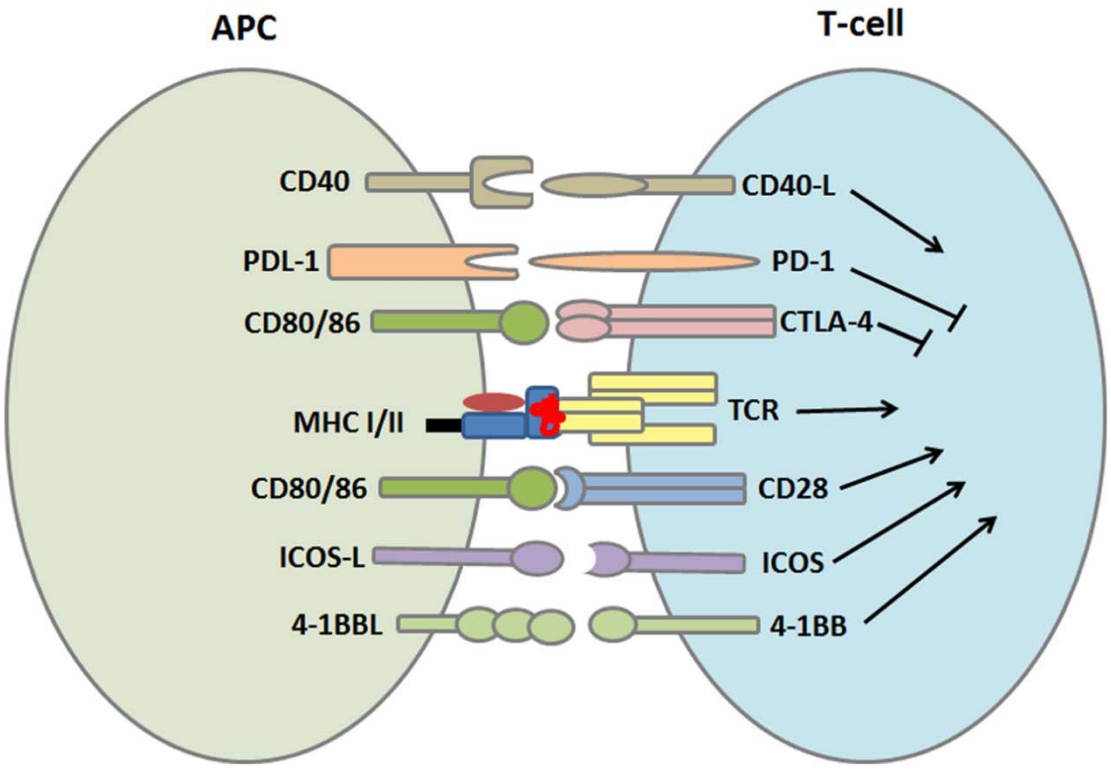

Figure 1. T-cell co-receptors that shape responsiveness to activation. T-cell co-receptors bind to ligands expressed on antigen presenting cells (APCs), left. CD40L, CD28, 4-1BB and ICOS are co-stimulatory molecules that enhance/activate downstream signaling upon TCR ligation. PD-1 and CTLA-4 are co-inhibitors that turn off and inhibit T-cell activation after TCR ligation.

CD28

CD28 structure. CD28 is a 44 kDa co-stimulatory receptor on T-cells that synergizes with the TCR to induce downstream intracellular signaling, cytokine production, actin rearrangement, and prevent apoptosis and anergy upon ligation with CD80/CD86 (B7-1/2) on APCs (121, 122). CD28 is expressed on the surface of T-cells as a glycosylated, disulfide-linked homodimer (123), with an extracellular ligand-binding domain, a transmembrane region, and an intracellular cytoplasmic tail containing several activation sequences, as CD28 lacks intrinsic kinase activity (**Figure 2**). The cytoplasmic tail of CD28 contains two immunoreceptor tyrosine-based activation motifs (ITAMs), namely a YMNM (i.e. YXXM) SH-2-binding domain and PYNPP SH-3-binding domain, that are essential for the binding of kinases and adapter molecules (124). The tyrosine (Y191) in the YMNM motif allows for binding of p85 subunit of PI3K and the guanine nucleotide exchange factor Vav1 through the Grb2 adaptor molecule (**Figure 2**) (124-126).

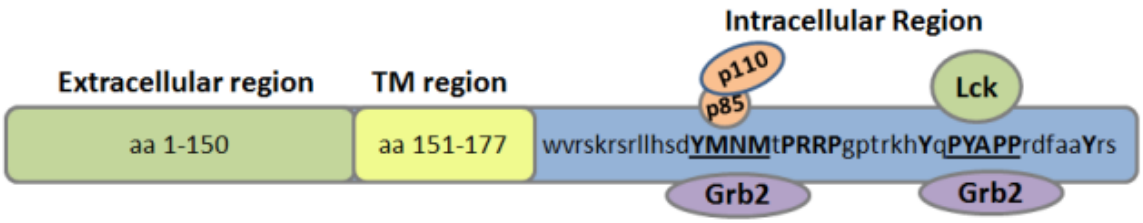

Figure 2. Schematic of CD28 receptor and signaling motifs. CD28 is a transmembrane receptor comprised of an extracellular ligand binding domain, a transmembrane domain (TM), and an intracellular tyrosine-rich region. The cytoplasmic tail contains 2 ITAMs (**bold**) that are phosphorylated upon activation (by Lck) to recruit signaling molecules, some of which are indicated above (Grb2 and p85).

CD28 signaling cascade. Knockdown of CD28 leads to decreased T-cell activation and reduces memory T-cell generation (127), and its expression is necessary for proliferation and homeostasis of regulatory T-cells (128). When the TCR encounters its respective antigen presented on MHC, tyrosines within the ITAM on the TCR/CD3 complex become phosphorylated by src-related kinases Lck and fyn (129, 130). Phosphorylation of ITAM motifs on the TCR recruits kinases Zap70, adaptor molecule Grb2, phosphoinositide 3-kinase (PI3K) and other SH-2 domain containing proteins to the TCR/CD3 complex, and further promote downstream activation and nuclear localization of transcription factors, like NF-kB and AP-1 (129, 131, 132). This activation cascade is essential, but not sufficient, for full TCR-induced signaling response.

Important kinases in the TCR signaling pathway, like lck, phosphorylate tyrosines within the YXXM and proline-rich PYAPP motif on the cytoplasmic region of CD28 leading to activation of PI3K, MAPK, and NF-κB pathways

(**Figure 3**) (124, 126, 133, 134). The PYAP motif also associates with Filamin A, an actin binding protein involved in scaffolding for lipid raft generation which is necessary for the formation of a functional immune synapse (135).

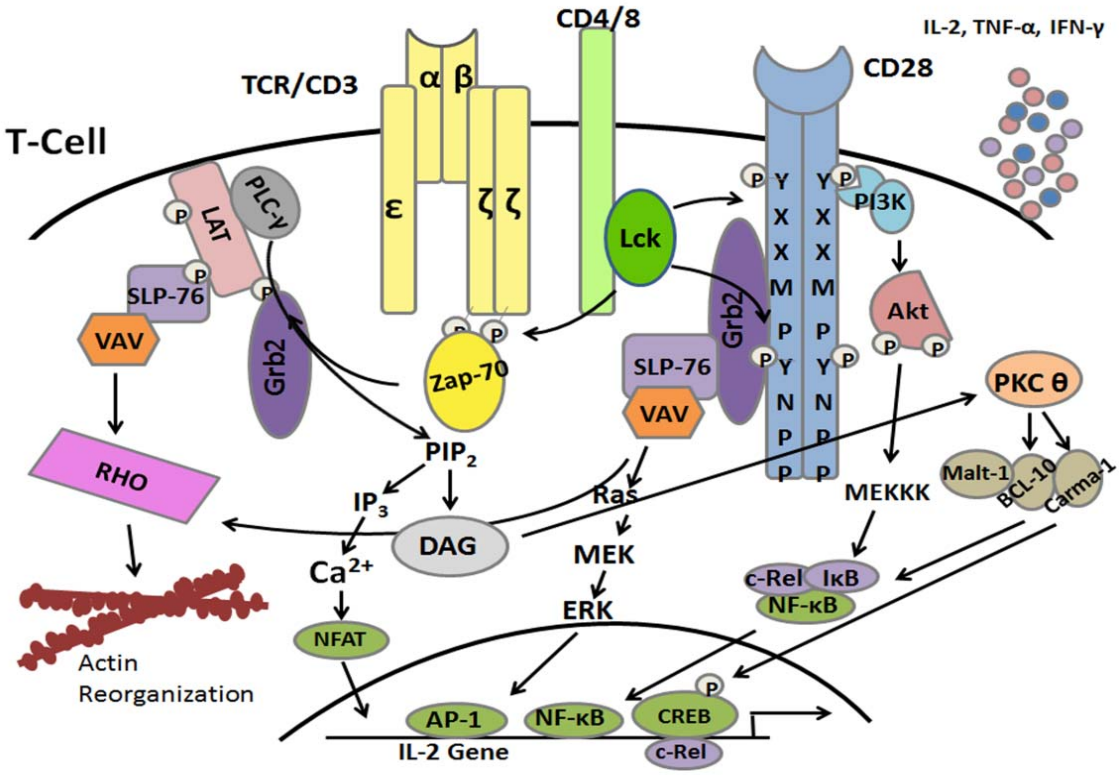

Figure 3. Schematic of T-cell signaling. TCR ligation induces activation of various kinases, including lck, which phosphorylates tyrosines within the intracytoplasmic tail of CD28, increasing downstream signaling and activation of PKC-θ, MAPK, and other signaling pathways. These pathways lead to the activation of classic T-cell transcription factors like AP-1, NFAT-1, and NF-κB that induce secretion of the T helper type 1 (Th-1) cytokines IL-2, tumor necrosis factor-α (TNF-α), and Interferon-γ (IFN-γ), as well as promote proliferation and prevent apoptosis.

There has been substantial difficulty in elucidating distinct signaling cascades emanating from TCR ligation versus CD28 ligation, as there is

significant cross-talk between the two pathways. Vav1 activation has been shown to be necessary for superagonistic CD28 antibody activation of T-cells in the absence of TCR ligation, alluding to its importance in CD28-mediated signaling (133, 136). Vav is extremely important in the activation of Rho GTP-ase and actin mobility/re-organization (137). The ability of CD28 to signal in the absence of the TCR has been hypothesized, as seen in microarray data of T-cells stimulated with anti-CD28 alone. Activation with only anti-CD28 results in an independent gene expression profile in comparison to anti-TCR signaling in the absence of CD28, but the significance of this remains controversial (138, 139). The relative importance of the ITAM motifs on the CD28 receptor in the activation of T-cells is also debated. Mutation of the proximal YXXM motif (Y170F) inhibited PI3K binding, but had no overt effects *in vivo* (140), although mutation of the proline-rich PYAP motif (to AYAA) abrogated proliferation and IL-2 and other cytokine expression (141). These data collectively suggest that signals emanating from the YXXM motif may be redundant, or that TCR ligation has sufficient activation. The activation of PYAP-binding proteins, however, appears to be essential for T-cell activation and most likely unique to CD28 signaling.

CD28 is necessary for Interleukin-2 production. The requirement of CD28 expression for transcriptional activation of the inerleukin-2 (IL-2) promoter is not contested. A 50-fold increase in IL-2 secretion by both transcriptional and post-transcriptional mechanisms occurs after CD28 co-stimulation compared to TCR activation alone (142). Moreover, CD28 knockout mice retain the ability to proliferate in response to stimulation, although reduced compared to WT

counterparts, but completely lack the ability to express IL-2(143). The p85 subunit of PI3K binding to the YMNM motif leads to activation of the Akt pathway, inducing phosphorylation of mTOR, IκB and other molecules that induce the activation of NF-κB, c-fos and c-jun (AP-1), and OCT-1 that induce IL-2 gene expression and the up-regulation of pro-survival proteins like Bcl-xL and Bcl-2 (144) (**Figure 3**).

Although PI3K activation is important to augment CD28 signaling, signaling through activation of PKCθ-Carma-1/Bcl-10/Malt-1 (CBM) complex pathway is specific and necessary for CD28-specific induced transcription factor activation of NF-κB and cAMP response element-binding protein (CREB) (145). Vav activation of Protein lipase Cγ1 (PLC-γ1) downstream of CD28 ligation activates diacylglycerol (DAG), that in turn induces PKC-θ activation and recruitment to the immunological synapse (146). Although Vav signaling is not specific to CD28, Vav activation is indispensible for CD28 mitogenic signaling (147). PKC-θ then phosphorylates caspase-recruitment domain-containing membrane associated guanylate kinase protein-1 (CARMA-1), allowing binding to Bcl10 creating the CBM complex that activates NF-κB and other transcription factors like CREB and NFAT to bind to the CD28 response element (CD28RE) on the IL-2 promoter (148, 149). NFAT, AP-1, and NF-κB have several DNA binding-sites on the IL-2 promoter, but it was discovered that the binding of these transcription factors to a specific region 180bp upstream of the IL-2 transcriptional start site was induced specifically by CD28 ligation (CD28RE), and were necessary to induce IL-2 expression (150). A transgenic mouse model of a

dominant negative form of T-cell specific promoter-driven CREB determined that dysfunctional CREB led to decreased proliferation and IL-2 production, cell-cycle arrest and apoptosis (151-153). Taken together, these discoveries suggest that the binding of transcription factors to the CD28RE is essential for IL-2 production and induced only through specific signals emanating from ligation of the CD28 receptor.

Negative regulation of CD28 signaling. There are several mechanisms inherent within T-cell biology to prevent hyper-activation of T-cells in the absence of co-stimulation, non-specific activation in the presence of low-affinity ligands, and to "turn off" signaling during T-cell contraction phase. Negative signaling regulation is necessary, in general, for prevention of autoimmunity.

Cbl-b. One protein in particular that contributes to CD28 regulation and is involved in anergy induction is casitas b-lineage lymphoma-b (cbl-b). Cbl-b is an E3 ubiquitin-ligase in the cbl-protein family that can either mono-ubiquitinate proteins to alter signaling cascades or poly-ubiquitinate proteins, targeting them for degradation via the proteasome pathway (154). The importance of cbl-b in inhibiting the activation of the CD28 signaling cascade was first discovered through the use of cbl-b knockout mice (155, 156). In 2000, two groups independently published results from germline cbl-b knockout mice. Homozygous cbl-b deficient (cbl-$b^{-/-}$) mice were hyper-responsive to TCR stimulation and intolerant to self-antigens, allowing the T-cells to proliferate and induce IL-2 cytokine production in the absence of CD28 ligation leading to generalized autoimmunity *in vivo*. Crossing the cbl-b-/- mice with CD28-/- mice reversed the

hyperactivation and autoimmune phenotype, demonstrating the importance of cbl-b in CD28 regulation (157).

In the absence of CD28 ligation, cbl-b mono-ubiquitinates the p85 subunit of PI3K, prohibiting its recruitment to the CD28 receptor and preventing activation of the PI3K pathway (158). PKC-θ is also mono-ubiquitinated and targeted for lysosomal degradation by cbl-b, inhibiting activation of NFAT and NF-κB (159). Cbl-b regulation of CD28 is extremely important in self-tolerance and prevention of non-specific T-cell activation, but these processes are also involved in anergy induction. Repeated TCR stimulation in the absence of co-stimulatory molecule ligation drives the expression of several anergy inducing cell cycle inhibitors, tyrosine phosphatases, and cbl-b which help establish anergy (160, 161). Up-regulation of cbl-b expression further inhibits the PI3K and PKC-θ pathways, as well as degradation-inducing ubiquitination of PLC-γ, leading to unstable immune synapses and defects in calcium mobilization (159, 162).

TCR ligation in the presence of CD28 activation is necessary to produce a strong enough signal allowing for sustained activation of PKC-θ and PI3K, inducing full T-cell activation. After CD28 ligation, activated PKC-θ phosphorylates cbl-b, increasing cbl-b auto-ubiquitination and proteosomal degradation demonstrated through PKC-θ-cbl-b co-IP experiments, and elevated cbl-b expression in PKC-$\theta^{-/-}$ T-cells (163, 164).

PP2A. Phosphatases have an important role in regulation of T-cell activation and reversion back to a resting phenotype upon stimulus removal, but

their study has been quite less extensive in the past years compared to that of kinases. CD45, SHP1, and SHP2 are protein tyrosine phosphatases (PTPs) with importance in both positive and negative regulation of T-cell signaling via the TCR, Zap-70, Syk, MAPK, and many other signal cascade molecules (129). Protein phosphatase 2A (PP2A) is a serine/threonine phosphatase that is also essential in regulating many cellular processes, including signal transduction pathways, cytoskeleton dynamics, cell-cycle regulation and cell mobility (165, 166), as well as negative regulation of T-cell signaling. Protein phosphatase 2A (PP2A) is a heterotrimeric holoenzyme composed of an “A” scaffolding subunit and “C” catalytic subunit that together comprise the PP2A enzymatic core. Proper function and regulation of PP2A is regulated through the association of the core heterodimer to variable “B” regulatory subunits (167, 168). The ability of the PP2A core to trimerize with the over 16 members of the four B subfamilies (B, B’, B’’, B’’’) confers substrate specificity (166, 169), which is essential for proper PP2A regulation as PP2A makes up over 1% of total cellular protein in some tissues.

PP2A has also been shown to have an important role in the negative regulation of IL-2 production in T-cells in systemic lupus erythematosus (SLE) (170, 171). Katsiairi et al, demonstrated that PP2A is over-expressed in SLE patients, and this over-expression leads to decreased CREB phosphorylation, inhibited CREB binding to the IL-2 promoter, and abrogated IL-2 expression. PP2A can directly de-phosphorylate CREB to affect its transcriptional regulation, as well as de-phosphorylate CARMA-1 to dissociate the CBM complex and

down-regulate T-cell activation (172, 173). PP2A interacts with both the CD28 and CTLA-4 receptors (174) to directly regulate their signaling pathways. It has recently been shown that the important motifs on CTLA-4 for interaction of the A and C PP2A subunits are a lysine rich region and amino acid Y182, respectively (175). Dissociation of this core a-c heterodimer from CTLA-4 likely inactivates downstream targets of TCR signaling (like Akt) because treatment with Okadaic Acid, a known inhibitor of PP2A, reverses this Akt inhibition (175). Since CTLA-4 and CD28 are structurally homologous and bind to shared ligands (174), it is likely PP2A that binds to similar motifs on CD28, however, this has not been experimentally verified. Although the exact binding motif is not determined, an *in vitro* kinase assay with lck and CD28 showed that the addition of PP2A removed lck-induced CD28 phosphorylation, indicating that PP2A may have a role in directly reversing CD28 ITAM activation (174) and inhibiting downstream signaling.

Tumor Immunology

Tumor immune surveillance (elimination). Immunosurveillance of cancer cells was first postulated by Dr. Burnet in the 1970s, and is now a well-established principle thought to contribute not only to the quantity, but also the quality, or immunogenicity, of a tumor during development (176-178). Dr. Robert Schreiber and colleagues first coined the 3 steps contributing to tumor immunoediting (elimination, equilibrium and escape) back in the early 2000s, and

the importance of proper immune regulation in cancer management continues to be experimentally supported today (179, 180). An active and healthy immune system is critical for managing tumor development at the sub-clinical level, evidenced through increased rate of spontaneous tumor generation in immunodeficient mice (177, 181), and in immune-suppressed patients. Immunosuppressed patients due to organ or bone marrow transplantation have a 3-100 fold increase in their susceptibility to develop certain malignancies (182, 183), including heart transplant patients who have shown a 25 times higher prevalence of lung tumors compared to the general population (184). Since the 1990s, there have been several studies demonstrating better prognosis and longer survival in patients who had evidence of tumor infiltrating lymphocytes (TILs) within solid tumor biopsies, indicating that the immune system plays a key role in eliminating tumors at the sub-clinical and clinically detectable level (185-189).

Tumor immune equilibrium. Mechanisms regulating innate and adaptive immune responses are carefully orchestrated to detect and remove infected, transformed, or erratically growing cells within the body on a daily basis, preventing tumor formation. Tumors eventually overcome immune detection through an equilibrium stage where the immune system actually “edits” tumors and selects cells that can evade immune detection. Tumor immune-editing has been demonstrated through several mouse tumor models. Transplantation of an immunogenic tumor through multiple rounds of immune-responsive hosts lead to reduced immunogenicity, and the eventual inability to eliminate the tumor (190,

191). The immune system is able to eliminate only those cells that it can recognize and kill, leaving behind tumor cells that have reduced immunogenicity and a survival advantage facilitated through inherent genetic instability within the tumor environment (192). Immune editing is also evidenced through tumors that have been grown in an immunocompromised host (i.e. $RAG^{-/-}$) that are rejected fairly easily when removed and transplanted into an immune-efficient host. This immunoediting process occurs prior to any clinically detectable lesion in humans, as the immune system is able to eliminate a majority of the abnormal cells, and the length of time that it takes for the tumor to eventually overcome the innate and adaptive immune systems is not known.

Tumor immune escape.

Mechanisms of escape. During the equilibrium stage, the immune system eventually becomes overwhelmed, or evaded, by the malignant cells and the tumor progresses into a clinically detectable lesion. At this point, the point at which clinicians and researchers are trying to eradicate the lesion, the tumor has already acquired several advantages to suppress the immune system and survive. Tumors can induce peripheral T-cell tolerance through antigen non-responsiveness (lack of co-stimulation) or skewing of the T-cell functional response towards a non-tumor cytotoxic phenotype (i.e.: Th2 or Th17) (193, 194).

Tumors have several mechanisms of inducing anergy/non-responsiveness and evading immune detection (**Figure 4**). These mechanisms include down-

regulation of class I human leukocyte antigen (HLA) molecules that allow for the detection of tumor associated antigens (195), increased recruitment of immature myeloid cells, called myeloid-derived suppressor cells (MDSCs), into the tumor environment (196, 197), increased recruitment of regulatory T-cells into the tumor (198), and various alterations in T-cell co-stimulatory/co-inhibitory molecules expressed on the tumor. One important mechanism of escaping T-cell recognition is through down-regulation of co-stimulatory molecules, leading to anergy or non-responsiveness towards the tumor cells (as reviewed in (199)). Lack of co-stimulation through B7-1 molecule even in the presence of low affinity antigenic stimulation (similar to that seen in the tumor environment) can also render CD8+ T-cells susceptible to PD-1/PDL-1 inhibition and suppress activation (200). Up-regulation of T-cell co-inhibitory molecules that shut down T-cell responsiveness and potentially increase apoptosis of tumor-reactive T-cells is another major mechanism of escaping immune recognition (201-203). B7-H1 (PDL-1) over expression is associated with tumor grade and staging in a variety of solid tumors and several hematologic malignancies (204-208), including myelodysplastic syndrome (209), and expression of B7-H2, a ligand for CTLA-4, is associated with poor prognosis in acute myeloid leukemia (AML) (210). Therefore, these co-stimulatory and co-inhibitory molecules have become attractive immunotherapeutic targets from both the T-cell and tumor cell vantage point.

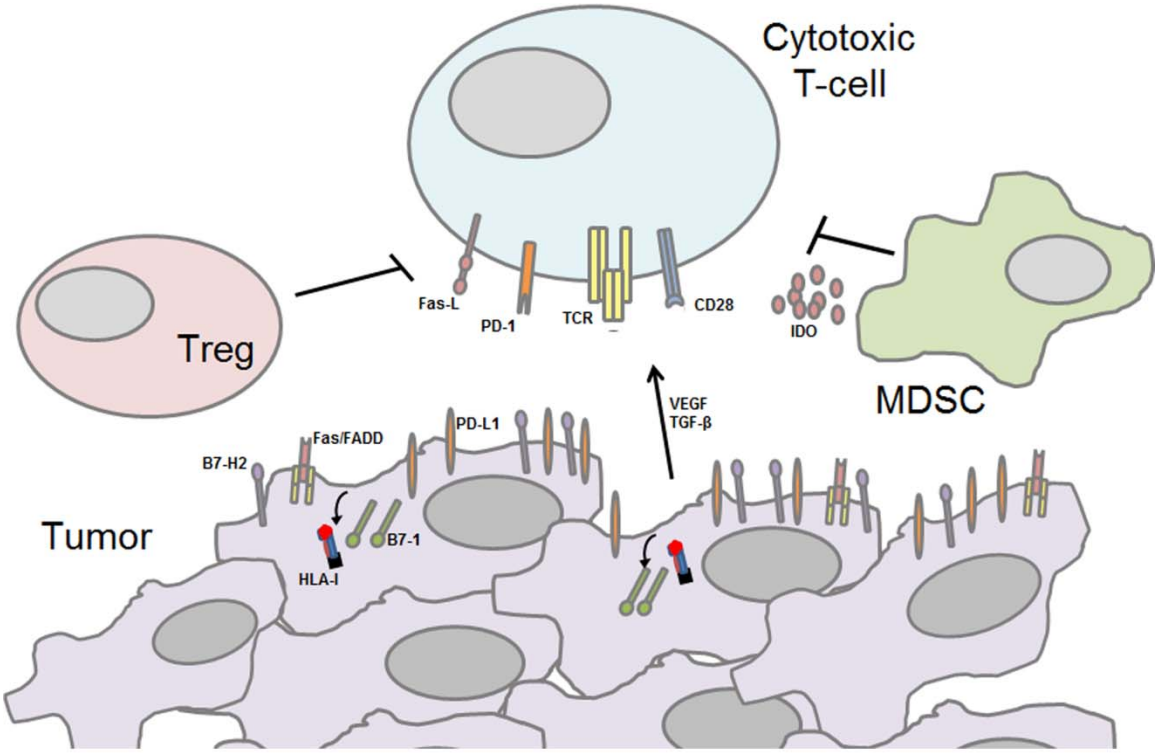

Figure 4. Mechanisms of tumor immune escape. Tumors utilize a variety of mechanisms to evade detection by the immune system. Tumors recruit regulatory T-cells, as well as immature myeloid cell (MDSCs) that suppress T-cell activation through a variety of mechanisms. Tumors also are able to down-regulate co-stimulatory molecules (B7-1) and HLA-I to evade antigen presentation, while up-regulating co-inhibitory molecules and Fas to induce T-cell suppression and apoptosis.

Immunotherapeutics. The importance of proper CD28 activation and the role of PD-1 and CTLA-4 activation in shutting down the immune response have translated into the development of several drugs targeting these pathways in cancer. As described above, tumor cells up-regulate co-inhibitory ligands like B7-H1 and B7-H2, which bind to and activate the T-cell inhibitory receptors CTLA-4 and PD-1. Recently, the use of a human anti-CTLA-4 monoclonal antibody (ipilimumab) has been approved for the treatment of

metastatic melanoma and is shown to increase tumor rejection and prolong survival, where it disrupts the CTLA-4/B7 interaction and increases CD4+ and CD8+ recruitment to the tumor environment (211-213). The blockade of PD-1 signaling also improves T-cell function, and the use of anti-PD-1 antibodies are currently in development and clinical testing in several hematologic and solid malignancies (214, 215).

Not only are monoclonal antibodies being developed to block the binding of inhibitory molecules to ligands on T-cells, but increasing the binding of co-stimulatory molecules has also been utilized. Cellular vaccine immunotherapies that over-express the B7-1 molecule have been tested and demonstrated some clinical efficacy in melanoma and non-small cell lung cancer (NSCLC) (216, 217) but this method does not account for increased expression of inhibitory molecules that dampen the activation signal. The success of these therapies support the idea that greater immunotherapy efficacy may be achieved with combination therapies, utilizing anti-inhibitory molecule antibodies, with co-stimulatory molecule engagement to produce a robust anti-tumor immune response, overcoming tumor-immune tolerance.

Lenalidomide and the immunomodulatory drugs (IMiDs®)

Thalidomide and the generation of IMiDs®. Lenalidomide (Revlimid, CC-5013) is a second-generation synthetic derivative of glutamic acid and thalidomide analogue with anti-angiogenic, anti-tumorigenic, and

immunomodulating activity. Thalidomide was initially discovered in the 1950s and was approved in Europe and Asia from 1957-1961 for distribution to pregnant women as an antiemetic drug, until it was banned because of an association with teratogenicity, including phocomelia, and peripheral neurophathy. The birth of thousands of children, dubbed “thalidomide babies” with severe limb malformations became popularly known as the “thalidomide tragedy”, and black-boxed usage of the drug worldwide. There are multiple proposed mechanisms for thalidomide-induced teratogenicity, some stemming from proposed differences in targets or off-target effects due to the chirality of the molecule. It was thought that the *S*-enantiomer of thalidomide induced teratogenic effects, whereas the *R*-enantiomer was non-toxic (218), leading to the idea that purification of enantiomer-specific molecules might have prevented the thalidomide tragedy. It was later discovered that thalidomide is able to interconvert between the *R* and *S* forms *in vivo*, suggesting that purification of a specific enantiomer would not have prevented this tragedy, and could not be used therapeutically in pregnant women (219).

Therapeutic usage of thalidomide was revitalized in the mid-1960s by Dr. J. Sheskin in Jerusalem who administered thalidomide as a sedative to a critically ill male patient with erythema nodosum leprosum (ENL), an extremely painful inflammatory complication of lepromatous leprosy (220, 221). Surprisingly, thalidomide healed the inflammatory skin lesions and overall disease burden of the patient. The ability of thalidomide to inhibit inflammation through down-regulation of TNF-alpha was ultimately discovered as the

mechanism for thalidomide efficacy and immune modulating effects in this disease (222, 223). The efficacy of thalidomide demonstrated through usage in ENL ultimately revitalized the drug, and stimulated development of several structurally-related compounds with anti-inflammatory activity for the treatment of autoimmune disorders such as Rheumatoid Arthritis (RA) (224), systemic lupus erthymatosus (SLE) (225), and Behcet's disease (226).

Creation of synthetic modifications to the thalidomide backbone led to the discovery of lenalidomide and pomalidomide, which demonstrate 500-fold greater immunomodulatory potency and safer side effect profile compared to the parent drug (227, 228) (**Figure 5**). Not only are lenalidomide and pomalidomide more potent immune-modulating drugs, but these agents are better tolerated (229).

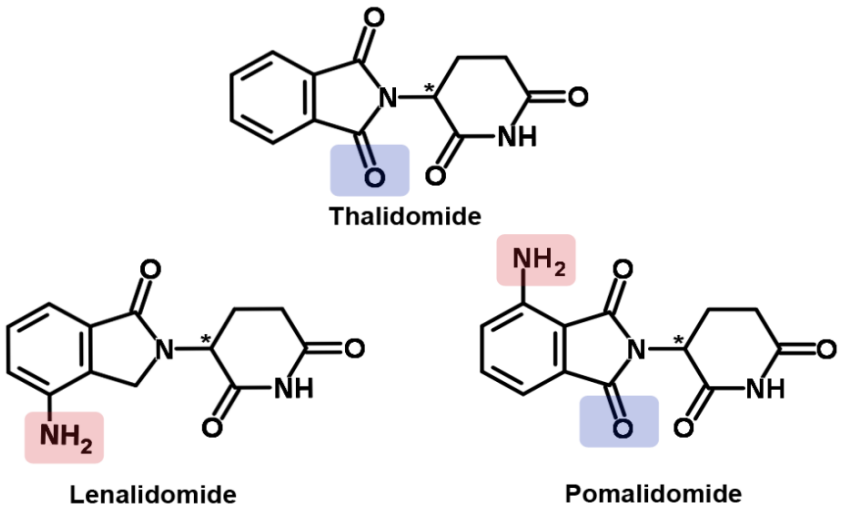

Figure 5. Structures of the immunomodulatory drugs. Thalidomide, $C_{13}H_{10}N_2O_4$, (thal, top), lenalidomide, $C_{13}H_{13}N_3O_3$, (len, bottom left), and pomalidomide, $C_{13}H_{11}N_3O_4$, (pom, bottom right) collectively make up a class of small molecules called immunomodulatory drugs or "IMiDs". The parent compound, thalidomide, was altered to generate len and pom. Light red boxes highlight an amine group added to the fourth carbon of the phthaloyl ring in the thal-derivatives. Light blue boxes indicate the carbonyl of the 4-amino-substituted phthaloyl ring shared by pom and thal. * indicates chiral carbon on all molecules.

Lenalidomide in Myelodysplastic Syndrome. Lenalidomide was first investigated in MDS because of its potent anti-TNF-α activity, and TNF is thought to contribute to disease pathogenesis in these patients. Lenalidomide is approved by the U.S. Food and Drug Administration for the treatment of low- or intermediate-1 risk and del(5q) Myelodysplastic Syndrome (MDS), and was the first karyotype-specific drug of its kind (230, 231). MDS is a heterogeneous grouping of bone marrow failure disorders characterized by ineffective hematopoiesis leading to various cytopenias, dysplasia in one or more myeloid lineages, and an increased risk of transformation to acute myeloid leukemia (AML). The International Prognostic Scoring System (IPSS) is used to stratify MDS into several risk categories (low, intermediate-1, intermediate-2, high) based upon cytologic features, blast counts, the number of cytopenias, and cytogenetics (232). These risk categories have distinct survival patterns and increased risk for AML progression as you go from low to high risk MDS. MDS with an interstitial deletion on the long arm of chromosome 5 (del(5q)) is one of the most common MDS chromosomal abnormalities that imparts a unique lower risk phenotype (233). Hematopoetic stem cell transplantation (HCT) is the only known curative treatment for MDS, but several other treatments such as 5-aza (234), anti-thymocyte globulin (ATG) (235), and lenalidomide have had great success in extending patient survival. Described herein are known effects of lenalidomide in mediating erythropoiesis and disease management.

***Effect on hematopoiesis*.** Anemia and red blood cell transfusion dependence ultimately leads to mortality in a significant portion of low, and high-

risk MDS patients. Lenalidomide was able to induce 63% transfusion independence in low-risk MDS patients, and 83% transfusion independence with a 75% complete response rate in the del(5q) subtype (230). There are many postulated mechanisms for lenalidomide's efficacy in MDS, namely associated with its ability to augment erythropoiesis and induce apoptosis within the myeloid clone. Reversal of anemia in these patients has potentially been attributed to expansion of hematopoietic progenitors and erythroid bursts (236, 237), as demonstrated through increases in these populations after treatment of normal CD34+ bone marrow progenitors with lenalidomide. An erythroid gene signature of responsiveness to lenalidomide in non-del(5q) MDS also indicated that patients who respond to lenalidomide therapy have decreased expression of erythroid differentiation genes that are enhanced through lenalidomide treatment, ultimately enhancing erythropoietin (EPO) signaling and reducing anemia (238).

*RPS14 and MDM2***.** RPS14 is among 44 genes within the CDR of MDS 5q-syndrome that is involved in the impaired erythropoiesis and macrocytic anemia phenotype. It was discovered through an a RNAi screen that the specific downregulation of RPS14 alone was able to promote the erythroid phenotype seen in del(5q) MDS patients, and this data along with the finding that del(5q) patients express half of the amount of RPS14 found in non-del(5q) MDS patients, suggests that haploinsufficiency of this gene alone is sufficient to induce impaired erythropoiesis (239, 240). Loss of RPS14 expression has been shown to lead to increased p53 expression in these patients, subsequently increasing expression of p53 target genes like p21 and other cell cycle regulators that prohibit

movement through the cell cycle and induce apoptosis (241). Loss of, or mutations within, ribosomal processing proteins leads to nucleolar stress, and sequestering and inducing degradation of murine double-minute protein 2 (MDM2), allowing for p53 stabilization (242). Recently, Wei et al. demonstrated that lenalidomide can promote p53 degradation in erythroid progenitors through inhibition of the auto-ubiquitination of MDM2 (243). MDM2 auto-ubiquitination prevents its binding to p53. Free and active MDM2 usually binds to, an poly-ubiquitinates p53, targeting p53 for proteosomal degradation and preventing it from halting the cell cycle progression. MDS patients with del(5q) were shown to have inherently increased p53 levels in the bone marrow that were then decreased after lenalidomide treatment. The upregulation of p53 expression after lenalidomide failure also suggests p53 induction could be involved in resistance to lenalidomide (243).

PP2Acα and Cdc25c. Lenalidomide has been shown to not only reverse apoptosis within the erythroid compartment, but also directly induce apoptosis of the myeloid clone in del(5q) MDS (244, 245), likely through inhibition of the haplo-insufficient phosphatases PP2Acα and Cdc25c located also within the CDR (246). PP2A and Cdc25c are key proteins that regulate the G_2-M cell cycle checkpoint. Through direct or indirect mechanisms, a decrease in PP2Ac and Cdc25c gene dosage allows for lenalidomide-induced inhibition of these phosphatases, preventing cell cycle progression and inducing apoptosis (246). It has also been demonstrated that lenalidomide upregulates the expression of the tumor suppressor SPARC (247), suggesting another means by which

lenalidomide induces apoptosis of the dysplastic myeloid clones in MDS, although the target in this pathway has not yet been determined.

*Cereblon***.** Recently, a direct target of IMiD-binding has been identified. Although the potential function of this protein in MDS is not clear, cereblon (CRBN) is a 51kDa protein that mediates thalidomide teratogenicity in humans (248). Ito and colleagues utilized thalidomide-conjugated beads in competitive binding assays to demonstrate that CRBN directly binds to thalidomide, and can induce teratogenic effects in zebrafish and chicken embryos, similar to those seen in humans. CRBN is a ubiquitously expressed highly conserved protein that was first discovered as an ionic channel regulator in the brain involved in memory and learning (249, 250). CRBN binds to calcium-activated potassium channels in the brain (250, 251), chloride channels in the retina (252), and AMP kinase in eukaryotic cells (253). The homozygous R419X nonsense mutation in CRBN deletes the C-terminus region and is associated with familial mild mental retardation (254). Forebrain-specific CRBN knockout mice also demonstrate memory and learning deficits (255). *In* vivo, CRBN forms a functional E3 ubiquitin ligase complex with DNA damage binding protein-1 (DDB1), Cul4A and Roc1 that bind to and poly-ubiquitinate target proteins, targeting them for proteosomal degradation.

Thalidomide was shown by Ito et al. to not necessarily disrupt formation of the CRBN-DDB1-Cul4A complex, but inhibit its inherent auto-ubiquitination and E3 ligase activity, disrupting downstream pathways involved in physical and mental development (248). Although the role of CRBN in thalidomide-induced

teratogenicity has been established, the role of CRBN in hematologic malignancies and immune cells is not completely clear. Decreased expression of CRBN was recently shown in two separate studies to be associated with resistance to lenalidomide- and pomalidomide-induced apoptosis in multiple myeloma cell lines (256, 257). Lopez-Girona et al. also demonstrated that knockdown of CRBN in primary T-cells reverses the ability of lenalidomide and pomalidomide to increase IL-2 and TNF-α production (257), implicating a potential role for the protein in immune modulating activity. Although these data support that CRBN is a lenalidomide target, the role of CRBN in proliferation and function of both hematologic cells and immune cells is not understood. Examination of the natural targets of CRBN in other cells besides the brain will help us to better understand how CRBN is affecting responses to lenalidomide, and potentially lead to generation of other compounds to counteract acquired lenalidomide resistance.

***Immune-modulating effects*.** The immunomodulating effects of lenalidomide are also thought to contribute to erythroid responsiveness in MDS, where it acts to alter immune homeostasis and modulate inflammation within the bone marrow microenvironment. The ability of thalidomide to down-regulate monocyte-derived TNF-α production originally explained the potent anti-inflammatory properties of the drug in SLE and other auto-immune disorders (222). Enhanced TNF-α secretion within the bone marrow microenvironment has been implicated in erythroid apoptosis in MDS, which is down-regulated through lenalidomide treatment (258). Increased expression of other inflammatory

cytokines, IL-6, IL-8, and IL-32, as a result of increased TNF-α levels are also implicated in bone marrow failure, and are decreased after lenalidomide treatment (259).

Early reports of the presence of bone marrow lymphoid aggregates in lenalidomide-responsive MDS patients also implicated immune modulation in hematological responses to this agent (231). Although the alteration of T-cell homeostatic regulation in MM by lenalidomide has been described, the direct effects on the lymphoid compartment in MDS patients, and the relationship to response, has not been studied (260). Neuber and colleagues show that lenalidomide can enhance antigen-specific activity of T-cells, as well as increase conversion of naïve antigen specific T-cells to memory cells. A similar increase in central memory T-cells was observed by Noonan et al (261) in MM patients that received lenalidomide in combination with the pneumococcal 7-valent conjugated vaccine (PCV) to establish the principle of vaccine combination therapy. Interestingly, the increase in PCV-specific antibody and cellular responses were specific to the vaccination schedule favoring administration of lenalidomide prior to PCV vaccine. Another hematologic malignancy, B-Chronic Lymphocytic Leukemia (B-CLL), is associated with dysfunctional T-cell activity (262, 263) with defects in actin polarization at the immune synapse (264). Treatment with lenalidomide in CLL restored IL-2 and IFN-γ secreting CD4+ and CD8+ T-cells to normal levels (253) and reversed the suppressive signals blocking lytic synapse formation (264). One of the aims of this dissertation was to examine the immune compartment within MDS patients to see if lenalidomide can alter immune

homeostasis and cytokine production *in vivo* to further elucidate the role of increased immune activation in the treatment of MDS.

Immunomodulatory capacity of lenalidomide in other hematologic malignancies. Lenalidomide is not only approved for the treatment of MDS, but has potent immune-modulating effects in a variety of other hematologic malignancies. Lenalidomide alone or in combination therapy enhances the co-stimulatory capacity of T-cells and antibody-dependent cell-mediated cytotoxicity (ADCC) by NK cells in diseases such as MM, B-CLL, and Non-Hodgkin's Lymphoma (NHL), although the direct molecular targets have yet to be elucidated. The known effect of lenalidomide in promoting immune detection and eradication of these diseases by lymphocytes is described in this section.

Enhanced T-cell co-stimulation and signaling. Lenalidomide is able to enhance the proliferative and functional capacity of T-cells, which augments immune activity through a variety of mechanisms. Thalidomide was first shown to augment T-cell proliferation and cytokine production in the absence of co-stimulatory molecules without direct mitogenic activity (265). When a T-cell encounters cognate tumor antigens presented by antigen presenting cell (APCs), there is an increase in a variety of co-stimulatory molecules, most importantly CD28, that enables a fully competent signal response by T-cells (266). As described above, the absence of CD28-APC interaction (Signal 2) in the presence of T-cell Receptor ligation (Signal 1) leads to inactivation or anergy of naïve T-cells. Thalidomide, and to a greater extent lenalidomide, induces IL-2, IFN-γ, and TNF-α secretion (265) in the absence of CD28 stimulation, suggesting

that the drug somehow activates the co-stimulatory-dependent signaling cascade initiated by Signal 2 (267).

Both Signal 1 (TCR) and Signal 2 (co-stimulation) are necessary for IL-2 production leading to the hypothesis that lenalidomide and the other IMiDs function somewhere within this co-stimulatory pathway (129, 268, 269) (**Figure 6**). LeBlanc et al. showed that lenalidomide acts to increase tyrosine-phosphorylation in the intracellular domain of the CD28 receptor in the absence of co-stimulatory molecules (270). Although it is not known if lenalidomide acts directly to induce phosphorylation, the presence of downstream signaling events after treatment such as NF-κB p65 translocation to the nucleus, and cytokine production, suggests that this pathway may be important for lenalidomide's immunomodulatory effect (270). Others have shown that the activation of PKC-ζ and NFAT-2 are important mediators of cytokine production after IMiD treatment (271). However, a conflicting report showed that PKC-θ activity and AP-1 DNA binding was increased, without an increase in NF-κB, OCT-1, and NFAT transcription factor binding, which adds to the controversy about lenalidomide's T-cell-associated molecular mechanism of action (272, 273) (**Figure 6**). These controversial results, however, may be attributed to the methods used for T-cell stimulation, namely TCR stimulation versus calcium channel activation, respectively. Görgün et al. showed that lenalidomide and pomalidomide reduce Suppressor of Cytokine Signaling-1 (SOCS1) expression in T-cells, which is an important negative regulator of cytokine signaling (274). Even when treated with IFN-γ to induce SOCS1 expression, the drug was capable of blocking this

inhibitory response and potentiating TCR/anti-CD28 co-stimulation in effector T-cells (274). Although reduction in a suppressive signal may be important, this would not be expected to generate unique responses, such as IL-2, that specifically replace the need for a co-stimulatory signal.

Effects on Tregs. In addition to the activation of effector T-cells, there is a valid concern about the potential effect of IMiDs on regulatory T (Treg) cells that may deter anti-tumor immunity by suppressing immunosurveillance (177, 275). In this regard, lenalidomide and pomalidomide were shown to inhibit the expansion and function of Tregs by downregulating the expression of FOXP3 (276, 277). **The preferential augmentation of CD8+ cytotoxic T-cells and inhibition of regulatory T-cells makes this drug a very interesting and potentially valuable therapeutic candidate to augment immunotherapy responses in cancer patients.**

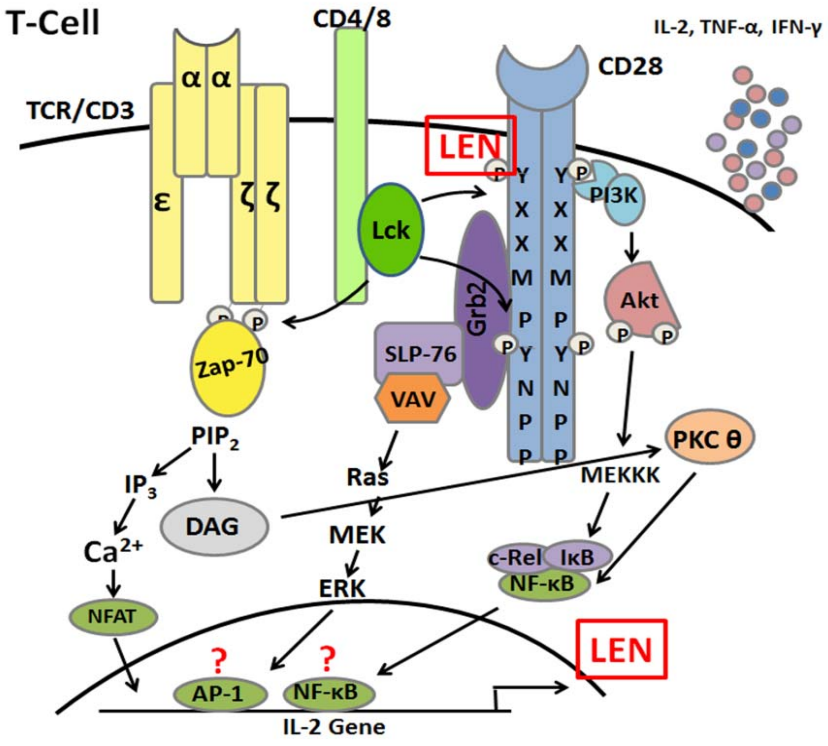

Figure 6. Schematic of various T-cell signaling pathways up-regulated after lenalidomide treatment. Lenalidomide is known to have no direct mitogenic activity, therefore it cannot induce proliferation upon TCR ligation alone. Upon TCR ligation, lenalidomide (LEN) increases phosphorylation of tyrosines within the intracytoplasmic tail of CD28, *through an unknown mechanism*, increasing downstream signaling and activation of PKC-θ, MAPK, and potentially other signaling pathways. It is controversial which transcription factors are ultimately increased upon lenalidomide treatment (indicated by a question mark). Up-regulation of these pathways potentially reverse T-cell defects, aid in breaking tolerance, and leads to greater CD4+ T-cell help to DCs, NK cells, and CD8+ T-cells, augmenting eradication of the tumor cells.

***Increased Natural Killer cell recognition and cytotoxicity of leukemia cells*.** In addition to the potentiating effect on T and B cells, immunomodulatory drugs have a profound effect on the innate immune response, namely Natural Killer (NK) cells. NK cells are an important component of the innate immune system where they play major roles in tumor rejection, viral clearance and DC regulation (278-280). Thalidomide was shown to enhance the cytotoxic effects of NK cells, as well as increase their cell numbers in MM patients (281). This enhanced killing effect requires cytokine support from accessory lymphocytes, like T-cells, as there is no measurable increase in direct killing of the K562 human leukemia cell line by purified NK cells in the presence of high doses of lenalidomide or pomalidomide (282). PBMCs depleted of NK cells were not able to kill K562 at all, nor were PBMCs in a transwell experiment, suggesting that NK cells and their contact with the tumor cell is a necessary component of lenalidomide-mediated tumor cell apoptosis (282). Support from T-cells, in the form of IL-2 secretion, is extremely important for NK cell mediated

cytotoxicity of MM after lenalidomide treatment (271). Although the combination of lenalidomide with dexamethasone has been shown to have significant activity, IL-2 production was abrogated *in vivo* when MM patients received this combination simultaneously (283). Hsu et al. demonstrated that dexamethasone treatment suppressed IL-2 production from CD4+ helper T-cells, impaired NK cell-mediated cytotoxicity and countered the immunostimulatory effects of lenalidomide in MM patients. Pharmocodynamic studies may maximize the efficacy of this combination therapy in MM.

There are multiple mechanisms postulated for increased NK cell killing in the various disease settings. Both pomalidomide and lenalidomide upregulate the expression of CD56, which normally decreases NK killing capacity, but in this setting had no detriment to NK cells (282). Carbone et al. showed that the expression of natural cytotoxic receptors (NCR) and NK receptor member D of the lectin-like receptor family (NKG2D) are necessary for myeloma cell recognition (284) and NKG2D blockade abrogated the effect of lenalidomide in solid tumors (285). It was recently shown by Benson et al. that the addition of a murine anti-inhibitory killer immunoglobulin receptor (KIR) antibody with concurrent lenalidomide therapy mediated rejection of lenalidomide-resistant tumors in a mouse model (286). This is similar to their IPH2101 human anti-inhibitory KIR antibody that also increases *in vitro* NK cell cytotoxicity specifically against MM cell targets, but not normal cells, suggesting that clinical testing in combination with lenalidomide is warranted (286).

A schematic of the various mechanisms of NK cell-mediated killing in MM after lenalidomide treatment in combination with various monoclonal antibodies is shown in (**Figure 7**). MM cells, like most tumor cells, express the programmed death receptor-1 ligand (PD-L1) which down-regulates the immune response against malignant cells through programmed death receptor-1 interactions on T-cells (287, 288). Recently, it was shown that NK cells from MM patients express PD-1, and the PD-1/PD-L1 interaction decreased NK cell-mediated killing (289). A novel anti-PD-1 antibody, CT-011, can increase NK cell-mediated killing of autologous MM cells from patients, without effecting normal cells (289). This new monoclonal therapy, along with lenalidomide's action of decreasing PD-L1 on MM cells, may improve response rates to this combination therapy.

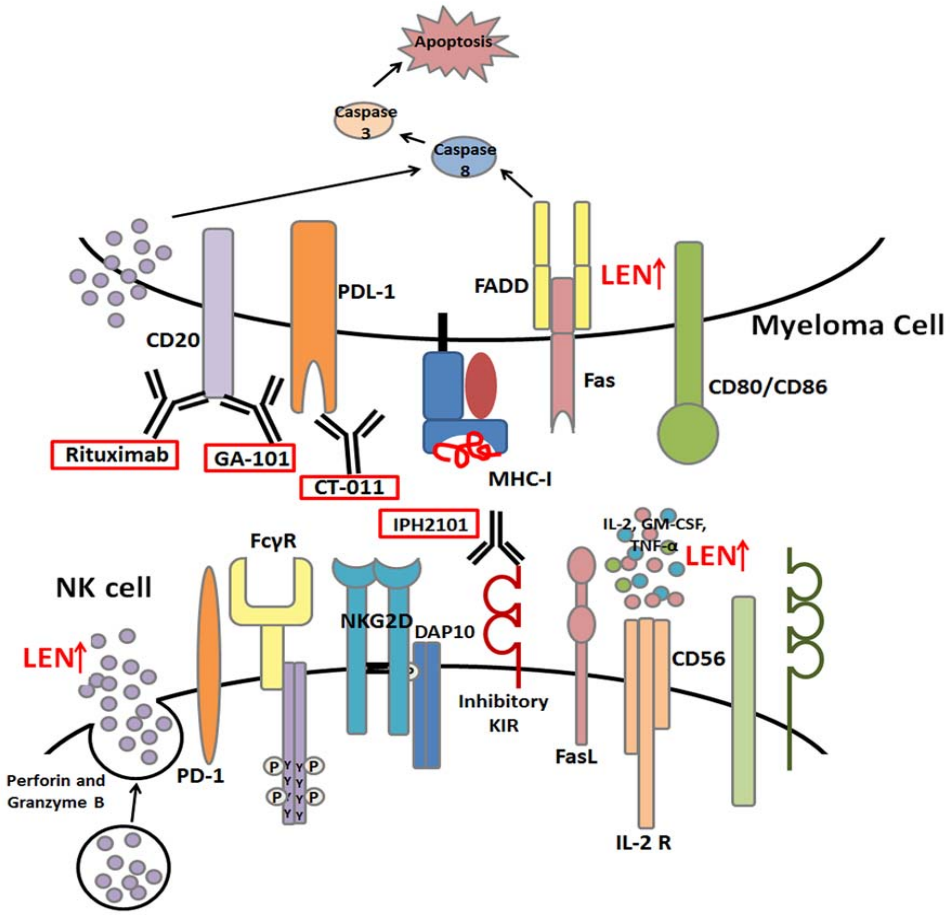

Figure 7. Lenalidomide alone, or in combination with a variety of therapeutic monoclonal antibodies, increases NK-cell mediated killing of multiple myeloma cells. Lenalidomide (LEN) up-regulates Fas expression and co-stimulatory molecules on MM cells leading to greater Fas-mediated apoptosis. Len has also been shown to augment the ADCC effect of various monoclonal antibodies like Rituximab (anti-CD20), GA-101 (glycoengineered anti-CD20), and CT-011 (anti-PDL-1). CT-011 blocks PD-1 ligand on the MM cells, interfering with binding to PD-1 and inhibiting NK cell activity. IPH2101 is an anti-inhibitory KIR that has been shown in combination with len to increase NK-cell killing as well, as blocking the inhibitory signals allows for NK activation and detection of the tumor cells.

Enhanced NK-cell ADCC by lenalidomide with combination antibody therapy. Enhanced antibody dependent cytotoxicity (ADCC) by NK cells is also an extremely important mechanism in IMiD function in CLL, MM, and even solid tumors (271, 285, 290). ADCC is a process where antibodies bind to their ligand antigens on target cells, which then bind to FcR-γ receptors on NK cells, and trigger cell lysis through perforin and granzyme-dependent pathways (291). Lenalidomide- and pomalidomide-induced killing via NK cells correlates with an increase in Fas Ligand (FasL) and granzyme B expression in NK cells, leading to increased ADCC in multiple tumor settings (290). Thalidomide plus rituximab (RTX), an anti-CD20 monoclonal antibody commonly used in CLL, was found to increase complete response rates in relapsed and refractory MCL patients (292). Further study of the mechanism showed that the drug-antibody combination increased growth arrest of MCL cell lines, as well as primary cells, compared to RTX alone (293). Mechanistically, they discovered that lenalidomide enhanced CD20-mAb-dependent apoptosis of the MCL cells by up-regulating activation of caspase-3, -8, -9 and the cleavage of PARP, as well as enhanced ADCC by

CD16 induction on NK cells (293). An increase in NK-mediated ADCC is also implicated in the success of RTX and lenalidomide combination therapy in CLL and NHL, although unproven *in vivo* (294, 295). Ofatumumab, another anti-CD20 monoclonal antibody, binds to a different epitope and induces greater complement dependent cytotoxicity and has shown evidence of activity in fludarabine and rituximab-refractory CLL (296, 297). Another CD20 mAb, the glycoengineered GA-101 antibody, induces greater ADCC *in vitro* than RTX, and has shown promising pre-clinical activity in animal models of NHL and B-CLL (298-302). Lenalidomide therapy is currently being tested with ofatumumab (303) and elotuzumab (304) in advanced, relapsed or refractory patients, and has shown therapeutic potential. Therefore, concurrent lenalidomide therapy with these antibodies may prove beneficial in refractory patients to augment anti-tumorigenic activity through NK cell potentiating effects.

As an immunomodulatory agent in solid tumors, lenaliomide has been used to reverse tolerance to tumor antigens (305, 306). As such, lenalidomide may prove beneficial as an adjuvant to vaccine therapies. Wu et al. demonstrated that lenalidomide enhances NK cell killing in a variety of solid tumor cell lines (breast, colorectal cancer, ovary, head and neck, lung cancer, bone sarcoma) treated with cetuximab or trastuzumab (285). The treatment of hematologic and solid tumors with specific monoclonal antibody therapy concurrently with lenalidomide could potently increase NK cell-mediated tumor lysis and enhance response rates. Lenalidomide induces NK cells to produce granulocyte-macrophage colony-stimulating factor (GM-CSF), TNF-α, and

various immune recruiting chemokines including RANTES, IL-8, MCP-1, and MIP-1α/β in response to antibody coated tumor cell lines, which contributes to a more effective immune response (285). The IMiDs enhance immunosurveillance in solid and liquid tumor settings through recruiting and activating T and NK cells to suppress malignant growth.

CHAPTER 2

Reversal of T-cell Tolerance in Myelodysplastic Syndrome through Lenalidomide Immune Modulation

A note to the reader: This work has been previously published in the journal *Leukemia*, McDaniel et al. 2012. (307) and has been reproduced here with permission from the publisher.

Introduction

Myelodysplastic syndromes (MDS) represent a spectrum of senescence-dependent, hematopoietic stem cell disorders (308). The classical clinical manifestations include dysplastic cytological features, ineffective hematopoiesis, and a propensity for transformation into acute myeloid leukemia (AML) (309-311). The prognosis varies, but recent FDA approved therapies, such as 5-azacytidine and the thalidomide analog lenalidomide, have significantly altered the natural history of disease (231, 312). In patients with non-del(5q) MDS, lenalidomide (Revlimid ®, Celgene Inc.) (230) improves hematopoiesis in a subset of patients in the absence of the clonal suppression observed in del(5q) MDS (313). Using micro-array gene expression analysis on bone marrow specimens from MDS patients treated with lenalidomide, Ebert et al. found that responsive patients display reduced expression of genes involved in erythroid differentiation (238). Moreover, treatment with lenalidomide restored differentiation potential accompanied by up-regulation of the natively suppressed

erythroid gene signature, indicating that lenalidomide may be effective in a select subgroup of anemic patients with non-del5q MDS (238).

In addition to its effects on erythropoiesis, lenalidomide is a potent immunomodulatory drug (IMiD®). All thalidomide derivatives suppress inflammatory cytokines produced by dendritic cells and activated macrophages, as well as enhance T and NK-cell proliferation and function (265, 270, 281). Although the direct erythroid-specific activity of lenalidomide may relate to effects on erythropoietin (EPO) receptor signaling, little is known about the effect of this drug and the mechanism of action *in vitro* and *in vivo* on the T-cell compartment (314). Immune dysregulation plays a critical pathophysiological role in the pathogenesis of MDS (315, 316), and a subset of low-risk patients with impaired hematopoiesis experience hematologic improvement after immunosuppressive therapy with cyclosporine A (CsA) and anti-thymocyte globulin (ATG) (317-319). T-cells generally lack the del(5q) chromosomal abnormality and are believed to be derived from the normal lymphoid progenitor compartment. Therefore, the immune dysregulation in MDS may be driven by activation against the abnormal myeloid clone. The hematologic remitting activity of lenalidomide in patients with del(5q) MDS is associated with an increase in bone marrow lymphoid aggregates, suggesting that activation of these cells may play a role in the clinical response (231). In an effort to understand lenalidomide's immunomodulatory activity, and to study the relationship between T-cell function and hematologic response in MDS patients, we evaluated T-cell activity before and after lenalidomide treatment, and correlated changes in immune parameters

with hematologic improvement. The results suggest that lenalidomide-mediated immunomodulatory function may contribute to hematologic improvement in MDS lower risk patients, and sheds light on the *in vivo* mechanism of action.

Results

Characteristics and hematologic response of lenalidomide-treated MDS cohort. One hundred patients with pathologically defined MDS were consented at Moffitt Cancer Center to evaluate immune responses. Thirteen of these were lower-risk, treated with lenalidomide, and had samples collected before and after treatment. Blood samples from an additional 5 patients with only lenalidomide pre-treatment samples available were used for *in vitro* treatment with lenalidomide, but were not included in the analysis to evaluate the relationship between T-cell response and hematologic response. Patient characteristics and hematologic response to lenalidomide are shown in **Table 1**. Seven patients exhibited a major erythroid response out of 13 (53.8%), as determined by international working group 2000 criteria. The median age of the group was 74 (range 49 – 83) and the median age of the responders was 72 years (mean 68.3, range 53 – 79 years), which did not differ from the non-responders (median 78.5, mean 73.5, range 49 - 83) ($p = 0.386$). Two patients with del(5q) were treated, one with del(2)(q11.2), one with complex abnormalities, and the remaining patients had a normal karyotype (n=9). All patients, including the patients with del(5q), were treated for severe anemia and

the responsive patients demonstrated a sustained increase in hemoglobin for at least 4 weeks duration. Patients with del(5q) also had a complete cytogenetic response with elimination of the clonal myeloid cells. The mean hemoglobin for the group was 9.1 g/dL, absolute neutrophil count 3.22 x 10^9 cells/L, and platelet count was required to be greater than 50,000 cells/L for eligibility on the clinical trial. There was no difference between responders and non-responders with regard to international prognostic score (IPSS) or World Health Organization

Table 1. Characteristics and hematologic response in MDS patients treated with lenalidomide.

MDS Pt ID	Samples	Sex	Age	WHO[2]	IPSS[3]	Karyotype	Hgb	ANC (1,000µl)	Platelets (1,000µl)	Response[4]
30	Pre/Post	M	74	RCMD-RS	Int-1	Normal	9.8	2.34	93	MER
8	Pre/Post	M	67	RCMD	Int-1	5q-, +21	11.1	1.33	70	MER/CCR
31	Pre/Post	M	53	RA-RS	Low	Normal	ND	4.3	354	MER
32	Pre/Post	F	55	RA	Low	5q-	ND	3.3	251	MER/CCR
33	Pre/Post	F	72	RCMD	Low	Normal	9.8	1.05	204	MER
20	Pre/Post	M	78	RAEB-1	Int-1	Normal	8	1.35	126	MER
4	Pre/Post	F	79	RCMD-RS	Int-1	Normal	9.9	1.51	182	MER
34	Pre/Post	F	73	RCMD-RS	Low	Normal	8.2	0.11	337	NR
35	Pre/Post	M	83	RCMD-RS	Int-1	del(2)(q11.2)	8.4	4.75	172	NR
36	Pre/Post	F	81	RA	Int-2	[1]complex	ND	12	196	NR
26	Pre/Post	M	83	RAEB-1	Int-3	Normal	9.8	3	62	NR
9	Pre/Post	M	76	RCMD	Int-4	Normal	7.4	2.84	274	NR
37	Pre/Post	M	49	RCMD	Low	Normal	8.8	4.04	227	NR

[1]Complex karyotype +19, 8, idem t(12;17)11
[2]Disease classified by the World Health Organization criteria.
[3]Disease classified by the International Prognostic Scoring System (IPSS).
[4]Hematologic response was classified by the International Working group (IWG) 2000 criteria.
MER=major erythroid response, CCR=complete cytogenetic response
ND=No data available at time of sample.
JAK2+ = $JAK2^{V617F}$ mutation by allele specific PCR.

(WHO) classification.

MDS patient T-cells were inherently tolerant to in vitro stimulation. To evaluate the proliferative response, the percentage of BrdU positive T-cells after anti-CD3 antibody-stimulation was compared in healthy donors and MDS patient PBMCs (n=13). **Figure 8A-B** shows that the percentage of proliferating T-cells

(both CD4 and CD8) after stimulation was significantly less in samples from patients (n=13, 6.91%±3.36) compared to controls (n=27, 17.91%±4.75) ($p<0.0001$ for both cell types). The difference in function was age-independent, as shown in **Figures 8A** (CD4 Healthy Donor Spearman r=0.009, MDS pt. r=0.341; both p=ns) and **8B** (CD8 Healthy Donor Spearman r=-0.212, MDS pt. r=0.316; both p=ns), indicating that MDS T-cells are anergic, or hypo-responsive, to T-cell stimulation.

Incompletely tolerant T-cells can potentially have their proliferative defects rescued by high doses of exogenous interleukin-2 (IL-2) (320). We therefore examined the TCR-induced proliferative response of healthy donor and patient T-cells in the presence of anti-CD3 stimulation and anti-CD3 stimulation with 100 U/ml recombinant IL-2. MDS patient T-cells (both CD4 and CD8) had a 57% recovery of proliferation with the addition of IL-2 in comparison to the normal T-cells stimulated with CD3 alone, but the proliferative capacity was still significantly below normal levels (data not shown). Also, the proliferation of T-cells in healthy donors and patient T-cells increased with the addition of IL-2, but the difference between groups remained unchanged (data not shown). This indicates that T-cells in MDS patients are anergic and only partially responsive to IL-2.

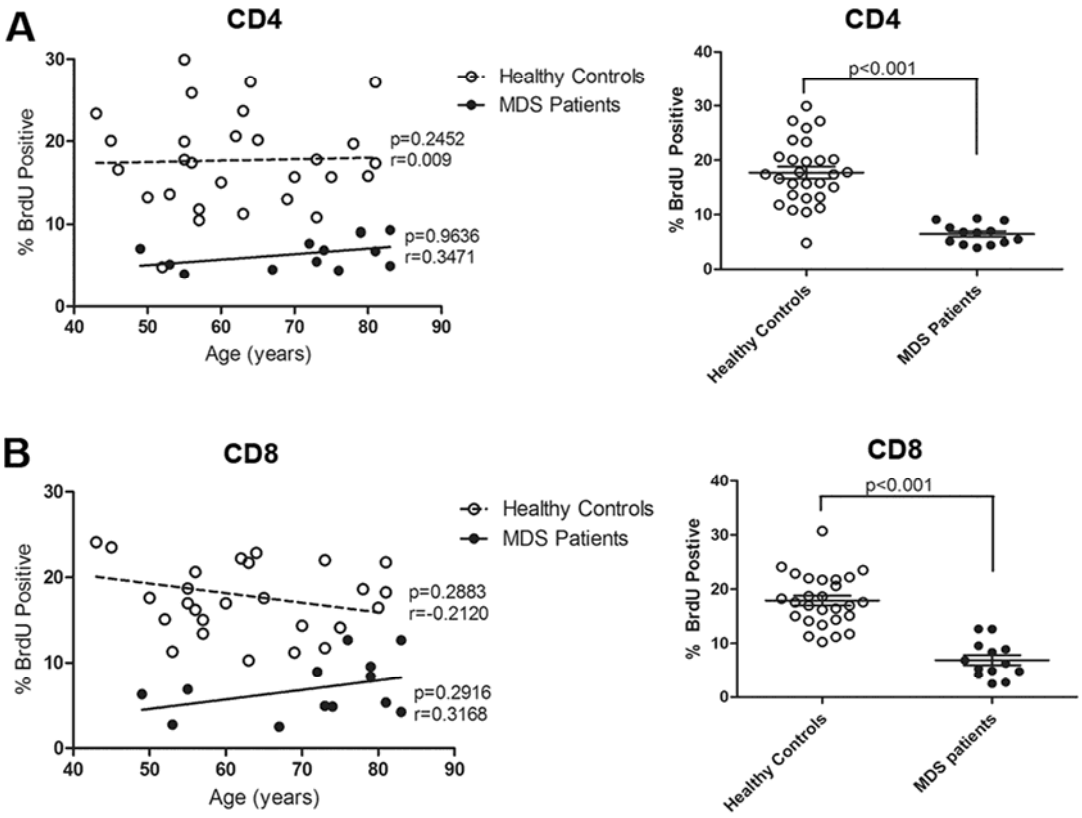

Figure 8. T-cells from MDS patients are inherently anergic. Proliferation of T-cells was measured by bromodeoxyuridine (BrdU) incorporation after 2-day culture in the presence of immobilized anti-CD3 antibody (10 µg/ml). The percentage of BrdU positive cells was determined in both CD4+ (**A**) and CD8+ T-cells (**B**) from 13 MDS patients (MDS) prior to lenalidomide treatment and 28 healthy donors (Controls). A Spearman Correlation was used to determine correlation of age and % BrdU incorporation, with insignificant p values (**A and B, left**). A Wilcoxon rank sum test was used to compare the mean proliferation between Healthy Donor and MDS patient samples (**A and B, right)**. P-values are shown. 9 patient samples pre-treatment were used to evaluate capacity to overcome proliferation defects after stimulation with both immobilized anti-CD3 and recombinant IL-2. The difference between the average of Control vs. MDS patients was analyzed using Wilcoxon Signed Rank test.

Lenalidomide recovers T-cell proliferation and augments Th1 cytokine production in vitro. Given the T-cell defects observed in MDS, we

next determined whether lenalidomide increased T-cell responses after culture with the drug *in vitro* during stimulation. PBMCs from 18 (untreated) MDS patients were stimulated *in vitro* with anti-CD3/CD28 antibodies in the presence of either 5 μM lenalidomide or vehicle control (DMSO), and proliferation was determined by BrdU incorporation. Data in **Figure 9A** shows that culture with lenalidomide *in vitro* augments TCR-induced proliferation compared to DMSO-treated cells in both CD4+ ($p<0.001$) and CD8+ ($p<0.001$) T-cells, in some cases up to that of healthy non-treated T-cells (shaded region) (**Figure 9A**). These results were compared to unstimulated cells (Unstim) also treated with lenalidomide. The fact that proliferation was not induced by lenalidomide in the absence of TCR stimulation indicates that lenalidomide alone has no direct mitogenic activity (**Figure 9A**).

In addition to proliferation, *in vitro* lenalidomide treatment increased cytokines induced by TCR stimulation, as shown in **Figure 9B-C**. The cytokine response favored T-helper 1 (Th1)-type cytokines including interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and IL-2, which are effectors of anti-tumor immunity and potentially important for the elimination of pre-malignant or dysplastic myeloid clones (20). As shown in **Figure 9B-C**, lenalidomide either decreased (IL-4) or induced no change (IL-10) in T-helper 2 (Th2) cytokines.

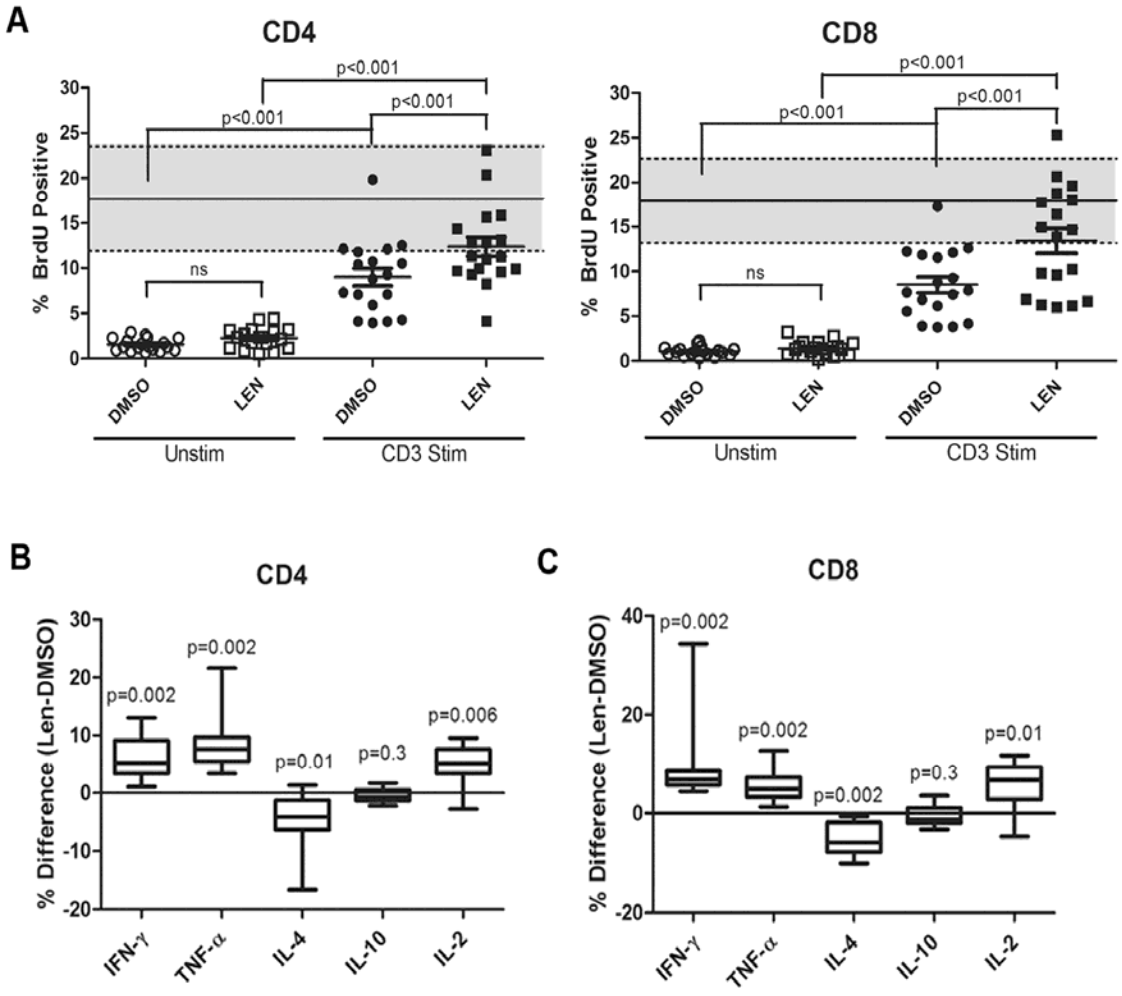

Figure 9. Lenalidomide augments Th1 type cytokine production and proliferation in MDS patient T-cells *in vitro*. MDS patient PBMCs were treated *in vitro* with either 5μM lenalidomide or vehicle control (DMSO) for 5 days and stimulated with anti-CD3/CD28 antibodies. On day 5, an aliquot of cells was taken and stained for BrdU incorporation in both CD4+ and CD8+ T-cells (**A**). The solid line at 17.71 (CD4+) and 17.90 (CD8+) represents the mean proliferation of untreated healthy donor T-cells. Gray shading indicates the normal range of one standard deviation above, and one standard deviation below the mean (**A**). Also on day 5, cells were stimulated with PMA/Ionomycin for 6 hours, with the last 4 hours in the presence of the protein transport inhibitor Brefeldin-A (BFA) for intracellular cytokine staining. Flow cytometry was used to determine the percentage of CD4+ (**B**) and CD8+ (**C**) Interferon-γ (IFN-γ), Tumor Necrosis Factor-α (TNF-α), Interleukin (IL) -2, -4, and -10 secreting cells. The difference between Len and DMSO treated samples for each patient is shown. **A, B, C.** Wilcoxon rank sum test was used to determine statistical difference

between Len and DMSO treated groups, with DMSO treatment used baseline proliferation/cytokine secretion; p values are indicated. A Wilcoxon Signed Rank test was used to determine statistical significance between stimulated and un-stimulated samples, **A**.

Improved homeostatic regulation after lenalidomide treatment in vivo. Next, the effect of lenalidomide was examined on T-cells in MDS patients who were treated with lenalidomide *in vivo* for anemia. The phenotype of T-cells in the peripheral compartment was examined using the surface markers CD45RA and L-selectin (CD62L) as phenotypic determinants of distinct naïve and memory T-cell subpopulations (321-323) with differential functional states. Multicolor flow cytometry was used to determine the percentage of CD4+ and CD8+ T-cells with a naïve (N), central memory (CM), effector memory (EM), and terminal effector memory (TEM) phenotype, and the change in T-cell phenotype (%post – pre) was then compared in responders (n=7) and non-responders (n=6). The percentage of T-cells within each of the memory compartments was compared in samples that were collected before (pre) and 16 weeks after lenalidomide therapy (post), as shown in the treatment schematic, **Figure 10A.** The flow cytometry gating strategy is detailed in **Figure 10B**. The T-cell compartment in MDS is dominated by EM and TEM T-cells compared to healthy controls, after adjustment for age (319). Memory phenotype skewing has been previously reported in MDS and correlated to chronic immune activation *in vivo* (77). Cells with the TEM phenotype represent a unique, poorly studied, population of effector cells that are generally senescent, lack the CD28 co-stimulatory molecule, and increase through aging and autoimmunity (44, 45). **Figure 10C**

shows that the percentage of naïve CD4 and CD8 T-cells increased significantly (p=0.004 and p=0.003, respectively), while CD4 and CD8 EM (p=0.046 and p=0.02, respectively) and TEM (p=0.0032 and p=0.015, respectively) decreased significantly after lenalidomide therapy. This improved composition within the T-cell compartment was significantly associated with an erythroid response in MDS. Lenalidomide significantly increased CD8+ CM T-cells (p=0.004, **Figure 10C**) with a similar trend for increased CD4+ CM cells (p=0.09). The changes in homeostasis of naïve and memory cells in responders were not due to an overall increase in total lymphocytes (**Figure 11**), indicating that lenalidomide alters the homeostasis of T-cells in the peripheral blood after *in vivo* therapy in association with hematologic improvement.

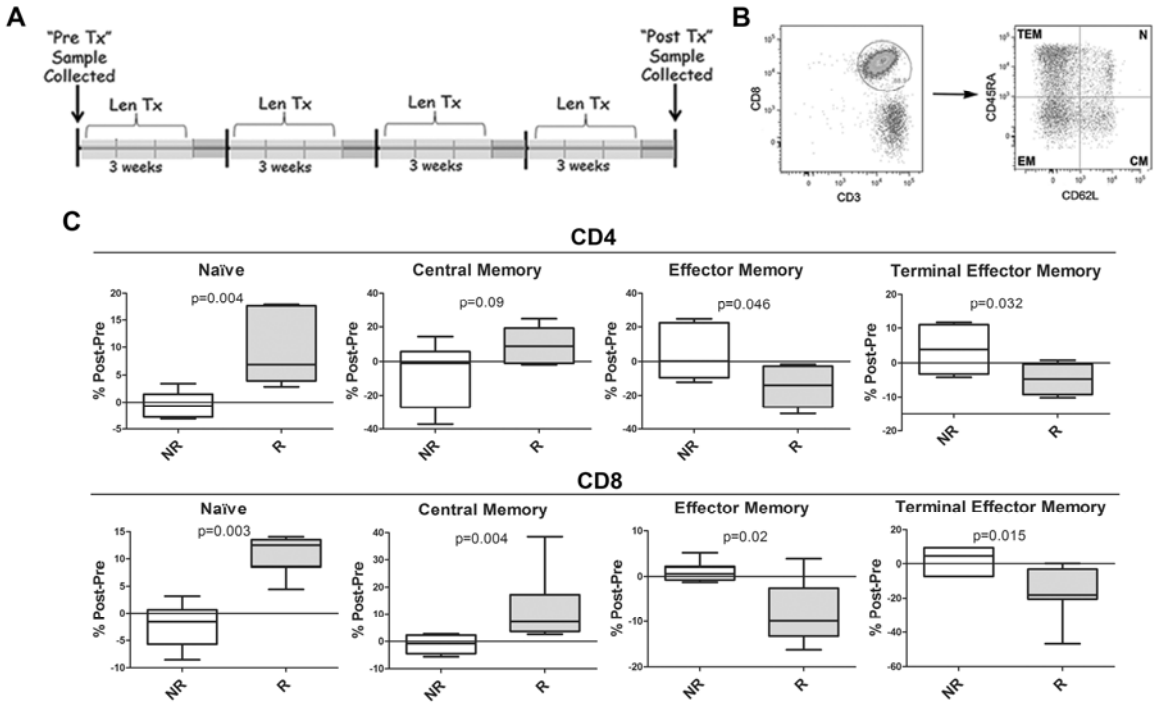

Figure 10. Naïve T-cells and immune reconstitution after lenalidomide treatment in hematologic responders. A. Schematic of patient sample collection and lenalidomide treatment during clinical trial. **B.** Flow cytometry gating strategy for memory phenotype on patient PBMCs. Cells were stained for CD3, CD8, CD45RA, CD62L, and DAPI as a viability marker. Cells were first gated on DAPI negative, then either $CD3^{+}CD8^{+}$ (CD8) or $CD3^{+}CD8^{-}$ (CD4). Memory phenotype of both CD4 and CD8 T-cells was then determined by CD45RA and CD62L expression, briefly: Naïve (N) cells are defined as $CD45RA^{+}CD62L^{+}$, Central memory (CM) are $CD45RA^{-}CD62L^{+}$, Effector Memory (EM) are $CD45RA^{-}CD62L^{-}$, and Terminal Effector Memory (TEM) are $CD45RA^{+}CD62L^{-}$. **C.** The proportion of Naïve, Central Memory, Effector Memory, and Terminal Effector Memory T-cells for both CD4 and CD8 was determined using flow cytometry. Naïve cells are described as $CD3^{+}CD62L^{+}CD45RA^{+}$, Central Memory as $CD3^{+}CD62L^{+}CD45RA^{-}$, Effector Memory as $CD3^{+}CD62L^{-}$ $CD45RA^{-}$, and Terminal Effector Memory are described as $CD3^{+}CD62L^{-}$ $CD45RA^{+}$.The difference Post-Pre of each of the phenotypes within Responding and Non-Responding patients is shown. (R n=7, NR n=6) Statistical analysis was performed using Wilcoxon Rank Sum. P values are indicated.

Reversal of functional defects in lenalidomide-responsive patients in vivo. Since lenalidomide is able to alter T-cell homeostasis and reverses functional T-cell defects *in vitro*, we assessed the functional impact of lenalidomide therapy *in vivo* in CD4 and CD8 T-cells. For this experiment, PBMCs were obtained before and after lenalidomide therapy and then stimulated *ex vivo* with anti-CD3 antibody to assess their proliferative capacity. The proliferative response was determined by the ability to enter S-phase on a single cell basis using BrdU staining and detection by flow cytometry. The change (% positive post-pre) in BrdU positive cells after *ex vivo* stimulation was then compared among responders and non-responders. Although responders had a significantly greater proliferative response after therapy compared to non-responders (**Figure 12A**, p=0.03 CD4; p=0.004 CD8), this difference was not associated with an increase in the absolute number of lymphocytes in this cohort

(**Figure 11**). The ability of T-cells to produce cytokines was also determined by intracellular cytokine flow cytometry analysis in responders and non-responders after *ex vivo* TCR stimulation with results shown In **Figure 12B-C.** IL-2 (p=0.05 CD4; p=0.01 CD8), IFN-γ (p=0.03 CD4; p=0.01 CD8), and TNF-α (p=0.004 CD4; p=0.01 CD8) secreting CD4 (**B**) and CD8 (**C**) T-cells were significantly increased by lenalidomide in responders compared to non-responders. Responders, however, had either no change or had a decrease in Th2-type cytokines (IL-4 and IL-10) compared to non-responders (**Figure 12B-C**), indicating that the type of T-cell immune response is similar to lenalidomide treatment *in vitro*.

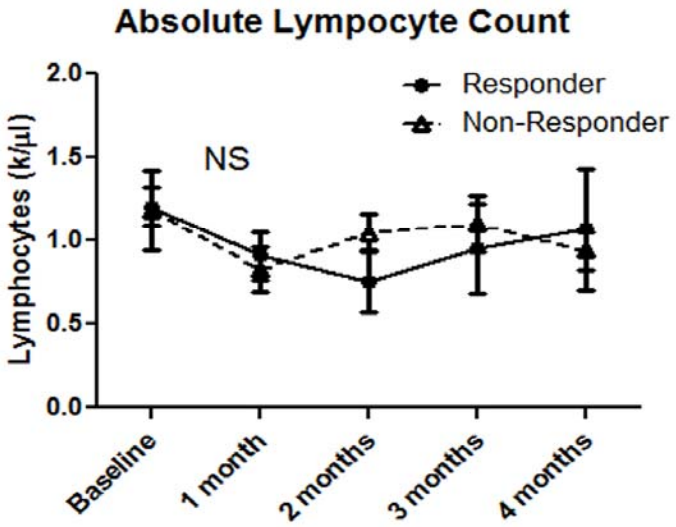

Figure 11: Absolute lymphocyte count remains unchanged during lenalidomide treatment. Absolute lymphocyte count of Non-Responders (NR) and Responders (R) at Baseline (pre-treatment), and 1 month intervals during 4 month course of treatment.

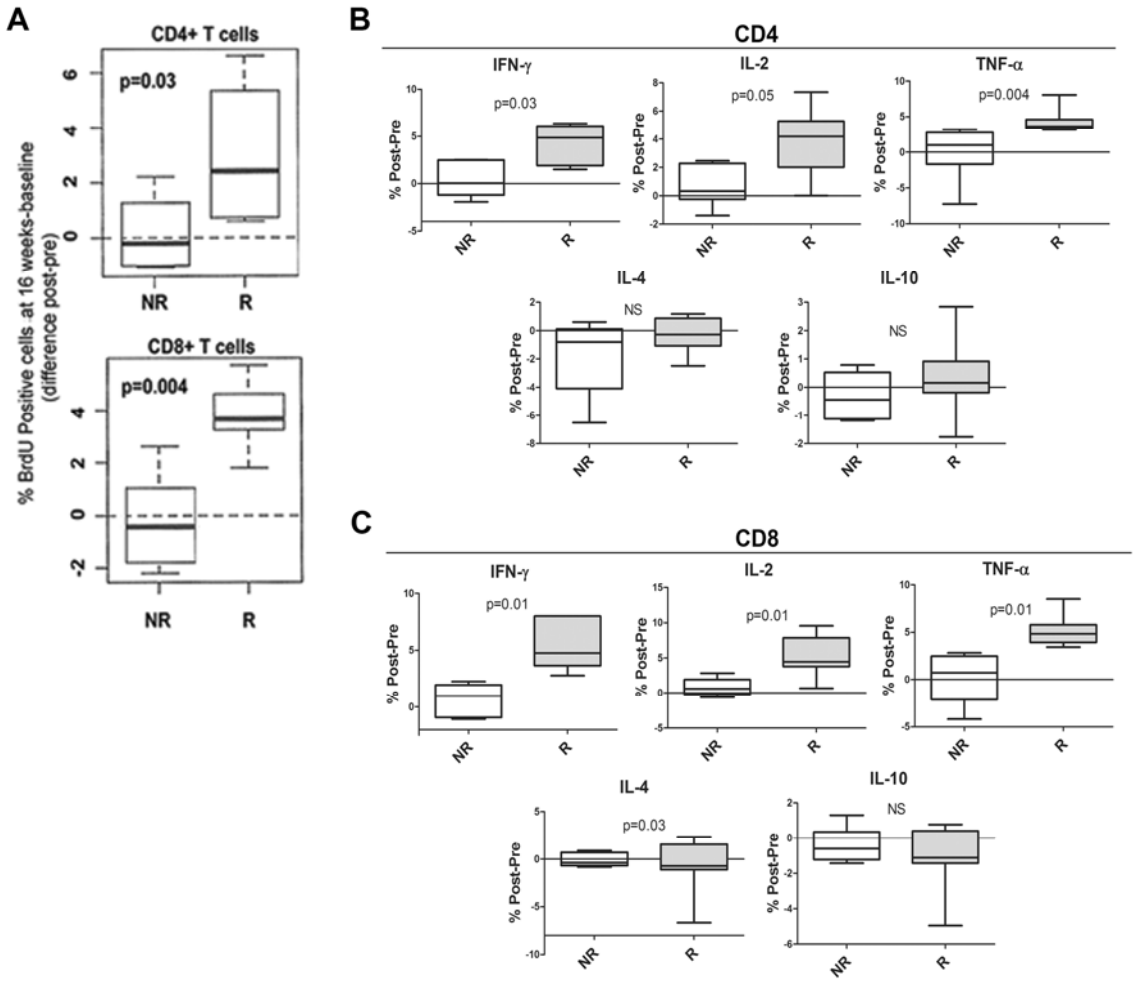

Figure 12. Lenalidomide reverses T-cell tolerance in MDS patients with hematologic response through increased proliferation and cytokine production *in vivo.* A. PBMCs were collected from MDS patients (N=13) prior to (pre) and 16 weeks after (post) receiving lenalidomide therapy. Patients were evaluated using the IWG 2000 criteria for response, with 7 responders (R) and 6 non-responders (NR). Cells were cultured in the presence of anti-CD3/CD28 antibodies for 48 hours before measurement of BrdU incorporation. Percentage of proliferating CD4+ (upper) and CD8+ (lower) T-cells was determined via flow cytometry. The difference in proliferation (post-pre) was analyzed using Wilcoxon Rank Sum Test, with p values indicated. **B-C.** *In vivo* cytokine production was determined from the same PBMC patient samples as collected in **A** for both CD4 (**B**) and CD8 (**C**) T-cells**.** PBMCs were stimulated for 48 hours with CD3/CD28 antibodies, and then for the last 6 hours with PMA/ionomycin. Golgi-block and cytokine staining was performed as described in **Figure 9B**.

Improved T-cell homeostasis is associated with functional improvement in lenalidomide-responsive patients. Next, the functional changes in proliferation and cytokine production in MDS T-cells were correlated with changes in naïve T-cells in patients treated with lenalidomide. Lenalidomide-induced increases in naïve T-cells was found to positively correlate with the improved proliferative response observed in both CD4 (p=0.0003) and CD8 (p=0.0220) T-cells, as shown in **Figure 13A**. IFN-γ production in CD8 T-cells was also positively correlated with the change in naïve T-cell percentage (p=0.0118), although this trend was not statistically significant in CD4+ T-cells (p=0.0941) (**Figure 13B**). Important to CD4+ T-cell helper function, the increase in IL-2 production was significantly associated with increased naïve T-cells in both T-cell subsets (CD4, p=0.0118 and CD8, p=0.0428, respectively). Collectively, these results suggest that one mechanism for lenalidomide-induced cytokine production and proliferation is mediated by reconstitution of the naïve T-cell compartment and removal of senescent/hypo-responsive EM and TEM T-cells in those MDS patients with hematologic improvement to lenalidomide.

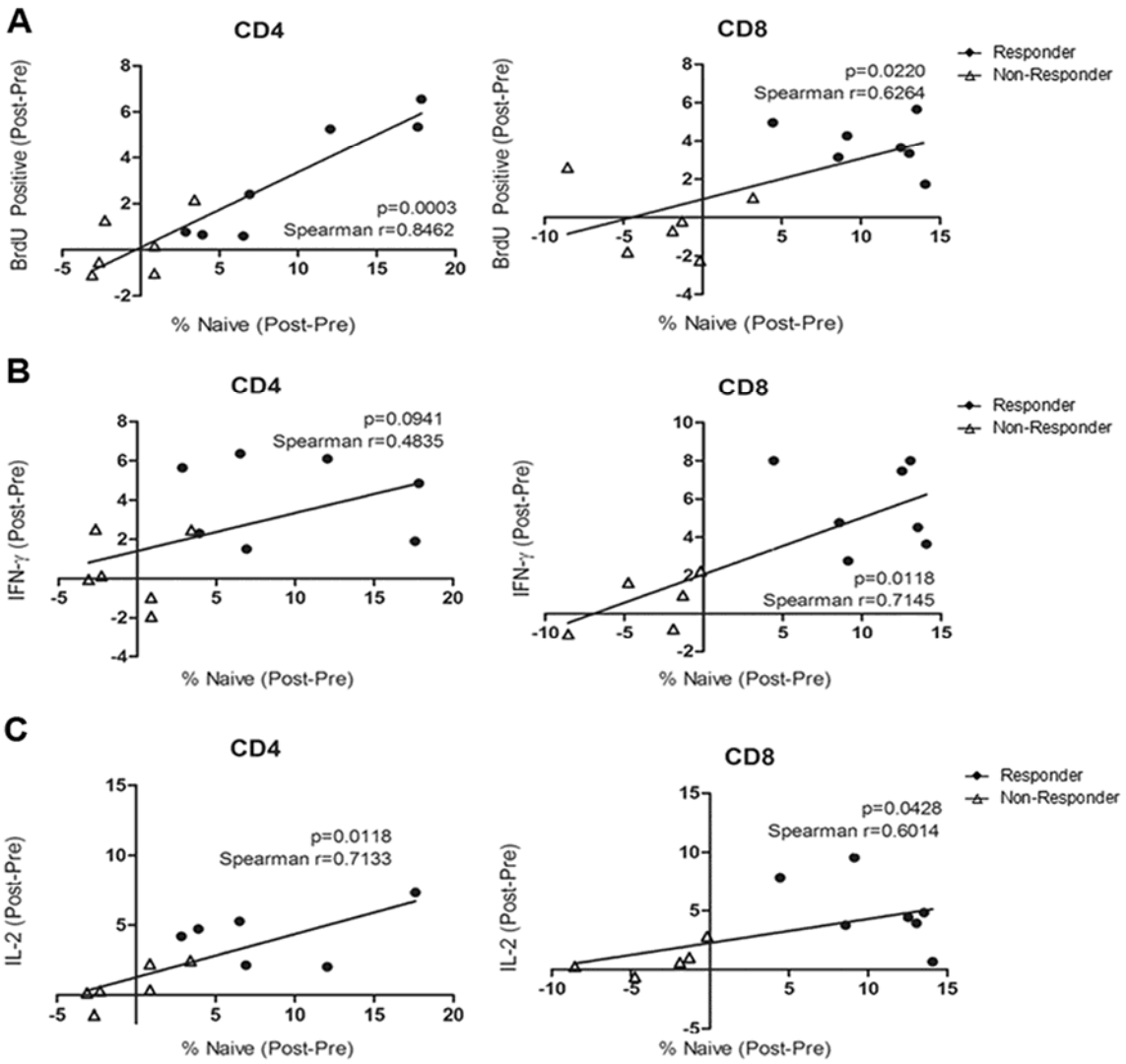

Figure 13. Increase in naïve cell production after lenalidomide treatment correlates with increased proliferation, IL-2, and IFN-γ production. A-C. MDS patient PBMCs were stimulated as previously described and the percentage of Naïve CD4+ and CD8+ cells after treatment was compared with BrdU incorporation (proliferation) **(A),** Interferon gamma production **(B)**, and interleukin-2 production **(C).** Naïve cells were determined as described in **3C**. A trend line was created for both CD4 and CD8 T-cells, and data analyzed via Spearman correlation. P values are indicated, as well as correlation of the data to the trend line (Spearman r), with 1 or -1 representing a perfect correlation. Non-Responders are represented by an open triangle symbol; Responders are represented by closed circle.

The mechanism for increased naive T-cell distribution after lenalidomide treatment differs in CD4+ and CD8+ T-cells. Thymic involution limits naïve T-cell output from the thymus and induces a steady decline in naïve T-cells in the peripheral blood with age (324). Thymic naïve T-cell production ceases after the age of 65 in humans, so maintenance of the peripheral naïve compartment rests almost exclusively with homeostatic proliferation (325). To determine if the lenalidomide-induced increase in naïve T-cells is age related, we correlated changes in phenotype to age in responders and non-responders. As shown in **Figure 14,** the percentage change in naïve CD4+ T-cells after lenalidomide treatment in hematologic responders (r=-0.92, p=0.007) showed a significant negative correlation with age; whereas, the change in naïve CD8+ T-cells was age-independent (**Figure 14**). These results suggest that naive CD4+ and CD8+ T-cells are differentially regulated by lenalidomide *in vivo.* Naïve CD4+ T-cells may be uniquely increased through an age-dependent release from the thymus after lenalidomide therapy; whereas, CD8+ T-cells increase through homeostatic proliferation.

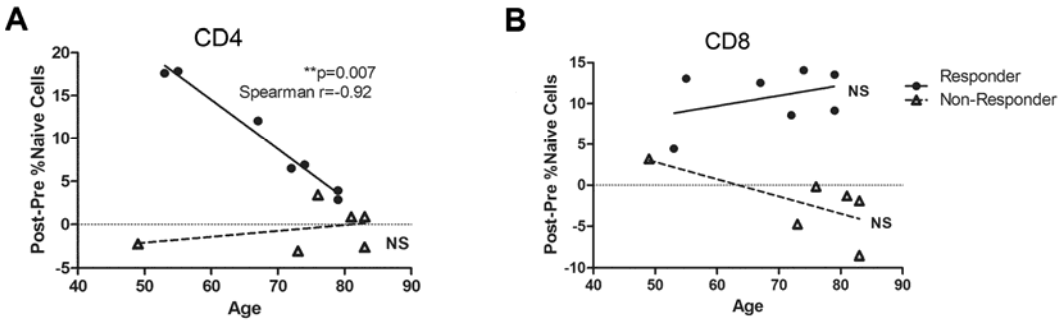

Figure 14. Naïve T-cell proliferation after lenalidomide treatment correlates with younger age in hematologic responders. MDS patient PBMCs were stimulated with antiCD3/antiCD28 antibodies and the percentage of naïve CD4+ (**A**) and CD8+ (**B**) T-cells after treatment with lenalidomide in both Responders and Non-Responders was correlated with age. A trend line was created for both Responders and Non-Responders in both the CD4+ and CD8+ populations. Data was analyzed via Spearman correlation. P values are indicated, as well as correlation of the data to the trend line (Spearman r), with 1 or -1 representing a perfect correlation. Non-Responders are represented by an open triangle symbol; Responders are represented by closed circle.

Discussion

The number of patients diagnosed with MDS annually is increasing as a result of the general trends in population aging (326). It is therefore critical to delineate response biomarkers and mechanisms of action of FDA approved therapies such as lenalidomide, to better tailor treatments to individual pathophysiological mechanisms. High rates of erythroid response in del(5q) patients are mediated by lenalidomide inhibition of the products of haplodeficient phosphatases encoded within the chromosome 5q CDR (246) . Nevertheless, responses in a subset of lower-risk patients lacking the del(5q) abnormality, and in patients with other hematologic malignancies, indicates that additional mechanisms may be important (230, 231). We previously reported that hematologic improvement to lenalidomide is associated with the appearance of bone marrow lymphoid aggregates, implicating immunologic changes associated with hematologic response (231). Thalidomide and structural analogues have potent immunomodulatory effects that are independent of del(5q), with documented changes in T-cells and NK-cells both *in vitro* and *in vivo* in multiple myeloma and Chronic Lymphocytic Leukemia (CLL) (260, 274, 327).

The effect of lenalidomide on immune activation in MDS has not been previously reported. Our current and previous data show that MDS T-cells are anergic or hyporesponsive to TCR stimulation prior to lenalidomide treatment compared to T-cells from healthy controls (**Figure 8**), and only partially responsive to high-dose exogenous IL-2 (data not shown). After treatment with lenalidomide *in vitro,* however, lenalidomide augmented T-cell function, indicating that the anergy is at least partially reversible and drug responsive. Lenalidomide is known to increase IL-2 production from T-cells *in vitro*, mimicking endogenous IL-2 production (265). In this case, exogenous IL-2 was only partially able to restore function in these anergic T-cells, suggesting that additional factors may be involved.

Based on these data, it is possible that lenalidomide directly improves signaling defects in anergic T-cells or improves immune composition. Age-dependent contraction of naïve cells and corresponding accumulation of effector and terminal effector memory cells (TEM and EM) impairs immune responses in the elderly (328). A significant improvement in the ratio of naïve-to-memory T-cells was evident in MDS patients with erythropoietic activity, increased cytokine production, and increased proliferation after lenalidomide *in vivo*. In responding patients, lenalidomide was able to reverse the skewed naïve/memory cell ratio by increasing naïve and central memory T-cells in the peripheral compartment after therapy, without increasing the total number of lymphocytes (**Figure 10C**)**.** The increase in naïve T-cells suggests that lenalidomide preferentially augmented naïve T-cell homeostatic proliferation, increased thymic output, or a combination

of both (329, 330). Since new naïve cell production deteriorates gradually over time as the thymus involutes, homeostatic proliferation becomes the main avenue for naïve T-cell expansion in the elderly (331-333). CD4+ and CD8+ T-cells differ in homeostatic regulation since CD4+ T-cells are less susceptible to memory switching than CD8+ T-cells, leading to longer preservation of the naïve CD4+ T-cell pool over time in healthy individuals (77, 334). Our results indicate that lenalidomide expands naive CD4+ T-cells through an age-related process that is greater in younger patients (**Figure 14**) and expands naïve CD8+ T-cells through an age-independent process. To confirm thymic involvement, examination of T-cell receptor excision circles (TRECs) to identify recent thymic emigrants is needed. However, the relationship between lenalidomide and therapeutic response preference in younger MDS patients has been documented in a large cohort suggesting that better immune modulation in younger patients may play a role (335). It is possible that a suboptimal immune response in the elderly may be an important determinant limiting the activity of lenalidomide in the aged.

Although there is differential regulation of naïve CD4+ and CD8+ T-cell subsets, lenalidomide is able to increase the liberation of effector cytokines and increase proliferation of both T-cell subsets in responders. Our analyses showed that Th1-type cytokine secretion (**Figure 12**) was increased *in vivo* in responders, suggesting that the effect of lenalidomide on hematopoiesis may be mediated by eradication of specific abnormal myeloblasts involved in clonal evolution in MDS (336). In support of this idea, Neuber et al. recently reported enhanced antigen-

specific T-cell activity *in vitro* and *in vivo* in multiple myeloma patients, indicating that lenalidomide has the ability to potentiate tumoricidal activity of effector T-cells (260). Given that MDS T-cells are anergic, and multiple mechanisms are typically involved in this process, it is possible that lenalidomide improves the suppressive microenvironment within the bone marrow of MDS patients, indirectly reversing anergy within the T-cell population. MDS blast cells have been shown previously to express higher levels of the inhibitory molecule B7-H1 that suppress T-cell proliferation and induce apoptosis of normal T-cells *in vitro* (209). Natural CD4+ Tregs, which impair T-cell activation (337), are also increased in some MDS patients. Therefore, lenalidomide may eliminate the suppressive populations within the bone marrow, which in turn improves T-cell responsiveness and acts in concert to restore hematopoiesis in lower risk responsive MDS patients.

Our findings indicate that hematologic response to lenalidomide is associated with homeostatic reconstitution and reversal of tolerance in T-cells in lower-risk MDS patients. We show that while activation of the immune compartment occurs after lenalidomide treatment, not all patients re-establish T-cell homeostasis and immune function, or display hematologic improvement after treatment. This variability in activity re-iterates the importance of identifying biomarkers predictive for response to enable more accurate selection of patients for lenalidomide therapy in non-del(5q) MDS. Our data provides a rationale to examine a larger cohort of patients to determine if basal T-cell function is predictive for response to lenalidomide in MDS.

Materials and Methods

Patients and healthy controls. MDS patients (n=100) were consented at H. Lee Moffitt Cancer Center in Tampa, FL to participate in a peripheral blood collection protocol approved by the University of South Florida Institutional Review Board from 2004-2009. All patients signed University of South Florida Institutional Review Board-approved informed consents for the collection of 40 ml of peripheral blood in heparin tubes. Samples were obtained from each patient at various times from 2004-2009 for immune monitoring studies and all cells were frozen in liquid nitrogen. Twenty-one of these patients were treated with lenalidomide, but only 13 patients (**Table 1**) treated with lenalidomide had samples obtained both prior to (Pre) and after (Post) therapy. Lenalidomide was administered at a dose of 10 mg for 21 out of a 28-day cycle for four cycles. In all patients, the post-treatment sample was drawn after 16 weeks of therapy when evaluated for hematologic response. Hematologic response was reported previously within a larger group of MDS patients (338).

Peripheral blood from buffy coats of healthy donors was obtained from the Southwest Florida Blood Services, St. Petersburg, FL, for use as controls (n=28) in some experiments. Healthy control T-cells for *in vitro* experiments were isolated from buffy coats using RosetteSep® Human CD3+ T-cell Enrichment Cocktail (StemCell Technologies, Vancouver, BC Canada) according to the manufacturer's protocol. Peripheral blood mononuclear cells (PBMCs) were isolated from patients and healthy donors by Ficoll-Hypaque (Amersham

Pharmacia Biotech, Piscataway, New Jersey, USA) gradient centrifugation, as previously described (319).

T -cell activation. To activate the T-cell receptor (TCR), PBMCs were stimulated with plate-bound anti-CD3 antibody (10 µg/ml) (BD Pharmingen, San Jose, CA USA) coated onto flat-bottomed polystyrene tissue culture plates overnight at 4°C. PBMCs (1 x 10^6/ml) were added to the coated 96-well plates in a 200 µl volume for 48 hours at 37°C. For intracellular cytokine experiments, soluble anti-CD28 (1µg/ml) (BD Pharmingen, San Jose, CA USA) was added to provide additional co-stimulation.

Proliferation. Proliferation was determined after *in vitro* activation by bromodeoxyuridine (BrdU) incorporation (BrdU flow kit, BD Biosciences, San Diego, CA). 10 µM of BrdU was added during the last 45 min of T-cell stimulation. BrdU-pulsed PBMCs were harvested and stained with anti-CD4-PE and anti-CD8-PE-Cy-5 (BD pharmingen, San Jose, CA USA). The cells were then fixed and permeabilized with BD Cytofix/Cytoperm buffer and incubated with DNase for 1 hour at 37°C. Cells were then stained with anti-BrdU-FITC antibody before flow cytometry analysis on an LSRII flow cytometer (BD Biosciences, San Jose, CA USA). The percentage of BrdU positive cells from each population was analyzed using Flow-Jo Software (BD Biosciences).

Intracellular cytokine staining. For intracellular cytokine staining in T-cells from MDS patients treated *in vivo* with lenalidomide, PBMCs from patients were stimulated on anti-CD3-coated antibody plates (10μg/ml) plus anti-CD28 (1μg/ml) for 6 hours and cytokines were examined by intracellular staining in gated CD4+ and CD8+ T-cells. Stimulation with anti-CD28 alone was used as a negative control, and stimulation with PMA (5ng/ml) and ionomycin (250ng/ml) (BD BioSciences, San Jose, CA USA) was used as a positive control. For intracellular cytokine staining of MDS patient cells cultured *in vitro* with lenalidomide, PBMCs were treated with/without 5μM lenalidomide for 5 days in the presence of CD3/CD28, as described above. On day 5, they were re-stimulated with PMA/ionomycin for 6 hours. In both cases, Brefeldin A (10 μg/ml, Sigma, CA USA) was added during the last 4 hrs of stimulation to block cytokine secretion. Cells were collected and incubated in EDTA (2 mM final concentration) for 15 min at room temperature, fixed with 2% formaldehyde for 20 min at room temperature, and washed with PBS containing bovine serum albumin (BSA). Cells were permeabilized with FACS permeabilization solution and triple-stained with CD3-Pe-Cy5, CD8-FITC and intracellular cytokines Interferon-γ (IFNγ), Tumor Necrosis Factor-α (TNFα), Interleukin (IL)-2, -4 and -10 (all PE-conjugated antibodies, BD Biosciences, San Jose, CA USA). Cells were subsequently washed, re-suspended in staining buffer, and run on a LSRII flow cytometer (BD Biosciences). Data were analyzed using Flow-Jo Software (BD Immunocytometry Systems, San Diego, CA). Quadrant markers were positioned to include >99% of immunoglobulin control stained cells in the negative quadrant.

Detection of naïve and memory T-cell populations. Naïve and memory CD4 and CD8 T-cell subtypes were detected after surface staining with anti-CD3-PE, anti-CD45RA-FITC, anti-CD62L-APC, and either anti-CD4- or CD8-PECy5 (all from BD Biosciences, San Jose, CA USA). Naïve and memory T-cell populations were distinguished by CD45RA and CD62L expression, as described previously by flow cytometry (319, 322, 323); briefly, the memory phenotype populations are characterized as naïve ($CD45RA^{+}/CD62L^{+}$), central memory ($CD45RA^{-}/CD62L^{+}$), effector memory ($CD62L^{-}/CD45RA^{-}$), and terminal effector memory ($CD45RA^{+}/CD62L^{-}$).

Preparation of lenalidomide for *in vitro* studies. Lenalidomide (Revlimid®) was kindly provided by Celgene Corporation, Warren, NJ. The drug was weighed and dissolved at the time of use in dimethyl sulfoxide (DMSO) and diluted 1:1000 in culture media to a final concentration of 5 μM because storage of stock solutions at −20°C resulted in variable loss in activity. An equal volume of DMSO was used as a control.

Statistical Analysis. To analyze the difference of paired samples, we used a Wilcoxon Signed Rank test to determine if the difference significantly differed from zero. A Wilcoxon Rank Sum test was also used for comparing two unpaired samples. To assess the relationships between age and the percentage of cells that were BrdU positive, as well as age and naïve cell percentage,

Spearman correlation coefficients were calculated. Relationship between lenalidomide and DMSO treated *in vitro* experiments were analyzed using a student T test. All p values were two sided tests with a significance value of $p<0.05$.

CHAPTER 3

CD28 expression is required for lenalidomide immune modulation: identification of a potential mechanism of drug resistance

Introduction

CD28 is a member of the B7 family of T-cell costimulatory molecules that binds to CD80/CD86 (B7-1/B7-2) expressed on APCs (266, 339, 340). When the T-cell receptor (TCR) CD3 complex is activated, proliferation can occur, but is slightly less in the absence of CD28. CD28 receptor co-stimulation, however, is essential for the induction of interleukin-2 (IL-2) gene expression and for the prevention of tolerance/anergy within a TCR-activated T-cell population (142, 341-343). CD28 is a transmembrane protein that contains two immuno-tyrosine activation motifs (ITAMs) within the cytoplasmic tail. Receptor-ligand interaction induces CD28 homo-dimerization, phosphorylation by src-family kinases lck and fyn, and the activation of downstream signaling mediators including phosphoinositide 3-kinase (PI3K) and extracellular signal-regulated kinases (ERK) 1/2 (ERK1/2) (129, 130). Absence of CD28 co-stimulation has been implicated in tumor immune evasion and is an important barrier to the success of tumor vaccine therapy and usually occurs due to B71/2 down-regulation or loss of CD28 expression(344) .

The necessity of CD28 for IL-2 production has been illustrated using CD28 homozygous deficient (-/-) mice, which lack the ability to induce IL-2 (143) in

response to antigen stimulation. CD28 is inherently expressed on both CD4+ and CD8+ T-cell subsets, and increases upon TCR stimulation. Aging of the immune system in humans, however, is associated with the accumulation of CD28-deficient (CD28-, $CD28^{null}$) T-cells, altering T-cell homeostasis and reducing pathogen and vaccine immune responses (345, 346). CD28 receptor down-regulation results from either chronic TNF-α exposure, as seen in bone marrow of MDS patients, IL-15 exposure in the absence of TCR ligation, or repeated TCR engagement indicative of robust proliferative *in vivo* history (87, 347).

Lenalidomide (LEN) is a second-generation thalidomide analogue with potent immune-modulating activity approved by the FD for treatment of MDS, multiple myeloma (MM), and lymphomas. Although known to replace the need for CD28 external ligation, the mechanism of LEN is poorly understood. The presence of an interstitial deletion on chromosome 5q (del(5q)) enhances apoptotic responses of myeloid clones in MDS and improves hematologic response rates, and approval for LEN in non-del(5q) MDS was issued based on erythroid improvement in a subset of patients (313).

It is currently unclear if LEN mediates hematologic improvement or anti-tumor activity in lymphomas, MDS, or MM through immune modulation. We previously showed that T-cells from MDS patients are inherently non-responsive/anergic to stimulation, and secrete less T-cell activating cytokines (307). Lymphoid aggregates increase in the bone marrow of MDS patients that improve hematologically, implicating immunologic changes in the response (231). Our previous findings indicated that patients with erythroid improvement have an

increase in T-cell proliferation and an increase in IL-2 and Th1 cytokine production after TCR activation as well as normalization of their naïve:memory ratio that may contribute to eradication of the myeloid clone (307).

Recently a direct molecular target of LEN was shown to mediate cytokine production in T-cells (257), but mechanism of LEN co-stimulation through the CD28 receptor is unknown. Therefore, the current study was conducted to determine if LEN requires CD28 expression for IL-2 production in T-cells (339). If the loss of CD28 renders cells resistant to LEN-induced immune modulation, then pre-treatment phenotyping for the expression of CD28 may be used as a biomarker to predict LEN immunomodulatory drug response.

Results

Lenalidomide induces robust interleukin-2 (IL-2) production in the absence of CD28 co-stimulation. LEN has been shown to enhance proliferation and IL-2 expression/secretion in T-cells. The expression of IL-2 is completely dependent upon CD28 activation. To determine if LEN increases IL-2 in the absence of APCs or without the addition of anti-CD28 antibody, primary purified CD3+ T-cells were isolated to greater than 95% purity from healthy donors and stimulated with increasing concentrations of anti-CD3 antibody in the presence or absence of LEN treatment (**Figure 15A**)**.** After simulation with plate-bound anti-CD3 antibody without ligating CD28, LEN increased the production of IL-2 in a dose dependent fashion. In contrast, there was little to no IL-2 produced without

CD28 ligation in cells treated with an equal volume of drug vehicle control (DMSO). These results indicate that there is a role for CD28 signaling in LEN immune modulation. LEN treatment was also able to increase, by an average of 6-fold, IL-2 production in T-cells that received both the TCR signal through anti-CD3 antibody stimulation plus anti-CD28 ($p<0.001$, **Figure 15B**) co-stimulation, showing that LEN augments the signal initiated by CD28. T-cells were then stimulated with a fixed dose (5μg/ml) of anti-CD3 alone with increasing concentrations of LEN to demonstrate the dose-dependency of IL-2 production (**Figure 15C**). In addition to protein secretion, we found that LEN enhances IL-2 mRNA expression. A dose-dependent increase in IL-2 mRNA was present in cells treated with anti-CD3 alone without CD28 after 18 hours compared to DMSO-treated cells, indicating that LEN transcriptionally activates the IL-2 gene (**Figure 15D**).

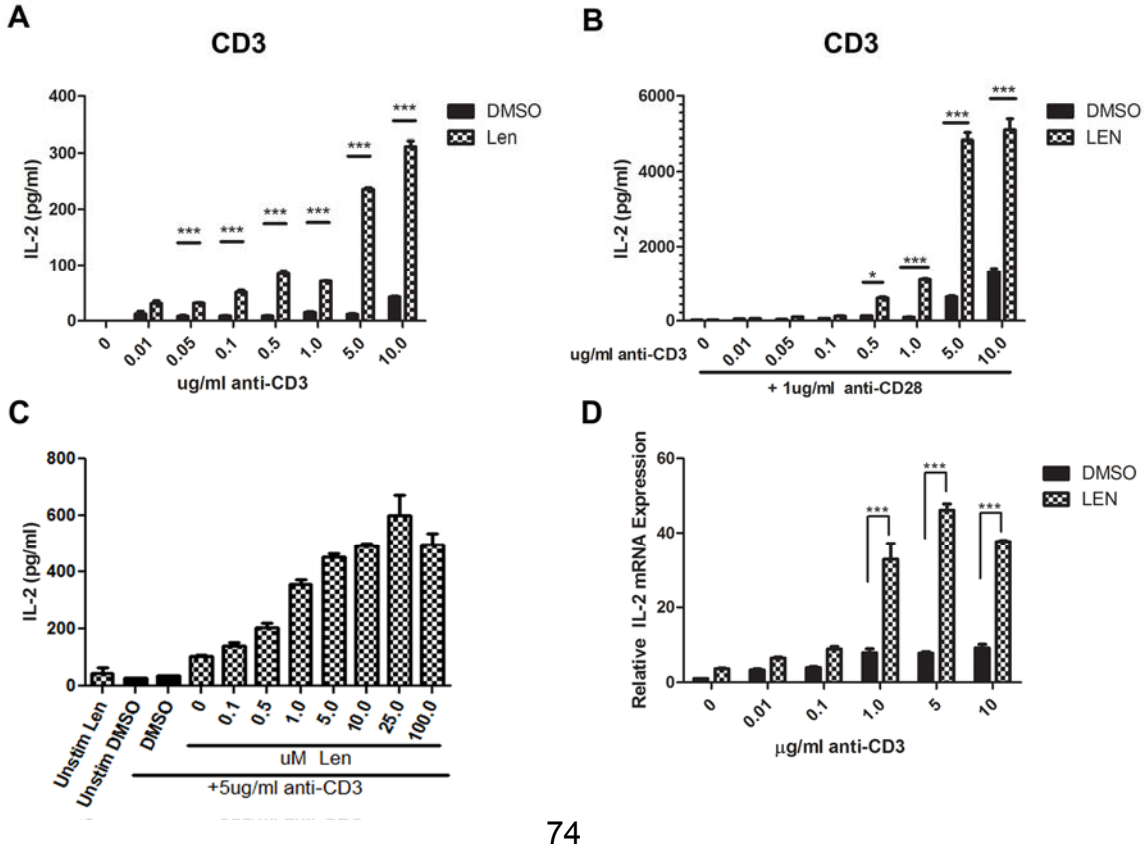

Figure 15. Lenalidomide increases IL-2 production in the absence of CD28 external co-ligation. T-cells purified from healthy donor PBMCs were stimulated in the presence of increasing concentrations of anti-CD3 in the presence of 10μM lenalidomide (Len) or vehicle control (DMSO). A subset of cells were stimulated with both anti-CD3+CD28 as a positive control. **A-B.** IL-2 production was measured in culture supernatant of cells stimulated with increasing concentrations of anti-CD3, (**A**) or anti-CD3+ anti-CD28 (1.0μg/ml) (**B**) via ELISA. **C**. Purified T-cells were stimulated in the presence of 5μg/ml anti-CD3 and increasing concentrations of Len. **D**. IL-2 mRNA expression was examined using RT-qPCR after 18 hours of increasing concentrations of anti-CD3 stimulation. 2-way non-parametric ANOVA. *=$p<0.05$, ***=$p<0.001$.

Since LEN increases *IL-2* gene expression, likely through the CD28 pathway, we determined if LEN augments the binding of a CD28-specific transcription factor to the IL-2 promoter. Multiple transcription factors are required to interact with the *IL-2* promoter transcription initiation (**Figure 16A**). Some transcription factors are regulated by the TCR signaling cascade, such as NFAT-1, AP-1, and NF-κB, but these are insufficient for transcription of the *IL-2* gene (150). Only binding of transcription factors such as pCREB (a CD28-Response Element specific binding factor), NF-κB and AP-1 within the CD28 response element following CD28 receptor ligation is capable of inducing IL-2 transcription. To determine if LEN treatment induces the binding of pCREB to the IL-2 promoter in the absence of CD28 co-ligation, we performed chromatin immunoprecipitation (**Figure 16A**). Results shown in **Figure 16B** indicate that with anti-CD3 treatment alone, LEN increases binding of pCREB to the IL-2 promoter compared to DMSO. These results indicate that LEN specifically activates the CD28 pathway in conjunction with TCR stimulation.

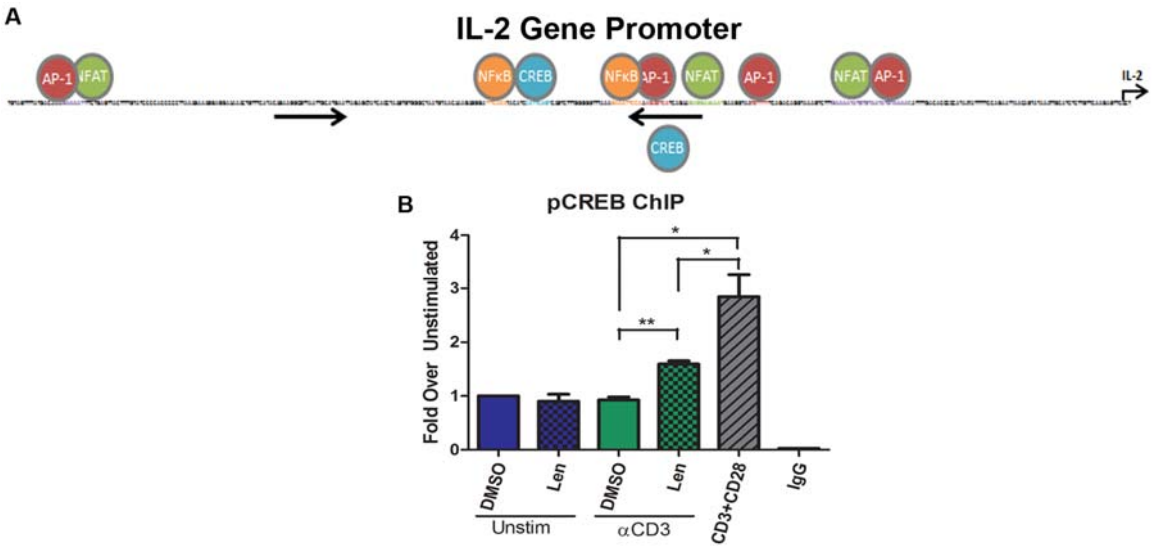

Figure 16. CD28 downstream transcriptional element is activated after LEN treatment. A. Schematic of transcription factor binding sites on the IL-2 promoter. Black arrows indicate forward and reverse primers used in Chromatin Immunoprecipitation (ChIP) to evaluate pCREB binding to the CD28-Response Element of the IL-2 promoter. **B**. pCREB binding to the IL-2 promoter after 18 hours stimulation with 1.0μg/ml anti-CD3 antibody in the presence of vehicle (DMSO) or 10μM Len treatment. CD3+CD28 stimulation and IgG pull-down were used as positive and negative controls, respectively. All values were calibrated to 10% input and calculated using ΔΔCT method relative to un-stimulated treated with DMSO. Graph is representative of 2 replicates. Statistical analysis was performed using an unpaired T-test. *p<0.05 **p<0.01.

Surface expression of CD28 is increased after LEN treatment upon TCR activation. Since LEN augments T-cell function through the CD28 pathway, it is possible that LEN alters CD28 surface expression or recruitment to the immune synapse. We therefore examined the surface expression of CD28 in primary T-cells from healthy donors that were either left un-stimulated or stimulated with anti-CD3 for 3 days (**Figure 17**). **Figure 17A** shows a representative flow plot of CD28 surface expression under these conditions. Treatment of cells with anti-CD3 in the absence of CD28 co-ligation results in significant increase in the number of CD28 molecules per cell compared to

unstimulated cells, as indicated by a shift in median fluorescence intensity (MFI). After treatment with LEN and anti-CD3, compared to DMSO plus anti-CD3, CD28 surface expression is significantly increased in both CD4 and CD8 LEN-treated T-cells, suggesting that CD28 expression may be an important determinant of LEN function (**Figure 17A-B**).

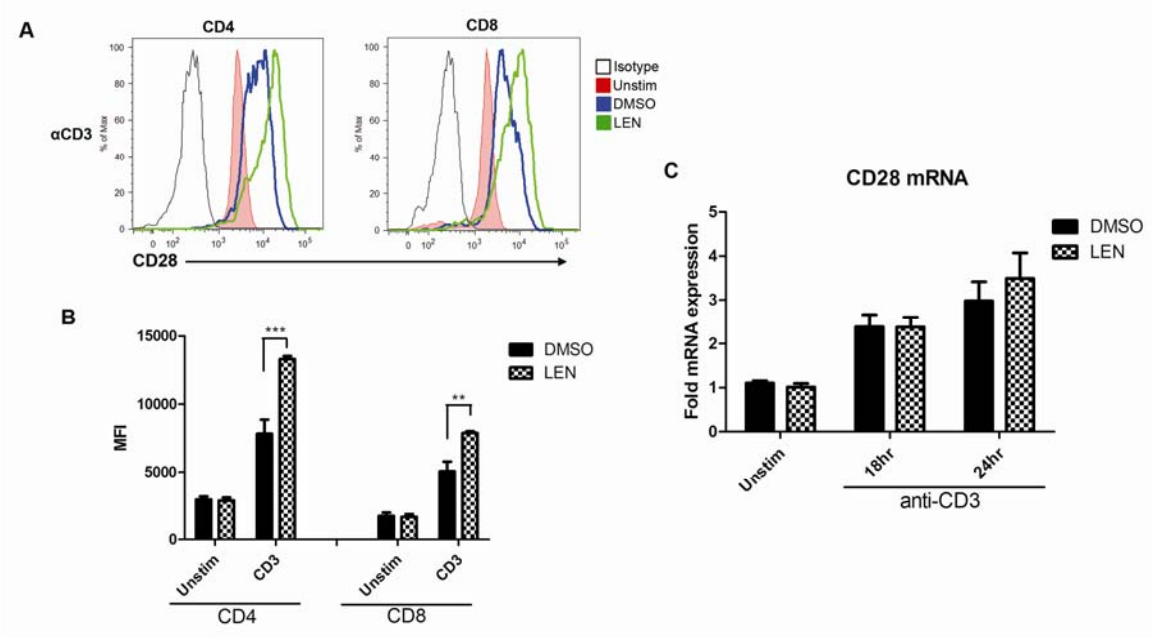

Figure 17. Surface expression of CD28 is increased after LEN treatment upon TCR activation. Isolated T-cells were treated with Len or DMSO and stimulated with polystyrene beads coated with anti-CD3 antibody alone. CD28 surface expression was measured 3 days after stimulation by flow cytometry, and an example histogram of CD28 surface expression after anti-CD3 stimulation is shown in **A.** The black line indicates PE-isotype (negative control), red shaded region represents un-stimulated T-cells, blue line represents DMSO treated, and green line is LEN treated T-cells. **B.** Graphs represent the CD28 MFI in one of four independent experiments on both CD4+ (left) and CD8+ (right) T-cells. The difference between Len and DMSO was determined using 2-way non-parametric ANOVA **=p<0.01, ***=p<0.001. **C.** CD28 mRNA expression was measured after 18 hours of anti-CD3 stimulation after LEN and DMSO treatment. P values in all instances are not significant.

CD28 surface expression is essential for LEN-induced IL-2 production. Ligation of the extracellular portion of the CD28 receptor with anti-CD28 antibody cross-linking abolishes CD28 receptor expression through internalization. Approximately 50% of CD28 is then targeted for degradation in the endosome while the remainder is recycled back to the surface. For this reason, surface expression of CD28 was reduced by treating with varying doses of anti-CD3 in combination with anti-CD28 antibodies to induce receptor internalization and degradation. Flow cytometry confirmed that CD28 expression, in the presence or absence of LEN, was significantly reduced after co-stimulation, with the rate and degree of surface recycling depending on the TCR signal strength. It was evident that 10 µg/ml of anti-CD3 significantly increased the rate (24 versus 48 hours) and proportion of cells that re-express CD28 (**Figure 18A** and **B**), but this was only slightly increased by treatment with LEN.

To elucidate whether the presence of the CD28 receptor is necessary for LEN-induced IL-2 production, we purified CD28+ T-cells by flow cytometry sorting and then subjected them to either CD28 siRNA or control siRNA. Knocking down CD28 expression in primary T-cells utilizing siRNA is technically challenging due to the long protein half-life and stable surface expression in the CD28. Therefore, we took advantage of receptor degradation in the experimental design. siRNA-treated cells were compared to siRNA control (**Figure 18**)-treated cells after 24 and 48 hours in the presence of 1.0 or 10 µg/ml of anti-CD3 plus anti-CD28 antibody stimulation. Re-expression of the receptor was slightly greater in cells treated with a higher dose of anti-CD3 (10 µg/ml

compared to 1.0 µg/ml) after 72 hours, but siRNA-CD28 treatment significantly reduced the expression of CD28 in all conditions. An example of the surface expression of CD28 by flow cytometry in the knockdown experiments is shown in **Figure 18A,** and quantified in **Figure 18B.** Production of IL-2 in LEN or DMSO treated cells with either siRNA-CD28 knockdown or control siRNA is shown in **Figure 18C**. Reduction in CD28 significantly diminished IL-2 and effectively blocked LEN-induced IL-2 release (decreased from a 7.5-fold increase to 4.9-fold increase on average compared to control siRNA), as shown in **Figure 18C**. This data suggests that the expression of CD28 on the surface imparts response to LEN.

CD28^{null} cells are resistant to LEN. As described previously, CD28 surface expression is lost on CD8+ memory T-cells as a function of aging, which contributes to the accumulation of a hypo-responsive CD8+CD28- T-cell population. The loss of CD28 expression is unique to the CD8 compartment (348), as there is little or no accumulation of CD4+CD28- T-cells in healthy individuals. We then determined if LEN could reverse functional defects of naturally occurring CD28^{null} T-cells. We sorted on CD8+CD28+ and CD8+CD28- T-cells from healthy donors, stimulated the cells *ex-vivo* with anti-CD3 and examined proliferation and IL-2 production from these two distinct T-cell subsets (**Figure 19A-B**). As shown in **Figure 19A,** LEN increased proliferation of CD8+CD28+ T-cells in a dose-dependent fashion with anti-CD3 stimulation alone. The CD8+CD28- T-cell population displayed an overall significant reduction in proliferation compared to the CD28+ T-cells, and displayed no

response to LEN compared to DMSO-treated cells after anti-CD3 stimulation (**Figure 19A**). Elaboration of IL-2 was also completely abolished in CD8+CD28- T-cells with no response to LEN. These results indicate that the surface expression of CD28 on T-cells is indispensable for LEN immunomodulatory response.

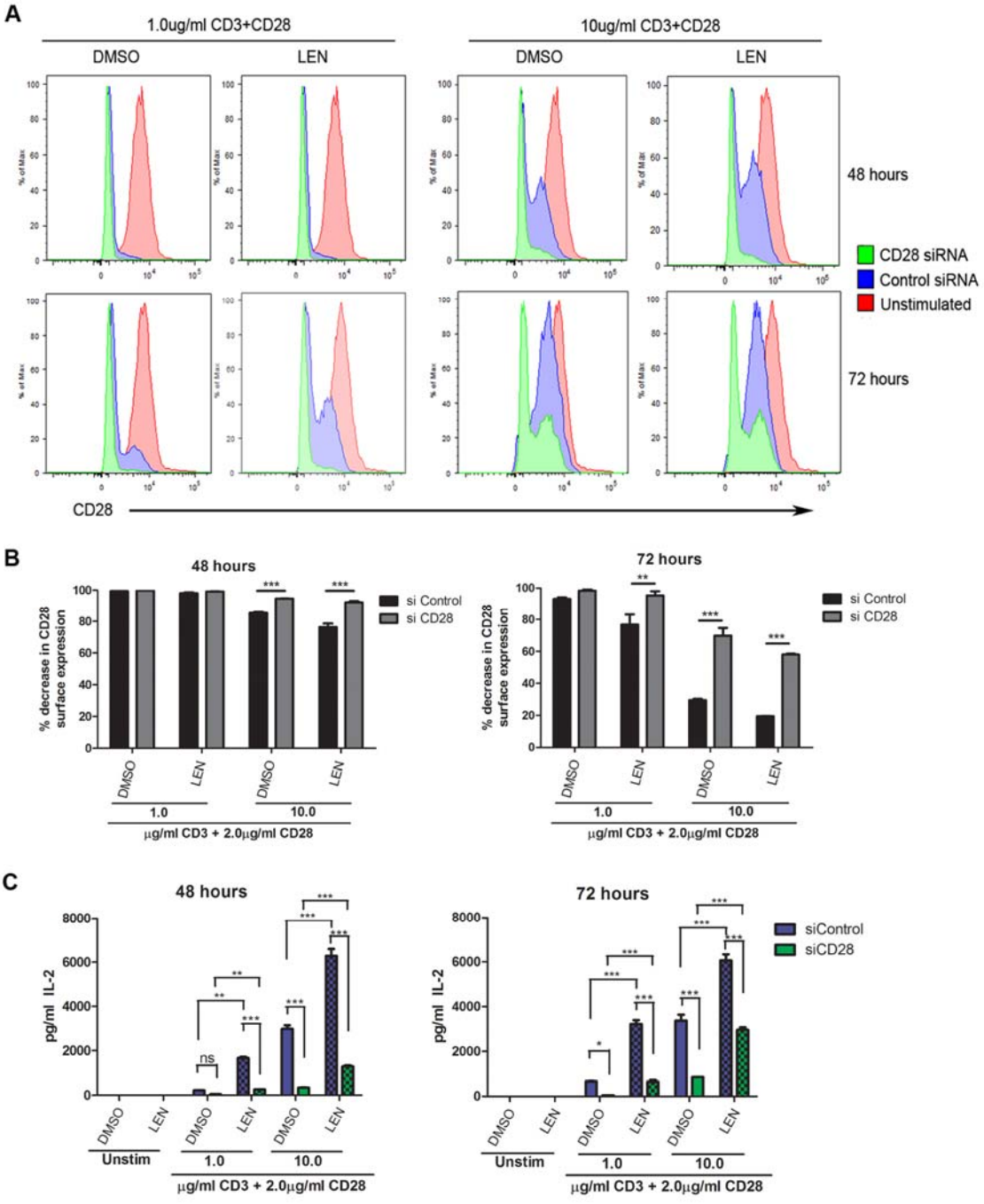

Figure 18. Knockdown of CD28 expression abrogates LEN activity in T-cells. CD28+ T-cells were sorted from healthy donors and transfected with either non-target (control siRNA) or CD28 siRNA. T-cells were then stimulated with either 1.0 or 10 µg/ml CD3 and 2.0 µg/ml CD28 for 48 and 72 hours in the presence of 10µM Lenalidomide or vehicle control (DMSO). **A**. Example of flow cytometry plot of one experiment to evaluate CD28 expression after siRNA infection and plate bound stimulation at 48 and 72 hour time points. Un-stimulated T-cells were used as positive control for CD28 expression. **B**. Bar graphs (right) quantitatively represent the average %$CD28^+$ T-cells at 48 and 72 hours from 3 independent experiments. **C**. IL-2 secretion in the supernatant in cells from A-B was collected after 48 and 72 hours of stimulation and evaluated by ELISA**. B,C**) Statistical analysis was performed using 2-Way ANOVA. **P<0.001 ***P<0.001.

Accumulation of CD28- T-cells in MDS patients is associated with LEN failure. Although LEN is approved for the treatment of MDS and MM, there are subsets of patients that are naturally resistant to the drug. Since CD28 expression is essential for LEN immunomodulatory effects in T-cells, we analyzed CD28 expression on T-cells from MDS patients who were treated with LEN (**Figure 19C-E**). Samples were taken before LEN treatment, and the percentage of CD28 positive cells was compared in responders (R) and non-responders (NR). Hematologic response was assessed after 16 weeks using IWG criteria for hematologic improvement. As shown in a representative R and NR patient (**Figure 19C**), the percentage of CD28+ cells was greater in the R compared to the NR. Data on this subgroup of MDS patients (**Figure 19D**) indicate that the proportion of CD28 expressing cells pretreatment is significantly associated with clinical outcome favoring more CD28+ cells in the responders. Although CD4 T-cells rarely lose CD28 expression in healthy donors, the NR MDS cohort had a significant accumulation in CD4+ CD28- T-cells (p=0.0061 **Figure 19D**). A similar significant decrease in the percentage of CD28+ T-cells in

NR patients was also seen in the CD8 compartment (p=0.0242) (**Figure 19E**). This data shows a correlation between accumulation of CD28- T-cells and resistance to LEN in MDS.

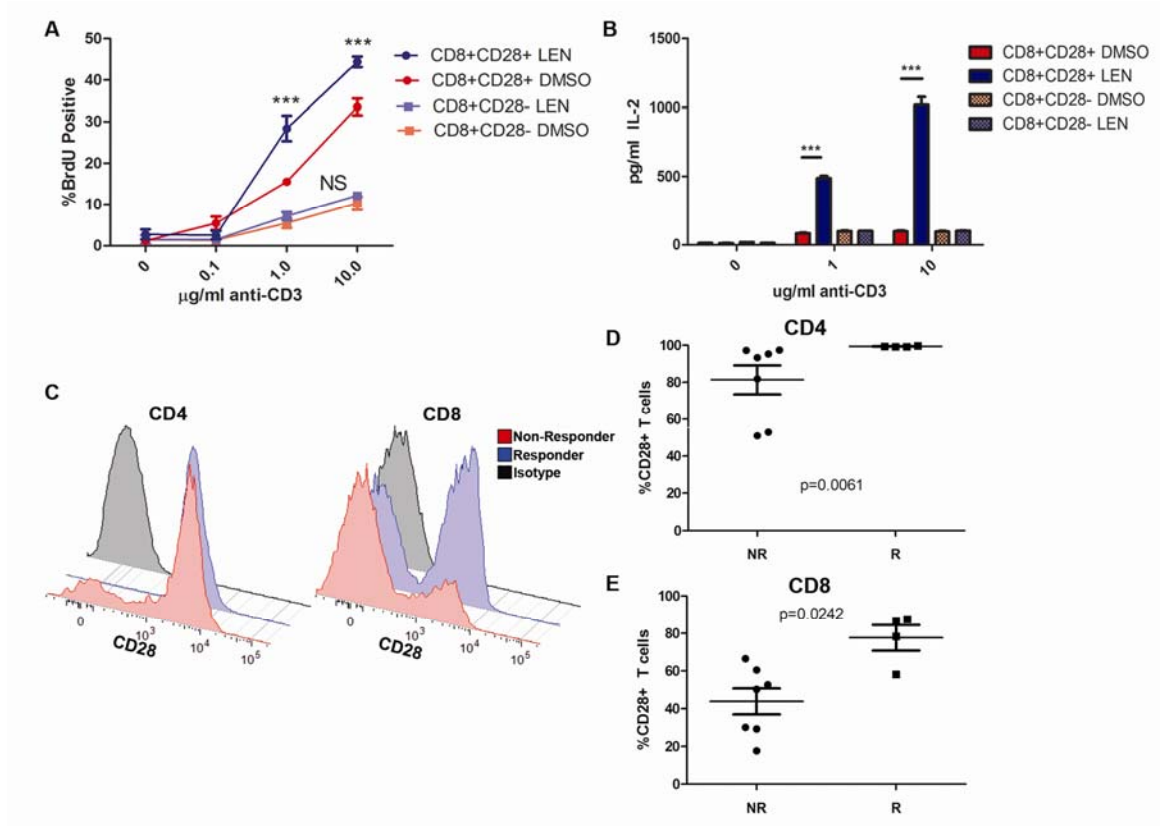

Figure 19. CD28^{null} T-cells are associated with LEN Response. A-B. Healthy donor CD8 T-cells were sorted into CD28^{+} and CD28^{-} populations before being stimulated for 3 days in the presence of increasing concentrations of plate bound anti-CD3 antibody (μg/ml) in the presence of DMSO (Vehicle) or 10μM Lenalidomide. **A**. BrdU incorporation was used to measure proliferation of sorted CD8+CD28+ and CD8+CD28- T-cells as determined via flow cytometry on day 3. **B**. CD8+CD28+, CD8+CD28- T-cells were stimulated as previously described, and supernatant was collected on day 3 of stimulation. IL-2 secretion was measured via ELISA. *** p<0.001. MDS patient PBMCs prior to lenalidomide treatment were analyzed for CD28 expression, and correlated with hematologic response. **C.** Representative histograms of CD28 expression on both CD4+ (Left) and CD8+ (Right) T-cells of erythroid Non-Responders (NR) and Responders (R). Black shaded region indicates isotype control staining. **D-E.** Percentage of CD28^{+} T-cells from both CD4^{+} (**D**) and CD8^{+} (**E**) populations was analyzed in 7 NR and 4 R. The difference between the two groups was determined using a Mann-Whitney T-test with p-values indicated on graphs.

We have previously shown that MDS patients who respond to LEN therapy have significant changes in the naive and memory T-cell compartment after LEN treatment (307). Cells with a CD28- phenotype generally express CD45RA but lack lymphoid-homing receptors such as CCR7, CD62L, and CD27, and these terminal effector memory (TEM) cells increase with age and are associated with autoimmunity (44, 45). The pre-treatment percentage of CD8+ naïve and memory T-cells (central memory, effector memory, and TEM cells) was then compared in LEN-treated MDS patients and correlated with response (R verses NR). **Figure 20A-C** are representative flow plots of CD28 expression in the different memory phenotypes on T-cells from a healthy donor (**Fig. 20A**), Responder (**Fig. 20B**), and Non-Responder (**Fig. 20C**). As shown in **Figure 20**, there was a clear distinction in the T-cell phenotype based on hematologic response. CD8+ TEM cells were significantly expanded in NR compared to R ($p=0.02$) (**Figure 20E**), where both naïve and central memory T-cells showed a tendency toward lower percentages ($p=0.06$ and $=p=0.07$). The difference in TEM CD8+ T-cells also reflects the reduction in the percentage of TEM expressing CD28 (**Figure 20D,** $p=0.03$**).** Although CD28-TEM CD8+ T-cells accumulate with age, the increase in these cells in NR was age-independent, as shown in **Figure 20F**. These results indicate that CD28 expression is necessary for immunologic responsiveness to LEN and loss of CD28 expression of CD8+ and CD4+ T-cells correlates with resistance to LEN in non-del(5q) MDS.

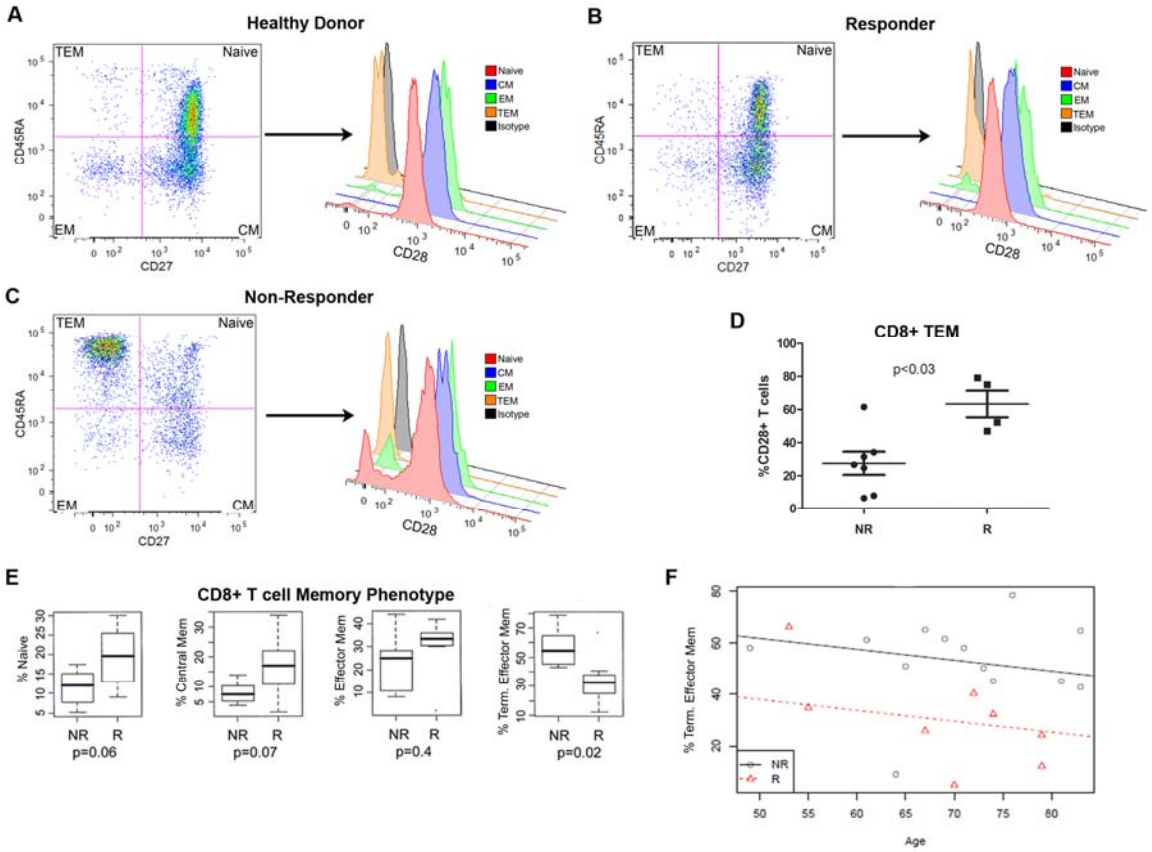

Figure 20. Non-Responder MDS patients have higher levels of Terminal Effector Memory (TEM) CD8+ T-cells and lower levels of CD28^{+} T-cells. The proportion of Naïve, Central Memory, Effector Memory, and Terminal Effector Memory T-cells for both CD4 and CD8 from MDS patients treated with Lenalidomide was determined using flow cytometry. **A-C**. Phenotype of CD3+ Healthy Donor (**A**) and MDS Patient Responders (**B**) and Non-Responders (**C**) and CD28 expression within each memory subset. **D**. Quantification of CD28^{+} T-cells within the TEM compartment in lenalidomide NR (n=7) and R (n=4) MDS patients. **E**. The proportion of each memory phenotype making up the CD8 T-cell compartment is shown for both Responding and Non-Responding patients prior to lendalidomide treatment, and is correlated with response after 16 weeks of *in vivo* treatment. Statistical analysis was performed using Wilcoxon Rank Sum Test. **F**. Proportion of CD8+TEM cells compared with age in MDS Responder (R) and Non-Responder (NR) patients (p=ns).

Discussion

LEN is a highly potent immunomodulatory drug (IMID®) used to potentiate T-cell and NK cell responses in a number of hematologic and solid malignancies. Anti-tumor activity of T- cells is increased by stimulating greater production of cytokines (307, 349), enhancing tumor antigen recognition (260), and activating T-cells by co-stimulation (265). Antibody-dependent cellular cytotoxicity (ADCC) (285, 290) and direct induction of tumor cell cytotoxicity (231, 350, 351) reportedly increases NK cells after LEN treatment. Since LEN enhances the activity of NK-, T- and B-cells, use of this agent is rapidly increasing in many settings and the direct molecular target responsible for enhancing immune function is under intense investigation. We and others have shown that LEN augments the production of IL-2 in the presence of anti-TCR activation alone (**Figure 15**), a function that is closely tied to CD28 signaling. Although LEN may replace the need for external ligation of the co-stimulatory molecule, we have demonstrated the requirement for, and the effect of LEN on CD28 surface expression. Ideally, the role of CD28 should be determined using T-cells from CD28 knockout mice, as receptor expression would be genetically eliminated, and these mice have been shown to lack IL-2 transcriptional activation after CD3 antibody stimulation. Unfortunately due to accelerated drug metabolism, altered pharmacokinetics, or differential regulation of LEN in murine T-cells, this experiment was not feasible. To evaluate the role of CD28, a knockdown approach was used along with evaluation of naturally-occurring CD28 deficient

cells. Our data reveal a novel aspect of LEN function and implicates the expression of the CD28 receptor in the molecular mechanism.

LEN and the thalidomide analog pomalidomide were recently shown to induce T-cell activation by inhibiting the activation of the E3 Ub ligase cereblon (CRBN). The function of CRBN in T-cells is undefined (257) and the only known role of the molecule is in brain and behavioral function. Casitas-B-lineage lymphoma protein-b (Cbl-b) and ITCH are RING-finger domain containing E3 Ub ligases known to regulate CD28 signaling. Cbl-b establishes the threshold for T-cell activation by regulating CD28 and TCRζ recruitment to lipid rafts and repressing PI3K and Vav1 signaling in the absence of CD28 co-stimulation (158). The activation of CD28 overcomes the repression by PKCθ-mediated phosphorylation, which targets Cbl-b for degradation (164). Cbl-b homozygous deficient mice exhibit lipid raft aggregation, sustained tyrosine phosphorylation of Vav1 and cytokine production in response to anti-CD3 stimulation without CD28 ligation, similar to LEN treatment (155, 156). Cbl-b -/- mice develop spontaneous autoimmune-mediated diabetes, increased susceptibility to experimental autoimmune encephalomyelitis, which is a mouse model of multiple sclerosis), and Cbl-b-null T-cells mediate more efficacious responses to tumors. Our data suggests that LEN modulate cells similarly to a deficiency in Cbl-b. Whether the drug can target other E3 Ub ligases or whether CRBN plays and undefined role in regulating CD28 signaling remains to be determined. It is clear, however, that LEN does not interfere with CD28 receptor internalization or recycling. CD28, CTLA4 and ICOS are regulated through receptor endocytosis and trafficking

through endosomes and while E3 Ub ligases are known to control receptor trafficking, their role in CD28 internalization is unknown.

CD28 ligation with CD80/CD86 on APCs in the presence of anti-TCR ligation enables a fully competent signal response by the T-cells, inducing cytokine production and proliferation, and preventing anergy (266). Anergy induction and T-cell non-responsiveness to tumor antigens are major obstacles to tumor immunotherapy (176). In solid tumors and hematologic malignancies, there is little expression of co-stimulatory molecules on the tumor cells themselves, as well as down-regulation of co-stimulatory molecules on DCs within the tumor environment (352). The lack of co-stimulation induces tolerance or ignorance by the immune system, preventing tumor-cell detection and eradication. LEN is being investigated as a combination therapy in a number of solid and hematologic malignancies due to its co-stimulatory function, while suppressing Treg expansion (276, 277). LEN's successes in hematologic malignancies like B-Chronic Lymphocytic Leukemia (B-CLL), Non-Hodgkin's Lymphoma (NHL) and MM are attributed to an immune-mediated anti-tumor effect, as well as direct anti-tumor activity. Clinical studies in solid malignancies such as melanoma and ovarian cancer have not demonstrated single-agent activity, and only nominal survival benefit (353-356). The combination of LEN with chemotherapy, however, has shown greater activity. For immunotherapy, benefit of the drug is evident in several solid tumor settings including metastatic melanoma (357), castration resistant prostate cancer (358, 359), pancreatic adenocarcinoma (360), and ovarian cancer (361).

Although LEN has had great successes in MDS and hematologic malignancies, and has shown potential in a variety of solid tumors, there are still subsets of patients that do not respond to treatment. Therefore, predictive biomarkers of response are necessary to help select, prior to therapy, patients that are likely to respond. We demonstrate in this study that CD28 expression on T-cells is likely an indicator of LEN responsiveness because of the necessity for CD28 expression for LEN-induced T-cell function (**Figure 18**). Augmented immune function is also implicated in the response of LEN in a subset of low-risk non-del5q MDS patients (307), and we show here that there is an accumulation of CD8+ TEM cells prior to LEN treatment in non-responding patients, which are predominantly $CD28^{null}$ (**Figure 20**). Hematologic response to LEN in MDS and MM is likely to involve the immune response (261, 307), and our data indicates that the accumulation of $CD28^{null}$ T-cells may be a factor in LEN resistance and suggests that additional clinical studies of biomarker analysis are necessary to confirm this as a predictive biomarker.

Materials and Methods

Healthy Donor T-cell isolation and activation. Peripheral blood from buffy coats of healthy donors was obtained from the Southwest Florida Blood Services, St. Petersburg, FL, for use as controls and for the purification of T-cells. T-cells were isolated from the buffy coats using RosetteSep® Human CD3+ T-cell Enrichment Cocktail (StemCell Technologies, Vancouver, BC Canada) according to the manufacturer's protocol. To activate the T-cell receptor (TCR), T-cells were stimulated with plate-bound anti-CD3 antibody (1 μg/ml or

indicated concentration) (BD Biosciences, San Jose, CA USA) coated onto flat-bottomed polystyrene tissue culture plates overnight at 4◦C. Purified T-cells (1 x10$_6$/ml) were added to the coated 96-well plates in a 200 µl volume for 48 hours at 37◦C. In some instances, 1.0 µg/ml anti-CD28 was added for co-stimulation.

Stimulation followed by measurement of CD28 surface expression was performed using anti-CD3 (10 µg/ml) alone or anti-CD3 (10 µg/ml) plus anti-CD28 (10µg/ml) coupled 4.5 micron polystyrene polybeads (Polysciences, Inc, Warrington, PA USA). 1x10^5 beads were added to 2x10^5 cells in u-bottom 96-well plates, as previously described (362). Cells were collected at 72 hours and 5 days, and CD28 surface expression was measured via flow cytometry on LSRII flow cytometer (BD Biosciences, San Jose, CA USA).

Preparation of lenalidomide for *in vitro* studies. Lenalidomide (Revlimid®) was provided by Celgene Corporation (Warren, NJ). The drug was weighed and dissolved at the time of use in dimethyl sulfoxide (DMSO) and diluted 1:1000 in culture media to a final concentration of 10 µM (or indicated concentration) because storage of stock solutions at −20°C resulted in variable loss in activity. An equal volume of DMSO was used as a vehicle control.

T-cell Proliferation. Proliferation was determined after *in vitro* activation by bromodeoxyuridine (BrdU) incorporation (BrdU flow kit, BD Biosciences, San Diego, CA USA). 10µM of BrdU was added during the last 45 min of T-cell stimulation. BrdU pulsed T-cells were harvested and stained with anti-CD4-PE and anti-CD8-PE-Cy-5 (BD Pharmingen, San Jose, CA USA). The cells were

then fixed and permeabilized with BD Cytofix/Cytoperm buffer and incubated with DNase for 1 hour at 37◦C. Cells were stained with anti-BrdU-FITC antibody before flow cytometry analysis on an LSRII flow cytometer (BD Biosciences, San Jose, CA USA). The percentage of BrdU positive cells from each population was analyzed using Flow-Jo Software (TreeStar Inc, Ashland, OR USA.).

Cell Sorting. T-cells were purified from healthy donor buffy coats as previously described, and were stained with 4',6-diamidino-2-phenylindole (DAPI) for viability, anti-CD8-APC-Cy7, CD4-PerCP-Cy5.5, and CD28-FITC antibodies (BD Pharmingen, San Jose, CA). Cells were then sorted into $CD8^+CD28^+$, $CD8^+CD28^-$ populations via FACS Aria Cell Sorter (BD Pharmingen, San Jose, CA).

siRNA Transfection and ELISA. For siRNA knockdown (KD), T-cells were transfected with siCD28 or control siRNA (Santa Cruz Biotechnology, Inc, Santa Cruz, CA USA) using Amaxa Nucleofection technology (Lonza, Basel, Switzerland). $10x10^6$ purified T-cells were mixed with either control or CD28siRNA in 100µl Human T-cell nucleofection solution, nucleofected, and then placed in Lymphocye Media (Lonza Basel, Switzerland). Cells were rested 24 hours before being stimulated with either plate bound anti-CD3, or anti-CD3+CD28, antibodies in the presence or absence of lenalidomide. CD28 surface expression was measured via flow cytometry at 48 and 72 hours after stimulation, at the same time supernatant was collected for ELISA analysis. Supernatant from stimulation experiments was frozen at -80ºC for analysis via Human IL-2 ELISA kit (eBioscience, San Diego, CA USA).

RT-qPCR. Total RNA was extracted from purified T-cells using the RNeasy Mini Kit (Qiagen, Chatsworth, CA USA). Reverse transcription was performed using the iScript cDNA synthesis kit (Biorad, Inc. Hercules, CA USA) in accordance with the manufacturer's suggestions. Relative target gene expression was measured by qRT-PCR using 18S rRNA expression as a reference gene. Expression levels of target mRNA and 18S rRNA were evaluated with Taqman Probes obtained from Applied Biosystems (Carlsbad, CA). All samples for both target genes (IL-2 or CD28) and 18S rRNA were measured in triplicate. Relative mRNA expression level for each sample was calculated using the $\Delta\Delta$Ct method (363).

Chromatin Immunoprecipitation (ChIP). Cells were isolated, fixed, lysed, and sonicated before chromatin-immunoprecipitation, as previously described (364). Briefly, after stimulation and drug treatment, primary T-cells were treated with 1% formaldehyde for 10min for cross-linking, followed by cell and nuclear lysis (50 mM Tris, pH 8.1, 10 mM EDTA, 1% SDS, 0.5 mM PMSF) and shearing. Chromatin was immunoprecipitated using 5µg anti-phospho-CREB antibody (Millipore, Temecula, CA USA) and protein A/G beads (Santa Cruz Biotechnology Inc., Santa Cruz, CA USA). Immunoprecipitated chromatin was collected and washed sequentially with TSE buffer (20mM Tris, pH 8.1, 50mM NaCl, 2mM EDTA, 0.1% SDS, 1.0% Triton X-100) and LiCl buffer (100mM Tris, pH 8.1, 50mM LiCl, 1% Nonidet P-40, 1% sodium deoxycholic acid, 1mM EDTA). DNA was then eluted from the beads with 50 mM $NaHCO_3$ containing 1% SDS

and cross-linking reversed at 65°C overnight followed by proteinase K treatment. DNA was then purified via QIAquick PCR purification kit (Qiagen, Germantown, MD USA). For each sample, 4µl DNA was amplified and measured using Sybr Green (Bio-Rad, Inc., Hercules, CA USA). Primers used to amplify the pCREB binding site -180 upstream of the transcription start site were: *forward: 5'-AGAAGGCGTTAATTGCATGAATT-3'* and *reverse: 5'-TCCTCTTCTGATGACTCTTTGGA-3'*.

MDS Patient Samples. MDS patients (n=100) were consented at H. Lee Moffitt Cancer Center in Tampa, FL to participate in a peripheral blood collection protocol approved by the University of South Florida Institutional Review Board from 2004-2009. All patients signed University of South Florida Institutional Review Board approved informed consents for the collection of 40 ml of peripheral blood in heparin tubes. Samples were obtained from each patient at various times from 2004-2009 for immune monitoring studies and all cells were frozen in liquid nitrogen. Twenty-one of these patients had samples that were collected within 4 weeks prior to LEN treatment. LEN was administered at a dose of 10 mg for 21 out of a 28-day cycle for four cycles. All patients were evaluated for hematologic response after 16 weeks according to 2006 International Working Group Criteria (IWG). Hematologic response was reported previously within a larger cohort of MDS patients (338).

Analysis of T-cell naïve and memory populations. Naïve and memory CD4 and CD8 T-cell subtypes in MDS patients and healthy donors were detected after surface staining with anti-CD3-PE-Cy7, anti-CD8-PerCP-Cy5.5, anti-CD45RA-FITC, anti-CD27-APC, anti-CD28-PE, and DAPI (all from BD Biosciences, San Jose, CA USA). Naïve and memory T-cell populations were distinguished by CD45RA and CD27 expression, as described previously by flow cytometry (319, 322, 323); briefly, the memory phenotype populations are characterized as naïve ($CD45RA^{+}/CD27^{+}$), central memory ($CD45RA^{-}/CD27^{+}$), effector memory ($CD27^{-}/CD45RA^{-}$), and terminal effector memory ($CD45RA^{+}/CD27^{-}$). Samples were run on an LSRII flow cytometer (BD Pharmingen) and populations were analyzed by FlowJo Software (Tree Star Inc., Ashland, OR USA).

CHAPTER 4

Lenalidomide and the IMiDs inhibit the negative regulatory activity of protein phosphatase 2A in T-cells to mediate T-cell co-stimulation

Introduction

Thalidomide is a potent immunomodulatory drug (IMiD) with anti-angiogenic and anti-TNF-α effects, as well as immune stimulating properties (222, 223). Although thalidomide was discovered to be a potentially important anti-cancer agent, severe side effects led to the development of the thalidomide analogues lenalidomide and pomalidomide, with greater immunomodulatory activity and less side effects such as peripheral neuropathy (227, 229). In 2005, lenalidomide was approved for the treatment of del(5q) myelodysplastic syndrome (MDS) because of its ability to induce karotype-specific transfusion independence and cytogenetic response (230). Isolated del(5q) MDS occurs in less than 15% of MDS cases where there is a 3 megabase (Mb) interstitial deletion on the long arm of chromosome 5 (5q31-5q32) coined the commonly deleted region (CDR) (365, 366). Patients with this specific chromosomal abnormality have a lower risk for leukemia transformation, improved survival, and a 75% complete response rate to lenalidomide treatment compared to other MDS patients without the deletion (230, 233). MDS is a clonotypic heterogeneous grouping of diseases characterized by ineffective hematopoiesis, multiple

cytopenias, dysplasia in the myeloid lineage, and a high risk for transformation to acute myeloid leukemia (AML).

Although lenalidomide is able to induce high rates of transfusion independence in a subset of MDS patients without del(5q), the mechanism of action of the drug in inducing apoptosis of the del(5q) myeloid clone and improving hematopoiesis is still unclear due to the complexity of the disease. Ribosomal abnormalities have been implicated in the pathogenesis of MDS, namely a loss of RPS14 gene expression, located within the 5q-CDR. Deletion of this gene through an siRNA screen was shown to be sufficient to induce the del(5q)-associated pattern of impaired hematopoiesis and the del(5q) syndrome in mice(239, 240). RPS14 loss, or inhibition of ribosomal processing proteins leads to nucleolar stress, degradation of murine double minute protein 2 (MDM2) and increased p53 expression in the erythroid blasts, subsequently increasing the expression of p53 target genes (like p21) that prevent cell cycle progression and induce apoptosis (241, 242). Wei and colleagues have recently demonstrated that lenalidomide can promote p53 degradation within the erythroid blasts through inhibition of the auto-ubiquitination of MDM2, allowing MDM2 to ubiquitinate p53 and induce its degradation (243).

Although these results suggest a mechanism for lenalidomide in promoting erythropoiesis, our group has shown that another set of genes within the CDR may be targeted by lenalidomide to induce myeloid apoptosis. Our group has shown that lenalidomide may inhibit the activity of the haplodeficient phosphatases cell division cycle 25c (Cdc25c) and protein phosphatase 2A

(PP2A) to prevent cell cycle progression and induce apoptosis within the myeloid clone (246). Reduction in phosphastase expression through siRNA in non-del(5q) MDS patient cell lines was sufficient to render them susceptible to lenalidomide-induced apoptosis through direct and indirect mechanisms. PP2A is a heterotrimeric, ubiquitously expressed serine/threonine phosphatase involved in a myriad of cellular processes, including cell cycle regulation, signal transduction, cytoskeleton dynamics, and cell mobility (165, 166).

Although lenalidomide may increase erythropoiesis and induce apoptosis within the del(5q) clone, the importance of immune activation against the myeloid clone has also been implicated in lenalidomide's mechanism of action in MDS. Lenalidomide has previously been shown to have potent immune stimulatory activity, likely through activation of the T-cell co-stimulatory pathway (265). The activation of immune system seems to be important in MDS patient response to lenalidomide treatment, as patients who responded to lenalidomide have an increase in immune infiltrates into the bone marrow (230), and increased T-cell proliferation and cytokine production *in vivo* (307). The immunomodulatory activity is also implicated in multiple myeloma and chronic lymphocytic leukemia (253, 260). Interestingly, PP2Acα is a key negative regulator of the CD28 co-stimulatory pathway in T-cells, reversing lck-induced phosphorylation of the cytoplasmic tail of the receptor (174). PP2A overexpression is implicated in decreased interleukin-2 (IL-2) production in systemic lupus erythematous (SLE) patients through increased de-phosphorylation of the pCREB transcription factor and carma-1 signaling molecule (171, 172). Lenalidomide has been shown to

increase phosphorylation of CD28 and secretion of IL-2 after anti-CD3 stimulation alone (270), alluding to the inhibition of a negative regulator of T-cell signaling in the drugs immune modulatory mechanism.

Therefore, we utilized multiple approaches to evaluate the interaction between PP2A and lenalidomide immune modulation. Both functional studies and molecular modeling approaches were used to evaluate the molecular mechanism of lenalidomide-induced T-cell proliferation related to PP2A inhibition.

Results

PP2A activity in T-cell lines and primary T-cells is inhibited with lenalidomide treatment.

PP2A is a negative regulator of IL-2 production through direct de-phosphorylation of the CD28 receptor, as well as de-phosphorylating and therefore inactivating several downstream signaling factors including CREB and CARMA-1 (171, 172). Both lenalidomide and pomalidomide are able to increase IL-2 production from primary T-cells stimulated with anti-CD3 antibody alone, in the absence of anti-CD28, although lower amounts of IL-2 are induced by thalidomide even when higher doses are used (0.05μg/ml POM and LEN versus 1.0 μg/ml THAL). It was evident that all three drugs significantly increase IL-2 production compared to DMSO vehicle-control treated cells (**Figure 21 A**).

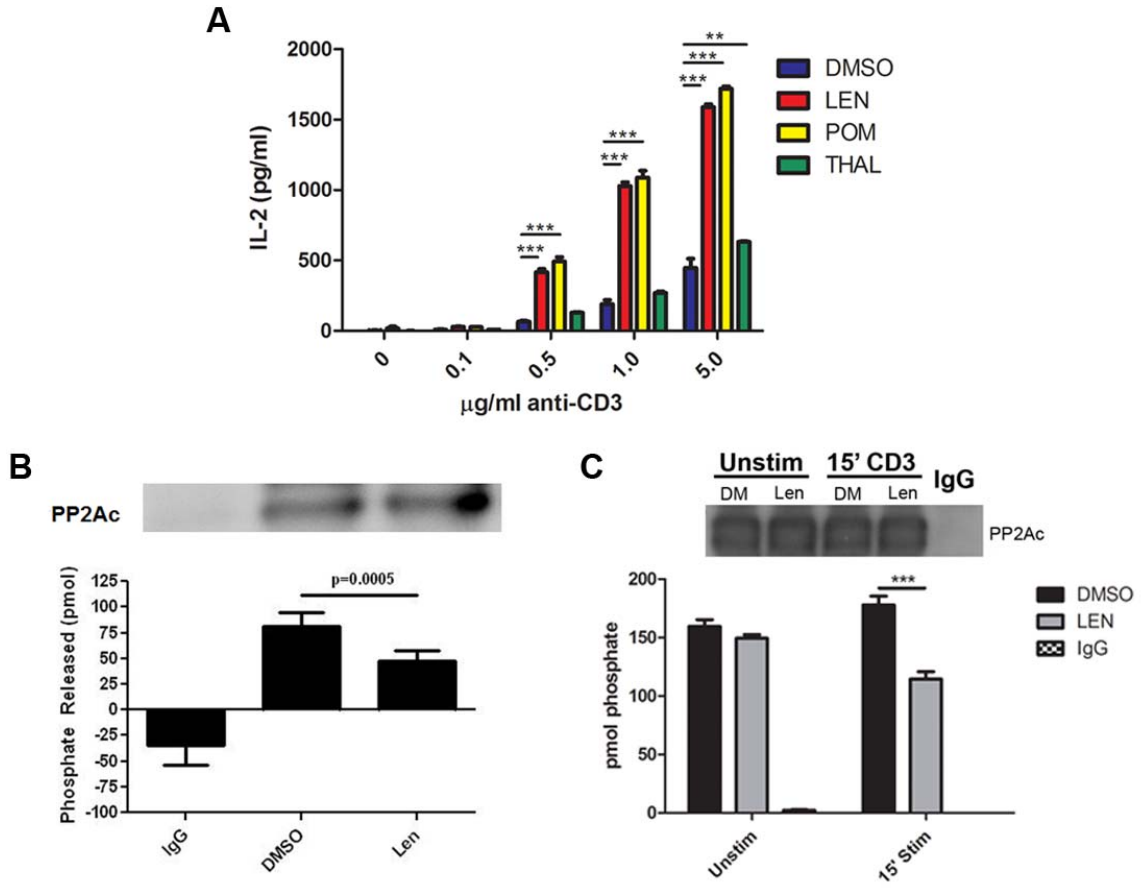

Figure 21. Lenalidomide increases IL-2 production and inhibits phosphatase activity of PP2A isolated from Jurkat and primary T-cells. A. All three IMiDs induce IL-2 production from primary T-cells with increasing doses of anti-CD3 stimulation relative to DMSO control measured by ELISA. Data analyzed using 2-Way ANOVA: **=p<0.01, ***=p<0.001. **B-C.** Measurement of endogenous PP2Ac activity from T-cells. PP2Ac was immunoprecipitated from cells treated with either DMSO (vehicle) or Len. Free phosphate released was then measured via phosphatase activity assay. Western blot in both **A** and **B** (upper panels) show equal pull-down of PP2Ac in each sample to ensure changes in activity were not due to unequal PP2Ac loading. IgG was used in each case as a negative control. **A.** PP2Ac catalytic activity was decreased in Jurkat cells treated with Len, p-value is indicated. **B.** PP2Ac activity from primary T-cells treated with len and stimulated in vitro with anti-CD3 antibody for 15 minutes was decreased compared to DMSO control. ***=p<0.001.

Although we have previously shown that lenalidomide can inhibit PP2A activity, it is unclear whether this occurs in primary T-cells. To analyze this, Jurkat T-cells and primary T-cells isolated from a healthy donor were treated with lenalidomide, PP2Ac was immunoprecipitated and subjected to an *in vitro* phosphatase activity assay. **Figure 21B** shows that lenalidomide can inhibit PP2Ac activity in Jurkat cells. The same can also be seen in primary T-cells after stimulation with anti-CD3 antibody (**Figure 21C**). These data indicate that lenalidomide inhibits PP2A phosphatase activity in T-cells after anti-CD3 activation, and this decrease in activity is associated with the release of IL-2.

PP2A catalytic activity is inhibited after in vitro treatment with the IMiDs. Although it was previously shown that lenalidomide can induce apoptosis of the del(5q) clone when PP2Ac expression is reduced, the ability of lenalidomide to inhibit PP2A catalytic activity was not established. To first determine if lenalidomide inhibits the catalytic activity of PP2A, ad293 cells were stably transfected with HA-tagged PP2Ac and treated with lenalidomide. After lenalidomide treatment, PP2Ac was immunoprecipitated and subjected to *in vitro* phosphatase enzymatic activity assay. As shown in **Figure 22A**, treatment with increasing concentrations of lenalidomide resulted in a dose-dependent decrease in PP2Ac enzymatic activity compared to DMSO vehicle control. Western blot analysis was performed to ensure equal HA-PP2Ac protein was present in each sample.

Since lenalidomide is able to inhibit PP2A enzymatic activity in vitro, we next determined whether other IMiDs display similar inhibitory function. **Figure 22B-D** shows that all three of the IMiDs, to varying degrees, inhibit PP2Ac phosphatase activity in a dose-dependent manner, with thalidomide being the least potent (**Figure 22D**). **Figure 22E** compares the percent activity of the PP2A enzyme in the presence of 10μM of the IMiDs, demonstrating that lenalidomide and pomalidomide are more potent inhibitors than thalidomide. This is consistent with the relative immunomodulatory effects of thalidomide and the various derivatives.

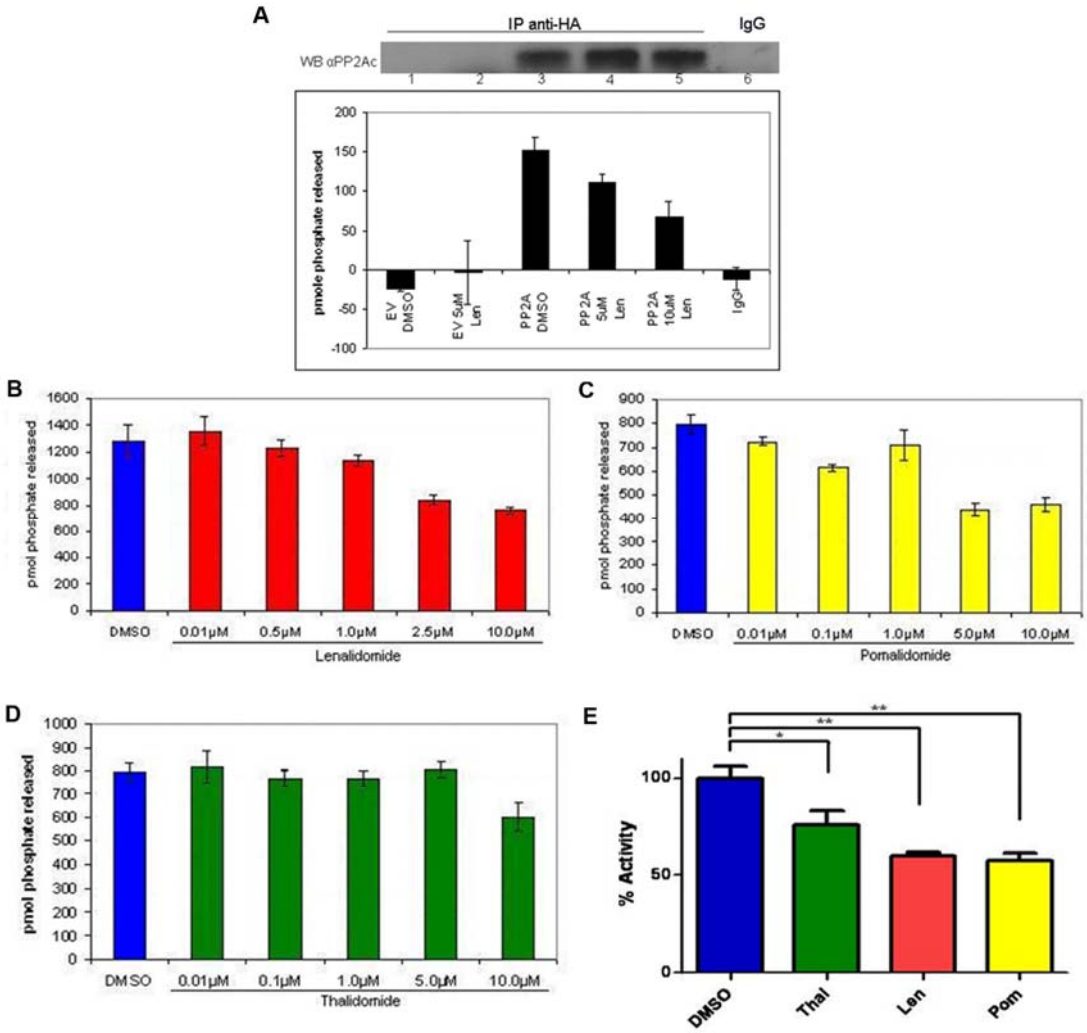

Figure 22. PP2A catalytic activity is inhibited after *in vitro* treatment with all of the IMiDs. **A.** For phosphatase activity assay, human embryonic kidney (HEK) cell line was stably transfected with Hemagluttinin (HA)-tagged PP2Acα. HA-PP2Ac was immunoprecipitated from ad293 cellular extracts treated with vehicle (DMSO) or 5-10μM lenalidomide (Len). Upper panel western blot shows equal pull-down of HA-PP2Ac in HA-transfected cells, and no pull-down in empty vector transfected (EV) and IgG pull-down negative controls. Graph represents dose-dependent decrease in free-phosphate released through PP2A activity with increasing doses of lenalidomide treatment. **B-E**. HA-PP2Acα expressing-HEK cells were treated with varying doses of IMiDs before HA-PP2A was then immunoprecipitated (IP), incubated with phospho-threonine peptide and phosphatase activity measured by Malachite green assay. Dose dependent inhibition of PP2Ac activity can be seen with lenalidomide (**B**) and pomalidomide (**C**). Inhibition of PP2A activity is seen at highest thalidomide dose (**D**). **E**. Graph represents inhibition of PP2A activity at 10μM dose of all three IMiDs. T-test: *= p 0.0002 **= p<0.0001.

Virtual computer modeling reveals a potential direct interaction of the IMiDs within the active site of the PP2A catalytic subunit. Recently, cereblon (CRBN) was shown to directly interact with IMiDs and to suppress its auto-E3-ubiquitination function. This is the only molecule known to directly interact with IMiDs. Next, we used a molecular computer modeling approach to estimate the potential for lenalidomide and the other IMiDs to directly bind to PP2A. Four compounds were prepared for docking to PDB 3K7V: lenalidomide, pomalidomide, thalidomide, and a known potent PP2Ac inhibitor, fostriecin. Each of these compounds possesses chiral carbons, therefore alternative stereoisomers were included in the docking. All of the IMiDs are administered as a racemic mixture, and cannot be administered in isolation as they can freely transform *in vivo* (219). Both the R and S forms of lenalidomide place the nitrogen bearing six-membered ring into the hydrophobic region of the binding site, and bind with similar estimated free energies of -6.52 and -5.80,

respectively. The R and S forms of pomalidomide actually dock significantly differently from one another, but have nearly identical docking energies (-6.39 and -6.06). The R and S forms of thalidomide binds similarly to thalidomide and lenalidomide , although the chirality causes significant differences in estimated binding free energy. The S enantiomer dockings of the IMiDs are shown in **Figure 23A-C**. As you can see, lenalidomide (**Figure 23A**) and pomalidomide (**Figure 23B**) form 2 hydrogen bonds within the catalytic pocket of the c-subunit; lenalidomide creates a hydrogen bond to W200 and R214 and pomalidomide to R214 and S120. The *S* binding pose of thalidomide on the other hand, only forms one hydrogen bond within the pocket, at W200, (**Figure 23C**) suggesting that the racemic mixture of the *R* and *S* forms of thalidomide would have significantly reduced binding capacity compared to the racemic mixtures of len and pom. Fostriecin is a significantly more potent and specific inhibitor of PP2A enzymatic activity and is effective in the nanomolar concentrations (367). The binding free energy for fostriecin was -9.17 kcal/mol and it was capable of making three hydrogen bonds, supporting the validity of the docking experiments. Further validation of the docking site can be seen through published co-crystallization of another PP2Ac inhibitor, okadaic acid, within the catalytic pocket of PP2A. Okadaic acid also shares all three potential interaction sites, H191, W200, and R214, within the PP2A active site (368). These data support the *in vitro* data that thalidomide is the weakest inhibitor of PP2A enzymatic activity, and suggest a potential direct inhibition of PP2A enzymatic activity in T-cells.

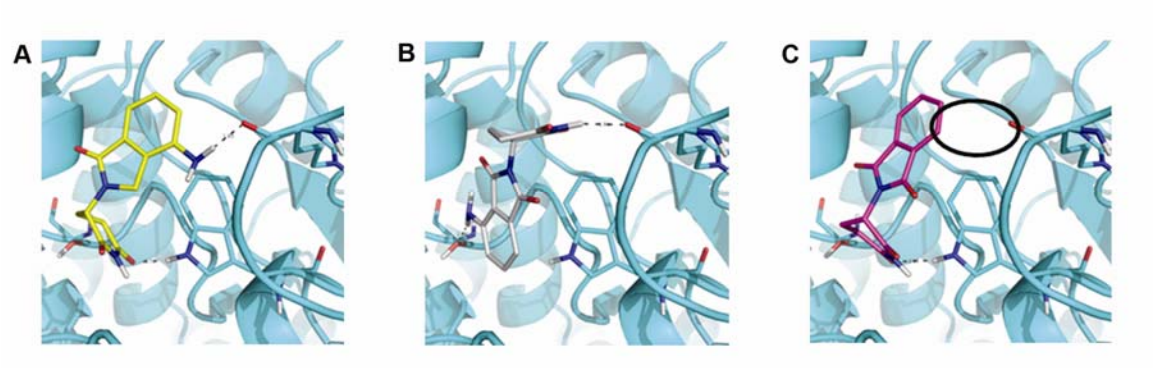

Figure 23. Virtual modeling of the IMiDs reveals potential interacting sites within the catalytic pocket of the PP2Ac subunit. Free energies of binding and poses for the molecules suggest that both the *R* and *S* enantiomers of all three immunomodulatory drugs bind to the same position within the catalytic pocket of the c subunit of the PP2A heterodimer, and thereby possibly inhibit phosphatase activity (**A-C**). The S enantiomers of both lenalidomide (**A**) and pomalidomide (**B**) have better binding poses and energies (-5.80 kcal/mol and -6.06 kcal/mol respectively) than thalidomide (**C**) (-5.34kcal/mol). *S* enantiomers of IMiDs Lenalidomide (**A**), Pomalidomide (**B**), and Thalidomide (**C**), are shown bound within the catalytic pocket of PP2Acα. Dashed lines indicate hydrogen bonds between the small molecule inhibitors and amino acid residues W200 and R214. Another hydrophobic interaction is shown to occur at H191. Black oval (**C**) indicates position where thalidomide lacks second hydrogen bond compared to the other compounds, owing to the lack of aromatic amine group seen in **A**.

Mutations in the lenalidomide-binding sites of the PP2A catalytic subunit render PP2A enzymatically inactive. Since virtual modeling predicted potential binding sites for lenaliodmide within the catalytic pocket of PP2A, it is possible that lenalidomide could directly bind to the active site and inhibit PP2A enzymatic activity. To confirm this prediction, we utilized site-directed mutagenesis of the estimated interaction sites that may be required for lenalidomide-mediated PP2A inhibition. According to the virtual modeling predictions, aaW200 and aaR214 may be important binding sites for lenalidomide within the PP2A catalytic pocket, forming a hydrogen bond with the lenalidomide *S* molecule. H191 was also implicated in lenalidomide binding

through formation of a hydrophobic interaction with the six-membered ring. These three amino acids in PP2Ac were mutated using site-directed mutagenesis and overexpressed in ad293 cells. As shown in Figure 2, a similar immunoprecipitation approach was used to selectively evaluate the function of the mutant proteins using immunoprecipitation with anti-HA antibody (IP anti-HA-PP2Ac). Stable cell lines of the HA-WT (wild-type) and HA-PP2Ac mutant proteins were established. **Figure 24A** shows the phosphatase activity and cellular expression of the different clones. WT-HA PP2A was used as the positive control (100% activity). Clones expressing W200F-D, R214A, and H191G did not retain HA-plasmid expression after selection, and therefore could not be utilized. Three independent W200F (A-C) and R214A (B-D) clones expressed mutated HA-PP2Ac protein but had no enzymatic activity, indicating that mutation of these amino acids renders PP2A catalytically inactive revealing novel amino acids within PP2A critical for function. Due to the importance of these amino acids in PP2A function, these mutant constructs were not useful to evaluate the effects of lenalidomide.

The only mutated clone retaining PP2A enzymatic activity was H191G-B, which was then utilized in lenalidomide experiments. Lenalidomide as well as an independent PP2A inhibitor (okadiac acid, OA) retained their suppressive function in the presence of the mutation (**Figure 24B**; $p<0.001$). Therefore, these mutant PP2A proteins cannot conclusively determine whether the direct interaction between lenalidomide and PP2A is important for the suppressive

response, although we have identified novel amino acids involved in the catalytic function of PP2A.

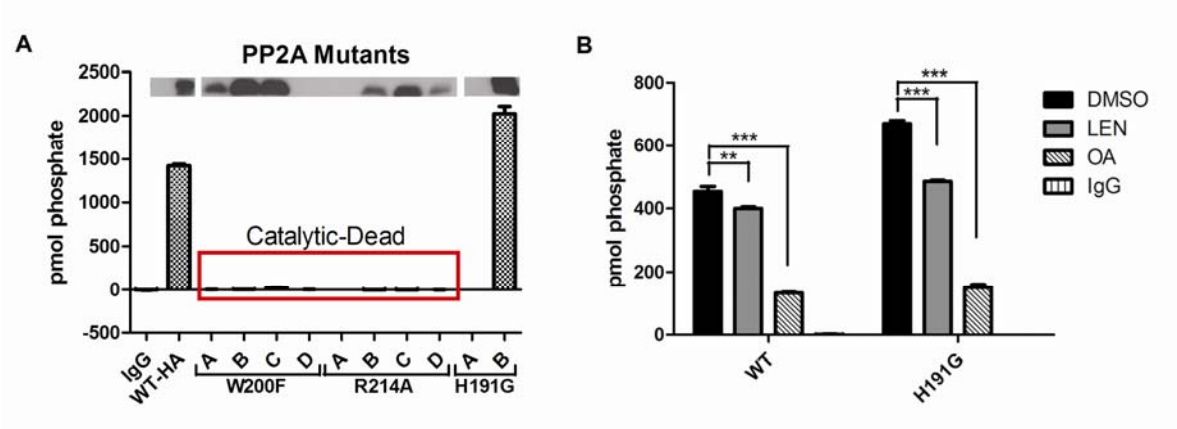

Figure 24. Mutations of theorized IMiD-interacting sites alters PP2A enzymatic activity. Virtual modeling suggested three potential amino acids within the catalytic pocket of PP2A to interact with lenalidomide. Mutations in these sites (W200F, R214A, and H191G) were created and the effect on the enzymatic activity of PP2A (**A**) and of lenalidomide's ability to alter PP2Ac enzymatic activity (**B**) were analyzed. **A.** WT-PP2Ac and mutants were immunoprecipitated from ad293 cells using anti-HA tag antibody and subjected to phosphatase activity assay. Red box highlights clones with no catalytic activity. One clone, H191G-B did retain enzymatic activity. Western blot (upper panel) of HA-PP2Ac expression used as positive indicator of mutant protein expression. **B.** H191G-B retained enzymatic activity, and was subjected to lenalidomide treatment and subsequent phosphatase activity assay. Mutation of histidine 191 to glutamate did not alter lenalidomide's ability to inhibit PP2Ac activity analyzed by 2-way ANOVA. **=p<0.01, ***=p<0.001.

Discussion

Lenalidomide and the other IMiDs are known to augment the proliferation and function of T-cells and NK cells by a mechanism that involves CD28 signaling and possibly involves suppression of CRBN. Previous work has shown that lenalidomide specificity for apoptosis induction in MDS del(5q) is mediated by haploinsufficiency of PP2Ac and Cdc25c. Reduction in these two

phosphatases alone are sufficient to render resistant myeloid clones susceptible to lenalidomide-induced apoptosis as shown by shRNA knockdown of these molecules in non-del(5q) hematopoietic progenitors and cell lines(246). Although these proteins are important cell cycle regulators, PP2Ac is essential to many cellular processes, including negative regulation of CD28 co-stimulatory signaling in activated T-cells. Previous studies in our lab have shown that lenalidomide treatment increases pCREB binding to the IL-2 promoter (307). PP2A can directly de-phosphorylate Carma-1, subsequently dissociating it from the CBM complex (172). Therefore, it is plausible that lenalidomide may stimulate IL-2 by inhibiting this negative regulator of T-cell signaling. Since CD28 stimulation is needed for de-phosphorylation of Carma-1, activation of this pathway may be mediated directly by lenalidomide leading to increased and prolonged signaling for proliferation and cytokine secretion in the absence of CD28 co-stimulatory ligation.

We have demonstrated through *in vitro* phosphatase activity assays that lenalidomide and the other IMiDs, to varying degrees, can inhibit PP2Ac enzymatic activity (**Figures 21-23)**. Based on previous experiments, lenalidomide has no direct inhibition of PP2Ac phosphatase activity. There are several plausible explanations, namely lack of proper PP2Ac subunit folding in baculovirus-generated protein, or improper PP2Ac holoenzyme subunit formation. It is difficult to generate properly-folded recombinant PP2Ac protein, as the catalytic subunit generated in bacteria is non-functional. Therefore, it is plausible that commercially available recombinant PP2A protein is altered in

confirmation compared to *in vivo*-expressed protein, and therefore lenalidomide cannot bind. Another plausible explanation is that the recombinant protein comes as an A-B-C heterotrimer with a fixed beta subunit. PP2Ac is a heterotrimeric enzyme composed of a scaffolding subunit (A α or β) , a catalytic subunit (Cα or β) and one of 16 different B-regulatory subunits (B, B', B'', or B''' family) with variable expression based on cell and tissue type (165, 166, 369). The A-C core enzyme has been purified from many different tissues, but its presence *in vivo* is slightly controversial and specific substrates of the dimer alone have not been identified (370). Since PP2A is ubiquitously expressed and involved in a myriad of cellular processes, that A-C heterodimer binds to the multiple different B-regulatory subunits that confer substrate specificity and prevent random de-phosphorylation activity of the PP2A enzyme (167, 371-373). The B subunit expressed with the PP2Ac heterotrimer in recombinant protein may not allow for proper lenalidomide binding to allow inhibition of enzymatic activity. Proteomics analysis of immunoprecipitated protein from lenalidomide treated lysates was unable to identify a particular ratio of B subunits distinct to lenalidomide-binding (data not shown).

Therefore, a molecular modeling approach was used to theoretically indicate the potential for lenalidomide direct binding interactions with PP2A, and found that all three of the IMiDs can potentially bind within the catalytic active site of the PP2A catalytic subunit in both the *R* and *S* enantiomeric forms (**Figure 23**). It was important to include both of the racemates in the virtual analysis, as it has been previously postulated that different forms of thalidomide (and

subsequently lenalidomide) have different *in vivo* activities (218, 219). Also, stereochemistry in virtual screening has been previously determined to significantly affect free binding energies (374). Interestingly, the lenalidomide racemates bound with similar docking poses and binding energies, while the thalidomide *R* and *S* enantiomers had dramatically different docking poses, with the *S* form of thalidomide losing a hydrogen bond within the pocket, reducing its binding energy. These results, combined with the expected higher binding energy of the known specific and potent PP2A inhibitor, forstriecin, indicate that it is possible for the IMiDs to directly bind to the PP2A active site to inhibit phosphatase activity.

To validate these predictions, mutation in the predicted interacting residues were made by site-directed mutagenesis, stably over-expressed, and tested for phosphatase activity. Mutation of only one of the potential interacting residues produced a functional protein. The other mutants either induced apoptosis or rendered the enzyme catalytically dead. Although this virtual modeling revealed novel amino acids with importance for PP2A catalytic function, the mutant proteins were unable to yield information about the nature of lenalidomide function. The only mutation to retain activity was H191G, which was predicted to generate only Van der Waals interaction with the lenalidomide compound, and mutation of this site failed to disrupt lenalidomide, OA, or fostriecin function.

Suppression of PP2A with lenalidomide in T-cells was evident only after anti-CD3 activation which is more consistent with an indirect mechanism of

suppression. Although proof of direct inhibition of PP2A phosphatase activity was not established, either direct or indirect inhibition of PP2A by lenalidomide and the other IMiDs is important for establishing the molecular mechanism of action of IMiDs in T-cells. Several other proteins such as E3 ubiquitin ligases murine double minute 2 (MDM2) and CRBN have recently been implicated as direct lenalidomide targets in MDS erythroblasts and multiple myeloma (243, 256, 257). The role of CRBN in T-cell proliferation and activation is unclear. Knockdown of CRBN expression in T-cells was shown to abrogate lenalidomide's cytokine-promoting activity, leading to the idea that CRBN expression is essential for lenalidomide's mechanism of action (257) but functional studies of CRBN in T-cells are lacking. Currently, the only E3 ubiquitin ligase known to regulate the response of CD28 is Cbl-b. More studies are necessary to confirm the role CRBN in PP2A function, Cbl-b, and CD28 signaling during T-cell activation.

Materials and Methods

Cell culture and creation of stably transfected cell lines. Ad293 cells were cultured in RPMI 1640 media (Gibco, Life Technologies, Grand Island, NY, USA) supplemented with 10% heat-inactivated FBS without antibiotic. Cells were transferred to 6-well plates at concentration of $0.8x10^6$ cells/well in 2ml media. The next day, each well was transfected with 4µg plasmid DNA pcDNA3 vector containing HA-PP2Acα, or empty vector, using Lipofectamine 2000 (Invitrogen, Grand Island, NY USA) according to the manufacturer's protocol for adherent

cells. 48 hours later, stably transfected cells were selected for using media supplemented with 1.0mg/ml G418 Sulfate (Cellgro, Manassas, VA USA). Cells were then diluted to 1 cell/well in a 96-well plate to isolate single clones expressing either HA-PP2Acα or empty vector. Stable expression of the HA transgenic protein was determined via western blot with anti-HA antibody (Sigma-Aldrich, St. Louis, MO USA), with empty vector serving as a negative control. Stable clones of HA-PP2Acα and EV were grown in RPMI 1640 supplemented with 10% FBS and 0.5mg/ml G418 in all further experiments.

Drug Treatment and Cell lysate preparation. Stably transfected ad-293 HA-PP2Ac and empty vector cells were treated with varying concentrations of lenalidomide, pomalidomide, and thalidomide dissolved in DMSO. Cells were grown at $0.8x10^6$/ml overnight in 35mm culture dishes before drug addition, or equivalent volume of DMSO for 5' at 37ºC before collection.

Cells were then washed with 1x TBS on ice and lysed in 150µl lysis buffer composed of 10mM Tris (pH 8), 50mM NaCl, 5mM EDTA, 1% Nonidet P-40, 1 µg/µl protease inhibitors leupeptin, aprotinin, and antipain, 1mM sodium orthovanadate, 5mM NaF, and 1mM DTT for 30' at 4ºC. Cellular extracts used in phosphatase activity assay was prepared using the lysis buffer adjusted to pH 7.0, omitting ser/thr phosphatase inhibitor NaF (171). Protein concentration was measured using Bradford reagent (Bio-Rad) and standardized with BSA. Cellular lysates for Western Blot were stored at -80ºC, while lysates for phosphatase assay were used immediately.

Phosphatase activity assay. Enzymatic activity of PP2Acα was determined following immunoprecipitation using a malachite green phosphatase assay (Ser/Thr Phosphatase Assay Kit 1; Upstate) and methods adapted from Katsiari et al. (171) as follows. 250ug whole cellular protein was incubated with anti-HA sepharose beads (Sigma-Aldrich) to pull down HA-PP2Acα, or a negative control mouse IgG antibody and Protein A/G agarose beads (Santa Cruz Biotechnology), at 4ºC rocking for 2 hours. Beads were then washed 3 times in 700µl TBS, and one time in 500µl Ser/Thr Assay Buffer before finally dissolved in 40ul Ser/Thr Assay Buffer. Samples were then incubated with 500µM of PP2Acα phosphor-peptide substrate (amino acid sequence: K-R-pT-I-R-R) for 10 minutes at 30ºC constantly shaking. Supernatants (25µl) were then added to a 96-well plate in triplicate and 100µl malachite green added to measure free-phosphate in solution. Color was allowed to develop for 15 minutes before read at 650nm on spectrophotometer. Phosphate concentrations determined using the equation from a standard curve.

After incubation with p-peptide, beads were then re-suspended in lysis buffer, boiled, and immunoprecipitated protein ran on an SDS-page gel (10%) to ensure equal pull-down of PP2Ac amongst samples.

T-cell isolation and stimulation. Peripheral blood from buffy coats of healthy donors was obtained from the Southwest Florida Blood Services, St. Petersburg, FL for purification of primary T-cells. T-cells were isolated using RosetteSep® Human CD3+ T-cell Enrichment Cocktail (StemCell Technologies, Vancouver, BC Canada) according to the manufacturer's protocol. Primary T-cells, or Jurkat T-cell leukemia cell line, were treated with 10μM Len or DMSO (vehicle control) for 1 day prior to stimulation. T-cells were then counted and coated with anti-CD3 antibody (5μg/ml) (BD Biosciences, San Jose, CA USA) on ice for 1 hour ($10x10^6$/sample group) before being washed and coated with anti-mouse Fc antibody (5.0μg/ml) (Thermo Scientific, Rockford, IL USA) on ice for 30 minutes. Cells were again washed and the TCR cross-linked through incubation at 37^oC for 15 minutes before lysis in PP2A lysis buffer described above. Subsequent phosphatase assay and western blotting were performed as previously described.

Virtual Modeling. Virtual chemical modeling of the three different IMiDs (thalidomide, lenalidomide, and pomalidomide) and a known positive control, fostriecin, was performed utilizing the PP2A crystal structure PDB 3K7V (2Å resolution). Prior to simulations and analysis, PDB 3K7V was prepared using the Schrödinger application Protein Prep (Schrödinger, L.L.C. Cambridge, MA USA) to remove waters, correct histidine orientations and hydrogen bonds, and provide "soft" energy minimization to relax protein structure. Protein structure was then analyzed via the SiteMap application (Schrödinger, L.L.C. Cambridge, MA USA) to identify potential binding sites, serving as a predictor to determine if the known

binding site for small molecules could be located, which found the catalytic pocket used for docking purposes.

The four compounds, lenalidomide, pomalidomide, thalidomide, and fostriecin were prepared for docking using the LigPrep application (Schrödinger, L.L.C. Cambridge, MA USA), creating a 3-dimensional geometry for the structures and providing for alternative tautomers and ionization states (between pH 5.0 and 9.0), alternative ring conformations, and diastereomers. Application GLIDE (Schrödinger, L.L.C. Cambridge, MA USA) was used to estimate the free energy of binding for fostriecin, thalidomide, lenalidomide and pomalidomide to the PP2Acα heterodimer. Fostriecin is a known PP2Acα inhibitor (367) and was used as a positive control in this experiment.

Site-directed mutagenesis. Site-directed mutagenesis of the HA-PP2Ac plasmid performed using the QuikChange Site-Directed mutagenesis kit from Agilent Technologies (Santa Clara, CA USA) per the manufacturer's instructions. Briefly, the pcDNA3 plasmid containing the HA-PP2Ac wild-type gene was denatured and primers containing the codon for the desired mutation for PP2Ac was annealed. Using *PfuTurbo* DNA polymerase, the mutagenic primers were incorporated into the plasmid, resulting in nicked circular strands. The circular dsDNA including the new mutation was then transformed into competent cells, colonies were selected, grown up, and isolated using plasmid mini-prep kit (Invitrogen, Carlsbad, CA USA). Plasmids were sequenced by the Moffitt Cancer Center Molecular Biology core facility to ensure expression of the mutation. The mutated plasmid DNA was then transfected into ad293 cells and stable clones

were isolated as previously described. Successful plasmid expression in the clones was measured through HA-tag expression via western blot. Evaluation of mutated PP2Ac functional activity was performed using phosphatase activity assays as previously described. Primers for generating the various PP2Ac mutants were: W200F: *forward: 5'-GAGGGTCCAATGTGTGACTTGCTGTTCTCAGATCCAGATG-3', reverse: 5'-CATCTG GATCTGAGAACAGCAAGTCACACATTGGACCCTC-3'*; R214A: *forward: 5'-GTGGTTGG GGTATATCTCCTGCAGGAGCTGGTTAC-3', reverse: 3'-GTAACCAGCTCCTGCAGGAGA TATACCCCAACCAC-3';* H191G: *forward: 5'-GCCTACAAGAAGTTCC CGGTGAGGGTCCAATGTGTG-3' reverse: 5'-CACACATTGGACCCTCACCGGGAACTT CTTGTAGGC-3'*

CHAPTER 5

Discussion

Therapeutic implications of thalidomide and the IMiDs have progressed significantly since the thalidomide tragedy of the 1960s. From use as a sedative, to anti-angiogenic and anti-cancer properties, to immune modulating mechanisms, there is great potential for lenalidomide and pomalidomide to be used in a variety of diseases and malignancies. The use of lenalidomide in del(5q) MDS revolutionized treatment of the disease and gave patients with few options other than repeated transfusions and a bone marrow transplantation a chance at long-term survival. Unlike most therapeutics that are designed to target a particular pathway or molecule, approval of lenalidomide was accepted based upon patient outcome without a complete understanding of the mechanism of action. Once clinical usage of the IMiDs became more widespread, research into the direct mode of action of the drugs in MDS and other malignancies escalated, and is still a topic of intense investigation today.

Lenalidomide is a small molecule therapeutic with a multitude of actions in several different diseases and cell types, contributing to the complexity and difficulty in determining the exact molecular target. Lenalidomide is able to induce apoptosis in MM and MDS dysplastic clones, as well as improve hematopoiesis through preventing apoptosis within erythroid blasts. Lenalidomide and the IMiDs also have strong immunomodulating capacity, where they can increase the

proliferation, activation, and cytokine production in T-cells and proliferation and ADCC in NK-cells. It is important for us to understand how lenalidomide has such powerful immune-harnessing potential, as knowledge of therapeutic mechanisms for immunomodulation could be used to create even better drugs with less side effects or threat of teratogenicity to offspring. This information would also be useful in determining whether lenalidomide would be a powerful immunotherapy adjuvant in different tumor settings/combination therapies.

For these reasons, we set out to determine how lenalidomide functions in augmenting T-cell proliferation and function in normal T-cells, and if improved immune function in patients with MDS due to lenalidomide contributes to their hematologic response. We were first able to discover that T-cells from MDS patients are inherently hypo-responsive to stimulation, and that lenalidomide can reverse this defect. Len can augment the Th-1 cytokine producing and proliferation capacity both *in vitro* and *in vivo* in MDS patient T-cells. We also discovered that an increase in immune activity through cytokine production, proliferation, and naïve T-cell production actually correlated with hematologic response in non-del(5q) patients (**Chapter 2**). These results are extremely important in recognizing the importance of an active and responding immune system in helping to eradicate the myeloid clone in MDS. This also raises the question of predictability of whether or not non-del(5q) patients will respond to lenalidomide therapy, as only 25% of them actually do? Ebert et al. described an erythroid gene signature that could help predict whether or not non-del(5q)

patients would respond to lenalidomide therapy, although the signature involves a costly microarray only presently utilized by their institution (238).

We then determined that the phenotype of the T-cell compartment in MDS patients prior to treatment with lenalidomide could potentially predict whether or not patients would respond to the drug. We found that patients who had high levels of Terminal Effector Memory T-cells, which are inherently $CD28^{null}$, were erythroid non-responders after lenalidomide treatment. We examined this further and determined that not only the percentage of T_{TEM} cells could potentially predict non-responsiveness, but also an overall decrease in CD28+ cells in the T-cell compartment, whether CD8+ or CD4+ (**Chapter 3**). A group in Australia has recently measured CD28 expression on MDS patient samples treated with lenalidomide at their institution, and we are currently collaborating to validate this predictive model. If true, testing for CD28 expression by flow cytometry in the peripheral blood of MDS patients prior to any therapy may help determine whether or not non-del(5q) patients should receive lenalidomide therapy, as they are less likely to respond to the drug the higher their CD28-%.

These results are not only important for influencing therapeutic decisions, but they point directly to a mode of action of lenalidomide in T-cells. Since aberrant accumulation of hypo-responsive CD28- T-cells may predict lenalidomide responsiveness, one can hypothesize that CD28 is important for lenalidomide mechanism of action in T-cells. Lenalidomide is known to possess the ability to induce proliferation of T-cells in the absence of co-stimulation or external ligation of the CD28 receptor, alluding to the importance of CD28 in

lenalidomide mechanism of action. We have shown that although lenalidomide can induce proliferation and function of T-cells in the absence of external receptor ligation, the presence of the CD28 receptor with its internal ITAM motifs is essential for lenalidomide action. Lack of the CD28 receptor, either in naturally occurring CD28- T-cells, or through *in vitro* shRNA knockdown, renders lenalidomide incapable of inducing a proliferative or cytokine response. It is therefore likely that receptor expression is necessary to act as a scaffold for CD28 downstream signaling molecules that lead to increased IL-2 expression and proliferation. Increased pCREB binding to the CD28-Response Element on the IL-2 promoter further supports this hypothesis (**Chapter 3**).

Although we have discovered that CD28 expression is necessary for len action, and that len likely acts through inhibition of a negative regulatory signal, the direct molecular target remains elusive. There are several potential molecules that could be targeted by lenalidomide, including phosphatases and E3-ubiquitin ligases. Thalidomide was shown to target the E3 ubiquitin ligase Cereblon in chicken and zebrafish embryos to cause teratogenesis (248). Although this group was able to prove direct binding of thalidomide to cereblon, therefore blocking the ability of the CRBN-Cul4-DDB1 E3-ligase complex to function, it is unclear whether this occurs in T-cells as well. Cereblon was first discovered in the brain to be involved in memory and learning, and there is actually no known function in T-cells. Interestingly, Lopez-Girona et al. have determined that CRBN expression in T-cells is essential for lenalidomide-induced IL-2 and TNF-α production (257). Preliminary results, however, from T-cells

isolated from cereblon knockout mice show an increase in IL-2 production in the presence of anti-CD3 stimulation, indicating that cereblon may play a role in negative regulation of IL-2 production (data not shown). Germline deletion of cereblon in mice has no detrimental effect on limb generation, lymphocyte populations, or overall T-cell phenotype/function. Therefore, it is unclear what role cereblon plays in T-cell function or what particular targets in the T-cell signaling pathway cereblon could bind to, and are topics of further investigation in our lab.

Another E3 ubiquitin ligase that plays a major role in T-cell anergy induction and preventing non-specific T-cell activation through ubiquitination of downstream CD28 targets is cbl-b. Although direct inhibition of cbl-b by lenalidomide was not identified (data not shown), T-cells isolated from cbl-b knockout mice are hyper-responsive to anti-CD3 stimulation through their ability to proliferate/function without anti-CD28 ligation (155, 156). The IL-2 production from these mouse T-cells stimulated with anti-CD3 antibody alone is nearly identical to cells treated with lenalidomide in the presence of anti-CD3 stimulation, indicating a potential link. Since there is solid evidence lenalidomide inhibits multiple E3 ubiquitin ligases (cereblon and MDM2) in a variety of cell types (multiple myeloma cells, erythroid cells, etc), other potential E3 ligases as targets are completely plausible.

Another known target of lenalidomide in apoptotic induction within the del(5q) myeloid clone is protein phosphatase 2A. Since PP2A is involved in a variety of cellular processes with varying roles, we have demonstrated that

lenalidomide not only inhibits PP2A activity within MDS to prohibit cell cycle progression, but in primary T-cells as well. PP2A is another negative regulator of CD28 signaling and over-expression has been implicated in the reduction of IL-2 secreting capacity in T-cells from SLE patients (170, 171). PP2A can directly bind to and de-phosphorylate the CD28 receptor, and inhibition through lenalidomide increases ITAM phosphorylation and prolongs downstream signaling. These data suggest multiple protein targets for lenalidomide not only in various cell types, but within T-cells as well, likely to share a negative regulatory role within the CD28 pathway.

Taken together, the data presented in this dissertation have several implications within cancer immunotherapy and MDS. Lenalidomide is likely to be a potent immunotherapy adjuvant, through its ability to augment T-cell proliferation and function in the absence of co-stimulatory signals that are often down-regulated on solid and liquid tumors. Lenalidomide has already been investigated and is used in several clinical trials in combination with cellular vaccines, but theoretically could also be used in combination with antibody therapies such as ipilimumab and anti-PD1 therapy that target the co-inhibitory pathway. Prevention of co-inhibitory signals while simultaneously activating co-stimulation could counteract problems of T-cell tolerance and non-responsiveness in cancer, and result in a robust antigen-specific cytotoxic T-cell response, while providing additional help through NK-cell mediated ADCC and killing.

Importantly, CD28 expression on primary T-cells from cancer patients could be used to identify those patients that would actually respond to the immunomodulating therapy. As described previously, CD28 expression is lost on CD8+ cytotoxic T-cells through the aging process. The greatest risk factor for cancer is age, and since the median age of onset of MDS is 76 and most cancers arise in the elderly, measuring the expression of CD28 inherent on the T-cell population would be advantageous to predict responses to lenalidomide therapy. Through our experiments we have determined the likely pathway for lenalidomide's mechanism of action, and a potential target that could be utilized by even other therapies to garner increases in T-cell function.

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ABOUT THE AUTHOR

Jessica Marie (Craig) McDaniel was born in Akron, Ohio and graduated valedictorian of the class of 2004 from Lake High School in Uniontown, Ohio. Jessica went on to earn a bachelor of science in Biology *Magna cum laude* from Marietta College in Marietta, Ohio. During the summer of 2007, Jessica worked as a research assistant at the Magee Women's Research Hospital in Pittsburgh, PA in the University of Pittsburgh School of Medicine Summer Undergraduate Research Program (SURP). After graduation in 2008, Jessica enrolled in the Cancer Biology PhD program at Moffitt Cancer Center at the University of South Florida. Jessica worked for four years in the laboratory of Dr. PK Epling-Burnette to determine the mechanism of action of lenalidomide in T-cells. During her tenure in Dr. Burnette's lab, Jessica was first author on an original research article and review paper, and first author of another manuscript pending review. Jessica attended the American Society of Hematology meeting in 2010 and 2011 where she presented her work both orally and in poster form. Jessica was also one of 42 students selected out of 1500 to attend the St. Jude Children's Research Hospital Graduate Student Conference in 2012.

During her PhD work, Jessica took classes towards a certificate in intellectual property and was an intern in the Office of Technology Management and Commercialization at Moffitt Cancer Center. Upon graduation, Jessica has accepted a postdoctoral position in the Office of Technology Commercialization and Knowledge Transfer at The Ohio State University.

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