Abstract

Short telomere (ST) syndromes are the most prevalent of premature aging disorders; they manifest as syndromic clustering of pulmonary fibrosis-emphysema, bone marrow failure and liver disease. We identified primary immunodeficiency (PID) as a first presentation in 20% (5 of 25) of telomerase (TERT, TR, DKCI) and telomere gene (RTEL1) mutation carriers under the age of 30. Asymptomatic ST patients had depleted naïve T cell subsets and accumulation of terminally differentiated effector cells, similar to older adults (OA) five decades older. However, ST shared only a subset of the immune aging immunophenotype such as CD95 and CD57 positivity, but not CD28 loss or upregulation of PD-1. We found evidence of intra-thymic failure and ST patients had abnormally low T cell receptor excision circles (TRECs). In one telomerase mutation diagnosed in infancy, TRECs were undetectable, pointing to telomere gene mutations as a cause of abnormal newborn screening. In mice, short telomeres caused T cell autonomous defects with a propensity to apoptosis during development in vivo and upon stimulation in vitro, but the thymic niche was generally intact. In addition to the T cell deficiency, we found the T cell receptor repertoire in ST patients was restricted by both flow cytometry and deep sequencing. There were, however, two major differences with OA T cells. The gene expression microarray of CD8$^+$ terminally differentiated effector T cells (TEMRA) was altered and showed upregulation of intrinsic apoptosis genes, in contrast to the extrinsic apoptosis pathways that were altered in OA. Moreover, the response to T cell stimulation in human and mouse T cells with short telomeres was marked by exaggerated and dysregulated cytokine release. Our data show that short telomeres cause a primary T
cell immunodeficiency that is often a first manifestation of short telomere syndromes.

Importantly, they implicate short telomeres as a driver of a unique T cell aging program that phenotypically resembles, but is molecularly distinct from the immune aging phenotype seen in OA with normal telomere lengths.

Advisor: Mary Armanios, MD

Readers: Mary Armanios, MD and Alan Friedman, MD
Acknowledgements

The work in this thesis is the result of many collaborative efforts and I am extremely grateful to all those who have contributed. This research would not have been possible without the gracious participation of the patients and their families, and to them I am very thankful. Additionally, much of this work was funded by the Turock Predoctoral Fellowship through the kind philanthropic support of Mr. David Turock.

I am very thankful to the Armanios lab members who contributed to this work in many ways. I would like to thank my advisor, Mary Armanios, for her support, tireless dedication, and guidance in this thesis work and beyond. I am also grateful to my fellow labmates Jon Alder, Erin Parry, Susan Stanley, and Dustin Gable for their intellectual and humorous assistance, and Sagar Hanumanthu for his flow cytometry expertise. I would also like to thank Carol Greider and the Greider lab for helpful discussions and encouragement.

Much of this work relied on collaborations across the Hopkins campus and beyond. I am especially thankful to Dr. Roshini Abraham of the Mayo Clinic for her contributions with the TREC assay and study design. I would also like to thank David Hamm of Adaptive Biotechnologies for assistance with Vβ sequencing analysis. I would also like to acknowledge Steve Desiderio, Leo Luznik, Chris Kanakry, Xuhang Li, and Connie Talbot of Johns Hopkins for helpful discussions and assistance.

Finally, I would like to thank my family and friends both near and far. Their support has been incredible and I am sincerely grateful for every note of encouragement along the way.
# Table of Contents

## Abstract

### ii

## Acknowledgements

### iv

## Table of Contents

### v

## List of Tables

### x

## List of Figures

### xi

## Chapter 1: Introduction

1.1 T cell dysfunction is a hallmark of immune aging 1

1.2 Acquired features which correlate with T cell aging 2

1.3 Multiple theories exist to define the mechanism of immune aging 4

1.4 Telomere shortening in T cells as a mechanism of immune aging 5

1.5 Short Telomere Syndromes are the most common premature aging syndromes 6

1.6 Mice with short telomeres show degenerative premature aging defects 7

1.7 Immunodeficiency is a phenotype associated with early manifestations of telomere syndromes 8

1.8 Therapeutic potential in immunodeficiencies and immune aging 10

1.9 Summary of thesis 11
Chapter 2: Short Telomeres Cause a Primary T cell Immunodeficiency and Are Sufficient to Cause T cell Aging

2.1 Introduction

2.2 Results

2.2.1 Individuals with short telomeres present with opportunistic infection and decreased peripheral blood counts

2.2.2 Short telomere immunophenotype recapitulates immune aging in individuals five decades older

2.2.3 Short telomeres correlate with premature thymic involution as shown through T cell receptor excision circle (TREC) quantification

2.2.4 Mouse model shows that the thymic niche in animals with short telomeres is capable of efficient T cell development

2.2.5 Deficiencies in the short telomere mouse hematopoietic hierarchy suggest compounding, cell intrinsic defects in T cell development

2.3 Discussion

2.4 Materials and Methods

2.4.1 Subjects and Controls

2.4.2 Sample Preparation for Immunophenotyping and Antibodies

2.4.3 Flow Cytometry and Analysis
Chapter 3: Analysis of T cell receptor repertoire diversity in young adults with short telomere syndromes

3.1 Introduction

3.2 Results

3.2.1 Deep sequencing of TCRβ genes shows differences in receptor repertoire of short telomere subjects

3.2.2 Flow cytometry analysis shows skewing in T Cell Receptor Vβ representation in short telomere patients

3.3 Discussion

3.4 Materials and Methods

3.4.1 Sample Preparation for Flow Cytometry, Sorting and Antibodies

3.4.2 TCR-Vβ Diversity Flow Cytometry Analysis

3.4.3 Cell Sorting

3.4.4 TCR-β Locus Sequencing

3.4.5 Statistics
Chapter 4: Terminally differentiated CD8+ T cells reveal a unique gene expression signature in the setting of short telomeres

4.1 Introduction

4.2 Results

4.2.1 Quantification of up- and down-regulated genes in ST compared to YC shows opposite trend to the OA to YC comparison

4.2.2 ST and OA gene expression patterns point to different mechanisms important for immune cell maintenance

4.3 Discussion

4.4 Materials and Methods

4.4.1 Sample Preparation for Flow Cytometry, Sorting and Antibodies

4.4.2 Cell Sorting

4.4.3 T_{EMRA} Gene Expression Microarray and Analysis

References

Chapter 5: Short Telomere and Older Adult T Cells Reveal Distinct Functional Responses to Stimulation

5.1 Introduction

5.2 Results
5.2.1 ST and OA T cells show increased rates of apoptosis following T cell receptor stimulation \textit{in vitro} 82

5.2.2 Stimulation of TCR with CD3/CD28 leads to dysregulated cytokine response in T cells with short telomeres 83

5.2.3 Short telomere mice recapitulate the human cytokine release phenotype 83

5.3 Discussion 91

5.4 Materials and Methods 93

5.4.1 T cell Stimulation and Proliferation 93

5.4.2 Secreted Cytokine Analysis 93

5.4.3 Mice 94

5.4.4 Mouse T cell Stimulation and Cytokine Studies 94

5.4.5 Statistics 94

References 95

Supplementary Tables 96

Curriculum Vitae 99
## List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplementary Table 1</td>
<td>Summary of qualitative and quantitative defects in ST and OA subjects described in this thesis</td>
<td>96</td>
</tr>
<tr>
<td>Supplementary Table 2</td>
<td>Human Flow Cytometry Antibodies Used</td>
<td>97</td>
</tr>
<tr>
<td>Supplementary Table 3</td>
<td>Mouse Flow Cytometry Antibodies Used</td>
<td>98</td>
</tr>
</tbody>
</table>
## List of Figures

| Figure 2.1 | Telomere lengths and immunological profile of subjects with short telomeres | 24 |
| Figure 2.2 | ST subjects replicate immune aging of OA with decreased naïve T cells and increased CD8$^+$ TEMRAs | 26 |
| Figure 2.3 | Immunophenotyping patterns of ST cells relative to OA and YC | 27 |
| Figure 2.4 | ST subjects show decreased thymic output suggesting premature thymic involution | 29 |
| Figure 2.5 | Congenic bone marrow transplant reveals thymic niche of short telomere mice is capable of normal thymocyte development and export of T cells | 30 |
| Figure 2.6 | Mice with short telomeres have significant defects in hematopoietic stem cell counts | 32 |
| Figure 2.7 | Young and adult short telomere mice show increased apoptosis in developing thymocytes by Annexin V staining | 34 |
| Figure 2.8 | Quantification of T cell apoptosis and peripheral lymphopenia in mTR$^{-/-}$ G5 mice | 36 |
| Figure 3.1 | n-nucleotide addition and CDR3 lengths are intact in short telomere subjects | 53 |
| Figure 3.2 | Diversity metrics show increased changes in DNA-level TCR repertoire in CD8$^+$ T cells of ST subjects | 55 |
Figure 3.3  Diversity metrics show slight skewing of DNA-level TCR repertoire in CD4+ T cells of ST subjects 56

Figure 3.4  Individual analysis of Vβ family expression by flow cytometry 57

Figure 3.5  Distribution of Vβ family expression by flow cytometry 58

Figure 3.6  Skewedness in Vβ family expression 59

Figure 4.1  Quantification and profile of up- and down-regulated genes in CD8+ T_EMRA cells 72

Figure 4.2  Ingenuity Pathway Analysis (IPA) reveals gene expression differences in common pathways 74

Figure 5.1  ST and OA T cells show increased apoptotic response to TCR stimulation 85

Figure 5.2  Cytokine release profiles after TCR stimulation 86

Figure 5.3  Quantification of dysregulated cytokine release in human T cells following CD3/CD28 stimulation 88

Figure 5.4  Mice with short telomeres recapitulate human results with increased cytokine release 90
Chapter 1:

Introduction

1.1 T cell dysfunction is a hallmark of immune aging

T cells are essential for adaptive immunity; they allow for long-term memory and distinguishing self from pathogens and cancer-directed antigens. The function of T cells, however, is finite, and a decline in immune function with aging, commonly referred to as immunosenescence or immune aging, is a major cause of morbidity and mortality. Recently, the term immune aging has gained notoriety as more is known about the complexities and heterogeneity of phenotypes and functions in adaptive immunity associated with aging; moreover, there is no evidence that T cells undergo senescence programs as classically understood (Hayflick and Moorhead 1961; Campisi 2005; Campisi and d'Adda di Fagagna 2007). Three of the top ten causes of death in Americans over the age of 65 may be related to immune aging: cancer, pneumonia, and septicemia (Heron 2015). Many of the infections are opportunistic and are only diagnosed in immunosuppressed patients and the elderly. In independent studies, increased rates of cancer, inflammation, and autoimmune phenomena correlate with decreased immune function with aging (Moro-García, Alonso-Arias et al. 2012). Despite medical advances that have allowed for increased lifespan, the true mechanisms by which the immune system ages, leading to decreased scavenging and cytotoxic T cell capacities, have not been defined and remain untargeted by modern medicine.
One hypothesized contributor to decreased peripheral T cell function is the development of stem cell failure with aging. The ubiquitous phenomenon of thymic involution is also well-documented. The driving forces are not understood, but thymic involution results in quantitative effects in the peripheral immune system, namely lymphopenia and decreased naïve T cells. Thymic involution is visible in thymic atrophy in humans and can be quantified using the T cell receptor excision circles (TRECs) PCR-based assay, and this decreased TRECs count is another hallmark of immune aging. Furthermore, CD4 and CD8 T cell counts are altered in aging, with decreases in CD4 T cells creating an inversion of the CD4:CD8 T cell ratio. An expanded population of memory T cells highlights the naïve T cell loss, especially in the CD8 compartment with the accumulation of oligoclonal T_{EMRA} cells. The work in this thesis aims to define the mechanisms underlying immune aging in the context of studies of humans with short telomere syndromes.

1.2 Acquired features which correlate with T cell aging

Many studies of changes with immune aging have identified several qualitative features that characterize an aged immunophenotype. Functional T cells are defined by an ability to activate quickly and proliferate extensively in response to antigenic stimulation. Changes in co-stimulatory signaling and cell surface expression have been correlated with age, though the mechanisms by which these changes occur are still undefined. Common T cell aging phenotypes describe decreased proliferative capacity, attributed to decreased expression of co-stimulatory receptor CD28 and increased expression of the
glycoepitope CD57 which marks replicative senescence (Brenchley, Karandikar et al. 2003). Loss of effector function is further identified by increased expression of inhibitory receptors such as the immunotherapeutic target PD-1, CTLA-4, LAG-3, and TIM-3 (Blackburn, Shin et al. 2009; Wherry 2011). Furthermore, increased rates of apoptosis are correlated with increased CD95 (FasR) expression (Aggarwal and Gupta 1998; Larbi, Fülöp et al. 2008).

Additional phenotypes associated with T cell dysfunction with aging include cytokine release and responses under the increasingly inflammatory environment of secondary lymphoid niches. Cytokines are essential for homeostatic maintenance and for proliferative recruitment and activation of peripheral immune cells. Various studies have shown the tipping of cytokine balances with aging, with most consensus showing decreased IL-2 and IL-2R, increased pro-inflammatory cytokine release, and skewing towards T helper (Th)17-derived cytokines (Weng 2006; Larbi, Fülöp et al. 2008; Montecino-Rodriguez, Berent-Maoz et al. 2013; Kared, Camous et al. 2014). IL-2 is important for proliferative responses to antigen while inflammatory milieus are hypothesized to drive development of autoimmune disease with aging. These factors combine to hamper the functions of T cells required for a healthy immune response.

A final characteristic of aging T cells is a reduction in the T cell receptor (TCR) repertoire, estimated to decrease from $10^8$ to $10^6$ over a lifetime (Weng 2006). Though the quantitative loss can be attributed to thymic involution and accumulation of oligoclonal memory T cells, the loss in repertoire diversity likely contributes to decreased immune function with aging through impaired protection from new immune challenges (Müller and Pawelec 2014; Qi, Liu et al. 2014). Altogether, there are many phenotypes
Chapter 1: INTRODUCTION

that have been linked to immune aging, and defining the mechanistic drivers of the phenotypes will improve clinical management of immune aging.

1.3 Multiple theories exist to define the mechanism of immune aging

Though the exact mechanisms of immune aging are not yet defined, a few theories exist to suggest possible drivers of decreased T cell function with aging. The predominant theory stems from the fact that the mean age of infection with the chronic antigen cytomegalovirus (CMV) in the American population is 28.6 years (Colugnati, Staras et al. 2007). It is hypothesized that long-lived chronic infections, especially CMV but also EBV and VZV, eventually exhaust the immune system, fill the immune space with oligoclonal virus-reactive cells, and trigger immune deficiency with time (Lelic, Verschoor et al. 2012; Castelo-Branco and Soveral 2014; Pawelec 2014).

Other theories describe dysfunction arising as a result of aging alterations in the surrounding environment. The existence of inflammatory mediators in various tissues is correlated with age and is hypothesized to act as a toxin in thymocyte development and to drive dysregulation in T cells and in their interactions with secondary lymphoid organs (Montecino-Rodriguez, Berent-Maoz et al. 2013). These inflammatory responses are also thought to drive autoimmunity, in which the aging immune system has increasing difficulty differentiating ‘self’ from ‘non-self’, a common disease state in aging. Furthermore, the endocrine system is an important mediator of immune function and of thymocyte development throughout a lifetime, however growth hormone (GH) and Insulin-like growth factor-1 (IGF-1) levels both decrease with age (Montecino-
Rodriguez, Berent-Maoz et al. 2013). This loss of inflammatory and endocrine control in the immune milieu may contribute to the phenotype of immune aging.

Finally, cell intrinsic defects are theorized to modulate aberrant immune responses with aging. The most important alteration in T cells is decreased signaling from the T cell receptor (TCR) in response to antigen recognition. The impaired TCR signaling results in decreased phosphorylation cascades, creating decreased activation especially in the MAPK and PI3K pathways (Larbi, Fülöp et al. 2008; Montecino-Rodriguez, Berent-Maoz et al. 2013). It is unclear how much of the TCR signaling defect is due to increased rigidity of the cell membrane, preventing robust immune synapses, or to diminished signaling cascades themselves (Larbi, Fülöp et al. 2008). Further hypotheses have suggested that telomere shortening with age may cause aberrant T cell function and senescence or apoptosis. In studies not focused on immune aging, the short telomere has been shown to affect mitochondrial function and cell cycle progression (Guo, Parry et al. 2011; Sahin, Colla et al. 2011). This thesis will examine the role of telomere shortening as a driver for T cell aging.

1.4 Telomere shortening in T cells as a mechanism of immune aging

One theory that has not been extensively explored is the T cell dysfunction caused by telomere shortening. Telomeres are tandem repeats of (TTAGGG)$_n$ in vertebrates bound by specialized proteins; they protect the ends of linear chromosomes. The end-replication problem prevents telomere sequences from complete replication during the cell cycle (Greider 1998; Blackburn, Greider et al. 2006). This issue is resolved by the
enzyme telomerase which uses an RNA template, hTR, and a reverse transcriptase, hTERT, to synthesize telomeres (Greider and Blackburn 1985; Greider and Blackburn 1987). When telomeres become critically short, this signals a DNA damage response resulting in apoptosis or cellular senescence (Hemann, Rudolph et al. 2001; d'Adda di Fagagna, Reaper et al. 2003).

Telomere length shortens with increasing age in humans (Harley, Futcher et al. 1990; Allsopp, Vaziri et al. 1992). Furthermore, telomerase activity is tightly regulated. Telomerase activity has been detected in hematopoietic cells, and in particular, in T cells (Buchkovich and Greider 1996). The activity of telomerase has been suggested to correlate with ‘stemness’ in hematopoietic hierarchy (Morrison, Prowse et al. 1996). This point is further supported in that telomere length also shortens with differentiation of peripheral T cell subsets; the telomere length of naïve cells is longer than the telomere length of memory cells from the same donor (Weng, Levine et al. 1995; Aubert, Baerlocher et al. 2012). Knowing the proliferative requirements placed on T cells during a lifetime, it is feasible to consider that telomere shortening could drive T cell aging, leading to dysfunction and disease susceptibility.

1.5 Short Telomere Syndromes are the most common premature aging syndromes

T cells are only one of the tissue types that have shown potential for telomere-mediated dysfunction. Over the past fifteen years, a framework of telomere shortening in human disease has emerged, showing connections between impaired telomere
maintenance and many degenerative disorders. Telomere length is heritable, and disease-causing mutations in telomere pathway genes are acquired through multiple modes of inheritance (Armanios and Blackburn 2012). Aside from hTERT and hTR, nine genes have been implicated in telomere-mediated disease, or telomere syndromes, which are important for telomerase function and recruitment to the telomere, and for telomerase biogenesis (Stanley and Armanios 2015). The resulting telomere syndromes manifest over broad age ranges from birth to adulthood with heterogeneous phenotypes. Some of the most common manifestations are in slow turnover tissues as pulmonary fibrosis and/or emphysema in the lung and develop in adults (Armanios and Blackburn 2012). More severe and rare presentations in tissues of high turnover such as aplastic anemia in the bone marrow and atrophy of the GI tract are diagnosed in children. All phenotypes seen in the short telomere syndromes are seen with normal aging and converge to describe a unique spectrum of degenerative defects all driven by a common mechanism: the short telomere (Armanios 2009).

1.6 Mice with short telomeres show degenerative premature aging defects

The role of short telomeres as a driver of degenerative diseases associated with aging has been previously investigated in mTR or mTERT mice with short telomeres. Successive breeding of C57/Bl6 mTR/− mice leads to short telomeres and severe defects presenting in highly proliferative organs that rely on telomerase activity to prevent apoptosis and senescence as a result of telomere shortening (Lee, Blasco et al. 1998).
Chapter 1: INTRODUCTION

Specifically, short telomeres were first shown to cause increased apoptosis in T lymphocytes in response to mitogens. Investigation of a model with shorter telomere lengths and a narrower telomere length distribution akin to humans with the \( mTR^{+/−} \) CAST/EiJ mouse revealed pathology showing degenerative defects in the gastrointestinal mucosa, increased rates of colitis, and bone marrow failure, which is the primary cause of death. Furthermore, quantitative immune defects manifested as decreased peripheral lymphocyte counts, altered 4:8 T cell ratio, and increased apoptosis after T cell stimulation (Armanios, Alder et al. 2009). However, whether these immune defects arise because of bone marrow failure or independently because of immune exhaustion was not known. The work in this thesis aims to clarify the effects of systemic and cell-intrinsic drivers of immune dysfunction under the settings of aging and the short telomere.

1.7 Immunodeficiency is a phenotype associated with early manifestations of telomere syndromes

Additional and more severe manifestations of T cell dysfunction occur in children with short telomeres, rendering them susceptible to opportunistic infection. The most severe onset, termed Severe Combined Immunodeficiency (SCID), manifests as immunodeficiency characterized by T and B cell lymphopenia and dysfunctional T cells (Al-Herz, Bousfiha et al. 2014). Most genetically characterized disorders are currently attributed to mutations in genes necessary for T cell development such as \( IL2RG, IL7RA, RAG1, \) and \( RAG2 \) (Buckley 2004). Further clinical presentations include failure to thrive, diarrhea, and serious infections; survival is commonly accepted to be dependent upon
reconstitution of immunity through bone marrow transplantation. Currently, SCID is the only heritable immune disorder that is tested for with newborn screening (Kwan, Abraham et al. 2014).

Common variable immunodeficiency, or CVID, is a milder primary immunodeficiency, characterized by lymphocyte dysfunction, recurrent sinus infections, and at times with autoimmune defects (Allenspach EJ 2013). These defects are not as severe as in SCID, and the CVID diagnosis is made usually by exclusion, when no other syndromic diagnosis is made to fit the immune symptoms. As a result, even fewer CVID cases have a known genetic cause.

Although not commonly recognized or documented, immunological abnormalities are some of the most severe manifestations in telomere syndromes and are often the primary phenotypes seen in children. Dyskeratosis congenita (DC) is a rare manifestation of short telomere syndromes presenting most often in children and is classically recognized by the triad of oral leukoplakia, nail dystrophy, and skin hyperpigmentation (Dokal 2000). Case reports of DC diagnoses have identified lymphopenia, decreased T cell function, and opportunistic infections as common disease manifestations (Solder, Weiss et al. 1998; Jyonouchi, Forbes et al. 2011). Another manifestation, Hoyeraal-Hreiderasson (HH) syndrome, presents in infancy as developmental delay, immunodeficiency, and cerebellar hypoplasia (de la Fuente and Dokal 2007). Additional immune defects in childhood-onset telomere syndromes include enteropathy, enterocolitis, and atrophy of and apoptosis in gastrointestinal mucosa (Jonassaint, Guo et al. 2013). Though these manifestations are rare at an estimated 1 in 1 million children, the incidence of immune defects in these cases is high since it is a defining feature of
Chapter 1: INTRODUCTION

Hoyeraal-Hreiderasson syndrome (Armanios and Blackburn 2012). In cases of early and severe-onset telomere syndromes, opportunistic infections are the biggest driver of morbidity and mortality. This thesis aims to show that short telomeres can function as a mechanism of immune dysfunction and immune aging, and telomere genes should be considered in clinical workup of immune abnormalities.

1.8 Therapeutic potential in immunodeficiencies and immune aging

In this thesis, we will examine the role of telomere shortening in immune aging. There has been some effort in the past to test ways to reverse T cell aging; however, these have been hampered by the lack of understanding of the exact driving mechanisms. There is no lack of consensus in clinical immunology that a better understanding of the mechanisms underlying immune aging phenotypes and causes is needed, especially as the size of the aging adult population continues to increase (Linton and Dorshkind 2004). Current theories and clinical trials have focused on rejuvenating thymic function and amending the peripheral naïve T cell deficit. Attempts and suggestions for targeting the thymic environment include clinical trials of lymphotrophic cytokine IL-7 to support T cell development (Okoye, Rohankhedkar et al. 2015), or endocrine hormones growth hormone (GH), insulin-like factor 1 (IFG-1), and fibroblast growth factor 7 (Fgf7) to target the thymic epithelial niche (Holländer, Krenger et al. 2010; Montecino-Rodriguez, Berent-Maoz et al. 2013).

Other theories suggest attempts to reverse aging of memory T cells in the periphery by using existing cells rather than reinvigorating the hierarchy. This concept
Chapter 1: INTRODUCTION

has already led to promising advances in immunotherapy by blocking function of PD-1 and CTLA4 receptors and reinvigorating immune responses (Nguyen and Ohashi 2014). Further defining the proliferative capabilities and gene signatures of peripheral cells will likely generate new therapeutic targets (Wherry 2011).

1.9 Summary of thesis

Immune aging is a well-accepted phenomenon with many potential mechanisms leading to infection and cancer susceptibility. Short telomeres are one mechanism that has not been extensively investigated as a primary driver of the immune aging phenotype. The work in this thesis is a detailed and mechanistic look at the short telomere-induced path to immune aging using cells and clinical data from a valuable cohort of individuals with short telomeres evaluated at Johns Hopkins. We compare the phenotypes and pathways in these subjects to young (age-matched) controls and older adults, five decades older. In Chapter 2, we explore quantitative defects that define common phenotypes of T cell aging and we show that young adults with short telomeres and telomerase mutations recapitulate the older adult phenotype in all categories. Furthermore, both short telomere and older adult subjects show increased apoptotic defects in vitro, suggesting that telomere length does limit replicative capacity and function of T cells. These data are also confirmed in our mouse models of telomere-mediated disease. In Chapter 3, we assess the T cell receptor (TCR) repertoire in two ways and show that both short telomere and older adult subjects show restricted repertoires in comparison to young controls. Here we posit a role for decreased TCR diversity in immune aging. In Chapter 4, we define gene
expression and cytokine release profiles in T cells and show that short telomere subjects differ from older adults in these mechanistic parameters. As a whole, our data define short telomere syndromes as a common cause of primary immunodeficiency and suggest that the short telomere is capable of driving immune aging in a subset but not all populations.
Chapter 1: INTRODUCTION

References


Chapter 1: INTRODUCTION


Chapter 1: INTRODUCTION


Chapter 1: INTRODUCTION


Chapter 2:

Short Telomeres Cause a Primary T cell Immunodeficiency
and Are Sufficient to Cause T cell Aging

Christa L. Wagner$^{1,2}$, Vidya Sagar Hanumanthu$^1$, Chris Kanakry$^1$, J. Brooks Jackson$^3$, Janet Siliciano$^4$, Roshini Abraham$^{5,6}$, Leo Luznik$^1$, Mary Armanios$^{1,7}$

Departments of Oncology$^1$, Program in Cellular and Molecular Medicine$^2$, Pathology$^3$, Infectious Diseases$^4$, and the McKusick-Nathans Institute of Genetic Medicine$^7$, Johns Hopkins University School of Medicine, Baltimore, MD

Departments of Laboratory Medicine and Pathology$^5$ and Allergy and Immunology$^6$, Mayo Clinic, Rochester, MN
Chapter 2: SHORT TELOMERES CAUSE A PRIMARY T CELL IMMUNODEFICIENCY AND ARE SUFFICIENT TO CAUSE T CELL AGING

2.1 Introduction

Immune aging is a major cause of morbidity and manifests as a susceptibility to infection along with decreased cancer surveillance, yet the mechanisms that drive this progressive process are not fully understood. Short telomere length is universally acquired with aging and telomere shortening has long been hypothesized as a mechanism of immune aging. Despite this hypothesis, whether or not telomere shortening in humans contributes to immune aging is not fully understood.

The short telomere syndromes are a heterogeneous collection of premature aging disorders (Armanios and Blackburn 2012). They are caused by inherited mutations in telomerase and telomere genes, and, because of their prevalence in adult populations, represent the most common of premature aging syndromes. Bone marrow failure is the canonical short telomere phenotype and it is linked with quantitative and qualitative stem cell defects in both humans and mice (Armanios and Blackburn 2012).

Few studies of immune dysfunction in telomere syndromes have been reported despite immunodeficiency being a primary presentation in many early-onset cases of telomere syndromes. Case reports and reviews that do exist show evidence of a range of immune phenotypes in children, including opportunistic infection, lymphopenia, and gastrointestinal crypt apoptosis (Solder, Weiss et al. 1998; de la Fuente and Dokal 2007; Jyonouchi, Forbes et al. 2011; Jonassaint, Guo et al. 2013). Molecular studies have focused on single families or bone marrow failure syndromes as a whole (Knudson, Kulkarni et al. 2005). These studies shed light on the immunologic phenotypes but do not encompass the entirety of telomere syndromes.
Chapter 2: Short Telomeres Cause a Primary T Cell Immunodeficiency and Are Sufficient to Cause T Cell Aging

Though previous studies do confirm immunodeficiency as a manifestation of disease in telomere syndromes, the concept of short telomeres driving the onset of immune dysfunction has not been systematically examined in humans. In this chapter, I study the hypothesis that young humans with short telomeres will display features of immune aging in a telomere-length dependent fashion. We show that a T cell-mediated primary immunodeficiency can be a manifestation of short telomere syndromes and is associated with fatal infections and cancers associated with impaired T cell surveillance. Through systematic analysis of immune phenotypes within these groups, I show that subjects with short telomeres replicate many quantitative and qualitative findings of the older adult cohort with normal telomere lengths. Further investigation of lymphopenia and proliferative defects in the short telomere mouse validate the conclusions from the human studies to show that short telomeres are sufficient to drive the disease-causing immune aging phenotype.

2.2 Results

2.2.1 Individuals with short telomeres present with opportunistic infection and decreased peripheral blood counts

To test the hypothesis that young short telomere (ST) subjects have premature aging of T cell immunity, we studied young ST patients (mean 19 y, range 8mo – 36y) and compared their immune profile to age-matched young controls (YC, mean 25y, range 14 – 31y) and healthy older adults five decades older (OA, mean 73 y, range 61 – 82y) (Fig. 2.1a,b). ST patients had abnormally short telomere length (TL), at or below the
age-adjusted 1st percentile, and carried mutations in telomerase (*TERT, TR, DKC1, n=15),
telomere genes (*RTEL1, n=1), or had features of a classic telomere syndrome (n=2). In
contrast, both YC and OA had normal age-adjusted lymphocyte TL as measured by flow
cytometry and FISH (Fig. 2.1a, b). Among 25 short telomere subjects, at least 5 presented
to medical attention with opportunistic infections including varicella zoster (n=2), CMV
encephalitis (n=1), enterocolitis with absent plasma cells (n=1) or human papiloma virus
associated squamous cell carcinoma (n=1) (Fig. 2.1d-f). Among these cases, CD4
lymphopenia was the most common abnormality and was often associated with
hypogammaglobulinemia especially of IgM (Fig. 2.1c). These data indicated that
primary immunodeficiency (PID) in severe cases and common variable
immunodeficiency (CVID) more commonly may be manifestations of short telomere
syndrome in 20% of individuals younger than age 30.

2.2.2 Short telomere immunophenotype recapitulates immune aging in individuals
five decades older

We first examined T cell subsets, and found that asymptomatic ST patients had
fewer naïve cells, both CD4+ helper and CD8+ killer cells as defined by
CD45RA+CCR7+. The extent of the naïve cell depletion was comparable to OA five
decades older (Fig. 2.2a-b). Similarly, like OA, ST subjects showed an expansion of the
terminally differentiated and non-functional CD8+ TEMRA population (Fig. 2.2c-d). Since
ST tended to have fewer circulating T cells due to lymphopenia, this analysis likely
underestimates the severity of naïve T cell loss in these young ST patients (Fig. 2.2b). An
interesting additional finding was an increase in the proportion of circulating CD4⁺CD8⁺ cells which is known to occur in aging humans and is correlated with increased immune activation (Fig 2.2e).

To test whether ST cells show the cell surface markers associated with aged T cells, we profiled peripheral T cells and found they shared the upregulation of CD57⁺ and CD95⁺ with OAs (Fig. 2.3a-d). However, in contrast to OA, there was no significant loss of CD28 or upregulation of the inhibitory receptor PD-1 expression (Fig. 2.3e-h). These data suggested that although young ST patients share loss of naïve populations with OA, their molecular profile may be distinct from OA with normal TL.

2.2.3 Short telomeres correlate with premature thymic involution as shown through T cell receptor excision circle (TREC) quantification

We examined whether the paucity of naïve T cells in ST patients is due to thymic failure and measured the fraction of naïve CD4⁺CD31⁺ T cells that are recent thymic emigrants (RTE) (Kilpatrick, Rickabaugh et al. 2008). We found this population was decreased in both ST and OA, although the defect was more severe in OA (Fig 2.4a). To examine this further, we prospectively recruited ST patients and quantified T cell receptor excision circles (TRECs) on fresh blood by real time PCR. We found a majority of ST patients (60%, 6 of 10), including all those above age 16 years (4 of 4), had counts less than the age-adjusted 5th percentile (Fig. 2.4b). The 5th percentile cutoff for ages 44+ is 172.7 TREC copies / 1 million CD3⁺ T cells, and the oldest subject we report measured 120 TREC copies / 1 million CD3⁺ T cells. In one infant with an uncharacterized PID
who carried a mutation in the dyskerin component of telomerase (DKC1, Ala308Gly), TREC numbers were undetectable (Fig 2.4b), indicating that telomere disorders may be diagnosed by TREC-based newborn screening for immunodeficiency.

2.2.4 **Mouse model shows that the thymic niche in animals with short telomeres is capable of efficient T cell development**

To test whether the thymic niche, T cell autonomous defects, or both, contributed to the decreased thymic output observed in ST patients, we took advantage of late generation telomerase RNA null mice (mTR−/−) which have short telomeres and faithfully recapitulate human telomere-mediated disease (Herrera, Samper et al. 1999; Armanios 2009; Alder, Guo et al. 2011; Guo, Parry et al. 2011; Armanios and Blackburn 2012; Alder, Barkauskas et al. 2015). We first tested the capacity of the short telomere thymic niche to support wild-type T cell development by performing an adoptive transfer of wild-type bone marrow progenitors into wild-type and mTR−/− generation 4 (G4) hosts after a small, sublethal dose of irradiation (scheme in Fig. 2.5a). Engraftment and homing rates of donor-derived cells in the bone marrow, thymus, and peripheral blood was comparable between wild-type and G4 hosts at 4 and 8 weeks post-transplant (Fig 2.5 b-d). More importantly, we found the proportion of donor-derived single positive CD4 and CD8 T cells in the thymus and periphery was similar to wild-type recipients indicating the short telomere thymic niche can support the homing, development and export of wild-type thymocytes (Fig. 2.5e-f).
2.2.5 Deficiencies in the short telomere mouse hematopoietic hierarchy suggest compounding, cell intrinsic defects in T cell development

To understand the mechanisms underlying the thymic export failure in ST humans, we first examined hematopoietic stem-progenitors in the mTR-/- G5 mouse and found they were severely compromised in number (Fig. 2.6a-d). These cells are also known to have compromised function in vivo in reconstitution assays, as has been previously shown (Allsopp, Morin et al. 2003; Choudhury, Ju et al. 2007). We then examined thymic T cell precursors and found an increased rate of apoptosis in CD4-CD8- double negative (DN) T cells, CD4+CD8+ double positive (DP) T cells, and in committed single positive (SP) cells by Annexin V expression (Fig. 2.7a-e). In vitro stimulation of peripheral T cells also showed they had increased rates of early and late apoptosis as defined by Annexin^+PI and Annexin^+PI^lo, respectively (Fig. 2.8a). These defects collectively led to a profound CD4^+ and CD8^+ lymphopenia, similar to what we saw in humans (Fig. 2.8b-f). However, in contrast, short telomere mice show a greater loss of CD8^+ cells relative to CD4^+ cells. We examined but did not find evidence of cell cycle arrest in mince, both in intrathymic developing T cells or after stimulation of peripheral T cells by morphology or EdU incorporation, respectively. These data indicated that short telomeres contribute to thymic failure by limiting bone marrow as well as intra-thymic progenitors in addition to compromising the survival of committed peripheral cells.
Chapter 2: Short Telomeres Cause a Primary T Cell Immunodeficiency and Are Sufficient to Cause T Cell Aging

Figure 2.1 Telomere lengths and immunological profile of subjects with short telomeres.

a. Lymphocyte telomere lengths (TL) of individuals in each cohort (YC, ST, OA) measured by flowFISH. Data is plotted by TL on y-axis and individual’s age on x-axis to show distribution of telomere length by age. Difference between individual TL and 50%-ile value for age is plotted in b. c. Profile of abnormal clinical blood counts in ST patients. Pie charts represent population of ST patients queried and compared to clinically accepted normal ranges. Percentage of ST patients with abnormal values is graphed in pink. Opportunistic infections observed in ST patients include pneumocystis (d), CMV encephalitis (e), and disseminated varicella zoster (f). CD4 T cell counts of individuals in e and f were below the AIDS criteria of 200 cells / mL. ****P < .0001.
Figure 2.1

Chapter 2: Short telomeres cause a primary T cell immunodeficiency and are sufficient to cause T cell aging
Chapter 2: Short Telomeres Cause a Primary T Cell Immunodeficiency and Are Sufficient to Cause T Cell Aging

Figure 2.2

Figure 2.2 ST subjects replicate immune aging of OA with decreased naïve T cells and increased CD8⁺ TEMRA⁺.

ST and OA cohorts show significant loss of CD4⁺ naïve T cells as assessed by flow cytometry (b, flow strategy in a). CD8⁺ T cells show same naïve loss (data not shown) with compensatory increase in terminally differentiated TEMRA population (d, flow strategy in e). e. ST subjects show significant increase in peripheral CD4⁺CD8⁺ (double-positive) T cells. *P <0.05; **P <0.01.
Chapter 2: **Short Telomeres Cause a Primary T Cell Immunodeficiency and Are Sufficient to Cause T Cell Aging**

**Figure 2.3 Immunophenotyping patterns of ST cells relative to OA and YC.**

Cell surface expression of immune aging-related markers on CD4⁺ T cells (a, c, e, g) and CD8⁺ T cells (b, d, f, h). ST recapitulates increased expression on OA cells of Fas-receptor CD95 (a, b) in total expression and in naïve T cells. A similar trend is seen in expression of the immunosenescence marker CD57 (c, d). OA show upregulation of inhibitory receptor PD-1 in CD4⁺ and CD8⁺ T cells (e, f) but this trend is not replicated in ST T cells. Similarly, OA cells show increased loss of stimulatory co-receptor CD28, but this trend is not replicated in ST T cells (g, h). Legends correspond to T cell subsets:
- **Total:** all CD4⁺ or CD8⁺ T cells.
- **Naïve:** CD45⁺CCR7⁺, CM: Central Memory, CD45RA⁻, CCR7⁺, EM: Effector Memory, CD45RA⁻CCR7⁺, TEMRA: Effector Memory RA⁺: CD45RA⁺CCR7⁺.

*P < 0.05; **P < 0.01.
Chapter 2: Short Telomeres Cause a Primary T Cell Immunodeficiency and Are Sufficient to Cause T Cell Aging

Figure 2.3
Figure 2.4

a. Quantification of CD4+ recent thymic emigrants (RTE) by flow cytometry, showing decreased thymic output in ST and OA. RTEs are marked by CD31+ naïve T cells.

b. Analysis of thymic output in ST subjects by quantification of T cell receptor excision circles (TRECs) by real time PCR. Y-axis shows TREC count per 1 million T cells and x-axis shows corresponding subject age. Shaded regions are defined from analysis of 254 healthy individuals, with pink representing TREC counts at or below the 5th percentile. Subject telomere gene mutations are indicated above data points. **P <0.01.
Figure 2.5 Congenic bone marrow transplant reveals thymic niche of short telomere mice is capable of normal thymocyte development and export of T cells.

a. Transplant scheme. Bone marrow from CD45.1\(^+\) wild-type mice was harvested and stem cells (HSCs) isolated by negative selection. CD45.2\(^+\) wild-type and mTR\(^{-}\) G4 mice were sublethally irradiated (1 Gy) and injected with equal numbers of donor-derived HSCs. Donor engraftment was monitored at 4 and 8 weeks post-transplant. Percentage of donor (CD45.1\(^+\))-derived cells was quantified in recipient bone marrow, thymus, and peripheral blood at each timepoint (b, c, d respectively). Further inspection of donor-derived thymocytes and T cells in peripheral blood was quantified at 4 and 8 week timepoints (e and f, respectively).
Chapter 2: Short Telomeres Cause a Primary T Cell Immunodeficiency and Are Sufficient to Cause T Cell Aging

Figure 2.5

[Diagram and graphs showing cell lineage and distribution over time in different compartments (Bone Marrow, Thymus, Peripheral Blood) for WT and mTR−/−G4 mice.]
Figure 2.6 Mice with short telomeres have significant defects in hematopoietic stem cell counts.

a, b. Flow cytometry strategy and quantification of lineage⁻ c-kit⁺ Sca-1⁺ (KSL) hematopoietic stem cells in age-matched wild-type and mTR⁻ G5 mice. c, d. Flow strategy and quantification of a more primitive HSC population (lineage⁻ c-kit⁺CD150⁺CD48⁻, SLAM) shows more significant loss in G5 mice compared to wild-type. ***P <0.001, ****P <0.0001.
Chapter 2: Short Telomeres Cause a Primary T Cell Immunodeficiency and Are Sufficient to Cause T Cell Aging

Figure 2.6

(a) WT and mTR-/-G5 cells are shown in Lin- vs. c-Kit and Sea-I vs. KSL dot plots. (b) Bar graph showing line-c-kit-sea+ (x10^3) for WT and G5 with error bars. (c) WT and mTR-/-G5 cells are shown in Lin- vs. c-Kit and CD150 vs. SSC dot plots. (d) Bar graph showing line-c-kit-CD150 (x10^3) for WT and G5 with error bars.
Figure 2.7 Young and adult short telomere mice show increased apoptosis in developing thymocytes by Annexin V staining.

**a.** Flow cytometry gating strategy for thymocyte subsets. CD3⁻ (pre- and during- VDJ recombination), CD3lo (CD4⁺CD8⁺ double positive stage) and CD3⁺ (single-positive) subsets were analyzed for Annexin V positivity. **b.** Percentage of CD3⁻ Annexin⁺ cells in double-negative (DN) populations undergoing VDJ recombination and proliferation. DN subsets are recognized by differential CD25 and CD44 staining as indicated. **c.** Annexin⁺ quantification of DP thymocytes following VDJ recombination and surface CD4 and CD8 expression. **d and e.** Annexin staining in SP (CD4⁺ or CD8⁺, respectively) thymocytes after negative and positive selection. Young mice = 6 weeks, adult = 18 weeks. *P <0.05.
Chapter 2: Short telomeres cause a primary T cell immunodeficiency and are sufficient to cause T cell aging

Figure 2.7

(a) Scatter plots showing CD3^+ CD4^+ and CD3^+ CD4^+ cells in the population.
(b) Bar graphs comparing the percentage of CD4^+ 4^+ Annexin in DN1, DN2, DN3, and DN4 stages.
(c) Bar graphs comparing the percentage of CD3^+ 8^+ Annexin in young and adult stages.
(d) Bar graphs comparing the percentage of CD4^+ 4^+ Annexin in young and adult stages.
(e) Bar graphs comparing the percentage of CD3^+ 8^+ Annexin in young and adult stages.

Legend:
- WT
- mTR^- G5
Chapter 2: Short Telomeres Cause a Primary T Cell Immunodeficiency and Are Sufficient to Cause T Cell Aging

Figure 2.8

**Figure 2.8** Quantification of T cell apoptosis and peripheral lymphopenia in mTR−/− G5 mice.

- **a.** Flow cytometry gating strategy and quantification of Annexin^+^PI^−^ (early) and Annexin^+^PI^lo^ (late) apoptosis following CD3/CD28 stimulation in murine T cells.

- Clinical blood counts for white blood cells (b) and absolute lymphocytes (c) quantified by Procyte Hematology Analyzer. CD4^+^ and CD8^+^ counts (d, e) were quantified from ALC (b) and percentages of CD4^+^ and CD8^+^ by flow cytometry. Counts in d and e were used to calculate the 4:8 ratio in f. *P < 0.05; **P < 0.01; ***P < .001.
2.3 Discussion

The phenomenon of immune aging has been well described for decades even though its basis remains incompletely understood. Defining the molecular mechanisms responsible for immunological decline with aging has important obvious implications for risk assessment and for potential intervention. A few hypotheses have been suggested as to the underlying mechanisms that drive immune aging, including telomere maintenance. Knowing that lymphocytes, specifically T cells, upregulate telomerase activity to be able to mount immunological responses (Buchkovich and Greider 1996) and that it is the telomere length that determines onset of disease (Armanios 2013), we designed a study to determine the role of short telomeres on the development of immune aging phenotypes. In this chapter, we show that young patients with telomerase mutations and short telomeres recapitulate many features of immune aging and present short telomeres as a driver of immune aging.

This study originated with clinical observations of severe opportunistic infections in patients with short telomeres (ST) and mutations in telomerase and telomere maintenance genes. Approximately 20% of ST subjects first presented with infections usually only observed in immune-deficient states such as HIV/AIDS. This observation is particularly important as these phenotypes have heretofore not been commonly recognized as primary presentations of telomere syndrome. I conducted immunophenotyping to show that the peripheral T cell compartment is prematurely aged, as shown through significantly fewer naïve T cells and increased CD8+ T EMRAS. Analysis of cell surface markers of immune aging showed increased expression of CD95 and
CD57 in both ST and OA, corroborating previous studies in a family with an \textit{hTR} mutation (Knudson, Kulkarni et al. 2005). Other aging markers of increased PD-1 and loss of CD28 were not as strongly correlated between ST and OA in this study, suggesting that immune aging moderated by the telomere may have a different T cell immunophenotype than OA with normal telomere lengths.

I queried thymic function and found, by flow cytometry, a trend towards decreased recent thymic emigrants, suggesting short telomeres cause premature thymic involution. The more refined TREC\textsc{s} assay in collaboration with Dr. Roshini Abraham’s laboratory at the Mayo Clinic showed that most ST subjects had abnormally low TREC counts, and those within the normal range had borderline low values. This result is of particular interest given the increasingly widespread universal incorporation of the TREC assay into national Newborn Screening (NBS) programs. Preliminary results from 3 million children screened show that 38\% of children with low TREC\textsc{s} do not obtain a genetic diagnosis before clinical intervention which most often involved bone marrow transplantation (Kwan, Abraham et al. 2014). Additionally, 2 cases of busulfan toxicity were reported in this review. Previous studies of bone marrow transplant in adults with telomerase mutations has shown that telomere syndromes cause explicit sensitivity to conditioning regimens such as busulfan and radiation, resulting in transplant-related complications and mortality (de la Fuente and Dokal 2007; Dietz, Orchard et al. 2010). Therefore, this work identifies short telomere syndromes as a cause of abnormal NBS and has implications for risk stratification in the hematopoietic stem cell transplant setting.
To follow up on the TRECs results, I incorporated studies in the short telomere mouse to show that the short telomere thymic niche is capable of supporting normal thymocyte development in an adoptive transplant assay. Though engraftment was not high in recipient mice, as would be expected for low doses of irradiation, it was sufficient to show that donor-derived hematopoietic stem cells were capable of differentiating, homing to the thymus, and creating viable CD4+ and CD8+ T cells in the periphery.

Intact thymic niche function pointed to a more severe T cell intrinsic defect playing a role in infection susceptibility and premature immune aging in peripheral cells. Using the same short telomere mouse model, I phenotyped each compartment of the hematopoietic system to look for evidence of defects in cell number and function. The bone marrow of mTR−/− G5 mice showed significantly fewer hematopoietic stem cells compared to age-matched wild-type controls. Absolute numbers of thymocytes was challenging to quantify, but assessment of apoptosis through Annexin V staining showed significantly higher rates of apoptosis in short telomere thymocytes. The apoptotic defect was recapitulated in peripheral T cells and corresponded to significantly decreased numbers of white blood cells, lymphocytes, and CD4+ and CD8+ T cells.

In summary, we found evidence that the short telomeres are sufficient to drive the immune aging phenotype. Our findings also establish short telomere syndromes as a cause of PID including in the setting of abnormal NBS. This result is important for patient care in several settings and provides new insights into the mechanisms of T cell aging.
2.4 Materials and Methods

2.4.1 Subjects and Controls. Cases were recruited from 2005 to 2015 as part of the Johns Hopkins Telomere Syndrome Registry (Jonassaint, Guo et al. 2013; Gorgy, Jonassaint et al. 2015). Clinical data were extracted from existing medical records. Mutation status was determined by Sanger sequencing (Alder, Guo et al. 2011; Parry, Alder et al. 2011), TruSeq Custom Amplicon sequencing followed by PCR verification as previously described (Stanley, Chen et al. 2015), or by exome sequencing (Alder, Stanley et al. 2015). Healthy controls were recruited from volunteers in the Baltimore area from 2009 to 2014. Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Paque from whole blood (SepMate, StemCell Technologies), and frozen until analysis. Telomere length was measured by flow cytometry and fluorescence in situ hybridization (flowFISH) as previously described (Baerlocher, Vulto et al. 2006). The study was approved by the Johns Hopkins Medicine Institutional Review Board and all the subjects gave written, informed consent.

2.4.2 Sample Preparation for Immunophenotyping and Antibodies. After thawing, PBMCs were treated to lyse contaminating red cells (Red Cell Lysis Buffer, eBioscience). Samples were washed and resuspended in 100 uL 2% FBS/PBS solution for antibody staining. The Live/Dead Fixable Stain conjugated with Aqua (Invitrogen) was used for viability. Samples were then washed and resuspended in 100 uL 2% FBS/PBS for antibody staining. Ideal antibody concentrations were determined by serial dilutions. Antibody staining was performed for 20 minutes in the dark at 4°C. Cell
surface phosphatidylserine (PS) was detected with APC-labeled Annexin V in Binding Buffer (BD Biosciences) and counterstained with propidium iodide (PI, BD Biosciences). The human (n=29) and mouse (n=18) antibodies, their conjugates, clone identifiers and suppliers are listed in Supplementary Tables 1 and 2, respectively.

2.4.3 Flow Cytometry and Analysis. Flow cytometry data were acquired using CellQuest Pro v.5.1.1 on a FACSCalibur and using the FACSDiva v.6.1.2 on an LSR II (BD Biosciences). Single color controls were collected for each antibody, and additional fluorescence-minus one (FMO) controls were collected for low abundance markers (CD25, PD1, CCR7). Analyses were performed using FlowJo (v.X, Treestar). Both Boolean and sequential gating strategies were used to analyze the data. A lymphocyte gate was created on a forward- vs. side-scatter plot, followed by doublet exclusion, and gating on CD3+ and CD4+ vs. CD8+ cells. Individual CD4+ and CD8+ sub-populations were then analyzed for markers of interest. At least 20,000 viable events were collected for each sample.

2.4.4 T cell Receptor Excision Circle (TREC) Assay. TRECs were measured by quantitative real time PCR (q RT-PCR) at the Mayo Clinic using previously described methods (Douek, Vescio et al. 2000) except T cell DNA was derived from peripheral blood. T cells were isolated using the RosetteSep (StemCell Technologies) except in pediatric cases where blood volume was limited, total lymphocytes were isolated. Absolute CD4+ and CD8+ T cells were counted by flow cytometry. Quantitative real time
Chapter 2: SHORT TELOMERES CAUSE A PRIMARY T CELL IMMUNODEFICIENCY AND ARE SUFFICIENT TO CAUSE T CELL AGING

PCR was performed using probes specific to signal-joint (sjTRECs) and the albumin gene as a control. TREC copy number was determined using standard curves, and reported as copies per million CD3+ T cells. Age-appropriate reference ranges were generated from pediatric (n=138) and adult (n=116) controls and in this study values <5th percentile were considered abnormal.

2.4.5 Mice. Mice were housed at the Johns Hopkins University School of Medicine campus, and all the procedures were approved by the Institutional Animal Care and Use Committee. mTR−/− mice on the C57BL/6J background were derived and maintained as previously described (Blasco, Lee et al. 1997). Wild-type donor mice were purchased from Charles River or Jackson Laboratories (B6.SJL-Ptprca Pepcb/BoyJ, males, 6 weeks, CD45.1). Recipient mice for adoptive transfer experiments were purchased from Jackson Laboratories (C57BL/6J, 16 weeks, male and female, CD45.2). For complete blood counts, 100 uL of blood from a cardiac puncture was analyzed on the Procyte Dx Hematology Analyzer (IDEXX Laboratories). Bone marrow and thymocytes were harvested using standard methods. Samples were prepared and analyzed by flow cytometry as described above.

2.4.6 Adoptive Transfer Experiments. Bone marrow from wild-type mice (CD45.1) was harvested and brought to single cell suspension in 2% FBS/PBS supplemented with 1 mM EDTA. Hematopoietic progenitors were enriched by negative selection using the EasySep Mouse Hematopoietic Progenitor Cell Isolation kit (StemCell Technologies).
Enriched progenitors \((9 \times 10^4)\) were injected via tail vein into 18-22 week old \textit{wild-type} or \textit{mTR}^- \(G4\) recipients (CD45.2) after a sublethal dose of irradiation (1 Gy, Cs-137 irradiator), as outlined previously (Serwold, Ehrlich et al. 2009). Recipient mice were sacrificed at preplanned timepoints and the donor-derived fraction in the blood, thymus and bone marrow were calculated by flow cytometry.

2.4.7 Mouse T cell Stimulation. Mouse CD3\(^+\) T cells or CD8\(^+\) T cells were stimulated as described for human T cells except using mouse antibodies (Dynabeads Mouse T-Activator CD3/CD28, Life Technologies). The viable cell count was measured using the colorimetric WST assay (Dojindo Molecular Technologies) and cell death was analyzed using Annexin/PI. 15,000 events were collected for each sample.

2.4.8 Statistics. We used GraphPad Prism v.6.05 (GraphPad Software) to generate the graphics, and unless otherwise noted, for statistical comparisons. Means were compared by Student’s \(t\)-test. Unless otherwise noted, all P-values shown are two-sided.
Chapter 2: SHORT TELOMERES CAUSE A PRIMARY T CELL IMMUNODEFICIENCY AND ARE SUFFICIENT TO CAUSE T CELL AGING

References


Chapter 2: SHORT TELOMERES CAUSE A PRIMARY T CELL IMMUNODEFICIENCY AND ARE SUFFICIENT TO CAUSE T CELL AGING


Chapter 2: Short Telomeres Cause a Primary T cell Immunodeficiency and Are Sufficient to Cause T cell Aging

Chapter 3:

Analysis of T cell receptor repertoire diversity in young adults with short telomere syndromes

Christa L. Wagner¹,², Vidya Sagar Hanumanthu¹, David Hamm³, Mary Armanios¹,⁴

Departments of Oncology¹, Program in Cellular and Molecular Medicine², and the McKusick-Nathans Institute of Genetic Medicine⁴, Johns Hopkins University School of Medicine, Baltimore, MD

Adaptive Biotechnologies³, Seattle, WA
Chapter 3: Analysis of T Cell Receptor Repertoire Diversity in Young Adults with Short Telomere Syndromes

3.1 Introduction

A diverse T cell receptor (TCR) repertoire is necessary for the ability to respond to the multitude of pathogenic and cancer antigens that one can experience over a lifetime. αβ T cells compromise approximately 95% of the peripheral T cell compartment. Diversity in the β chain is generated through VDJ recombination in thymocytes, with the greatest diversity in the DNA sequence showing in the antigen-binding complementarity-determining region 3 (CDR3) (Danska, Livingstone et al. 1990; Robins, Campregher et al. 2009). The repertoire is established within the first two decades of life before thymic involution reduces the output of new T cells with unique receptor sequences, and peripheral diversity must be maintained through homeostatic proliferation (Qi, Liu et al. 2014). The combined thymic involution and persistent peripheral challenge from pathogens leads to a decreased TCR repertoire over time with aging. This time-dependent phenomenon has been hypothesized to be a contributor to increased rates of opportunistic infection in the elderly and immune compromised state with aging (Weng 2006).

Two methods have commonly been used to assess TCR repertoire diversity: the study of the Vβ gene sequences and their protein products. A more refined and detailed look at receptor repertoire uses deep sequencing of each Vβ gene locus. Sequencing of the CDR3 region of each Vβ gene allows for analysis of n-nucleotide additions and deletions, lengths of the CDR3 region products and distributions relative to a normal curve, as well as richness and clonality measurements to assess redundancy within a given repertoire (Robins, Campregher et al. 2009; Carlson, Emerson et al. 2013). Flow
cytometry allows for an overview of different Vβ families expressed as functional receptors on the surface of different cell subsets (Woodsworth, Castellarin et al. 2013).

Based on the observed lymphopenia, decreased TRECs, and increased apoptotic susceptibility in individuals with short telomeres, we worked to assess the potential role of a limited TCR repertoire as a contributor to their observed telomere-mediated immunodeficiency. We used deep sequencing and flow cytometry to define the TCR repertoire of short telomere subjects (ST) in comparison to young controls (YC) and older adults (OA). Within these groups, we assessed CD4+ and CD8+ T cells independently as they have separate replicative histories. In this chapter, I show using deep sequencing analyses and flow cytometry that short telomeres decrease diversity of the TCR repertoire in both CD4+ and CD8+ T cells.

3.2 Results

3.2.1 Deep sequencing of TCRβ genes shows differences in receptor repertoire of short telomere subjects

The T cell quantitative defects of ST patients were similar to OA, but the prevalence of opportunistic infections was significantly higher and the lymphopenia-associated infections more severe (Chapter 2). Despite the evidence of cell autonomous defects in T cell development, I hypothesized that additional defects were contributing to the immunodeficiency and the cancer prone state. To test whether increased infection susceptibility may be in part due to a restricted TCR repertoire, we performed deep sequencing on the Vβ genes’ complementarity-determining region 3 (CDR3) using
Chapter 3: Analysis of T Cell Receptor Repertoire Diversity in Young Adults with Short Telomere Syndromes

Adaptive Biotechnology’s immunoSEQ platform. PBMCs were sorted as CD4+ for all three groups and CD8+ T cells for young and short telomere groups only. Repertoire diversity is created at V-D and D-J junctions with n-nucleotide additions created by DNA polymerase Terminal deoxynucleotidyl transferase (TdT) (Davis and Bjorkman 1988). This random addition leads to a normal distribution of CDR3 lengths within the population. In my analysis, ST T cells showed both nucleotide addition at V-D and D-J junctions and the distribution of CDR3 length to be comparable to controls, consistent with intact VDJ recombination (Fig 3.1).

Multiple mathematical relationships can shed light on TCR repertoire diversity at the sequence level. A straightforward analysis is the clonality score, which estimates diversity by quantifying how much of the total repertoire is dominated by a few clones, represented as a scale of 0 to 1. A clonality score of 0 represents a diverse distribution and 1 represents a completely oligoclonal repertoire. A second measure, richness, quantifies the breadth of unique sequences in a repertoire, adjusted for input number of T cells. A value closer to 1 represents a distribution closer to 1 sequence per T cell (Dewitt, Lindau et al. 2014). A third diversity metric is Pielou’s evenness index. Also on a scale of 0 to 1, Pielou’s J’ is equal to 1 minus the clonality, and therefore approaches 1 as the population approaches its maximum possible value (Pielou 1966). In this study, the CD8+ T cell compartment of ST subjects showed increased repertoire clonality and a corresponding decrease in richness of the queried sequences (Fig 3.2 a,b). Furthermore, ST subjects show decreased Vβ gene usage diversity as calculated by Pielou’s J’ and increased frequencies of the use of top clones in the CD8+ compartment in comparison to
Chapter 3: Analysis of T Cell Receptor Repertoire Diversity in Young Adults with Short Telomere Syndromes

YC subjects (Fig 3.2 c,d). These results are consistent with reduced effector cell diversity with oligoclonality in immune aging.

Additionally, I analyzed CD4+ T cell TCR repertoires in the three groups. With aging, CD4+ T cells do not approach oligoclonality and terminal differentiation as quickly as CD8+ T cells. This pattern of differential aging in CD4+ and CD8+ was replicated in the repertoire results as a decreased clonality score in CD4+ T cells in comparison to sample-matched CD8+ TCR clonality scores (Fig 3.3 a). Within the CD4+ compartment, both the clonality of ST and OA samples was increased in comparison to YC, with ST subjects showing intermediate clonality to the two controls. Interestingly, however, the richness of ST and OA samples was slightly increased in comparison to YC in the CD4+ compartment. This may be a result of global, unbiased loss of TCR sequences in these groups which already display T cell lymphopenia. CD4+ TCR repertoires are not oligoclonal and thus more diverse than CD8+ in all contexts, so global sequence loss from a population that already has fewer T cells in ST and OA may be reflected by a slight increase in repertoire richness. When analyzing Vβ gene usage, however, the ST group showed the same pattern in CD4+ T cells as CD8+ T cells, with a decrease in usage, whereas OA samples show a slight increase in Vβ gene usage in comparison to the YC group (Fig 3.3 c). The sum of the top four most represented clones in the ST group showed greater frequency in comparison to the top four from both YC and OA (Fig 3.3 d). Together, these data indicate that although the ability to create a diverse repertoire in ST subjects is intact, T cell loss contributes to a selective restriction of the TCR repertoire in both CD4+ and CD8+ cells.
3.2.2 Flow cytometry analysis shows skewing in T Cell Receptor Vβ representation in short telomere patients

To validate whether the restricted T cell repertoire I saw at the sequence level affects the expressed TCR, I analyzed the frequency of T cells expressing each of 24 TCR Vβ family proteins by flow cytometry. This assay examines approximately 70% of the available TCR repertoire. I compared individuals in the three experimental groups (YC, ST, and OA) to expression data from 85 controls. An example analysis for a ST individual is shown in Figure 3.4. In this comparison, I quantified skewing in test subjects that fell between -1 to -2 or +1 to +2 SD and for more severe skewing ≤ -2 or ≥ +2 SD from the mean and SD values of the 85 controls. Results from individual subjects by each Vβ are shown in Figure 3.5. In analyzing grouped data, I found ST subjects and OA had considerable divergence, showing a significantly higher proportion of over- and under-represented Vβ families compared to YC (Fig 3.6). The frequency of divergent Vβ family expression was statistically significant and more severe in ST than YC in total CD3 T cells and the CD8 subsets, but not in CD4 T cells (P=0.06, P=0.02, P=0.31, two-sided Kruskal-Wallis test). The increased TCR repertoire skewing in CD8 T cells corroborated the deep sequencing data which showed increased clonality and decreased diversity in these cells at the DNA level.
Figure 3.1  n-nucleotide addition and CDR3 lengths are intact in short telomere subjects.

a-b. Quantification of n-nucleotide additions in CD4 (a) and CD8 (b) TCRs between V-D genes (left) and D-J genes (right) in YC, ST, and OA subjects. c. Distribution of lengths of CDR3 sequences, measured by number of nucleotides, by individual in YC (top), ST (middle), and OA (bottom).
Figure 3.1
Figure 3.2  Diversity metrics show increased changes in DNA-level TCR repertoire in CD8⁺ T cells of ST subjects.

a. Clonality scores for YC and ST groups. A clonality score of 1 represents a completely oligoclonal population. b. Richness metric for YC and ST groups. A larger value converges on a 1:1 ratio of unique sequence to T cell. Value is normalized to input T cell number. c. Diversity of Vβ gene usage, scored as the evenness metric Pielou’s J. d. Usage frequency of different Vβ genes, ranked by most to least frequent usage.
Figure 3.3  Diversity metrics show slight skewing of DNA-level TCR repertoire in CD4⁺ T cells of ST subjects.

**a.** Clonality scores for all 3 groups. **b.** Richness metric for all 3 groups. **c.** Diversity of Vβ gene usage, scored as the evenness metric Pielou’s J. **d.** Usage frequency of different Vβ genes, ranked by most to least frequent usage.
Chapter 3: ANALYSIS OF T CELL RECEPTOR REPERTOIRE DIVERSITY IN YOUNG ADULTS WITH SHORT TELOMERE SYNDROMES

Figure 3.4

**Figure 3.4 Individual analysis of Vβ family expression by flow cytometry.**

Comparison of Vβ expression in a short telomere subject (red bars) with mean and standard deviation of 85 healthy control values provided by the flow cytometry assay (black bars). *: -1 to -2 or +1 to +2 SD; **: ≤ -2 SD or ≥ +2 SD.
### Figure 3.5

**Distribution of Vβ family expression by flow cytometry.**

Summary of flow cytometry data of Vβ family usage. Individual subjects are represented in separate columns, individual Vβ protein families are represented in separate rows. Results are divided into CD3, CD4, and CD8 T cells from left to right. Individual data was compared to 85 healthy control values provided by the assay. Grey boxes indicate deviations within ± 1 SD from the 85 controls mean. Red boxes indicate decreased expression of a given Vβ family, and blue boxes indicate increased expression. Lighter shades correspond to -1 to -2 SD or +1 to +2 SD respectively. Darker shades correspond to ≤ -2 SD and ≥ +2 SD Vβ family skewing, respectively.
Figure 3.6 Skewedness in Vβ family expression.

Quantification of Vβ family usage skewing shown in Figure 3.5. Grey bars represent % of receptors skewed in each experimental group by -1 to -2 or +1 to +2 SD in comparison to the mean and SD of 85 healthy controls. Black bars represent percentage of Vβ family expression with greater skewing of ≤-2 SD and ≥+2 SD. YC = Young Control n=6, ST = Short Telomere n=7 for CD3 and CD4 n=6 for CD8, OA = Older Adult n=5.
3.3 Discussion

A diverse TCR repertoire is required for successful immune function, and a decreased repertoire is associated with aging. Development of a diverse repertoire is dependent upon intact VDJ recombination in both B and T cells and the long-term maintenance and survival of those clones. Defects in the VDJ recombination pathway are a common genetic cause of severe-combined immunodeficiency (SCID), and in milder cases may present as Common Variable Immunodeficiency (CVID) (Shearer, Dunn et al. 2014). Immunodeficiencies have previously been reported in telomere syndromes without an in-depth analysis of contributing mechanistic factors (Jyonouchi, Forbes et al. 2011; Walter, Armanios et al. 2015). In this chapter, I found evidence of premature restriction of TCR repertoires in young short telomere subjects by both deep sequencing and flow cytometry, suggesting that decreased receptor diversity may contribute to the observed immune dysfunction.

Deep sequencing analysis of the CD8+ TCR repertoire in ST subjects shows a consistent loss in diversity by all measures including an increased variance in comparison to age-matched controls. This DNA-level analysis however did not include a comparison with older adults. Decreases in clonality and richness, in the presence of intact VDJ recombination, indicate dropout of the most rare clones in the ST repertoire dominated by terminally differentiated Effector Memory CD45RA+/TEMRA cells. The observed Vβ usage restriction at the DNA level was confirmed through flow cytometry analysis of cell surface expression of Vβ families. Though protein analysis covers roughly 70% of the available repertoire, the widespread skewing of repertoire diversity in the ST subjects
shows a more fragile balance of diversity maintenance. Again CD8⁺ TCR repertoires are further skewed, and this analysis shows ST subjects meet or exceed the repertoire skewing seen in individuals five decades older.

In matched samples, CD4⁺ T cells show greater diversity than CD8⁺ T cells, which is expected based on previous observations of oligoclonality in CD8⁺ T cells. Vβ family protein expression again showed ST subjects trend towards OA findings, with a skewed repertoire diversity. Deep sequencing revealed that ST and OA subjects show increased clonality in comparison to YC in the CD4⁺ compartment, with greater variance than YC. Increases in the richness metric may reflect the impact of lymphopenia in ST and OA, which creates a smaller input population, and thus a different repertoire shape in comparison to YC. Interestingly, ST and OA results diverge here in that ST subjects show decreased Vβ gene usage in CD4⁺ T cells. The reason for this difference is not completely clear, but may indicate a short telomere-specific effect. Small sample sizes in this analysis (n=4/group) prevented statistically-significant findings however, the trends favor the hypothesis that short telomeres prevent long-term maintenance of an age-appropriate TCR repertoire and drive early repertoire restriction likely due to apoptotic dropout.

In summary, individuals with short telomeres and telomere gene mutations show restriction in CD4⁺ and even more so in CD8⁺ TCR repertoires. The restriction is equal to or greater than individuals representing immune aging, at approximately five decades older than ST individuals. Previous studies have not investigated CD4⁺ and CD8⁺ T cells sequence data separately, but analysis of naïve and memory cells have shown that TCR
repertoires show restriction but remain highly diverse with immune aging, and therefore suggest that additional changes in peripheral maintenance can cause age-associated immune dysfunction (Qi, Liu et al. 2014). Our data therefore suggest that replicative aging or short telomeres contribute to a more accelerated restriction of the repertoire in some T cell subsets than would normally occur with aging. An additional limitation is that the advances brought on by deep sequencing are still not perfect, as this assay is not capable of sequencing every cell in a given individual’s repertoire. Despite the decreased repertoire diversity in our cohort, repertoire production during development is intact. This points to peripheral loss of T cells as the main driver of repertoire restriction rather than problems in TCR production. Indeed, our data in previous studies and in Chapter 2 of this thesis support that view because of the propensity of short telomere T cells to undergo apoptosis. Altogether, these results indicate that decreased antigen-binding diversity plays a role in the development of opportunistic infection and potentially in defective cancer surveillance, in the setting of short telomeres.
3.4 Materials and Methods

3.4.1 Sample Preparation for Flow Cytometry, Sorting and Antibodies. After thawing, PBMCs were treated to lyse contaminating red cells (Red Cell Lysis Buffer, eBioscience). Samples were washed and resuspended in 100uL 2% FBS/PBS solution for antibody staining. Ideal antibody concentrations were determined by serial dilutions. Antibody staining was performed for 20 minutes in the dark at 4°C. Antibodies, their conjugates, clone identifiers and suppliers are listed in Supplementary Table 1.

3.4.2 TCR-Vβ Diversity Flow Cytometry Analysis. Analysis of TCR-Vβ diversity was performed by flow cytometry using IOTest Beta Mark Kit (Beckman Coulter) which targets 24 Vβ proteins as previously described (Degauque, Boeffard et al. 2011). Supplementary Table 1 lists the details for the CD3+ APC, CD4+ PerCP Cy5.5, and CD8+ V450 antibodies used to distinguish the 24 Vβ families expression on T cell subsets. Flow cytometry data were acquired using CellQuest Pro v.5.1.1 on a FACSCalibur and using the FACSDiva v.6.1.2 on an LSR II (BD Biosciences). Single color controls were collected for each antibody. Analyses were performed using FlowJo (v.X, Treestar). Both Boolean and sequential gating strategies were used to analyze the data. A lymphocyte gate was created on a forward- vs. side-scatter plot, followed by doublet exclusion, and gating on CD3+ and CD4+ vs. CD8+ cells. Individual CD4+ and CD8+ sub-populations were then analyzed for markers of interest. At least 80,000 lymphocyte events were analyzed for each sample.
3.4.3 **Cell Sorting.** For the T cell receptor-β (TCR-β) sequencing studies, PBMCs were sorted using a MoFLo Legacy (Beckman Coulter). The sorted populations CD3^+^CD4^+^CD56^-^ or CD3^+^CD8^-^CD56^-^ (100,000 cells) were prepared for DNA extraction using Gentra Puregene Blood Core kit (Qiagen).

3.4.4 **TCR-β Locus Sequencing.** The TCR-β Complementarity Determining Region 3 (CDR3) was sequenced using the immunoSEQ platform survey level (Adaptive Technologies, Seattle) as previously described (Carlson, Emerson et al. 2013). For CD4 T cell immunoSEQ, a mean of 7,354 T cells were analyzed at a mean coverage of 47X. For CD8 T cell immunoSEQ, a mean of 8,335 T cells was analyzed at a mean coverage of 31x. The clonality score was calculated based on Shannon’s entropy (H) as previously described (Sherwood, Emerson et al. 2013; Ramesh, Hamm et al. 2015).

3.4.5 **Statistics.** We used GraphPad Prism v.6.05 (GraphPad Software) to generate the graphics, and unless otherwise noted, for statistical comparisons. Means were compared by Student’s t-test. TCR Vβ flow cytometry data were compared using the Kruskal-Wallis test. Unless otherwise noted, all P-values shown are two-sided.
Chapter 3: ANALYSIS OF T CELL RECEPTOR REPERTOIRE DIVERSITY IN YOUNG ADULTS WITH SHORT TELOMERE SYNDROMES

References


Chapter 3: ANALYSIS OF T CELL RECEPTOR REPERTOIRE DIVERSITY IN YOUNG ADULTS WITH SHORT TELOMERE SYNDROMES

Sherwood, A. M., R. O. Emerson, et al. (2013). "Tumor-infiltrating lymphocytes in colorectal tumors display a diversity of T cell receptor sequences that differ from the T cells in adjacent mucosal tissue." Cancer Immunology, Immunotherapy 62(9): 1453-1461.


Chapter 4:

Terminally differentiated CD8\(^+\) T cells reveal a unique gene expression signature in the setting of short telomeres

Christa L. Wagner\(^{1,2}\), Vidya Sagar Hanumanthu\(^1\), Hao Zhang\(^3\), Connie Talbot\(^4\), Leo Luznik\(^1\), Mary Armanios\(^{1,4}\)

Departments of Oncology\(^1\), Program in Cellular and Molecular Medicine\(^2\), and the McKusick-Nathans Institute of Genetic Medicine\(^4\), Johns Hopkins University School of Medicine, Baltimore, MD

Department of Molecular Microbiology and Immunology\(^3\), Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD
4.1 Introduction

One hallmark of immune aging is the accumulation of oligoclonal, dysfunctional CD8+ terminally differentiated effector memory CD45RA+ (TEMRA) cells. This subset is one of four functionally distinct subpopulations that emerge with T cell differentiation, and is distinguished by CD45RA+ and CCR7− expression (Moro-García, Alonso-Arias et al. 2012). Despite decreased thymic output of both CD4+ and CD8+ T cells with age, CD8+ T cells specifically show differentiation of naïve cells to the dysfunctional memory populations at about twice the rate of CD4+ T cells (Saule, Trauet et al. 2006). This results in the accumulation of a large CD8+ TEMRA compared to a small or completely absent CD4+ TEMRA population. This phenomenon has sparked interest in studying the terminally differentiated TEMRA population with the hope of finding ways of improving its functional capacity.

Previous studies have attempted to functionally define exhaustion from senescence in differentiated cells through gene expression studies in mice. Since it is challenging to control for the heterogeneity of immune phenotypes in humans, infection with lymphocytic choriomeningitis virus (LCMV) in mice has served as a useful animal model. In these models, ‘immune exhaustion’ has been defined as virus-specific cells with no effector function (Kahan, Wherry et al. 2015). Conditions of acute and chronic infection have found that exhausted CD8+ T cells show increased expression of multiple inhibitory receptors, and downregulated expression of both TCR signaling molecules and metabolic intermediates (Wherry, Ha et al. 2007). Other studies in mice have also found
that there are changes in transcription factor expression and enrichment for apoptosis and programmed cell death components (Doering, Crawford et al. 2012).

In this study, we analyzed the gene expression profile of young, short telomere, and older adults to test the relevant pathways involved in telomere-mediated CD8$^+$ T<sub>EMRA</sub> survival and dysfunction. With most previous studies in mouse models or in human cancers, we chose to use our three groups to study gene expression in CD8$^+$ T<sub>EMRAS</sub> in hopes of finding differences that more robustly represent transcriptional changes in immune aging and as a result of the short telomere rather than in an animal model. As discussed in Chapter 2, young individuals with short telomeres ubiquitously replicate the aging phenotype of significantly increased CD8$^+$ T<sub>EMRAs</sub>. With this surprising finding and evidence of increased apoptosis in the ST and OA cohorts, we hypothesized that gene expression analysis of all three groups would point to important drivers of the exhausted CD8$^+$ T cell phenotype in a relevant human model. In this Chapter, however, I found that ST subjects show a divergent pattern of gene expression in comparison to OA and YC, which are unexpectedly more alike.

4.2 Results

4.2.1 Quantification of up- and down-regulated genes in ST compared to YC shows opposite trend to the OA to YC comparison

I sorted T<sub>EMRA</sub> populations from 4 individuals per experimental group (YC, ST, and OA) and studied their gene expression profiles using microarray. We hypothesized that CD8$^+$ T<sub>EMRA</sub> cells from young ST subjects and OA would be more similar to each
other, and would show distinct gene expression patterns compared to YC due to the skewed \( T_{EMRA} \) population representation by immunophenotyping shown in Chapter 2. First we examined the number of genes that are differentially expressed compared to YC. We found that ST \( T_{EMRA} \) had twice as many genes downregulated compared to YC (Fig. 4.1a). In contrast, OA had comparable numbers of down and up-regulated genes (defined as 2 SD different from the mean, Fig. 4.1c). These data are consistent with short telomeres downregulating gene expression as has been seen previously in prior mouse studies (Guo, Parry et al. 2011; Alder, Barkauskas et al. 2015). When over- and under-expressed all groups are compared simultaneously, \( T_{EMRA} \) cells from both normal telomere YC and OA groups are more similar to one another (Fig 4.1d), pointing to evidence that short telomeres have a distinct expression profile.

### 4.2.2 ST and OA gene expression patterns point to different mechanisms important for immune cell maintenance

We next classified the up- and down-regulated genes using the Ingenuity Pathway Analysis (IPA) to better understand how they might interact in common networks in vivo and contribute to immune aging. This IPA analysis classifies the differentially expressed genes represented in Fig.4.1 (> or < ~2SD depending on comparison) into pre-existing canonical pathways. We then analyzed the output of statistically different pathways that were both up- and down-regulated in one-to-one comparisons. We independently compared both ST and OA to YC. Among the top 20 statistically up-and down-regulated pathways from each comparison, we found one common up-regulated pathway and 2
common down-regulated pathways (Fig. 4.2). Among the top 5 pathways in the ST vs. YC analysis, 3 involved p53 function including Cyclins and Cell Cycle Regulation, p53 Signaling, and Cell Cycle: G1/S Checkpoint Regulation. In contrast, among the top 5 pathways in OA vs. YC, one involved extrinsic apoptosis and in the top 20, 3 involved extrinsic apoptosis including Apoptosis Signaling, Death Receptor Signaling, Myc Mediated Apoptosis Signaling, and Calcium-Induced T Lymphocyte Apoptosis.

Surprisingly, there was no overlap of cell-cycle or apoptosis-mediated pathways in the upregulated IPA comparisons of ST and OA vs. YC. Therefore, these gene expression data suggested that, though ST and OA subjects share commonalities of immune aging immunophenotypes and propensity to apoptosis in vitro, the underlying mechanisms that are causing the immune deficient state are likely inherently different and short telomeres can drive an independent mechanism of immune aging.
Chapter 4: TERMINALLY DIFFERENTIATED CD8+ T CELLS REVEAL A UNIQUE GENE EXPRESSION SIGNATURE IN THE SETTING OF SHORT TELOMERES

Figure 4.1 Quantification and profile of up- and down-regulated genes in CD8+ T_{EMRA} cells.

a-c. Number of genes up- and down-regulated in individual ANOVA analyses.

Differential gene expression in ST compared to YC (A), ST compared to OA (B), and OA compared to ST (C). d. Heatmap displaying supervised clustering of differentially expressed genes from all subject groups. n = 3,188 probes analyzed.
Chapter 4: TERMINALLY DIFFERENTIATED CD8⁺ T CELLS REVEAL A UNIQUE GENE EXPRESSION SIGNATURE IN THE SETTING OF SHORT TELOMERES

Figure 4.1

A

ST vs YC

n=982
n=2131

2SD Up
2SD Down

B

ST vs OA

n=732
n=2237

C

OA vs YC

n=1760
n=1386

D

Telomere Syndrome
Older Adults
Young Controls

Log₂-Fold Change

-4.9
4.9
Chapter 4: TERMINALLY DIFFERENTIATED CD8⁺ T CELLS REVEAL A UNIQUE GENE EXPRESSION SIGNATURE IN THE SETTING OF SHORT TELOMERES

Figure 4.2

Ingenuity Pathway Analysis (IPA) reveals gene expression differences in common pathways.

Red bars show upregulated pathways in ST compared to YC (left) and OA compared to YC (right). Bar length represents significance as –log(p-value). Blue bars show downregulated pathways in the same comparisons.
4.3 Discussion

Immune aging is a ubiquitous mechanism of decreased ability to respond to pathogens and cancer. A well known immunophenotype of this phenomenon is the accumulation of CD8+ TEMRAs, a terminally-differentiated population of effector cells with decreased cytotoxic capacity and propensity towards apoptosis. Determining the molecular drivers of this terminal differentiation in hopes of reversing the loss of cytotoxic capacity is a broad goal in the immunology field (Duraiswamy, Ibegbu et al. 2011). Previous work in mouse models of chronic LCMV infection showed upregulation of inhibitory receptors in virus-specific CD8+ T cells. These data have been transformed into cancer immunotherapy targeting a few inhibitory receptors with some success. In Chapter 2, we found evidence of similar accumulation in young ST subjects and OAs. However, here we found evidence suggesting divergence in the gene expression program in these TEMRAs from OAs.

As shown in Chapter 2, both ST and OA CD8+ populations show significantly more TEMRA cells than YC subjects. Independent analyses of ST and OA gene expression in comparison to YC surprisingly showed more differences than similarities in ST than in OA gene expression in CD8+ TEMRAs. In ST subjects, this profile shows a larger percentage of genes with significantly decreased expression. However, the upregulated genes are involved in cell cycle checkpoints and the p53 pathway, which are known responses to short, dysfunctional telomeres (Chin, Artandi et al. 1999). In contrast, OA subjects upregulated genes in pathways important for immune defense and extrinsic apoptosis.
We have found that both ST and OA T cells show increased CD95 expression are prone to stimulation-induced apoptosis (Chapter 2 and Chapter 5). As noted in previous studies, short telomeres have been shown to cause cell death and senescence by inducing a DNA damage response (Lee, Blasco et al. 1998; Hemann, Rudolph et al. 2001; d'Adda di Fagagna, Reaper et al. 2003). Our studies here indicate that dysregulated genes from both ST and OA groups enrich for apoptotic pathways, but favor different mechanisms, suggesting that different apoptotic pathways contribute to the short telomere apoptosis and aging associated apoptosis that occurs in humans with normal telomere length.

The experiments presented in this chapter are limited in that we have not validated the gene expression changes seen with microarray by other quantitative methods. One experiment that would test our model is to identify whether TCR stimulation is more likely to upregulate a telomere-associated DNA damage response. In contrast, our model would predict that stimulated T cells from OAs would more likely be prone to extrinsic apoptosis possibly because of altered TCR signaling. Future studies will undoubtedly shed light on how T cells age in the setting of normal and short telomere length.
4.4 Materials and Methods

4.4.1 Sample Preparation for Flow Cytometry, Sorting and Antibodies. After thawing, PBMCs were treated to lyse contaminating red cells (Red Cell Lysis Buffer, eBioscience). Samples were washed and resuspended in 100uL 2% FBS/PBS solution for antibody staining. Ideal antibody concentrations were determined by serial dilutions. Antibody staining was performed for 20 minutes in the dark at 4°C. Antibodies, their conjugates, clone identifiers and suppliers are listed in Supplementary Table 1.

4.4.2 Cell Sorting. For the gene expression microarray, an average of 20,000 CD3⁺CD8⁺CD45RA⁺CCR7⁻ (CD8⁺ TEMRA) cells were sorted from PBMCs using a MoFlo XPD (Beckman Coulter) directly into TRIzol (Invitrogen) and stored at -80°C. Total RNA was extracted using the Agencourt RNAdvance Tissue kit (Beckman Coulter).

4.4.3 TEMRA Gene Expression Microarray and Analysis. RNA was amplified and reverse-transcribed using the Ovation® Pico WTA System V2 (NuGEN). Transcriptional profiling was performed at the Johns Hopkins University Deep Sequencing and Microarray Core Facility using Affymetrix GeneChip® PrimeView™ Human Gene Expression Array. CEL file data were extracted and normalized with Partek Genomics Suite software using the Robust Multi-array Average (RMA) algorithm. To ensure better understanding of the transcriptome, the array’s Affy probeset annotation
was updated to contemporary HUGO/NCBI nomenclature and those that could not be aligned to the genome were excluded.

A Student’s t-test ANOVA was used to compare transcript expression levels between young controls, old controls, and affected subjects, and the 48,880 aligned transcripts with gene-level annotation were selected for further analysis. These genes’ log2-fold change distribution was then evaluated to determine their standard deviation (SD) up or down from the mean of 0 (i.e. no change). Genes with greater than 2SD change in a given comparison were considered to have significant differential expression. This corresponded to a linear fold change of approximately +/- 2.61 fold for Short Telomere versus Young Control, 2.58 for Short Telomere versus Older Adult and 2.10 for Older Adult versus Younger Control ANOVAs, resolving to 962 up and 2,131 down regulated transcripts for the first, 732 up and 2,237 down for the second and 1,760 up and 1,386 for the last. These probeset transcripts were uploaded to the Ingenuity Pathway Analysis (IPA) platform to evaluate their functional relevance in canonical pathways. P-values for pathway studies were calculated using Fisher’s exact test. Our MIAME-compliant microarray data have been deposited in the NCBI GEO database (Series GSE77525).
Chapter 4: TERMINALLY DIFFERENTIATED CD8+ T CELLS REVEAL A UNIQUE GENE
EXPRESSION SIGNATURE IN THE SETTING OF SHORT TELOMERES

References


Chin, L., S. E. Artandi, et al. (1999). "p53 deficiency rescues the adverse effects of
telomere loss and cooperates with telomere dysfunction to accelerate


Connected Genes and Pathways Involved in CD8+ T Cell Exhaustion versus

profiles of programmed death-1(hi) CD8 T cells in healthy human adults." Journal
of immunology (Baltimore, Md. : 1950) 186(7): 4200-4212.

Guo, N., E. M. Parry, et al. (2011). "Short telomeres compromise β-cell signaling and


Saule, P., J. Trauet, et al. (2006). "Accumulation of memory T cells from childhood to
old age: central and effector memory cells in CD4(+) versus effector memory and
terminally differentiated memory cells in CD8(+) compartment." Mechanisms of
ageing and development 127(3): 274-281.
Chapter 5:

Short Telomere and Older Adult T Cells Reveal Distinct Functional Responses to Stimulation

Christa L. Wagner¹,², Xuhang Li³, Mary Armanios¹,⁴

Departments of Oncology¹, Program in Cellular and Molecular Medicine², Medicine³, and the McKusick-Nathans Institute of Genetic Medicine⁴, Johns Hopkins University School of Medicine, Baltimore, MD
Chapter 5: Short Telomere and Older Adult T Cells Reveal Distinct Functional Responses to Stimulation

5.1 Introduction

Immune responses are complex cascades involving many different cell types and signaling pathways. Cytokines and chemokines are particularly important intermediates that orchestrate responses to a range of stimuli including pathogens and cancer. They act as the link between the innate and adaptive immune system to prime for a response and continue to organize and execute adaptive immune responses. Measuring levels of certain cytokines can give hints as to the immune status of a patient, and therefore previous work has been conducted to define a cytokine profile associated with immune aging. Most studies quantify plasma or circulating cytokine levels and have agreed on a profile of decreased IL-2 and IL2R, and increased IL-6 and TNF-α as characteristic features of immune aging (Pawelec, Wagner et al. 1999; Prelog 2006).

General trends suggest a shift in the Th1/Th2 cytokine axis and increased circulating inflammatory cytokines with aging (Montecino-Rodriguez, Berent-Maoz et al. 2013). Autoimmune conditions mediated by chronic inflammation, especially rheumatoid arthritis (RA), have increased incidence with aging and have defined an “autoimmune-risk phenotype” (Costenbader, Prescott et al. 2011). Several studies have shown correlations between autoimmunity and telomere length (Andrews, Fujii et al. 2010; Hohensinner, Goronzy et al. 2011). Our group has anecdotally observed several incidences of autoimmunity in our ST cohort (unpublished). Despite these correlations, the hypothesis of short telomeres driving immune aging including its associated cytokine release patterns remains unstudied.
Our observations of quantitative and qualitative characteristics resembling immune aging in infection-prone ST patients directed us to test the hypothesis that ST T cell functional would resemble OA. To test T cell functionality, I stimulated T cells \textit{in vitro} and assessed rates of apoptosis and quantity and quality of cytokine release in a short term culture. My results show an overactive response solely in ST cells, supporting our hypothesis that short telomeres drive a distinct pattern of immune aging that is different in normal telomere aged T cells.

5.2 Results

5.2.1 ST and OA T cells show increased rates of apoptosis following T cell receptor stimulation \textit{in vitro}

To study potential mechanisms underlying the immune aging defect, we stimulated T cells from all groups with antibodies against the T cell receptor (TCR, CD3 and CD28). As was previously shown, lymphocytes from telomere syndrome subjects showed a rapid decline in proliferative capacity correlating with increased time in culture (Knudson, Kulkarni et al. 2005). In a short term experiment, we found ST and OA samples can maintain proliferative competency in culture for 2 days, but by day 4, the rate of culture expansion of ST T cells is significantly lower than YC (Fig. 5.1a). To test whether this effect is due to apoptosis, we analyzed T cells by Annexin V and PI staining after 2 days in culture and discovered that both ST and OA showed higher rates of cell death than YC (P< .01 and P <.001 respectively, Fig. 5.1b-c).
5.2.2 Stimulation of TCR with CD3/CD28 leads to dysregulated cytokine response in T cells with short telomeres

We next tested whether ST limit T cell viability because of defects in cytokine production. As a control for apoptosis-induced cytokine responses, we incubated select YC samples with camptothecin (CPT). CPT is a topoisomerase I inhibitor that induces single-stranded DNA breaks, leading to apoptosis through a p53-dependant DNA damage response (Siu, Lau et al. 2004). Surprisingly, we found an extremely skewed and upregulated cytokine release profile in ST patients compared to both control groups. While it is known that cytokine release is affected in aging with increases in inflammatory cytokines being most prominent (Costenbader, Prescott et al. 2011), ST subjects showed increased cytokine release in multiple types of cytokines (Fig. 5.2, 5.3b). ST subjects showed increases in comparison to YC in almost half of the cytokines queried, while OA subjects showed up-regulation in only 1 of 24 and down-regulation of 2 cytokines compared to YC (Fig. 5.3a,c). The upregulation of the cytokines subsets in ST subjects appeared random and did not follow a specific T helper axis. This result indicated that a dysregulated stimulatory response may reflect the inability of the cells to fight off infections in vivo.

5.2.3 Short telomere mice recapitulate the human cytokine release phenotype

To confirm that the dysregulated cytokine response in human cells was T cell-specific and an effect of short telomeres, I tested whether mice with short telomeres (mTR−/− G5) may show a similar pattern as ST patients. In this experiment, I used separate
cultures of total CD3$^+$ and enriched CD8$^+$ T cells to study subset-specific responses. I analyzed only 3 cytokines by ELISA in this analysis. IL-2 as the most important cytokine for mounting an immune response, IL-6 as a pro-inflammatory cytokine, and IL-5 as a CD4$^+$-specific cytokine. In striking similarity to the human results, short telomere mice showed significant increases in cytokine release in both CD3$^+$ and CD8$^+$ T cells in comparison to wild-type following two days of stimulation (Fig. 5.4).
Chapter 5: Short Telomere and Older Adult T Cells Reveal Distinct Functional Responses to Stimulation

Figure 5.1

Figure 5.1 ST and OA T cells show increased apoptotic response to TCR stimulation.

a. Quantification of cell expansion by the WST assay. D2 % of D0 represents expansion of given populations after 2 days of stimulation with CD3/CD28. D4 % of D2 represents expansion of given populations from day 2 to day 4 with continued CD3/CD28 stimulation. D2 data represents results from 5 independent experiments. D4 data comes from one experiment in which OA samples were not included. b. Flow cytometry gating strategy for assessment of apoptosis with Annexin V and PI. c. Quantification of Annexin$^+$PI$^-$ (early) and Annexin$^+$PI$^{lo}$ (late) apoptosis following two days (D2) of CD3/CD28 stimulation in human T cells.
Chapter 5: Short Telomere and Older Adult T Cells Reveal Distinct Functional Responses to Stimulation

Figure 5.2 Cytokine release profiles after TCR stimulation.

Quantification of 24 cytokines in cell culture supernatant from YC, ST, and OA groups after 2 days (D2) of TCR stimulation. YC+CPT represents select YC samples incubated with 10μM camptothecin as a control for an apoptotic response. Grey circles indicate cells incubated without stimulation, black squares indicate cells incubated with antibodies against CD3 and CD28. Cytokine levels were quantified using a human multiplex panel with Luminex technology. P-values are indicated above stimulated sample comparisons where P-values ≤ 0.1. The 25th cytokine from the multiplex analysis, Eotaxin, was omitted due to all readings falling below the limit of detection.
Chapter 5: Short Telomere and Older Adult T Cells Reveal Distinct Functional Responses to Stimulation

Figure 5.2

No stimulation

+CD3/CD28

Figure 5.2
Figure 5.3 Quantification of dysregulated cytokine release in human T cells following CD3/CD28 stimulation.

a. Number of cytokines with skewed release profiles in ST and OA in comparison to YC values, respectively. b. Magnitude of skewing of cytokines upregulated in ST to YC comparison. c. Venn diagram of individual group comparisons. First group listed in the comparison shows the skewing, indicated by ↑ for increased and ↓ for decreased cytokine release. n= 12 YC, 7 ST, and 5 OA per group.
Chapter 5: Short Telomere and Older Adult T Cells Reveal Distinct Functional Responses to Stimulation

Figure 5.3

**a**

Number of cytokines out of 24

- **n = 11** of 24
- **n = 3** of 24

**b**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Fold Change</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>↑ IL-1β</td>
<td>1.46</td>
<td>0.1</td>
</tr>
<tr>
<td>↑ IL-1RA</td>
<td>1.33</td>
<td>0.01</td>
</tr>
<tr>
<td>↑ IL-2</td>
<td>1.61</td>
<td>0.08</td>
</tr>
<tr>
<td>↑ IL-12</td>
<td>1.57</td>
<td>0.09</td>
</tr>
<tr>
<td>↑ IL-15</td>
<td>1.23</td>
<td>0.08</td>
</tr>
<tr>
<td>↑ IL-17</td>
<td>1.88</td>
<td>0.09</td>
</tr>
<tr>
<td>↑ MIG</td>
<td>1.82</td>
<td>0.06</td>
</tr>
<tr>
<td>↑ MIP-1α</td>
<td>1.87</td>
<td>0.005</td>
</tr>
<tr>
<td>↑ MIP-1β</td>
<td>1.43</td>
<td>0.06</td>
</tr>
<tr>
<td>↑ RANTES</td>
<td>1.83</td>
<td>0.02</td>
</tr>
</tbody>
</table>

**c**

Venn diagram showing cytokines upregulated in ST vs. YC, OA vs. YC, and ST vs. OA conditions.

- ↑ IL-1β
- ↑ IL-1RA
- ↑ IL-2
- ↑ IL-12
- ↑ IL-17
- ↑ MIG
- ↑ MIP-1β
- ↑ RANTES
- ↓ IL-6
- ↓ IL-7
- ↑ IL-2R
- ↑ IL-15
- ↑ MIP-1α
- ↑ IL-6
- ↑ IL-10
Figure 5.4 Mice with short telomeres recapitulate human results with increased cytokine release.

Cytokine levels quantified in cell supernatant after 48 hours of CD3/CD28 stimulation. Release of cytokines IL-2, IL-5, and IL-6 from a. CD3+ T cells and b. CD8+ T cells, both isolated with negative selection. Cytokine levels were quantified using ELISA. Samples are pooled n= 3 mice per group. *P <.05; ***P <.001.
Chapter 5: Short Telomere and Older Adult T Cells Reveal Distinct Functional Responses to Stimulation

5.3 Discussion

Cytokines are ubiquitous immune molecules and are required for coordinating and orchestrating immune responses to pathogens and cancer. Previous research has been conducted to find a connection between altered cytokine profiles and immune aging. Most of these studies consider only basal levels of cytokines in the plasma, which has made a definitive cytokine-related immune aging phenotype hard to find (Pawelec, Wagner et al. 1999). However, alterations in cytokine release as a response to stimulation of the T cell receptor can be indicative of problems with proper T cell functionality. In this chapter, we queried the functionality of ST T cells in vitro through TCR stimulation and found evidence that short telomeres may drive premature apoptosis of peripheral T cells.

For a snapshot of T cell functionality, I conducted experiments with 48 hours of TCR stimulation using antibodies against CD3 and CD28 to generate a non-specific immune response. As in previous studies, proliferative capacity of ST T cells was not inhibited within 2 days of stimulation as we observed through equal EdU incorporation and similar numbers of viable cells in ST and YC cultures (Knudson, Kulkarni et al. 2005). However, the design of this study allowed for comparison to the immune aging phenotype, and analysis of apoptosis in all groups showed significant cell death in ST and OA T cells after 2 days of TCR stimulation. Additionally, shrinking of the ST viable cell pool between days 2 and 4 of stimulation indicated that, though ST cells are capable of responding quickly to stimulation, the response is not sustainable and cells quickly
undergo apoptosis. This could contribute to the lymphopenia and infection susceptibility we documented in ST patients.

We had hypothesized that ST T cells would show changes in cytokine release in response to stimulation mirroring changes in the OA cohort. Surprisingly, ST subjects showed paradoxical upregulation of cytokine responses while OA cells showed changes in only 3 cytokines, with 2 of the 3 showing decreased levels compared to YC. ST responses were not directed in any one lineage (i.e. Th1, Th2, Th17), suggesting stimulation of the TCR causes a non-specific hyperactive response. Concerns that the negative selection used to isolate human T cells wasn’t selective enough were abated with confirmatory results of upregulated cytokines in the G5 mouse model, in both CD3⁺ and CD8⁺ cells.

How short telomeres cause an increase in cytokine release is currently unclear. It may be that this response reflects an upregulated DNA damage response that occurs with stimulation and is associated with this program. Short telomeres cause transcriptional changes in senescence but we found no evidence of senescence here. One way to test whether this effect is caused by a DNA damage response versus a telomere dysfunction response would be to see if gamma-irradiation or DNA damaging chemotherapy would induce similar patterns of cytokine release. What is clear from our data is that the cytokine dysregulation is not a feature of immune aging in normal telomere T cells and is unique to both short telomere human and mouse cells.
5.4 Materials and Methods

5.4.1 T cell Stimulation and Proliferation. CD3\(^+\) T cells were isolated from PBMCs (EasySep Human T Cell Enrichment Kit, StemCell Technologies). Cells were resuspended in media in flat-bottomed 96-well plates (Corning) at 8 \(\times\) 10\(^5\) cells/mL. Anti-CD3 and anti-CD28 covalently coupled to magnetic beads at a ratio of 1:1 were used for stimulation (Dynabeads Human T-Activator CD3/CD28, Life Technologies). The viable cell count was measured using the colorimetric WST assay (Dojindo Molecular Technologies). Cell death was analyzed with flow cytometry with detection of cell surface phosphatidylserine (PS) with APC-labeled Annexin V in Binding Buffer (BD Biosciences) and counterstained with propidium iodide (PI, BD Biosciences). 15,000 events were collected for each sample.

5.4.2 Secreted Cytokine Analysis. Secreted cytokines were measured in media collected after two days of T cell stimulation alongside an identical aliquot of cells grown in culture without stimulation. Media from triplicate samples (450 uL total) were pooled and frozen until analysis. T cells treated with camptothecin were used as a control to test for the specificity of cytokine release after an apoptotic response. We used the Cytokine Human Magnetic 25-Plex Panel and the Luminex multiplex system (Luminex Platform, ThermoFisher) to simultaneously quantify 25 secreted cytokines and chemokines in duplicate as previously described (Alex, Zachos et al. 2009). Because IL-2 readings fell above the standard curves, this cytokine was measured by ELISA (Human IL-2 kit, Invitrogen).
5.4.3 Mice. Mice were housed at the Johns Hopkins University School of Medicine campus, and all the procedures were approved by the Institutional Animal Care and Use Committee. *mTR*⁻/⁻ mice on the C57BL/6J background were derived and maintained as previously described (Blasco et al. 1997).

5.4.4 Mouse T cell Stimulation and Cytokine Studies. Mouse CD3⁺ T cells or CD8⁺ T cells were stimulated as described for human T cells except using mouse antibodies (Dynabeads Mouse T-Activator CD3/CD28, Life Technologies). Cell numbers and apoptosis fractions were then analyzed as outlined for the human studies. Media from stimulated mouse T cells were harvested as for human studies and secreted cytokines were measured by ELISA using the IL-2 and IL-6 LEGEND MAX kits and IL-5 ELISA MAX kit (BioLegend).

5.4.5 Statistics. We used GraphPad Prism v.6.05 (GraphPad Software) to generate the graphics, and unless otherwise noted, for statistical comparisons. Means were compared by Student’s *t*-test. Unless otherwise noted, all P-values shown are two-sided.
References


Supplementary Table 1. Summary of qualitative and quantitative defects in ST and OA subjects described in this thesis.

<table>
<thead>
<tr>
<th>Category / Immune phenotype</th>
<th>Short Telomere</th>
<th>Older Adults</th>
</tr>
</thead>
<tbody>
<tr>
<td>↓ CD4 T cell count</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Skewed CD4 : CD8 T cell ratio</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>↓ Naïve T cells</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>↓ TREC</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>↓ TCR repertoire</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>↑ T cell apoptosis</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>↑CD95 protein expression</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>↑PD-1 inhibitory receptor expression</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Unique $T_{EMRA}$ gene expression profile</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>De-regulated cytokine response</td>
<td>X</td>
<td></td>
</tr>
</tbody>
</table>
Supplementary Table 2. Human Flow Cytometry Antibodies Used (n=29)

Antibodies were individually titrated for optimal staining concentration.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Fluorochrome</th>
<th>Clone</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>AF700</td>
<td>UCHT1</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD3</td>
<td>APC</td>
<td>UCHT1</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD3</td>
<td>FITC</td>
<td>UCHT1</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD4</td>
<td>AF488/FITC</td>
<td>RPA-T4</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD4</td>
<td>BV605</td>
<td>OKT-4</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD4</td>
<td>PE-CY5</td>
<td>RPA-T4</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD4</td>
<td>PerCP/Cy5.5</td>
<td>RPA-T4</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD8</td>
<td>AF700</td>
<td>OKT-8</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD8</td>
<td>PE-CF594</td>
<td>RPA-T8</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD8</td>
<td>V450</td>
<td>RPA-T8</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD8a</td>
<td>FITC</td>
<td>RPA-T8</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD8a</td>
<td>PE</td>
<td>RPA-T8</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD19</td>
<td>BV421</td>
<td>HIB19</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD19</td>
<td>BV560</td>
<td>HIB19</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD25</td>
<td>PE/CY7</td>
<td>BC96</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD27</td>
<td>BV560</td>
<td>O323</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD28</td>
<td>PE-CY7</td>
<td>CD28.2</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD31</td>
<td>FITC</td>
<td>WM59</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD31</td>
<td>PE</td>
<td>WM59</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD45RA</td>
<td>APC/CY7</td>
<td>HI100</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD56</td>
<td>PE-CF594</td>
<td>B159</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD56</td>
<td>PE-CY7</td>
<td>HCD56</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD57</td>
<td>FITC</td>
<td>HCD57</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD95</td>
<td>PE-CF594</td>
<td>DX2</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD197 (CCR7)</td>
<td>BV421</td>
<td>G043H7</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD197 (CCR7)</td>
<td>PE</td>
<td>3D12</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD197 (CCR7)</td>
<td>V450</td>
<td>150503</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD279 (PD-1)</td>
<td>APC</td>
<td>ebioJ105</td>
<td>eBioscience</td>
</tr>
<tr>
<td>Ki67</td>
<td>PerCP Cy5.5</td>
<td>B56</td>
<td>BD Biosciences</td>
</tr>
</tbody>
</table>
Supplementary Table 3. Mouse Flow Cytometry Antibodies Used (n=18)

Antibodies were individually titrated for optimal staining concentration.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Fluorochrome</th>
<th>Clone #</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>AF700</td>
<td>17A2</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD3e</td>
<td>PE</td>
<td>145-2C11</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD4</td>
<td>FITC</td>
<td>RM4-5</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD8a</td>
<td>APC</td>
<td>53-6.7</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD8a</td>
<td>BV605</td>
<td>53-6.7</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD19</td>
<td>PECF594</td>
<td>1D3</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD19</td>
<td>PECY7</td>
<td>6D5</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD25</td>
<td>BV421</td>
<td>BC61</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD44</td>
<td>PECY7</td>
<td>IM7</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD45.1</td>
<td>AF700</td>
<td>A20</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD45.1</td>
<td>PE</td>
<td>A20</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD45.2</td>
<td>PECY7</td>
<td>104</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD45.2</td>
<td>PerCP-Cy5.5</td>
<td>104</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD48</td>
<td>PECY7</td>
<td>HM48-1</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD117 (c-kit)</td>
<td>APC</td>
<td>2B8</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD150</td>
<td>PE</td>
<td>TC15-12F12.2</td>
<td>Biolegend</td>
</tr>
<tr>
<td>Lineage panel:</td>
<td>FITC</td>
<td>n/a</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD3/GR1/CD11b/CD45R (B220)/Ter-119</td>
<td>FITC</td>
<td>n/a</td>
<td>Biolegend</td>
</tr>
<tr>
<td>Ly-6a/e (sca-1)</td>
<td>PE</td>
<td>E13-161.7</td>
<td>Biolegend</td>
</tr>
</tbody>
</table>
Christa L. Wagner
Curriculum Vitae • March 21, 2016

Johns Hopkins University School of Medicine
725 N. Wolfe St.
PCTB 607
Baltimore, MD 21205
E-mail: cwagner@jhmi.edu

EDUCATION

2010-Feb 2016 PhD Candidate, Cellular and Molecular Medicine, Johns Hopkins University School of Medicine, Baltimore, MD

2008 BA in Biochemistry with Honors, Minor in Religion, Oberlin College, Oberlin, OH

EXPERIENCE

2010-Feb 2016 Graduate Student Researcher, Department of Oncology and Institute of Genetic Medicine, Johns Hopkins University School of Medicine, Baltimore, MD
Principle Investigator: Mary Armanios, MD

• Spearheaded independent research project investigating the immunodeficiency phenotype in short telomere syndromes, a group of premature aging disorders associated with increased risks of cancer and lung disease.
• Coordinated effort to recruit and procure clinical samples from human subjects for my thesis project.
• Drafted a first-author publication while collaborating on several human genetics projects with colleagues, resulting in 3 co-authorships.
• Received my graduate stipend through philanthropic support as a result of presenting before private financial donors to advocate for my research.
• Mentored newer PhD candidates and undergraduate interns.

Sep-Oct 2015 Public Policy Fellow, Ovarian Cancer National Alliance (OCNA), Washington, DC

• Engaged policymakers from all 50 states to prioritize ovarian cancer care as part of OCNA’s state report cards.
• Drafted summary of the Notice of Proposed Rulemaking (NPRM) for the Common Rule to assist senior staff in writing an organizational response.
• Represented OCNA at Congressional briefings, hearings, and patient advocacy organization meetings; briefed senior office staff and drafted communication with constituents following these meetings.
• Managed website content for patient and advocate outreach on various topics.
• Advocated for medical research during September Hill Day; raised awareness of ovarian cancer and the importance of basic science and cancer research among multiple congressional staff.
2008-2010 **Research Technician**, Departments of Anesthesiology and Pharmacology, Weill Cornell Medical College, New York, NY  
Principle Investigator: Paul Heerdt, MD, PhD  
- Contributed to clinical research on understanding the mechanisms of adverse events related to anesthesia.  
- Managed coordination with clinical staff for sample and data collection.  
- Established protocols and oversaw compliance for use of large animal models.  
- Reported our findings as a poster presentation at an international meeting.

2007-2008 **Undergraduate Researcher and Honors Student**, Department of Biochemistry, Oberlin College, Oberlin, OH  
Principle Investigators: Jason Belitzky, PhD and Catherine Oertel, PhD  
- Honors Thesis topic: Amino Acids as Ligands for Chiral Metal-Organic Frameworks  
- Undergraduate research project: Synthetic melanin as an environmental remediation agent

2007 **Summer Talent Identification Program Intern**, Human Target Validation, GlaxoSmithKline, King of Prussia, PA  
- Independent research project assessing efficacy of new drugs targeting high cholesterol in a cell culture model system.

2005 **Summer Research Intern**, Lankenau Institute for Medical Research, Wynnewood, PA  
Principle Investigator: Thomas O’Brien, PhD  
- Collaborated with senior researchers to study a mouse model of melanoma through genome sequencing analysis.

**HONORS AND AWARDS**

- April 2016: **Clinical Immunology Society Travel Award**, CIS Annual Meeting, Boston, MA  
- October 2014-Present: **Turock Predoctoral Fellowship**, Johns Hopkins University School of Medicine  
- 2014: **Outstanding Journal Club Presentation Prize**, Johns Hopkins Department of Molecular Biology and Genetics  
- 2013: 1st place **Translational Research Poster Session**, Johns Hopkins Department of Oncology, Fellows Research Day
PROFESSIONAL MEMBERSHIPS

• Clinical Immunology Society (2016-Present)
• American Society for Cell Biology (2016-Present)
• Association for Women in Science (2015-Present)
• American Society of Hematology (2014-Present)
• AAAS (2010-Present)
• American Chemical Society (2008-Present)
• Sigma Xi (2008-Present)

SERVICE AND LEADERSHIP EXPERIENCE

• Encouraged alumni financial contributions to support health and wellness of current students as a board member for the Heisman Club of Oberlin College (September 2015-Present).
• Coached novice female runners in a Baltimore-based program, leading to personal bests and at-capacity enrollment (2013-Present).
• Volunteered in numerous diverse programs including outreach to the homeless, nursing home programming, tutoring high school students, and Oberlin College representative to local high school students (2009-Present).

PUBLICATIONS


ORAL PRESENTATIONS

Wagner, CL, Hanumanthu VS, Kanakry CG, Applegate CD, Talbot Jr C, Luznik L, Armanios M; T cell Immunosenescence and Primary Immunodeficiency in Telomerase Mutation Carriers; Clinical Immunology Society Annual Meeting; Boston, MA; planned April 2016.

POSTER PRESENTATIONS


Heerdt PM, Wagner CL, Vladagina O, Fontes M; Nitrosative Stress in Cardiac Surgical Patients: The Potential Role of Intraoperative Hyperglycemia; American Society of Anesthesiologists Annual Meeting, New Orleans, October 2009; Poster #A1109.


Wagner CL, Heerdt PM, Sunaga H, Savarese JJ; The variant contribution of histamine to the hemodynamic effects of CW002, a cysteine-reversible neuromuscular blocking drug; International Anesthesiology Research Society (IARS) Annual Meeting, San Diego, March 2009; Poster #S-291.