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Elucidating Molecular Changes in $CD8^+$ T cells During Aging

by

Mark Yungjie Jeng

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biomedical Sciences

in the
GRADUATE DIVISION
of the
UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

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by
Mark Yungjie Jeng

DEDICATION

I dedicate this dissertation to my parents, Peter and Sharon Jeng. I don't say this enough, but I am forever grateful for the unfailing love and support you both have given me throughout my life. I owe so much of who I am to you two. Thank you for everything—I love you both so much!

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I have so many people I want to acknowledge for their continued help and support throughout my graduate program. To my advisor, Melanie: thank you for being such a fiercely strong and inspiring mentor during the last 5 years. I have grown so much as scientist, and as a person, because of you. To the other members of my thesis committee, Eric and Shomy: thank you both for your insightful advice and deep knowledge—I've learned an incredible amount from my discussions with both of you. To all the members of the Ott lab, past and present: thank you for sharing everything with me, your wisdom and advice, your failures and achievements, your tears and laughter. You make me look forward to coming into lab everyday, even when science does its best to discourage me. Special shout-outs to Holly and Gregory, for recruiting me to the lab and ensuring I survived first few years here, as well as Krystal and Ibs, for all the wildly inappropriate conversations we have and ensuring my blood glucose levels never dropped too low. To all my friends and fellow classmates at UCSF: you have all contributed hugely to my imposter syndrome, but also helped me through it. I am so proud of all of you and can't wait to celebrate all of your future accomplishments! I want to especially thank my 'big brothers' Sai-wing and Richard: thank you for welcoming me into your family, for pushing me out of my comfort zone, for keeping me safe, and for always being there for me—you've both made San Francisco feel like home to me. Finally, to my parents, Peter and Sharon, and my brothers, Jack and Brian: thank you guys for being such a solid and unfailing support system throughout all of this.

Elucidating Molecular Changes in $CD8^+$ T cells During Aging

Mark Yungjie Jeng

Thesis Committee:

Melanie Ott (Advisor)

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Abstract

One of the consequences of human aging is an overall decline in immune function. A hallmark of this aging process, termed immunosenescence, is the downregulation of co-receptor CD28 from the surface of $CD8^+$ T cells. This population of terminally differentiated $CD8^+CD28^-$ T cells accumulates during aging and chronic infections, and is linked to many age-related diseases observed in elderly individuals. In this thesis, we explore the molecular factors regulating $CD8^+CD28^-$ T cell fate and function. In **Chapter III**, we find that resting $CD8^+CD28^-$ T cells possess a unique glycolytic profile that is associated with their enhanced cytotoxicity and decreased expression of NAD^+-dependent protein deacetylase SIRT1. Global gene–expression profiling identified the transcription factor FoxO1 as a SIRT1-target involved in the transcriptional reprogramming of $CD8^+CD28^-$ T cells. FoxO1 is proteasomally degraded in $CD8^+CD28^-$ T cells, and inhibiting its activity in $CD8^+CD28^+$ T cells recapitulates the metabolic and cytotoxic phenotype of resting $CD8^+CD28^-$ T cells. In **Chapter IV**, we explore the signaling and functional consequences of IL-15, a pro-memory cytokine that serves as a TCR-independent activation signal for $CD8^+CD28^-$ T cells. We find that IL-15 signals through mTOR to upregulate a metabolic program conducive for effector T cell function. Targeting $CD8^+CD28^-$ T

cells with a SIRT1 activator or glucose-starvation suppresses IL-15-induced cytotoxicity and proliferation. Altogether, this body of work identifies new molecular pathways that regulate the function of human $CD8^{+}CD28^{-}$ T cells—providing deeper insight into the mechanisms of immune aging, as well as novel therapeutic opportunities to target it.

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CHAPTER I

INTRODUCTION

Clinical Consequences of Human Aging

As an outcome of dramatically improved sanitation and technological advances in medical care, there has been a near doubling of the global life expectancy within the last 50 years. However, with this remarkable achievement comes a new set of challenges specific to the healthcare needs of the aging population. Within the US, while the elderly (individuals over the age of 65) constitute approximately 12% of the population, they are responsible for greater than 35% of visits to general internists, 34% of prescription drug use, and 50% of hospital stays *(1)*. In 2011 alone, $414.3 billion in total health care expenses was reported for elderly care—a figure that was $100 billion higher than inflation-adjusted expenses from 2001 *(2)*. As the proportion of the world's population over 60 rises to 22% in the next few decades, as predicted by the World Health Organization (WHO), understanding and addressing the diseases associated with aging becomes not only a social and political priority, but an economic one as well.

One of the most striking changes that occur during aging is the loss of overall immune function. Not only are the elderly more susceptible to new infections, but they also respond poorly to various treatments and vaccination attempts *(3)*. As a result, they are vulnerable to even common infectious diseases—80-90% of deaths associated with influenza occur in individuals greater than 65 years old *(4)*. Other age-associated comorbidities include increased susceptibility to cancer and autoimmunity, lending further evidence that the elderly experience an overall decline of proper immune function. While mouse models have proven invaluable for identifying and establishing many immunological principles, the evolutionary distance between mice and

humans remains a major barrier when it comes to studying specific immunological processes such as immune aging. Beyond the significant differences in organismal lifespan (mouse = 2-3 years; human = 75-100 years), the disparities experienced from different infections and comorbid conditions also cannot be ignored, due to their effects on the repertoire and replicative history of the immune system *(5)*. As a result, many of the molecular mechanisms regulating immune aging in humans remain unknown.

Overview of Immunosenescence: A Paradox of Immunodeficiency and Inflammation

Aging is a multi-faceted process that affects a number of tissues and organ systems in different ways. In the immune system, age-related changes are collectively referred to as "immunosenescence", a term that does not necessarily reflect a functional or mechanistic designation. From a clinical perspective, the consequences of immunosenescence primarily manifest through a paradoxical expression of both immune deficiency and chronic inflammation. As previously mentioned, elderly individuals experience an overall decline in immune protection, demonstrated by an increased susceptibility to new infections and decreased response to vaccinations. Simultaneously, a majority of aged individuals undergo a phenomenon known as "inflamm-aging"—a low-grade chronic level of inflammation characterized by elevated levels of pro-inflammatory cytokines such as IL-6, IL-18, IL-15, and TNFα, as well as increased serum concentrations of clotting factors and acute phase reactants *(6)*. Given that most age-related diseases share an inflammatory pathogenesis, this process poses a highly significant risk factor for both human morbidity and mortality *(7)*. While the mechanisms contributing to this age-related dysregulation of the immune system are complex and poorly understood, it is clear that

immunosenescence affects the immune system at every level—including alterations in hematopoietic stem cells (HSCs), innate and adaptive immune cells, and surrounding tissue microenvironments.

To start, many of the immunologic changes observed in the periphery arise as a result of alterations in hematopoietic stem cell (HSC) and progenitor cell populations. In elderly humans and some strains of mice, HSCs exhibit impaired adherence to stromal cells, a loss of regenerative capacity, as well as an overall shift from lymphocyte production to myeloid cells *(8)*. Lymphoid progenitors, such as pro-B cells and early T lineage progenitors, also experience an age-associated decrease in proliferative potential, exacerbating the decline in lymphopoiesis. On a molecular level, transcriptional and genomic analysis of aged vs young HSCs reveal differences in cell-cell interaction molecules, DNA-damage, and a general downregulation of lymphoid genes coupled with a general upregulation of myeloid genes, thereby corroborating the functional defects that have been observed *(9)*. While less understood, the upregulation of several NF-kB response genes has also been reported in aged HSCs—possibly contributing to the overall inflamm-aging phenotype in the elderly *(10)*. Recently, there has been interest in studying the epigenetic signature promoting HSC aging. Changes in the histone epigenetic state (e.g. H4K16 acetylation), as well as differing levels of chromatin/epigenetic modifiers (e.g. Sirtuins) have been observed in HSCs from aged mice—linking environmental effects to molecular alterations in HSC function *(10, 11)*.

One of the consequences of impaired HSC regenerative capacity and reduced lymphoid-based differentiation is a decline in peripheral naïve B cells during aging. In addition to a declining antigen recognition repertoire, B cells in the elderly also exhibit reduced co-stimulatory molecules and impaired class switch recombination—resulting in overall weaker

antibody responses *(12)*. Concomitantly, the levels of poly-specific and auto-reactive antibodies increase significantly with age as well, thus contributing to several inflammatory disorders *(13)*. However given that changes in the T cell compartment tend to precede the age-associated changes in the B cell compartment, it is thought that many of the humoral defects observed in the elderly occur secondary to T cell deficiencies. Naïve T cells not only decline in numbers with age (from impaired HSC function and thymic involution), but they also experience a reduced capacity to activate, proliferate, and differentiated into robust effector cells *(5)*. When examining effector functions in aged subjects, $CD8^+$ T cells exhibit a wide variety of defective responses, including deceased antigen-specific activation and proliferation, quantity and diversity of secreted cytolytic molecules, and ability to lyse target cells *(14)*. In aged $CD4^+$ T cells, alterations in several signaling pathways—such as decreased phospho-ERK and c-Jun N-terminal kinase (JNK) activity—have been implicated *(15)*. Restoration in some of these pathways in elderly $CD4^+$ T cells has proven effective in rescuing T helper activity for B cell differentiation and antibody production *(16)*. Altered T cell properties during aging have also been implicated in the inflamm-aging process. The expansion of terminally differentiated memory T cells, in both $CD4^+$ and $CD8^+$ T cell compartments, occurs during normal aging. These cells are resistant to apoptosis, acquire heightened cytolytic and pro-inflammatory functions, and are linked to numerous autoimmune diseases such as rheumatoid arthritis *(17)*.

While the size of most innate immune cell compartments remain more or less stable over an individual's lifespan, age-associated defects in the migration and effector function of innate immune cells have been reported. For example, neutrophils and natural killer (NK) cells in aged humans and mice demonstrate a diminished migratory and killing capacity *(18)*. Moreover, age-associated downregulation of numerous toll-like receptors (TLRs), as well as its associated

signaling pathways, have been observed in monocyte and dendritic cells (DCs) and consequently linked to increased susceptibility to pathogenic challenges *(18-20)*. Conversely, the altered expression of pattern recognition receptors (PRRs), coupled with increased exposure to antigens from reactivated latent viruses or endogenous damage-associated PRR ligands, can result in the excessive and unregulated secretion of inflammatory cytokines observed during inflamm-aging *(18)*. Furthermore, deleterious effects of aberrant PRR activation occurs on non-immune cells as well. For example, excessive TLR4 signaling on aged vascular smooth muscle cells is linked to increased circulating levels of pro-inflammatory cytokine IL-6 and accelerated arteriosclerosis progression *(21)*.

While cell-intrinsic events clearly contribute to certain aspects of immunosenescence, age-related alterations in the tissue microenvironment are also thought to play a significant role. The bone marrow niche undergoes several changes during aging, including reduced osteogenesis, increased reactive oxygen species (ROS) levels, and increased secretion of chemokines such as CCL5; all of which may impact HSC survival, proliferation, self-renewal and differentiation *(9)*. Regarding changes within the adaptive immune system, B-cell and T-cell development is potentially disrupted as a result of decreasing IL-7 levels within the bone marrow and a dysregulated thymic milieu that accompanies thymic involution *(8)*. As previously alluded to, innate immune cells may aberrantly respond to damage-associated signals (e.g. macromolecules or cells) that accumulate during aging. Finally, non-immune cells can also serve as a source of inflammation. After accumulating several markers of DNA-damage, senescent cells from various tissues can acquire a senescence-associated secretory phenotype (SASP) and continuously secrete numerous pro-inflammatory cytokines such as IL-6 *(22, 23)*.

Aging of the $CD8^+$ T Cell Compartment: Accumulation of $CD8^+CD28^-$ T Cells

The processes associated with immunosenescence are complex and interconnected. In this thesis, we focus specifically on $CD8^+$ T cells, and the molecular changes that occur in this compartment during aging. Not only are $CD8^+$ T cells vital immune defenders against intracellular pathogens and tumor cells, but they also experience one of the largest immunological changes during human aging. Firstly, as a result of stem cell alterations and thymic involution, there is a severe reduction in circulating $CD8^+$ naïve T cells. This reduction is coupled with an expansion of oligoclonal $CD8^+$ memory T cells, which is thought to occur from both homeostatic proliferative signals as well as a persistent antigenic stimulation (i.e. CMV infection). As a consequence of these changes in T cell populations, there is an overall decline in TCR diversity that contributes to the increased susceptibility to new infections (Figure 1-1).

A hallmark of this process is the downregulation of co-receptor CD28 from the surface of T cells—most dramatically observed within the $CD8^+$ compartment (Figure 1-2) *(24, 25)*. Unlike the transient loss of CD28 that occurs immediately after normal T-cell activation, this age-related decline in CD28 expression occurs transcriptionally and is more permanent *(25)*. Generally, $CD8^+CD28^-$ T cells are considered functionally defective due to their shortened telomeres and impaired antigen-induced proliferative response. In addition to the loss of CD28, these cells experience a downregulation of other important co-stimulatory receptors such as CD27 and CTLA-4 *(26)*. At the same time, $CD8^+CD28^-$ T cells can play a more active role in the age-related decline of immune function. Given their oligoclonal nature, the expansion of $CD8^+CD28^-$ T cells constrains the adaptive repertoire by competing for immunological "space". Furthermore, a subset of $CD8^+CD28^-$ T cells acquire immuno-suppressive functions *(27)*, and their presence is linked to the defective humoral responses observed in elderly individuals *(28)*. Accordingly, the

frequency of $CD8^+CD28^-$ T cells is an important component of a patient's immune risk profile, and serves as a strong predictor of impaired vaccination responses and an increased risk of human mortality *(29, 30)*.

One defining characteristic of $CD8^+CD28^-$ T cells is their enhanced cytotoxicity. They produce significantly higher levels of the effector molecule, granzyme B (GZMB), a serine protease that induces caspase-mediated apoptosis in target cells. However, GZMB has many biological roles beyond its potent cytotoxic effects. In the plasma, GZMB retains its enzymatic activity and has a normal concentration of 20-40 pg/mL; whereas in several disease states, GZMB levels are found to be elevated, including the plasma of patients with chronic viral infections and the synovial fluid of patients with rheumatoid arthritis *(31)*. Through its role in degrading extracellular matrix proteins, GZMB is involved in chronic inflammation, impaired wound healing, and age-related skin fragility *(32)*. Furthermore, GZMB can generate auto-antigens by cleaving and modifying susceptible host proteins, thereby initiating and amplifying the disease progression of several systemic autoimmune diseases *(33)*. The tissue-damaging effects of $CD8^+CD28^-$ T cells is exacerbated through their acquisition of receptors more commonly associated with NK cells. Many of these NK-cell (NKC) receptors, such as NRC1 and CD244, are considered activating receptors—suggesting that $CD8^+CD28^-$ T cells can elicit cytotoxic killing in an antigen-independent manner *(25)*. What conditions driving this heightened effector profile in $CD8^+CD28^-$ T cells remain largely unknown.

THESIS OBJECTIVE

Given the accelerating trends projected for global aging demographics, it is becoming increasingly imperative that we better understand the consequences of immune aging and its underlying mechanisms. A hallmark of this process is the accumulation of $CD8^+CD28^-$ T cells, a population of terminally differentiated memory T cells that accumulate during aging and chronic infections. Characterized by shortened telomeres and defective antigen-induced proliferation, these cells are often called "senescent" and are linked to many age-related diseases. However because their accumulation is not observed in mice, many of the mechanisms governing their fate remain unknown. In this thesis, we aim to identify key molecular pathways regulating the effector function of $CD8^+CD28^-$ T cells. We focus especially on the role of protein deacetylase, SIRT1, a highly conserved factor linked to aging, immune function, and metabolism. The data presented in this thesis not only provide deeper understanding into the molecular changes that occur during immune system aging, but also identify potential therapeutic opportunities to reverse or delay its effects.

The Role of SIRT1 in T Cells

Sirtuin 1 (SIRT1) belongs to a highly conserved family of (NAD)+-dependent protein deacetylases that regulate a wide variety of cellular processes associated with organismal longevity. In mammals, SIRT1 maintains energy homeostasis and protects against the metabolic stresses of aging through the deacetylation of downstream transcription factors such as peroxisome proliferator-activated receptor (PPAR)γ and PPARγ coactivator 1α (PGC-1α) *(34)*. Therefore targeting SIRT1—either through diet, genetic, or pharmacological manipulation—can affect numerous metabolic pathways such as lipolysis, gluconeogenesis, glycolysis, fatty acid

oxidation, and mitochondrial biogenesis *(35)*. Conditions that activate SIRT1 activity not only improve symptoms associated with metabolic dysfunction, but can also can also exert protective effects against other age-related diseases such as cancer, neurodegeneration, and cardiovascular disease *(36)*. In some model organisms, sirtuin activation has even elicited modest, yet reproducible extension in lifespan *(34, 35)*. As such, a number of clinical trials are now focusing on the beneficial effects of SIRT1-activating compounds in humans *(37)*.

Recent studies suggest that SIRT1 may also play a critical role within the immune system. SIRT1 negatively regulates the activity of transcription factors NF-kB, AP-1, and STAT3, therefore suppressing inflammatory pathways found in both innate and adaptive immune cells *(38)*. Furthermore, global deletion of SIRT1 in mice results in loss of peripheral tolerance and the development of autoimmunity *(39, 40)*. More recent studies using T-cell specific knockouts, however, suggest that SIRT1 may also possess pro-inflammatory functions by positively regulating the balance between T helper 17 (Th17) effector cells and T regulatory (Treg) cells through deacetylation of signature transcription factors RAR-related orphan receptor y-t (RORyt) and Foxp3, respectively *(41-44)*. While SIRT1 activity has recently been linked with effector $CD8^+$ T cell differentiation via its association with transcription factors BATF and T-bet *(45)*, its role in T cell metabolism and immune aging remains unclear.

In recent years, there has been a rapid accumulation of research demonstrating the integral role of metabolism over the course of an immune response *(46, 47)*. Naïve T cells are primarily dependent on mitochondrial oxidative phosphorylation (OXPHOS) and fatty acid oxidation (FAO) to support their basal levels of nutrient uptake. Upon antigen engagement, T cells markedly upregulate aerobic glycolysis and glutaminolysis to generate the biosynthetic precursors necessary for clonal proliferation and effector function *(48)*. Certain glycolytic

enzymes, such as GAPDH, have also been shown to directly regulate effector function. GAPDH binds and suppresses the translation of IFNγ (IFNG) in resting T cells—a mechanism that is released when activated T cells undergo a dramatic switch toward aerobic glycolysis *(49)*. Finally, after the immune response contracts and memory T cells form, there is a metabolic switch back towards catabolic metabolism and OXPHOS. These memory T cells retain an 'imprinted' metabolic potential, marked by an increased glycolytic capacity and mitochondrial mass, that provides a bio-energetic advantage to support rapid recall responses *(50, 51)*. Understanding these metabolic pathways has not only provided greater insight into basic T cell biology, but also new opportunities to therapeutically enhance or suppress immune responses in a wide range of clinically settings *(52)*.

Hypothesis

SIRT1 is a versatile protein deacetylase that impacts a wide range of cellular processes, including lifespan and metabolism. While SIRT1 has recently emerged as an important regulator of T cell differentiation, its role in immune aging remains unknown. Therefore given the longstanding connection of SIRT1 with aging and age-related diseases, we hypothesize that SIRT1 may regulate the function of $CD8^{+}CD28^{-}$ T cells—a population of terminally differentiated memory T cells that accumulate in humans during aging and chronic infections. Specifically, we focus our investigation on the downstream metabolic effects of SIRT1 activity, as metabolic reprogramming controls many aspects of T-cell effector function.

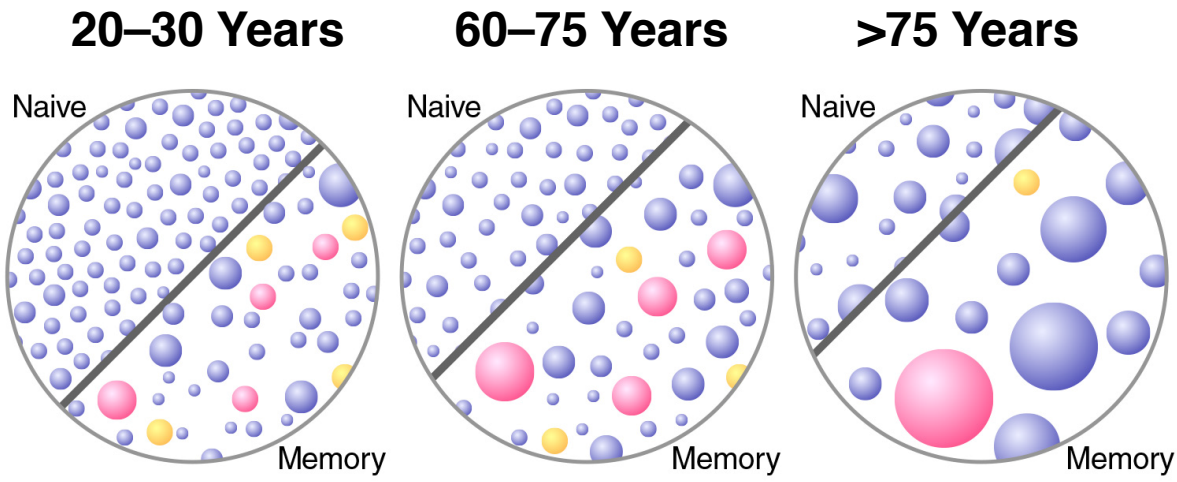

Figure 1-1. Restructuring of T cell compartment during aging.
There is a decrease in circulating peripheral naive T cells with age, primarily due to involution of the thymus. Concomitantly, there is an expansion of oligoclonal memory T cells (the size of the circle represents the number of cells within that clonal population), thought to arise from the increasing antigen burden that an individual is exposed to over the course of his or her lifespan. In support of this, a significant number of memory T cells in adults are reactive to persistent viral infections (represented as yellow or pink circles) such as CMV. Together, this restructuring of T cell populations with age results in a reduced T cell repertoire, contributing to the overall decline in immune function observed in the elderly.

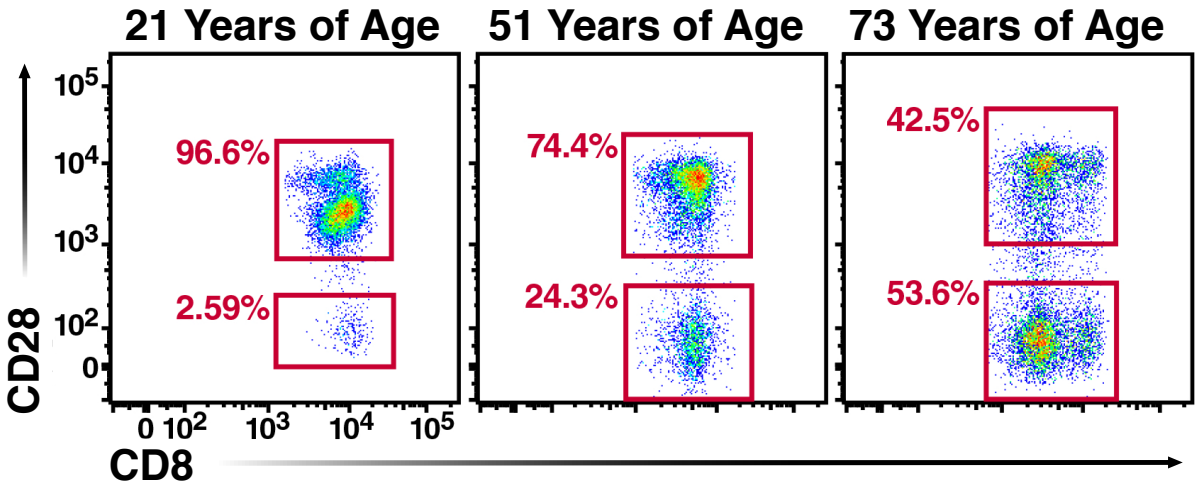

Figure 1-2. Accumulation of $CD8^+CD28^-$ T cells in humans during aging.
A hallmark of immunosenescence is the irreversible downregulation of co-receptor CD28 from the surface of T cells (more dramatically within observed within the $CD8^+$ T cell compartment), as indicated by these representative FACs plots analyzing the blood of donors from varying ages.

CHAPTER II

MATERIALS AND METHODS

Human donors and T cell–population sorting

Blood samples were collected at the Blood Centers of the Pacific in San Francisco, California (all donors were kept anonymous and provided written informed consent). Total human $CD8^+$ T cells were enriched via negative selection using the RosetteSep Human $CD8^+$ T–Cell Enrichment cocktail (15063; Stemcell). The following antibodies were used to stain $CD8^+$ Naïve, $CD8^+CD28^+$ Memory, and $CD8^+CD28^-$ T cells: CD3-PECy5 (555334; BD Biosciences), CD8-V450 (560347; BD Biosciences), CD28-PE (12-0289; eBioscience), CD45RA-APC (17-0458; eBioscience), and CCR7-PECy7 (557648; BD Biosciences). Flow cytometry was performed on a LSRII or Calibur DxP8 and analyzed with FlowJo software. Sorting was performed on an ARIA II flow cytometer.

T-cell culture and treatment conditions

T cells were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and penicillin-streptomycin. Activation with αCD3/28 dynabeads (11161D; ThermoFisher) were used at a 1:1 bead:cell ratio. Activation with recombinant human IL-15 (570302; Biolegend) was used at 50 ng/mL. Resveratrol (R5010; Sigma) was used at 25 μM and 50 μM for 48 hours. FoxO1 inhibitor AS1842856 (344355; Calbiochem) was used at 25 nM, 50 nM, and 100 nM for 48 hours. Mg132 (M7449; Sigma) was used at 20 μM for 6 hours.

Nicotinamide riboside was obtained from Anthony A. Sauve's Lab (Weill Cornell Medical College) and used at 0.5 μM and 1 μM for 48 hours. Rapamycin was used at 20 nM.

Lentivirus Production and Transductions

293T cells were co-transfected with the transfer plasmid encoding shRNA or overexpression constructs, an HIV-based packaging construct (pCMVΔR8.91), and a construct expressing the glycoprotein of vesicular stomatitis virus (VSV-G) (pMD.G). Culture supernatants containing pseudotyped lentiviral particles were stored at -80° C. Cells were transduced in the presence of 8 μg/ml Polybrene (Sigma) for 24 hours at 37° C.

$CD8^+CD28^+$ memory T cells were sorted and stimulated with αCD3/28 dynabeads (11161D; ThermoFisher) and IL-2 (30 IU/ml). 2 and 3 days after stimulation, cells were transduced with lentivirus-expressing scramble shRNA, or 2 different SIRT1-targeting shRNAs (see Table below for plasmid information). On day 6, transduced mcherry+ cells were sorted by FACS, and restimulated again with αCD3/28 beads with IL-2. On day 9, RNA was isolated from the cells and gene expression was examined by qRT-PCR. Jurkat T cells were stably transduced with SIRT1-myc overexpression lentiviral constructs via spinfection and selected with puromycin for 7 days.

Plasmid Name	Ott ID	Plasmid Details
shRNA scramble (m880)	PMO1112	psicoR-(U6-scramble-hEFL-HTLV-mcherry-T2A-puro)
SIRT1 shRNA #1 (2123)	PMO1115	psicoR-(U6-sh2123-hEFL-HTLV-mcherry-T2A-puro)
SIRT1 shRNA #2 (1425)	PMO1114	psicoR-(U6-sh1425-hEFL-HTLV-mcherry-T2A-puro)
Control Vector	PMO1132	hEFL-HTLV-IRES-puro
SIRT1 Over Expression	PMO1131	hEFL-HTLV-KOZAK-SIRT1-myc-his-IRES-puro

Metabolic assays

OCR and ECAR were determined using a Seahorse XF96 Extracellular Flux Analyzer (Seahorse Bioscience). Cells (5.0 x 10^5 per well) were seeded onto Cell-Tak (354240; Corning)-coated wells in non-buffered RPMI 1640 media (R1383; Sigma) supplemented with 11 mM glucose and 2 mM sodium pyruvate. Measurements were obtained under basal conditions and after addition of 1 μM oligomycin, 0.5 μM carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), and 0.5 μM rotenone/antimycin A (103015-100; Seahorse Bioscience).

Lactate measurements were detected using the primary metabolism screen conducted by the West Coast Metabolomics Center. Briefly, sorted T cell populations were treated with oligomycin (1 μM) and harvested at various time points (0, 15, 60 min). Samples were run on an ALEX-CIS GC-TOF mass spectrometer, and metabolites are quantified by peak heights and reported by retention index, quantification mass, biochemical database identifiers, and full mass spectra. Refer to *(53)* for further details regarding data acquisition and processing.

Mitochondrial mass was determined by flow cytometry using Mitotracker Green (M7514; ThermoFisher), according to the manufacturer's instructions.

Microarray and Ingenuity Pathway Analysis

$CD8^+CD28^+$ and $CD8^+CD28^-$ T cells were sorted from 3 human donors and total RNA was extracted using the RNeasy Micro Kit (Qiagen), according to the manufacturer's instructions. Microarray experiments were performed using the Affymetrix™ platform, and 159 significant genes were identified using a cut-off of ≥ 1.0-log2(fold-change) and ≤ 0.05 false-discovery rate

(FDR, adjusted P-value). Using the Ingenuity Pathway Analysis (IPA) software on the differentially expressed genes, a list of possible upstream regulators was generated based on the literature compiled in the Ingenuity Knowledge Base. The list of upstream transcription factors was ordered from most significant to least significant *P* value, via a Fisher's Exact Test to assess the significance of enrichment of the gene expression data for the genes downstream of an upstream regulator. The activation z-score infers the activation states of the predicted transcriptional regulators (positive = activating; negative = inhibiting). Refer to the manufacturer's website (http://www.ingenuity.com) for further details.

Cell viability

Using the AlamarBlue Cell Viability Assay (88951; ThermoFisher), cells were incubated with 100 µL of alamar blue reagent for 6 hours following various treatment conditions. The percent reagent reduction was calculated according to the manufacturer's instructions.

Cell proliferation

Sorted human T cells were incubated with 1 µM Vybrant CFDA-SE Cell Tracer (V12883; Invitrogen) prior to activation with αCD3/28 or IL-15. Proliferation was assessed by flow cytometry on a Calibur DxP8.

RNA extraction and qRT-PCR

Total RNA from samples was extracted using the RNeasy Plus Mini Kit (74136; Qiagen). cDNA was generated using 50–500 ng of total RNA with Superscript III Reverse Transcriptase (18080-044; ThermoFisher) and oligo$(dT)_{12-18}$ (18418-012; ThermoFisher). The SYBR green qPCR reactions contained 5 µl of 2x Maxima SYBR green/Rox qPCR Master Mix (K0221; ThermoFisher), 5 µl of diluted cDNA, and 1 nmol of both forward and reverse primers. The reactions were run using the following conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. See Table 2-1 for qRT-PCR primer list.

For miRNA detection, RNA was isolated easy miRNeasy Mini Kit (217004; Qiagen). cDNA was generated using QuantiMIR RT Kit (RA420A-1; SBI) according to manufacturer's instructions. See Table 2-1 for qRT-PCR primer list.

Western blot

Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, supplemented with protease inhibitor cocktail; Sigma) for 30 min at 4°C or subcellular fractions were extracted using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (78835; ThermoFisher) according to the manufacturer's instructions. Samples re-suspended in Laemmli buffer for SDS-PAGE. For chemiluminescent detection, we used ECL and ECL Hyperfilm (Amersham).

Antibodies

The following primary antibodies were used: SIRT1 (ab104833; abcam), SIRT1 (8469S; Cell Signaling Technology), SIRT6 (12486; Cell Signaling Technology), SIRT7 (5360; Cell Signaling Technology), β-actin (A5316; Sigma), FoxO1 (2880S; Cell Signaling Technology), Sp1 (sc-14027; Santa Cruz), and GZMB-FITC (515403; Biolegend).

Affinity-Purification Mass-Spectrometry

AP-MS protocol was adapted from *(54)*. Briefly, cells were lysed in IP buffer with detergent (150 mM NaCl, 50 mM Tris pH 7.4, 1 mM EDTA, 0.5% NP-40 substitute) for 30 min at 4°C and passed 10 times through a G23 needle. Clarified lysates were affinity-purified with anti-myc conjugated beads at 4°C overnight. Resin was washed four times in IP buffer with detergent, followed by two washes in IP buffer without detergent (150 mM NaCl, 50 mM Tris pH 7.4, 1mM EDTA). Resin was resuspended in 40µl IP buffer without detergent containing c-myc peptide and 0.05% Rapigest (Waters) at 4°C for 30 minutes, with agitation. Protein eluate was digested with trypsin and analyzed by LC-MS/MS. 20 µl of the eluate was reserved for analysis by SDS-PAGE followed by either western blotting or silver staining (24600; ThermoScientific). SIRT1-interactome was compiled through selecting bait-prey pairs with a mass spectrometry interaction statistic (MiST) score $\geq$ 0.75 (See Table 5-1 for list), computed with the previously published feature weights for reproducibility, abundance, and specificity.

Statistical analysis.

Comparisons for two groups were calculated using a paired (comparing populations derived from

the same donor), two-tailed Student's *t*-test. Comparisons for more than two groups were calculated using one-way ANOVA followed by Tukey's multiple comparison tests. Data are presented as mean ± SD for technical replicates or mean ± SEM for biological replicates. Statistical significance is indicated in all figures by the following annotations: **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001.

Table 2-1. qRT-PCR Primer List

Gene Name	Forward Primers	Reverse Primers
RPL13A	CCTGGAGGAGAAGAGGAAAGAGA	TTGAGGACCTCTGTGTATTTGTCAA
SIRT1	GCCTCACATGCAAGCTCTAGTGAC	TTCGAGGATCTGTGCCAATCATAA
GZMB	CCCTGGGAAAACACTCACACA	GCACAACTCAATGGTACTGTCG
PRF1	GGCTGGACGTGACTCCTAAG	CTGGGTGGAGGCGTTGAAG
IFNG	TCGGTAACTGACTTGAATGTCCA	TCGCTTCCCTGTTTTAGCTGC
CCR7	TGAGGTCACGGACGATTACAT	GTAGGCCCACGAAACAAATGAT
CD62L	CTTTCACCAAGGGCGATTTA	GGCATTTATCATTTGGCTGG
IL7R	CCCTCGTGGAGGTAAAGTGC	CCTTCCCGATAGACGACACTC
KLRG1	TCCATGTTAGAGTTGCCTACGG	AAGTGGAGTAGTTGGAGCCCT
FOXO1	TCGTCATAATCTGTCCCTACACA	CGGCTTCGGCTCTTAGCAAA
IL15RB	CAGCGGTGAATGGCACTTC	GGCATGGACTTGGCAGGAA
TFAM	ATAGGCACAGGAAACCAGTTAG	GCAGAAGTCCATGAGCTGAATA
CPT1A	TCCAGTTGGCTTATCGTGGTG	TCCAGAGTCCGATTGATTTTTGC
HIF1A	GAACGTCGAAAAGAAAAGTCTCG	CCTTATCAAGATGCGAACTCACA
GLUT1	CCAGCTGCCATTGCCGTT	GACGTAGGGACCACACAGTTGC
PFK1	GGCAACCTGAACACCTACAAGC	CGAAGCCGTCAAAGCCATCATAG
PKM2	CCATTACCAGCGACCCCACAG	GGGCACGTGGGCGGTATCT

miRNA	Universal Forward Primer (U6)	Forward Primers
mir22 (3p)	CGCAAGGATGACACGCAAATTC	AAGCTGCCAGTTGAAGAACTGT
mir34a (5p)	CGCAAGGATGACACGCAAATTC	TGGCAGTGTCTTAGCTGGTTGT
mir93 (5p)	CGCAAGGATGACACGCAAATTC	CAAAGTGCTGTTCGTGCAGGTAG
mir181a (5p)	CGCAAGGATGACACGCAAATTC	AACATTCAACGCTGTCGGTGAGT
mir181b (5p)	CGCAAGGATGACACGCAAATTC	AACATTCATTGCTGTCGGTGGGT
mir181c (5p)	CGCAAGGATGACACGCAAATTC	AACATTCAACCTGTCGGTGAGT

CHAPTER III

A SIRT1-FoxO1 Axis Couples Metabolic and Cytotoxic Reprogramming in $CD8^+$ T Cells During Aging

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Summary

The accumulation of $CD8^+CD28^-$ T cells—a population of terminally differentiated memory T cells—is one of the most consistent immunologic changes observed in humans during aging. $CD8^+CD28^-$ T cells are highly cytotoxic, and their frequency is linked to many age-related diseases. As their accumulation is not observed in mice, many of the molecular mechanisms regulating their fate and function remain unclear. Here we found that human $CD8^+CD28^-$ T cells under resting conditions have an enhanced capacity to use glycolysis, a function linked to increased cytotoxic gene expression and decreased expression of the NAD^+-dependent protein deacetylase SIRT1. Global gene–expression profiling identified the transcription factor FoxO1 as a SIRT1 target involved in the transcriptional reprogramming of $CD8^+CD28^-$ T cells. FoxO1 is proteasomally degraded in $CD8^+CD28^-$ T cells, and inhibiting its activity in $CD8^+CD28^+$ T cells recapitulates the metabolic and cytotoxic phenotype of $CD8^+CD28^-$ T cells. These data identify the evolutionarily conserved SIRT1-FoxO1 pathway as a new regulator of immune aging in humans.

Introduction

Virtually all $CD8^+$ T cells in umbilical cord blood express the co-receptor CD28 *(24)*. However, with repeated exposure to antigens over the course of an individual's life, many $CD8^+$ T cells in human peripheral blood become $CD28^-$, mostly in response to persistent viral infections, such as CMV and HIV *(25, 55)*. The accumulation of $CD8^+CD28^-$ T cells is not observed in laboratory mice and is considered a predictor of human mortality linked to several age-related disorders, including autoimmunity, cancer, an increased susceptibility to infections, and a reduced response to vaccinations *(30, 56, 57)*. Functionally, $CD8^+CD28^-$ T cells have an impaired proliferative response to antigen-specific activation, but they remain very cytotoxic, acquiring high expression of natural killer (NK)–cell receptors and producing greater levels of effector molecules, such as granzyme B (GZMB), perforin (PRF1), and interferon-gamma (IFNG) under resting and activated conditions *(25, 58)*. GZMB, in particular, has a wide range of biological activities that can contribute to disease pathogenesis *(32)*. When circulating in the blood stream, GZMB retains its proteolytic activity and can create auto-antigens by cleaving the unstructured and linker regions of antigens, thereby exposing new epitopes that may initiate and propagate autoimmunity *(33)*. Given the ubiquitous presence of $CD8^+CD28^-$ T cells and their connection to autoimmunity and immune aging, a better understanding of the molecular mechanisms driving their uncontrolled production of effector molecules is needed.

Metabolism is a key driver of T-cell function. While naïve and resting memory T cells primarily depend on mitochondrial oxidative phosphorylation (OXPHOS) and fatty acid oxidation, activated T cells rapidly shift their metabolism toward aerobic glycolysis to support their full effector function *(59)*. Human sirtuins (SIRT1–7) are highly conserved proteins that regulate cellular processes linked to metabolism and organismal longevity *(34, 37)*. SIRT1 is a

nuclear NAD^+-dependent protein deacetylase involved in different metabolic pathways—such as lipolysis, gluconeogenesis, glycolysis, fatty acid oxidation, and mitochondrial biogenesis—in a number of tissues, including heart, liver, adipose tissue, and endothelium *(35)*. Although several fate-determining functions of SIRT1 have recently emerged in $CD4^+$ and activated $CD8^+$ T cells *(41-45)*, its role in T-cell metabolism remains unknown.

Here we identify SIRT1 as a new regulator of $CD8^+$ T-cell metabolism and cytotoxic gene expression. Our data support a model where SIRT1—through the downstream transcription factor FoxO1—restricts $CD8^+$ T cells from readily switching from OXPHOS to glycolysis under resting conditions. We find this restricted metabolic potential linked to the suppression of spontaneous cytotoxic gene expression in naïve $CD8^+$ and $CD8^+CD28^+$ memory T cells, and inactivated in $CD8^+CD28^-$ T cells—contributing to greater basal production of GZMB.

Results

Because SIRT1 levels decrease with age in the brain, liver, skeletal muscle, and white adipose tissue of rodents *(60-62)*, we examined SIRT1 expression in human $CD8^+CD28^-$ T cells. We found SIRT1 protein expression markedly downregulated in freshly isolated $CD8^+CD28^-$ T cell populations when compared to $CD8^+$ naïve or $CD8^+CD28^+$ memory T cells derived from the same donor (Figure 3-1A). This observation was consistent across samples from multiple individuals (Figure 3-1B). *SIRT1* mRNA levels were not significantly different (Figure 3-1C), implicating that SIRT1 downregulation occurred post-transcriptionally. Importantly, expression of other nuclear sirtuins, SIRT6 and SIRT7, were unchanged in $CD8^+CD28^-$ T cells (Figure 3-1D).

Next, we investigated whether low SIRT1 expression changed the metabolism of $CD8^+CD28^-$ T cells. Using Seahorse technology, we first measured the oxygen consumption rate (OCR) with a standard mitochondrial stress test. In this test, sorted subsets of resting human $CD8^+$ T cells were challenged with oligomycin (an inhibitor of ATP synthase), carbonyl cyanide-4(trifluoromethox)phenylhydrazone (FCCP; an uncoupling agent), and a combination of antimycin A and rotenone (inhibitors of complex III and complex I, respectively). We found no difference in OCR between resting $CD8^+$ naïve, $CD8^+CD28^+$, and $CD8^+CD28^-$ T cells (Figure 3-2A), despite previous reports of defective mitochondrial function in 'senescent' effector memory RA T cells (TEMRA) *(63)* and the known role of SIRT1 in regulating mitochondrial function in skeletal muscle *(64)*. In contrast, $CD8^+CD28^-$ T cells exhibited a distinctive glycolytic profile, highlighted by their markedly elevated extracellular acidification rate (ECAR) after exposure to oligomycin (Figure 3-2B).

The ECAR response to oligomycin, called 'glycolytic capacity', measures how well cells are primed to use glycolysis when ATP production from OXPHOS is impaired. One function of this metabolic potential is to support the glycolytic gene program in $CD8^+$ effector memory T cells immediately after activation *(50)*. In resting $CD8^+CD28^-$ T cells, we found that the glycolytic capacity (maximum ECAR after oligomycin injection) was consistently increased among various donors (Figure 3-2C), producing a unique energy profile (OCR vs ECAR plot) defined by a characteristic oligomycin-induced shift in ECAR, but not OCR (Figure 3-2D). Importantly, this capacity was associated with increased lactate production (Figure 3-2E) and was abrogated under glucose deprivation (Figure 3-2F), indicating that the increased ECAR-response is mainly driven through glycolysis.

To determine whether low SIRT1 levels and high glycolytic capacity were linked in

$CD8^+CD28^-$ T cells, we treated the cells with resveratrol, a natural compound that increases SIRT1 activity on specific substrates *(65)*. With little toxicity (Figure 3-3A), resveratrol lowered the glycolytic capacity (Figs. 3-3B, 3-3C) and shifted the energy profile of $CD8^+CD28^-$ T cells back to that observed in $CD8^+$ naïve and $CD28^+$ memory T cells (Figure 3-3D). Similar results were obtained after addition of nicotinamide riboside *(66)*, a precursor of the SIRT1 cofactor NAD^+, independently linking the control of glycolytic capacity to SIRT1 activity in $CD8^+CD28^-$ T cells (Figure 3-3E).

As the switch to a glycolytic program in activated T cells is associated with drastic changes in gene expression *(47)*, we performed microarray analysis to define the global gene–expression profiles of freshly isolated $CD8^+CD28^+$ and $CD8^+CD28^-$ T cells from three individual blood donors. This analysis showed up- and downregulation of 159 genes within resting $CD8^+CD28^-$ T cells (log2-fold change ≥ 1.0, FDR ≤ 0.05). Upregulated genes included NK-cell receptors and cytolytic effector molecules, while expression of CD28 and several T-cell homing receptors was downregulated (Table 3-1, left and middle). Supporting previous reports *(67)*, we found that under non-activated conditions, $CD8^+CD28^-$ T cells expressed markedly higher *GZMB* mRNA and intracellular protein levels using quantitative RT-PCR (qRT-PCR) and flow cytometry analysis, respectively (Figures 3-4A, 3-4B). To a lesser extent, *PRF1* and *IFNG* transcripts were similarly increased in $CD8^+CD28^-$ T cells (Fig. 3-4A).

Using Ingenuity Pathway Analysis (IPA), we generated a list of upstream transcription factors (ordered from most to least significant *P* value) that were potentially involved in the variable gene expression between the two populations (Table 3-1; right). The top candidate was FoxO1 (*P* value = 2.97×10^{-6}), a known SIRT1 substrate implicated to regulate multiple metabolic pathways, including glycolysis, in $CD8^+$ T cells *(68)*. FoxO1 also binds and regulates the

expression of multiple genes that control T-cell homing, homeostasis, tolerance, and memory differentiation *(69-73)*.

With qRT-PCR, we verified the downregulation of canonical FoxO1-target genes in $CD8^+CD28^-$ T cells, including lymph node homing receptors *CCR7* and *CD62L* (also known as *SELL*), and the growth factor interleukin 7 receptor *(IL7R) (69)* (Figure 3-5A). $CD8^+CD28^-$ T cells also expressed increased killer-cell lectin-like receptor G1 (*KLRG1*), a marker of T-cell differentiation that is inversely related to FoxO1 expression in $CD8^+$ T cells *(72)* (Figure 3-5A). A similar dysregulated expression of FoxO1-target genes and KLRG1 was observed in human $CD8^+CD28^+$ T cells transduced with lentiviral shRNA constructs targeting SIRT1, linking SIRT1 expression with FoxO1 activity in human $CD8^+$ T cells (Figure 3-5B).

FoxO proteins are known targets of the deacetylase function of SIRT1 *(74, 75)*. When SIRT1 activity is inhibited, FoxO1 is more sensitive to phosphorylation mediated by protein kinase B (PKB; also known as Akt), thereby attenuating its DNA-binding affinity and facilitating its nuclear export and degradation *(76-78)*. We performed nuclear and cytoplasmic fractionation in sorted subsets of human T cells and found that FoxO1 protein expression was decreased in both cellular compartments of $CD8^+CD28^-$ T cells (Figure 3-5C). The downregulation of FoxO1 was consistently observed among different donors and exhibited a pattern of gradual decline in protein expression (naïve>$CD28^+$>$CD28^-$), similar to SIRT1 in the same T-cell populations (Figures 3-5D, 3-1B). No significant differences in *FoxO1* mRNA levels were observed, supporting that FoxO1 is post-transcriptionally downregulated in $CD8^+CD28^-$ T cells (Figure 3-5E). Interestingly, treatment with proteasome inhibitor MG132 rescued protein expression of FoxO1, but not SIRT1, in $CD8^+CD28^-$ T cells (Figure 3-5F), implicating that expression of FoxO1 and SIRT1 proteins is distinctly regulated in these cells.

To determine whether FoxO1 regulates glycolytic activity and cytotoxicity in $CD8^+$ T cells, we treated resting $CD8^+$ naïve or $CD8^+CD28^+$ memory T cells with the compound AS1842856, which blocks FoxO1-transcriptional activity by selectively binding its dephosphorylated active form *(79)*. AS1842856 consistently enhanced the glycolytic capacity and energy profile in both cell populations (Figures 3-6A, 3-6B, 3-6C), replicating the phenotype observed in $CD8^+CD28^-$ T cells with reduced FoxO1 expression (Figures 3-2B, 3-2C, 3-2C).

With little effect on cell viability (Fig. 3-7A) or T cell activation (Fig. 3-7B), AS1842856 effectively suppressed expression of the FoxO1-target genes *CD62L* and *IL7R* in resting human $CD8^+$ T cells (Figure 3-7C). In contrast, AS1842856 significantly and dose-dependently increased basal *GZMB* mRNA expression (Figure 3-7C), indicating that FoxO1 activity controls cytotoxic gene expression in resting $CD8^+$ T cells. The AS1842856-mediated increase in GZMB expression was observed in both $CD8^+$ naïve and $CD8^+CD28^+$ memory T cells (Figure 3-7D) and required glucose consumption (Figure 3-7E), further linking glycolytic and cytotoxic capacities of $CD8^+$ T cells under resting conditions.

Discussion

Collectively, these results identify the SIRT1-FoxO1 axis as a novel regulator of metabolic and cytotoxic reprogramming in human $CD8^+$ T cells during aging. We propose a model in which reduced levels of SIRT1 destabilize the expression and function of FoxO1 to enhance the glycolytic and cytotoxic capacities of resting $CD8^+CD28^-$ T cells, thereby contributing to immune dysfunctions observed in the elderly (Figure 3-8).

While this represents a new role for the SIRT1-FoxO1 axis in human $CD8^+$ T cells,

previous studies showed that the homologous SIR2-DAF16/FOXO pathway, when strengthened, extended organismal lifespan in *Caenorhabditis elegans (80)*. More recent studies in mammals found this axis involved in regulating cellular processes such as maintenance and growth of skeletal muscle *(81)*, apoptosis in senescent cardiomyocytes *(82)*, and intracellular signaling in both insulin-sensitive and insulin-producing tissues *(68, 83)*. It remains unknown whether these functions also depend on metabolic control by the SIRT1-FoxO1 axis. Our findings newly connects this evolutionarily conserved pathway with T–cell metabolism and immune aging, underscoring the growing evidence that emphasizes strategies to strengthen SIRT1 activity as a therapeutic opportunity to delay human aging *(34, 37, 84)*.

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provided the use of her laboratory. The manuscript was written by M.Y.J. and M.O.

Competing interests: The authors have declared that no conflict of interest exists.

Table 3-1. Global gene–expression profiling of human $CD8^+CD28^-$ vs $CD8^+CD28^+$ T cells.

Top Microarray Genes: $CD28^-$ vs $CD28^+$				Pathway Analysis	
Up-regulated	**log2(Fold-change)**	**Down-regulated**	**log2(Fold-change)**	**Upstream Regulators**	**p-value of overlap**
GPR141	3.05	CD28	-3.21	FOXO1	2.97E-06
FCGR3A*	2.92	RCAN3	-3.09	ID3	3.42E-06
CX3CR1	2.91	LRRN3	-3.02	BACH2	1.13E-05
KLRF1*	2.70	LEF1	-2.88	ID2	2.13E-05
TXNDC3	2.39	SCML1	-2.85	ETS1	8.25E-05
GNLY**	2.31	NELL2	-2.76	PRDM1	1.18E-04
PLEK	2.04	CCR7	-2.75	EBF1	2.32E-04
PDGFD	2.03	CCR4	-2.74	ARNT	1.09E-03
ZEB2	2.02	CD62L	-2.72	IRF8	1.76E-03
FCRL6	1.82	VSIG1	-2.64	TP63	1.85E-03
GZMB**	1.78	IFI44L	-2.44	ERG	2.04E-03
KLRD1*	1.69	SERINC5	-2.41	STAT6	2.20E-03
GZMH**	1.65	TXK	-2.41	SPIB	2.50E-03
KIR2DS2*	1.60	ITGA6	-2.26	IRF4	3.02E-03
KIR2DL1*	1.56	FCER1G	-2.21	CREB1	3.16E-03
PRSS23	1.52	HOOK1	-1.98	STAT3	4.96E-03
TGFBR3	1.49	GCNT4	-1.97	TP53	5.80E-03
NKG7*	1.47	GZMK	-1.96	HOXA10	7.33E-03
TNFRSF9	1.47	ZNF204	-1.94	SP1	9.95E-03

Top genes from microarray (n = 3 human donors) ordered by log2fold-change (Refer to Appendix Table 1 for full list of 159 significant differentially regulated genes). * = NK-cell receptor; ** = cytolytic molecule. Potential upstream regulators generated via Ingenuity Pathway Analysis and ordered by P-value (Fisher's Exact Test).

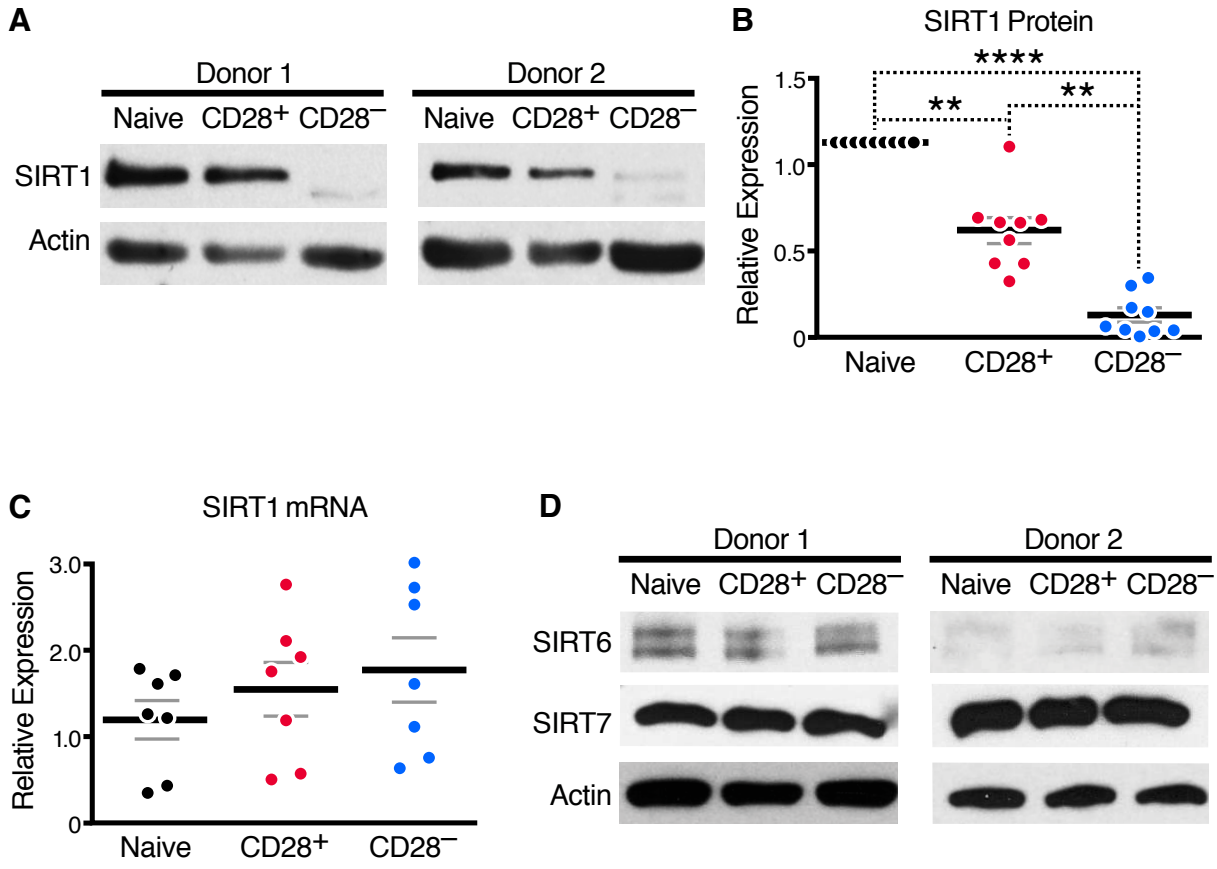

Figure 3-1. SIRT1 is post-transcriptionally downregulated in CD8$^+$CD28$^-$ T cells.
(A–D) CD8$^+$ naïve, CD8$^+$CD28$^+$ memory, and CD8$^+$CD28$^-$ T–cell populations were sorted from blood of healthy human donors based on the surface markers CD3, CD8, CD28, CCR7, and CD45RA. **(A–C)** SIRT1 expression was assessed by **(A,B)** immunoblot and **(C)** qRT-PCR, normalized to *RPL13A*. **(D)** SIRT6 and SIRT7 expression was assessed by immunoblot in sorted human T cells (n = 2 donors). Data are mean ± SEM of **(B)** 9 donors and **(C)** 7 donors. **P<0.01, ****P<0.0001; paired one-way ANOVA.

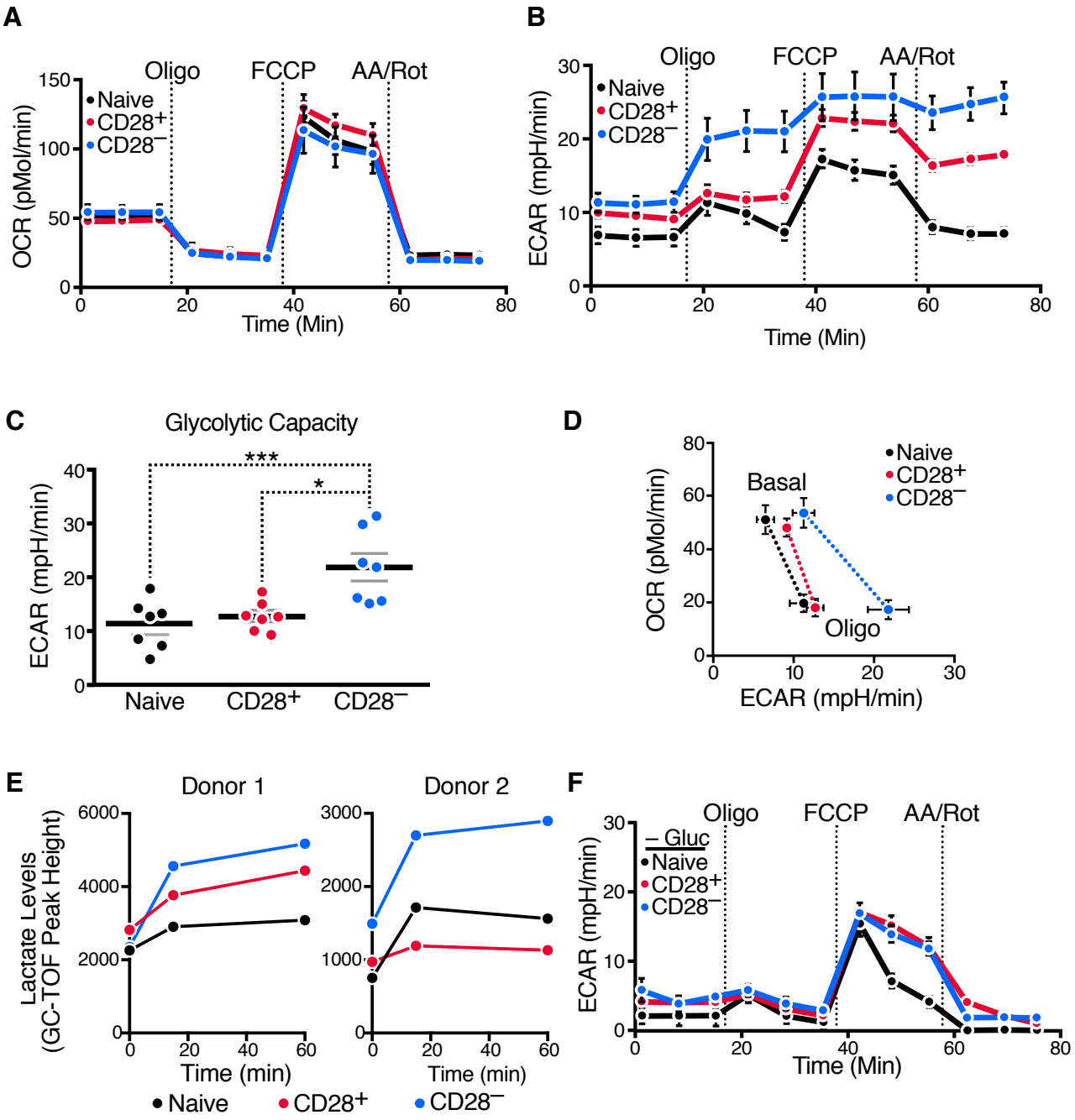

Figure 3-2. Resting CD8⁺CD28⁻ T cells have enhanced glycolytic capacity.
(A-D, F) Metabolism of sorted human CD8⁺ T cell subsets were measured with Seahorse XF Analyzer. Using a standard mitochondrial stress test [see Materials and Methods for more details], **(A)** Oxygen consumption rate (OCR) and **(B)** extracellular acidification rate (ECAR) was assessed. **(C)** Glycolytic Capacity (Max ECAR after oligomycin injection). **(D)** Energy Profile (OCR vs ECAR) plot. **(F)** ECAR was assessed under glucose deprivation. **(E)** Lactate concentrations in sorted T cell populations were analyzed by GC-TOF following oligomycin treatment (n = 2). Data are mean ± SEM of **(A-D)** 7 donors. *P<0.05, ***P<0.001; **(C)** paired one-way ANOVA. **(F)** Representative donor (n = 2).

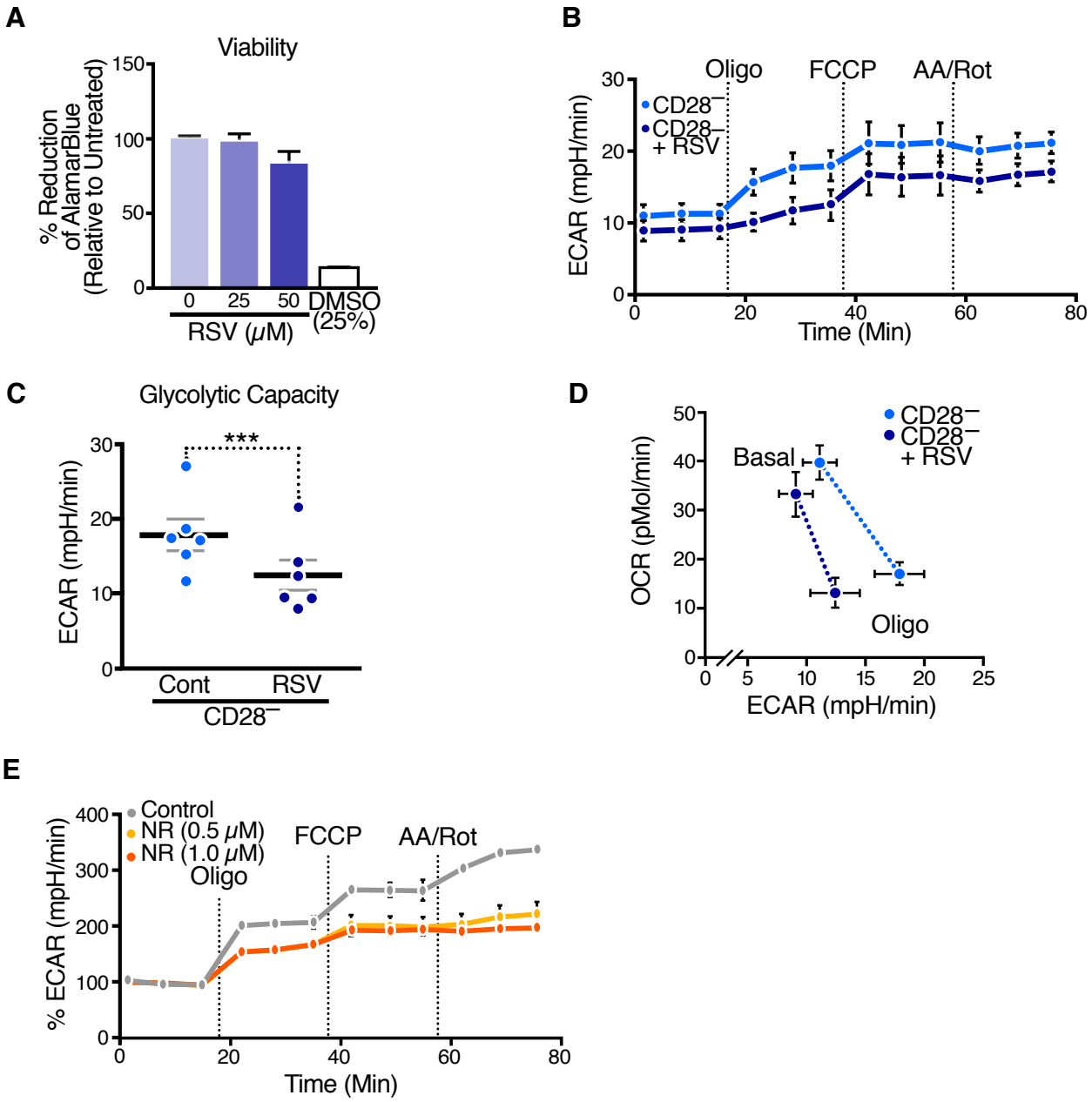

Figure 3-3. SIRT1 activation corrects glycolytic capacity.

(A) Viability of human $CD8^+$ T cells assessed by alamar blue assay after resveratrol treatment for 48 hours; 25% DMSO was used as toxicity control. **(B-E)** Metabolism of sorted human T cell populations was assessed using an extracellular-flux (XF) analyzer. **(B,C)** ECAR measured after 48 hours in 50 μM resveratrol-treated human T cells. **(D)** Energy Profile (OCR vs ECAR) after 48 hours in 50 μM resveratrol-treated human T cells. **(E)** % ECAR (normalized to 100% baseline) of $CD8^+CD28^-$ T cells treated with nicotinamide riboside for 48 hours. Data are mean ± SEM of **(A)** 2 donors and **(B-D)** 6 donors. ***P<0.001; **(C)** paired two-tailed student's t-test. **(E)** Representative donor (n = 2).

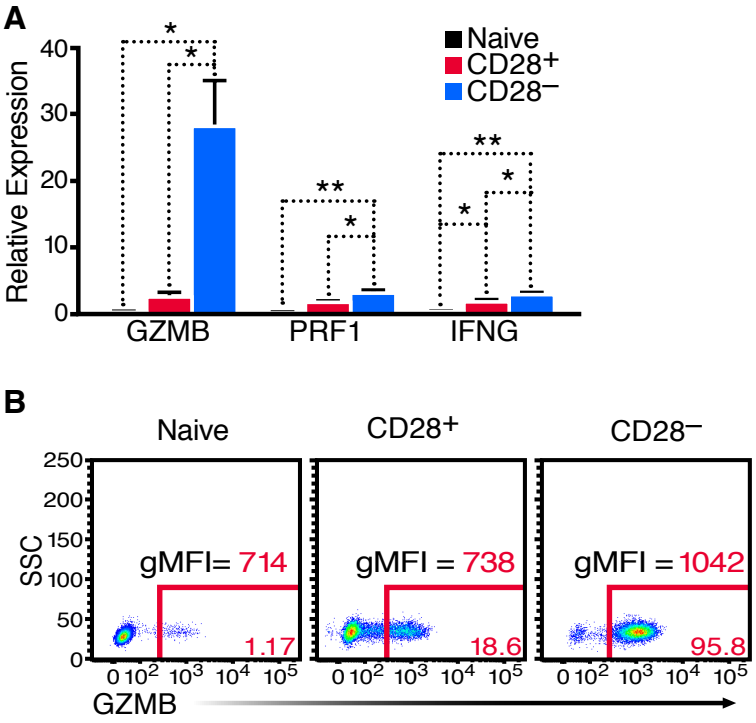

Figure 3-4. CD8⁺CD28⁻ T cells possess enhanced cytotoxicity.
(A) Cytotoxic gene expression assessed by qRT-PCR in freshly isolated human T cells relative to the average expression in $CD28^+$ cells. Data are mean ± SEM of 7 donors. *P<0.05; **P<0.01 (paired one-way ANOVA). **(B)** GZMB intracellular staining was measured by flow cytometry in resting T cell populations treated with brefeldin A for 4 hours (Representative data, n = 3).

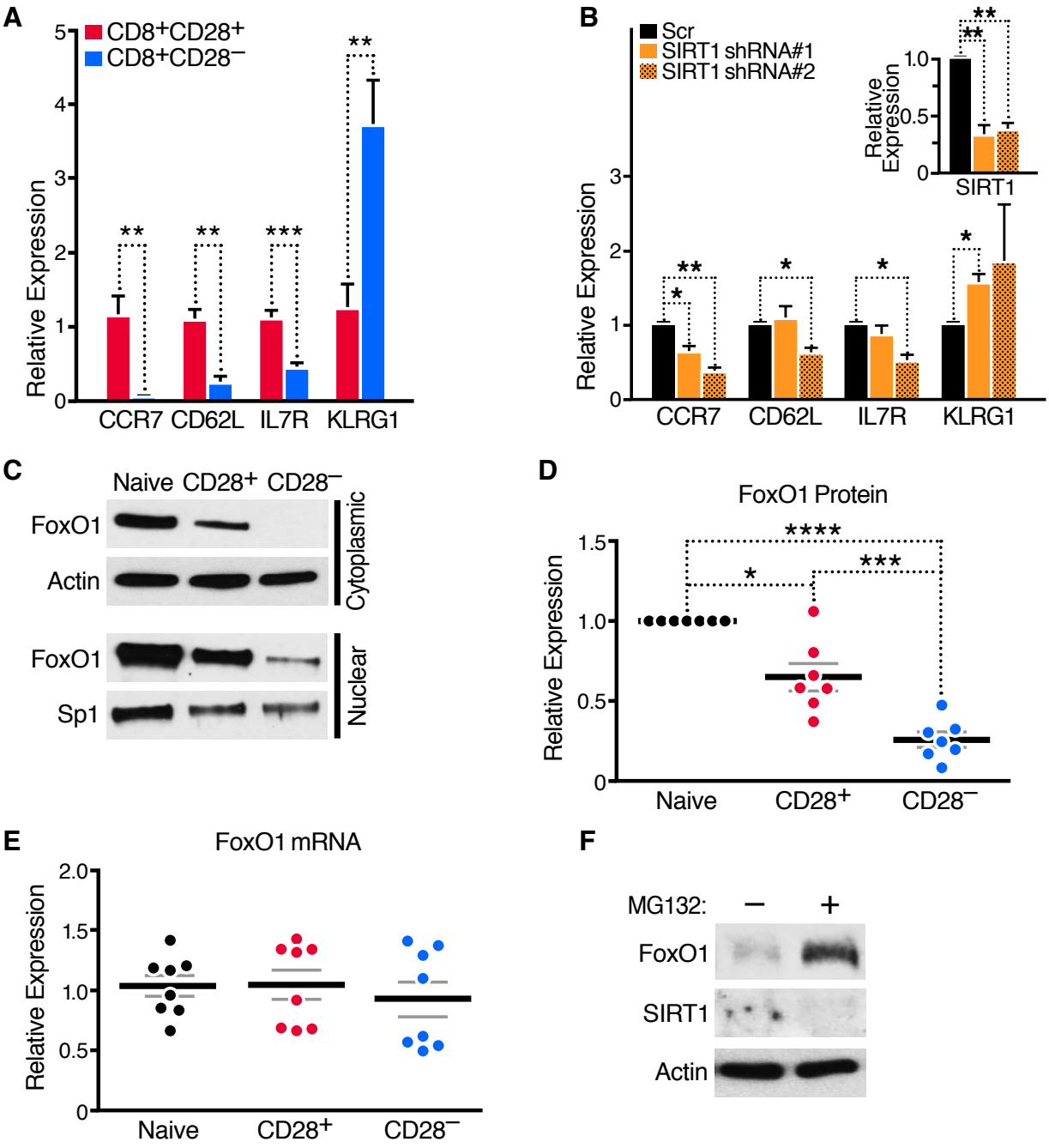

Figure 3-5. SIRT1 regulates FoxO1 target–gene expression in CD8⁺ T cells.
(A,B) CCR7, CD62L, IL7R and KLRG1 mRNA was assessed by qRT-PCR and normalized to RPL13A mRNA from **(A)** sorted human T cell populations and **(B)** $CD8^+CD28^+$ T cells transduced with either scramble or SIRT1-targeting shRNAs. **(C–E)** FoxO1 expression in sorted human T cells was measured by **(C,D)** immunoblot and **(E)** qRT-PCR, normalized to *RPL13A*. **(F)** FoxO1 expression was measured after treatment with 20 μM MG132 for 6 hours. Data are mean ± SEM of **(A)** 5 donors, **(B)** 4 donors/condition, **(D)** 7 donors, and **(E)** 8 donors. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; **(A,B)** paired two-tailed student's t-test or **(D,E)** paired one-way ANOVA. **(C,F)** Representative blots (n = 2).

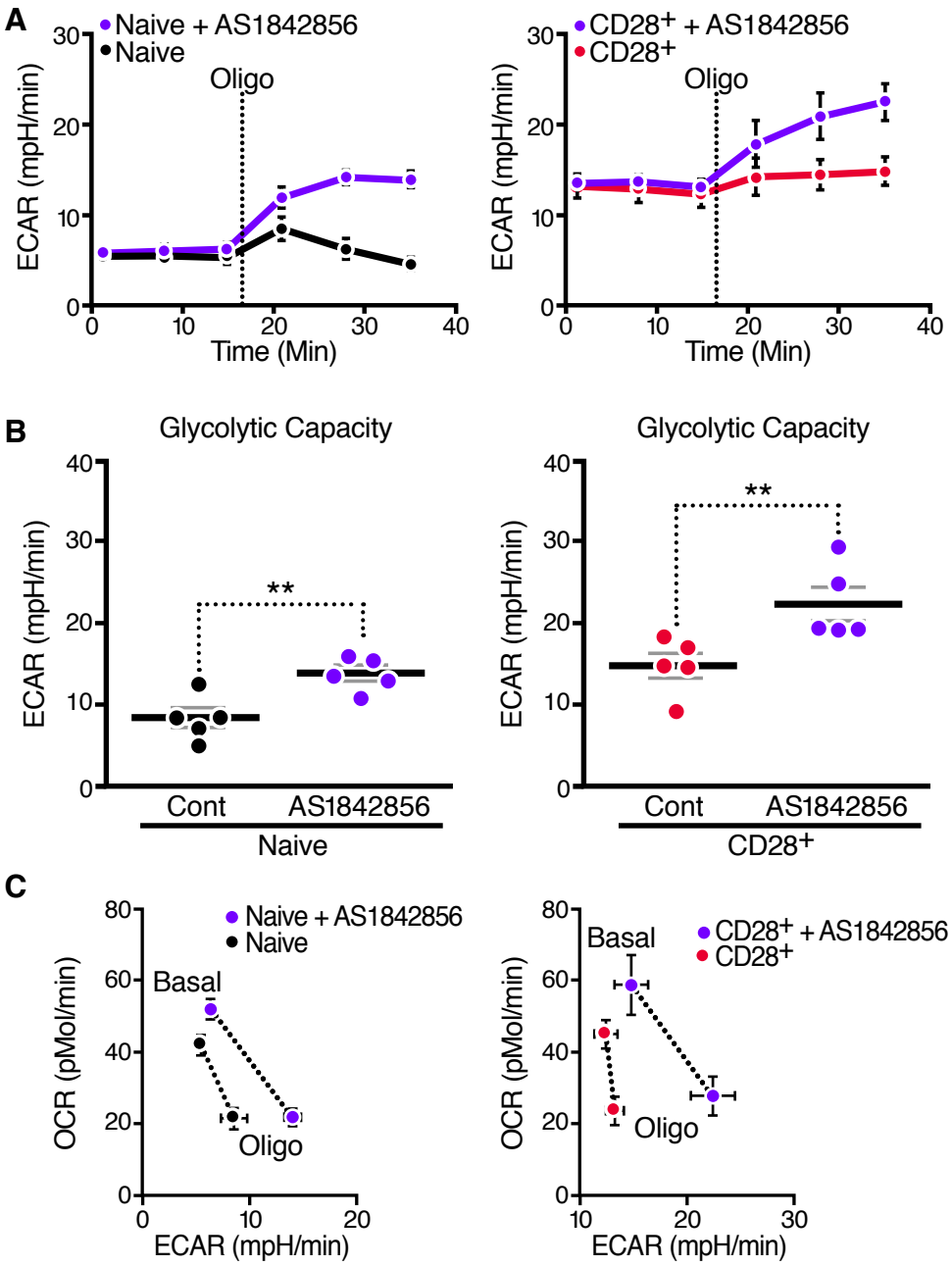

Figure 3-6. Downregulation of FoxO1 reprograms CD8⁺ T–cell metabolism.
(A,B) ECAR of sorted human T–cell populations treated with 100 nM AS1842856 for 48 hours was assessed with an extracellular-flux (XF) analyzer. **(C)** Energy Profile (OCR vs ECAR) was measured in sorted human T–cell populations treated with 100 nM AS1842856 for 48 hours. Data are mean ± SEM of **(A-C)** 5 donors. **P<0.01; paired two-tailed student's t-test.

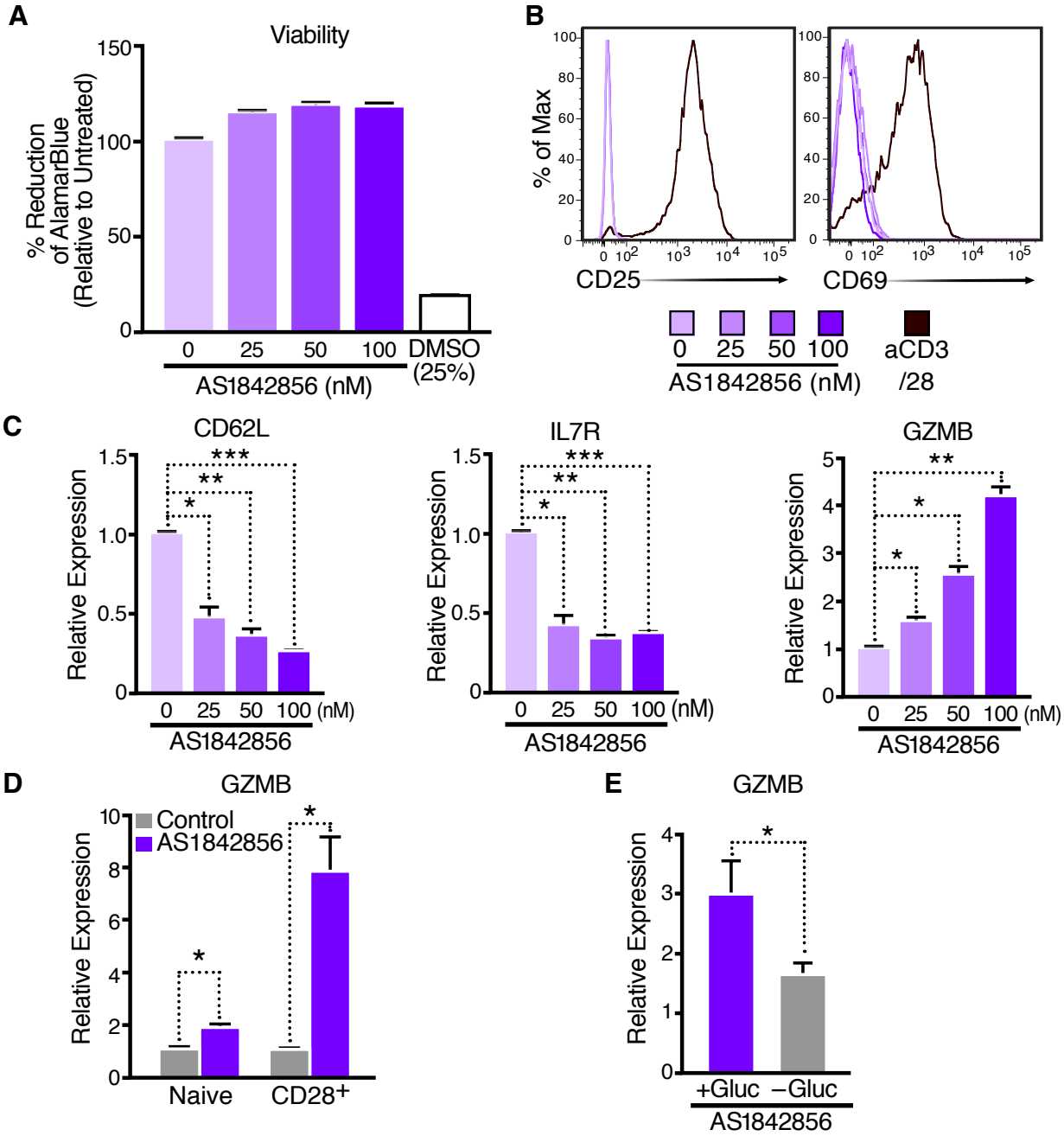

Figure 3-7. Downregulation of FoxO1 reprograms CD8⁺ T–cell cytotoxicity.
(A) Viability of human CD8⁺ T cells was assessed with an alamar blue assay after AS1842856 treatment for 48 hours; DMSO (25%) served as toxicity control. **(B)** Activation markers CD25 and CD69 were assessed by flow cytometry in CD8⁺ T cells following 48 hour treatment of AS1842856 or aCD3/28 activation (representative, n = 2). **(C-E)** Gene expression was measured by qRT-PCR, normalized to *RPL13A* and relative to untreated samples in **(C)** human CD8⁺ T cells after increasing doses of AS1842856 for 48 hours, **(D)** human CD8⁺ naïve and CD8⁺CD28⁺ T cells after treatment with 100 nM AS1842856 for 48 hours, **(E)** human CD8⁺ T cells after treatment with 100 nM AS1842856 for 48 hours, with and without glucose. Data are mean ± SEM **(A)** 2 donors/condition, **(C)** 3 donors/condition, **(D)** 3 donors/condition, and **(E)** 5 donors/condition. *P<0.05; **P<0.01; ***P<0.001, paired two-tailed student's t-test.

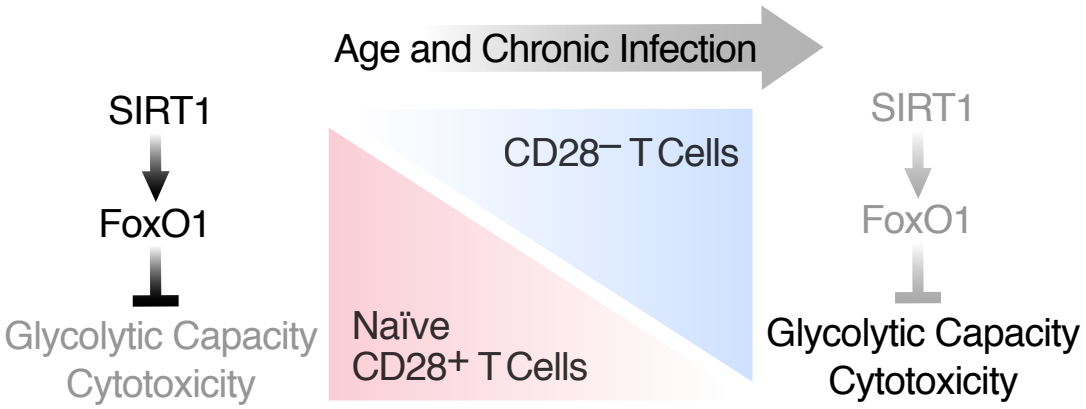

Figure 3-8. Model: A dynamic SIRT1-FoxO1 axis regulates metabolism and cytotoxicity in $CD8^+$ T cells. This axis is lost in $CD8^+CD28^-$ T cells, resulting in increased T-cell glycolytic capacity and cytotoxicity.

CHAPTER IV

IL-15 Promotes TCR-independent Activation of $CD8^+CD28^-$ T cells through mTOR

Summary

A defining characteristic of terminally differentiated $CD8^+CD28^-$ T cells is their impaired responsiveness to antigen stimulation. Previous reports, however, have indicated that $CD8^+CD28^-$ T cells retain proliferative capacity in response to certain cytokine stimulus, such as the pro-memory cytokine IL-15. In this chapter, we explore the signaling and functional consequences of IL-15 treatment on $CD8^+CD28^-$ T cells. We find that IL-15, signaling through the mTOR pathway, induces a metabolic state supportive of effector and memory T cell function. We also demonstrate that both glucose deprivation and SIRT1 activation suppress IL-15-mediated activation, thus connecting the SIRT1-FoxO1 axis identified in **Chapter III** to activated $CD8^+CD28^-$ T cells as well.

Introduction

CD28 is a co-stimulatory receptor that is critical for T cell activation. Without CD28 signaling, generated by the binding of B7 family members on antigen presenting cells (APCs), TCR engagement of naïve T cells alone cannot effectively induce T cell proliferation and effector function. Therefore given the importance of this co-receptor, it is not fully understood what signals support the maintenance and cytotoxicity of $CD8^+CD28^-$ T cells, a population of age-acquired memory cells that have permanently lost the expression of CD28.

One factor of particular interest is IL-15, a pleiotropic cytokine important for the development, maintenance, and function of naïve T cells, memory T cells, NK cells, NKT cells, and intestinal epithelial cells *(85)*. On the surface of monocytes/macrophages and dendritic cells, IL-15 forms a complex with receptor IL-15Rα. The IL-15/IL-15Rα complex is then presented in *trans* to the heterotrimeric IL-15 receptor (composed of IL-2R and IL-15Rb subunits) on the surface of NK and T cells *(85)*. In $CD8^+$ memory T cells, comparative and kinetic analysis on the effects of IL-15 vs TCR stimulation revealed surprisingly similar responses regarding proliferation, gene expression changes (77% overlap), synthesis of effector molecules, and cytotoxicity *(86)*. A subsequent study reported that IL-15 could induce similar proliferative responses in both $CD8^+CD28^+$ and $CD8^+CD28^-$ T cells *(87)*.

Here, we investigated the downstream consequences of IL-15 treatment on $CD8^+CD28^-$ T cells. We found that IL-15 signals through the mechanistic target of rapamycin (mTOR) complex, a master growth regulator, to induce a metabolic profile (high glycolytic and oxidative capacity) found in activated effector memory T cells *(46)*. We demonstrated that IL-15-mediated induction of proliferation and effector gene expression was affected by the presence of glucose

and SIRT1 activity. Together, this study provides a better understanding of how $CD8^+CD28^-$ T cells are maintained and respond to extracellular signals, affording greater physiological context to their activity in the human immune system.

Results

A defining characteristic of $CD8^+CD28^-$ T cells is an impaired proliferative response to TCR stimulation *(25)*. In line with previous reports, we found that $CD8^+CD28^-$ T cells were unresponsive to αCD3/28 activation; however, they proliferated at similar rates as their $CD28^+$ counterparts when treated with the cytokine IL-15 (Figure 4-1A). This result supports the notion that $CD8^+CD28^-$ T cells are not "senescent", in the sense of being in a state of permanent cell-cycle arrest, but instead, represent a population of terminally differentiated memory T cells responsive to selective TCR-independent signals such as IL-15. As such, we find that $CD8^+CD28^-$ T cells consistently have higher expression of IL-15 receptor (*IL15Rb*) in comparison to $CD8^+$ naïve and $CD8^+CD28^+$ memory T cells, suggesting that these cells may have adapted to receiving other signals (Figure 4-1B).

Increased metabolic activity is a hallmark of T cell activation *(59)*. To test whether IL-15 induced similar changes in metabolism, we treated sorted T cells with IL-15 for 48 hours and measured metabolic output using an extracellular flux (XF) analyzer. We observed that IL-15 treatment increased several measurements of mitochondrial respiration in T cells, including basal respiration (before oligomycin injection) and spare respiratory capacity (SRC; after FCCP injection) (Figure 4-2A). SRC—the extra mitochondrial capacity available to produce ATP under stressed conditions—is an important regulator of memory T cell formation and function *(88)*. By

analyzing their ECAR responses, we found that glycolytic activity was also upregulated, as indicated by increased basal glycolysis (before oligomycin injection) and glycolytic capacity (after oligomycin injection) (Figure 4-2B). While increased aerobic glycolysis is a primary metabolic characteristic of activated effector T cells, increased glycolytic capacity is a feature more specific to effector memory T cells *(50)*. Interestingly, we found that the increased IL-15-induced metabolic activity was greater in $CD28^-$ T cells than $CD28^+$ T cells (Figure 4-2A,B), possibly from higher expression of IL15Rb. Together, we demonstrate that IL-15-stimulated $CD8^+CD28^-$ T cells possess a metabolic profile similar to activated effector memory T cells, as indicated by increased basal metabolic activity, as well as increased metabolic potential (SRC and glycolytic capacity).

Consequently, we investigated whether the increased metabolic activity is associated with changes in metabolic gene expression. Previous reports have demonstrated that IL-15 promotes oxidative metabolism and SRC by increasing mitochondrial biogenesis and fatty acid oxidation (FAO)-associated factors *(88)*. In line with previous reports, we found that IL-15 increased the expression of mitochondrial transcription factor, TFAM, in both $CD8^+CD28^+$ and $CD8^+CD28^-$ T cells (Figure 4-2C). As expected, we observed an increase in mitochondrial mass after IL-15 treatment in $CD28^+$ and $CD28^-$ memory T cells (Figure 4-2D). Notably, the increase in mitochondria mass was greater in $CD28^-$ than $CD28^+$ T cells, correlating with the previous trends in TFAM expression and OCR activity (Figures 4-2C and 4-2A). Furthermore, we found that the rate-limiting FAO-transferase protein, CPT1α, was also upregulated after IL-15 treatment (Figure 4-2E), indicating that multiple OXPHOS components are stimulated with this cytokine. Next, we measured changes in genes associated with glycolysis. We found that transcription factor HIF1α, a master regulator of T cell glycolysis *(89)*, was upregulated after IL-

15 treatment in $CD28^-$ T cells (Figure 4-2F). Concordantly, we found that expression of direct HIF1α-target genes such as glucose transporter 1 (GLUT1), and rate-limiting glycolytic enzymes such as PFK1 and PKM2, were also upregulated in $CD8^+CD28^-$ T cells following IL-15 treatment (Figure 4-2G). These data demonstrate that changes in $CD8^+CD28^-$ T cell metabolic activity after IL-15 stimulation is associated with changes in appropriate metabolic gene programming.

After TCR engagement, PI3K-Akt signals through mTOR in order to activate downstream changes in T cell metabolism. Given that IL-15 has been shown to signal through mTOR in NK cells, we wanted to investigate whether mTOR was activated after IL-15 treatment in $CD28^-$ T cells. We found that mTOR activity, as assessed by downstream phosphorylated S6 (pS6) ribosomal protein expression, was increased after IL-15 treatment in $CD28^+$ and $CD28^-$ memory T cells, and abrogated in the presence of mTOR inhibitor, rapamycin (Figure 4-3A). These differences in IL-15 responsiveness are in line with the previously observed metabolic differences (Figure 4-2) as well as the varying levels of IL-15Rb (Figure 4-1B). To assess whether the enhanced metabolic activity associated with IL-15 is mTOR dependent, we treated sorted T cells with IL-15 in absence or presence of rapamycin, and then performed measurements using XF analysis. We found that all IL-15-induced metabolic features (basal respiration, SRC, basal glycolysis, glycolytic capacity) were impaired in the presence of rapamycin, indicating that the metabolic effects of IL-15 signal through mTOR (Figure 4-3B,C). Consequently, as increased metabolic activity supports T cell proliferation, we found that IL-15-induced proliferation in $CD8^+CD28^-$ T cells was inhibited with rapamycin (Figure 4-4D). Together, these results demonstrate the metabolic program mediated by IL-15 is dependent on mTOR.

$CD8^+CD28^-$ T cells are highly cytotoxic, even under resting conditions (See **Chapter III**). Here, we found that activation with IL-15 further upregulated cyototoxic genes such as GZMB, IFNG, and PRF1 in a glucose-dependent manner, further connecting the metabolic profile of $CD8^+CD28^-$ T cells with its cytotoxicity (Figure 4-5A). The upregulation of cytotoxic genes with IL-15 was also abrogated after treatment with SIRT1 activator, resveratrol (Figure 4-5B). These results demonstrate that the previously identified SIRT1-FoxO1 axis affects IL-15 activation of $CD8^+CD28^-$ T cells as well. Finally, IL-15 induced proliferation is similarly impaired with glucose deprivation as well as resveratrol treatment (Figure 4-5C), indicating that IL-15-mediated activation of $CD8^+CD28^-$ T cells is dependent on glucose and SIRT1 activity.

Discussion

IL-15 is a pleiotropic cytokine that is crucial for the development, proliferation, survival, and differentiation of cells within both the innate and adaptive immune system. Given its selective activation properties (i.e. minimal effect on regulatory T cell expansion) and relatively low toxicity index compared to other cytokine therapies, IL-15 is being heavily pursued in several clinical trials to treat cancer and HIV *(85)*. Therefore, as IL-15 treatment continues to move to the forefront of immunotherapy, it becomes critical to deepen our understanding of IL-15 and the impact it has on different T cell subsets in the immune system.

In this study, we investigate the effects of IL-15 on $CD8^+CD28^-$ T cells. In **Chapter III**, we demonstrate that $CD8^+CD28^-$ T cells, even in the resting state, are highly cytotoxic, upregulating many NKC receptors and producing excessive amounts of cytolytic molecules. Here, we establish IL-15 as an antigen-independent activation signal that induces proliferation

and heightens cytotoxicity in $CD8^+CD28^-$ T cells. We find that IL-15 signals through the mTOR pathway and upregulates metabolic activity and associated metabolic genes. Finally, activation of SIRT1 with resveratrol suppresses IL-15-mediated activation of cytotoxicity and proliferation—implicating a role for the SIRT1-FoxO1 axis in both resting and activated $CD8^+CD28^-$ T cells.

IL-15 plays several important roles in autoimmune disease and inflammation. Elevated IL-15 signaling has been implicated in inflammatory diseases such as rheumatoid arthritis, celiac disease, multiple sclerosis, and inflammatory bowel disorder *(90)*. Interestingly, a recent study reported that IL-15 levels not only increased in the spleens of aging mice, but also in several peripheral lymphoid organs following LPS-induced inflammation *(91)*—further connecting IL-15 to $CD8^+CD28^-$ T cells, which expand in humans during aging and chronic infections. In support of this, administration of intravenous IL-15 was shown to stimulate a 100-fold *in-vivo* expansion of $CD8^+CD28^-$ memory T cells in rhesus macaques *(92)*. Therefore as the utilization of IL-15 therapies progress in clinical trials, we advise that the frequency and activity of $CD8^+CD28^-$ T cells in patients be closely monitored. Because of the highly cytotoxic nature of $CD8^+CD28^-$ T cells and their responsiveness to IL-15, we anticipate that the degree of their expansion may correlate with the inflammation-associated toxicity of IL-15 immunotherapy, and possible serve as a novel biomarker in future IL-15 trials.

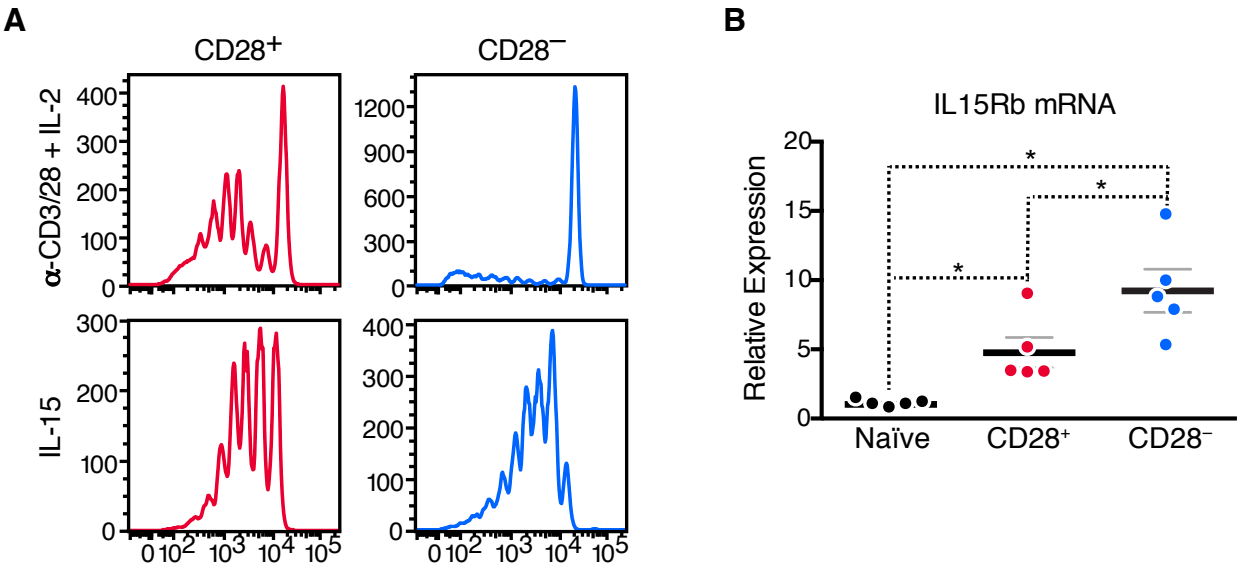

Figure 4-1. IL-15 induces proliferation in CD8⁺CD28⁻ T cells.
(A) Cell proliferation of sorted human T cells activated for 7 days with αCD3/28 + IL2 or 14 days with IL-15, as assessed by CFSE dilution (representative donor, n = 2). **(B)** *IL15Rb* mRNA was measured by qRT-PCR and normalized to *RPL13A* in sorted human T cells. Data are mean ± SEM of **(B)** 5 donors. *P<0.05; paired one-way ANOVA.

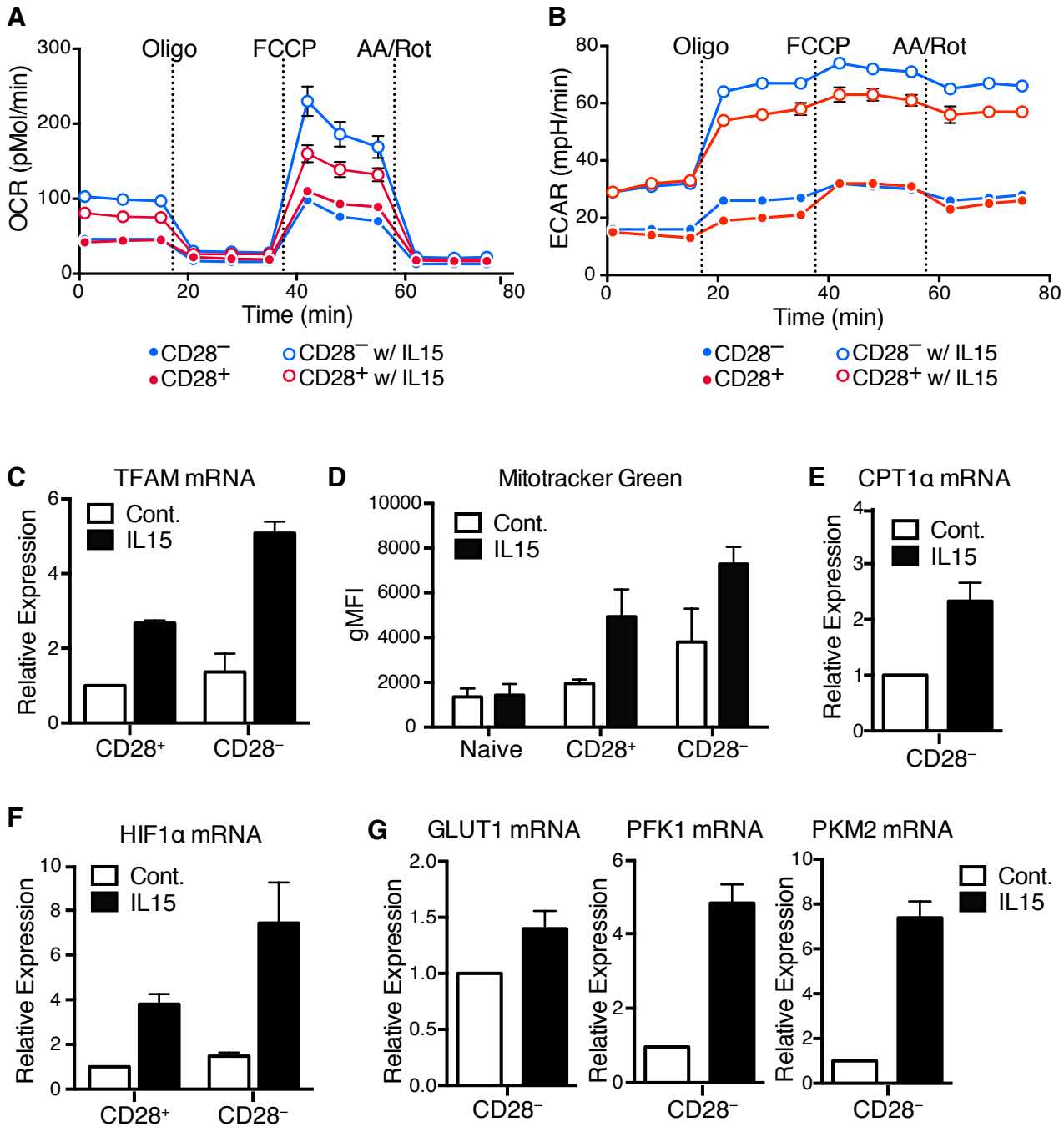

Figure 4-2. IL-15 activates metabolic program in CD8⁺CD28⁻ T cells.
(A) Metabolism of sorted human CD8⁺ T cell subsets was measured with Seahorse XF Analyzer. Using a standard mitochondrial stress test [see Materials and Methods for more details], **(A)** Oxygen consumption rate (OCR) and **(B)** extracellular acidification rate (ECAR) was assessed. **(C, E-G)** Gene expression was measured by qRT-PCR, normalized to *RPL13A* in sorted human T cells treated with IL-15 for 48 hours. Data are mean ± SEM of **(C-G)** 2 donors. **(A-B)** Representative data, n = 2.

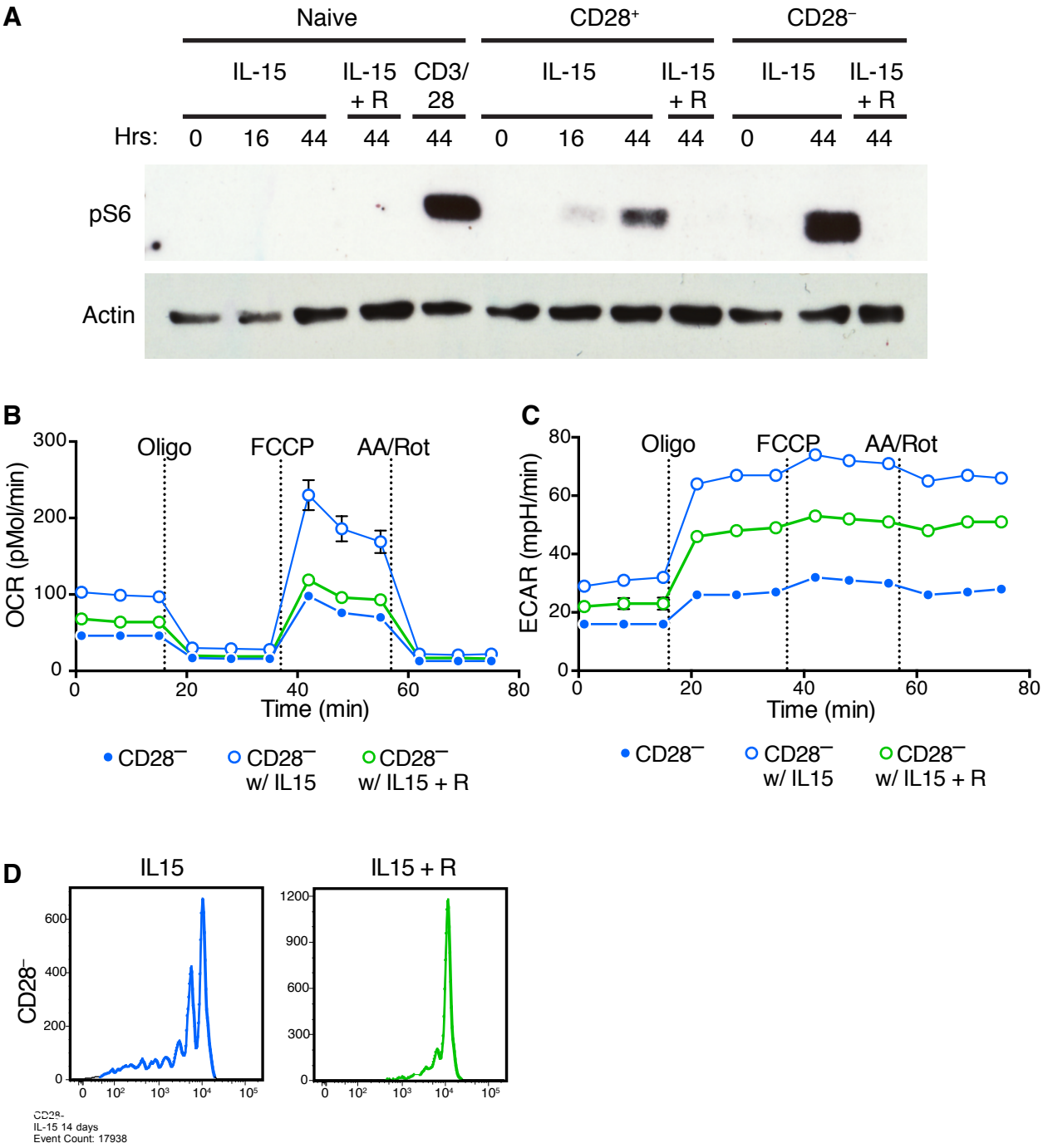

Figure 4-3. IL-15 signals through mTOR in CD8⁺CD28⁻ T cells.
(A) pS6 was measured by immunoblot in sorted T cell subsets under various activating conditions. **(B,C)** Metabolism of CD8⁺CD28⁻ T cells treated with IL-15 ± Rapamycin (R) for 48 hours were measured with Seahorse XF Analyzer. **(B)** Oxygen consumption rate (OCR). **(C)** extracellular acidification rate (ECAR). **(D)** Cell proliferation of CD8⁺CD28⁻ T cells treated with IL-15 for 14 days ± Rapamycin (R), as assessed by CFSE dilution. **(A-D)** Representative data, n = 2.

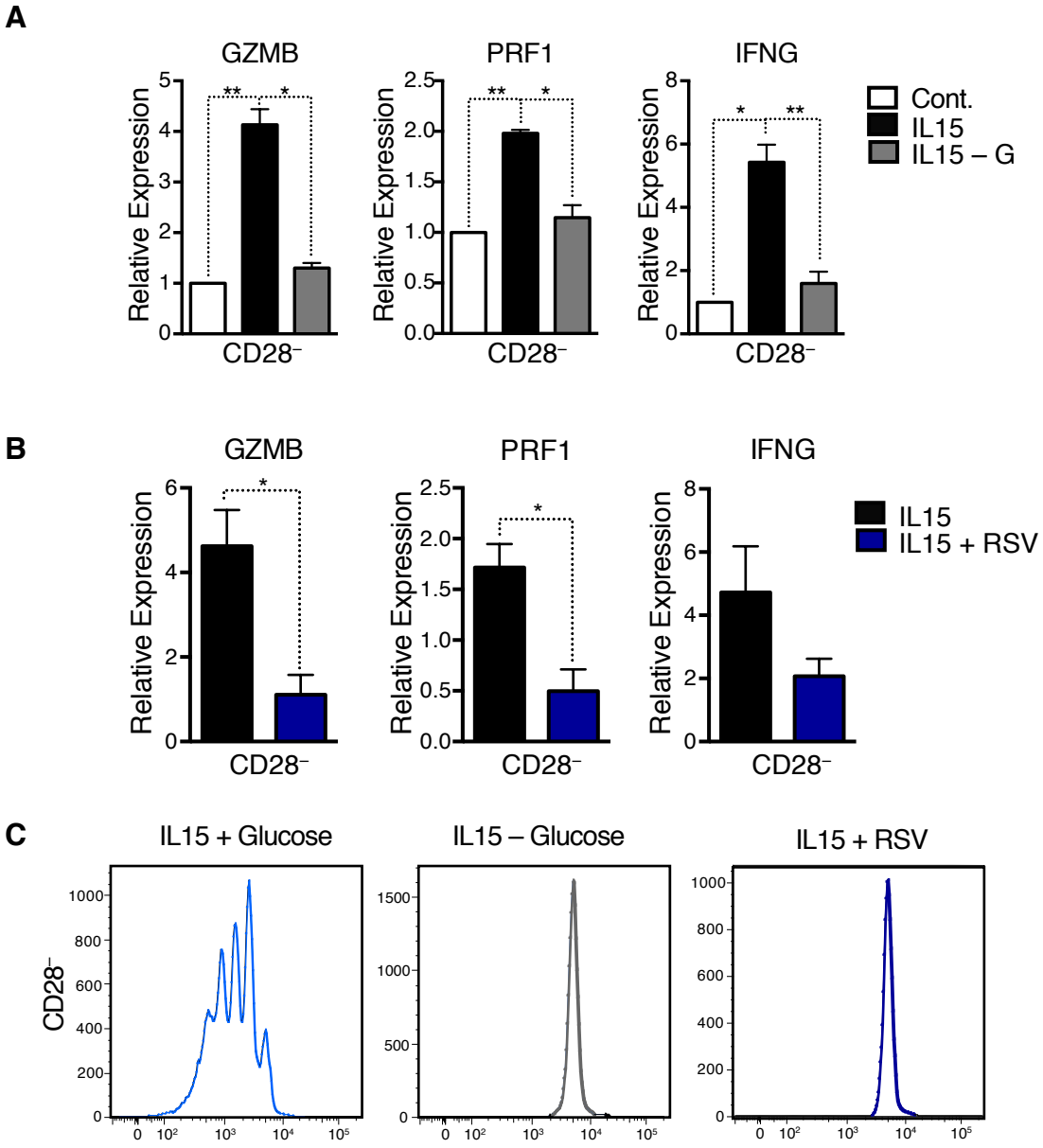

Figure 4-4. IL-15-mediated activation of CD8$^+$CD28$^-$ T cells is dependent on glucose and SIRT1.

Gene expression was assessed by qRT-PCR and normalized to *RPL13A* in **(A)** CD8$^+$CD28$^-$ T cells treated with IL-15 ± glucose (48 hours) relative to unstimulated cells, and **(B)** CD8$^+$CD28$^-$ T cells treated with IL-15 ± RSV (48 hours) relative to unstimulated cells. **(C)** Cell proliferation of CD8$^+$CD28$^-$ T cells activated with IL-15 for 7 days, as assessed by CFSE dilution (representative donor, n = 2). Data are mean ± SEM of **(A)** 3 donors/condition or **(B)** 4 donors/condition. *P<0.05, **P<0.01; paired two-tailed student's t-test.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

Summary

In this thesis, we investigate the molecular changes that occur within $CD8^+$ T cells during aging. In particular, we focus on $CD8^+CD28^-$ T cells, a population of terminally differentiated memory cells that accumulate during aging and linked to several age-related diseases. While unresponsive to TCR engagement, $CD8^+CD28^-$ T cells are instead supported by the pro-memory cytokine, IL-15. We find that IL-15 signals through mTOR and activates their proliferation and cytotoxicity in a TCR-independent manner. However even in their resting state, $CD8^+CD28^-$ T cells remain highly cytotoxic. By analyzing the metabolic state of resting $CD8^+CD28^-$ T cells, we find they possess an enhanced capacity to utilized glycolysis—a metabolic potential linked to the downregulation of SIRT1 protein. Through global gene profiling, we identify the transcription factor, FoxO1, as a downstream target of SIRT1 in $CD8^+$ T cells. We demonstrate that inhibition of FoxO1 activity in $CD8^+$ T cells increases GZMB production in a glucose-dependent manner, thereby connecting the metabolic profile of resting $CD8^+CD28^-$ T cells with their excessive cytotoxic activity. While intact in $CD8^+$ naïve and $CD8^+CD28^+$ memory T cells, this SIRT1-FoxO1 axis is severely weakened in $CD8^+CD28^-$ T cells, resulting in reprogramming of T cell metabolism and effector function. Intriguingly, treatment with SIRT1 activator, resveratrol, reverses many of the functional properties of resting and IL-15-activated $CD8^+CD28^-$ T cells. Accordingly, we propose that agents that strengthen the SIRT1-FoxO1 could serve as potential therapeutics to delay or reverse immune aging in humans.

Conclusions

The downregulation of co-receptor CD28 is a hallmark of T cell immunosenescence and serves as an important component of an individual's immune risk profile. Additional markers—including the loss of CD27 and gain of CD57 and KLRG1—are thought to further identify the most terminally differentiated population of T cells, which are functionally characterized by a weak proliferative capacity and high cytotoxic activity *(93, 94)*. However, both disease-mediating and protective roles have been attributed to $CD28^-$ T cells. Recent work in rhesus macaques demonstrate that $CD28^-$ T cells represent a highly effector-like memory population that can be targeted and expanded using a CMV-derived vector, enhancing immune protection against viruses such as HIV *(95)*. It is important to note that the accumulation of $CD28^-$ T cells during aging does not occur in mice, possibly because of the sterile conditions laboratory mice are kept in *(96)*. Given their ubiquitous presence in humans, as well as their increasingly appreciated role in both immune protection and human disease, there is much interest in understanding the molecular factors governing their fate and function.

Here, we find that resting $CD8^+CD28^-$ T cells possess a distinctly enhanced glycolytic capacity, driven by the loss of the metabolic regulator, SIRT1. This energy profile is reminiscent to the metabolic features of $CD8^+$ T effector memory (Tem) cells, in which their 'imprinted' glycolytic capacity supports their immediate-early glycolytic reprogramming after TCR activation *(50)*. However, in addition to what was shown with Tem cells, we demonstrate that $CD8^+CD28^-$ T cells utilize glycolysis to promote cytotoxicity under both resting and cytokine-mediated activation. This distinction points to a possible role for $CD8^+CD28^-$ T cells in executing antigen-independent effector functions, promoting innate-like protective responses, or—when present in excessive and unregulated conditions—autoimmunity. While metabolic

priming has been previously documented in memory T cell populations, the molecular pathways driving this reprogramming have largely remained unclear. From this study, we propose that the loss of SIRT1 promotes glycolytic 'imprinting' in terminally differentiated $CD8^+CD28^-$ T cells through the destabilization of the downstream transcription factor, FoxO1. In resting $CD8^+$ naïve and $CD8^+CD28^+$ T cells with high levels of SIRT1-FoxO1, inhibiting Foxo1 activity with AS1842856 enhances glycolytic capacity. Conversely in $CD8^+CD28^-$ T cells that have low levels of SIRT1-FoxO1, activating SIRT1 activity with resveratrol reduces the glycolytic capacity. How the loss of the SIRT1-FoxO1 axis translates into this specific metabolic profile in resting $CD8^+CD28^-$ T cells remains to be determined. Both SIRT1 and FoxO1 possess multiple and complex regulatory functions in cellular and organismal metabolism. In the case of FoxO1, several pathways—including gluconeogenesis, glycolysis, lipolysis, and insulin signaling—are affected by this transcription factor *(68, 97, 98)*. The FoxO1-regulated glycolytic programs can either be the result of promoter binding directly onto these genes or via the regulation of another metabolic transcription factor such as HIF1α or Myc *(99, 100)*.

Interestingly, we find that the metabolic effects of shifting the SIRT1-FoxO1 axis simultaneously promotes changes in T cell cytotoxicity as well—further highlighting the intimate relationship between cellular metabolism and effector function. Previous studies have shown that glycolysis gene expression is not only important to support the energetic demands required to produce cytotoxic molecules, but can also work through metabolism-independent effects, such as the ability of GAPDH to bind and block translation of *IFNG* mRNA *(49)*. Our data demonstrate that FoxO1 may be playing a central role in coupling cytotoxicity and glycolytic capacity in $CD8^+$ T cells. It remains to be seen whether the FoxO1-regulated cytotoxicity in $CD8^+CD28^-$ T cells is driven primarily through its effects on metabolism, or via

its previously observed interactions with the transcription factor T-bet *(71)*. Nonetheless, we find that the metabolic profile of $CD8^+CD28^-$ T cells is intrinsically tied to their heightened cytotoxicity—possible driving their activity in autoimmune diseases. In this study, we demonstrate that resting and IL-15-activated $CD8^+CD28^-$ T cells produce significantly higher amounts of the serine protease, GZMB. Beyond its well-characterized role of inducing apoptosis in virally infected cells, GZMB can also degrade extracellular matrix proteins and propagate inflammatory signaling, thereby playing an important pathological role in several age-related, chronic inflammatory diseases *(32)*. Amplifying the situation, we and others have also reported the acquisition of several activating NK cell (NKC) receptors on the surface of $CD28^-$ T cells (Table 3-1, left side) *(58)*. The presence of these receptors fundamentally augments the specificity of T cell cytotoxicity, and contributes to inflammatory disorders such as rheumatoid arthritis *(101-103)*.

This work also builds upon previous studies characterizing the essential role of FoxO1 in regulating T cell homing and differentiation *(69, 72, 73)*. As such, it is tempting to envision FoxO1 seated at the heart of a complex reprogramming network which synchronizes several aspects of T cell biology: coordinating the differentiation of T cell subsets with where they traffic to, how they respond to metabolically restrictive environments, and their cytotoxic capabilities. A deeper understanding of these connections may provide greater insight into the contributions of cytotoxic $CD8^+CD28^-$ T cells within inflammatory or tumor microenvironments, which are typically hypoxic and glucose-restricted *(59)*. And as pursued in many cancer eradication strategies, the unique metabolic pathways within normal and disease-causing T cells may also be effectively targeted to support or suppress immune function, respectively *(52, 104)*.

Future Directions: The Mechanism of SIRT1 downregulation

In **Chapter III**, we observed that SIRT1 is post-transcriptionally downregulated in resting human $CD8^+CD28^-$ T cells (Figures 3-1, 3-2, 3-3). However unlike FoxO1, which we find degraded primarily by the proteasome (Figure 3-5F), the mechanism behind SIRT1 downregulation remains largely unclear. As a central regulator of several important cellular processes, it is unsurprising that SIRT1 is tightly regulated at multiple levels under various conditions *(105)*. In $CD4^+$ T cells, SIRT1 expression has been linked to the cell's activation state *(40, 44)*. Here, we found that treatment of $CD8^+$ memory T cells with αCD3/28 antibodies resulted in increased SIRT1 levels in the $CD28^+$, but not the $CD28^-$ subset (Figure 5-1A), indicating that T cell activation and SIRT1 expression is similarly linked in $CD8^+$ T cells. Given that CD28 receptor engagement is a critical component of T cell activation, we investigated whether the loss of CD28 co-receptor was directly linked to the downregulation of SIRT1. To test this, we utilized a previously establish *ex-vivo* culture system to generate $CD8^+CD28^-$ T cells from $CD8^+CD28^+$ T cells *(87)*. We performed a long-term culture of sorted $CD8^+CD28^+$ T cells (99.9% purity) in the presence of IL-15, which led to significant and stable loss of CD28 surface expression (Figures 5-1B, 5-1C). When $CD8^+CD28^+$ and $CD8^+CD28^-$ T cells were re-sorted after 20 days of culture, we did not observe differences in SIRT1 expression, indicating that the downregulation of SIRT1 in $CD8^+CD28^-$ T cells is not causally linked to the loss of CD28 surface expression (Figure 5-1D). It remains to be determined what additional signals present in human body are necessary to mediate the downregulation of SIRT1 within *in vivo*–generated $CD8^+CD28^-$ T cells.

Another well-characterized mechanism of SIRT1 post-transcriptional regulation is

through miRNA binding to the 3' untranslated region (3'UTR) of SIRT1 mRNA. There are currently more than 16 documented SIRT1-targeting miRNAs, many of which have been shown to affect various parameters of metabolism and aging in mammals *(106)*. Among these, miR-34a has been the most studied and has been demonstrated to target and downregulate SIRT1 in normal tissues as well as numerous types of cancers. Interestingly, not only does miR-34a expression increase in the heart and spleen of older mice, it has also been shown to drive cellular senescence by increasing the acetylation of a downstream SIRT1-target, FoxO1 *(107)*. Other miRNAs of interest include miR-217 and miR-146b, which have been shown to promote FoxO1-acetylation through SIRT1 downregulation in endothelial and adipocytes, respectively *(108, 109)*.

Here, we screened the expression of several known SIRT1-targeting miRNA in sorted human $CD8^+$ T cell subsets. While many miRNAs were either undetectable or had equivalent/lower expression (data not shown), a select list of candidate miRNAs—mir22, mir93, and mir181a/b/c—had greater expression in $CD8^+CD28^-$ T cells (Figure 5-2). In numerous types of cancers, mir22 expression is deregulated, affecting cellular processes including apoptosis, proliferation, and migration *(110)*. Found to be highly expressed in senescent fibroblast cells, mir22 acts as a tumor suppressor by directly targeting SIRT1 expression and thus, restoring a cellular senescence program in tumor cells *(111)*. The expression of mir93 is inversely correlated to decreasing levels of SIRT1 in the liver of aging rats and when transfected into 293T cells, led to the modest decrease of SIRT1 3'UTR reporter intensity *(112)*. The mir181 family (mir181a/b/c/d) regulates numerous aspects of T cell biology, including thymic development and T cell activation signaling *(113)*, and has been shown to target both SIRT1 3' UTR reporters, as well as endogenous levels of SIRT1 *(114)*. Whether any of these identified candidate miRNAs

regulates SIRT1 expression in $CD8^+CD28^-$ T cells remains an area of active investigation in our laboratory. Identifying and blocking the specific miRNA(s) responsible for the downregulation of SIRT1 in $CD8^+CD28^-$ T cells may prove a more effective strategy than the use of SIRT1 activators such as resveratrol, which are limited by the levels of SIRT1 present and may possess off-target effects *(115)*.

Future Directions: SIRT1 Interactome in Jurkat T Cells

SIRT1 is a versatile deacetylase that regulates a broad range of cellular processes by targeting an ever-growing list of histone (e.g., H3K9, H3K56, H4K16, etc) and non-histone targets (e.g., p300, p53, NF-kB, c-Jun, Myc, HIF1α, Ku70, PGC1 α, SREBP-1c, E2F1, Tau, etc) *(116)*. Furthermore, recent advances in high resolution mass-spectrometry have identified over 3600 novel lysine-acetyl sites on a wide range of proteins *(117)*. As a result, the loss of SIRT1 expression in $CD8^+CD28^-$ T cells is likely to disrupt many cellular pathways beyond the transcriptional activity of FoxO1. While previous SIRT1 interactomes have revealed novel mechanisms of SIRT1 activity—such as its recruitment and regulation of the SAGA transcriptional coactivating complex through its interaction with deubiquitinating enzyme ubiquitin-specific protease 22 (USP22)—these screens have been limited to 293T cells *(118, 119)*. Therefore to better understand the role of SIRT1 in T cells, we utilized an affinity tag-purification mass-spectrometry (AP-MS) approach to generate a high-confidence SIRT1 interactome in Jurkat T cells (see **Chapter II: Materials and Methods** for more detail). With a 0.75 cut-off MIST scoring threshold, as previous defined *(120)*, we identified 66 unique SIRT1 interactors (Figure 5-3 and Table 5-1). The resulting hits include many previously identified

SIRT1 interactors, such as KIAA1967 (also known as DBC1) and TRIM28 (also known as KAP1), confirming our experimental approach *(121-123)*. At the same time, this screen revealed a wide array of novel SIRT1 interactors that have interesting implications for T cell biology and immunosenescence.

During aging, alterations in several TCR signaling pathways have been observed *(124)*. One factor in particular is lymphocyte-specific protein tyrosine kinase (Lck), a well-characterized member of the Src family of tyrosine kinase. Lck associates with the cytoplasmic tails of CD4 and CD8 co-receptors and is critical for initiating signaling cascades downstream of TCR engagement. Recently, increased activity of the phosphatase SHP-1 was observed in T cells from elderly individuals, thereby favoring the inactive form of tyrosine-phosphorylated Lck (Y505) and disrupting T cell signaling processes *(125)*. While post-translational modifications such as phosphorylation and ubiquitination have proven important in the activation and inhibition of Lck activity, the role of acetylation has remained unexplored. Given that SIRT1 expression and activity is connected with T cell activation, the SIRT1-Lck interaction identified in this Jurkat T cell interactome merits further investigation.

Another interaction of interest that has emerged from this interactome is with the autophagy-associated adaptor protein p62/sequestosome-1 (SQSTM1). Autophagy—the cellular function responsible for degradation and recycling of intracellular components—plays a key role within numerous cellular contexts, including starvation, apoptosis, protein repair mechanisms, and immune function *(126)*. Consequently, the decline of proper autophagy function during aging is thought to contribute to many of the deleterious effects observed in the elderly. In $CD8^{+}CD28^{-}$ T cells, autophagy activity is impaired in both resting and TCR-activated states *(127, 128)*. Given that SIRT1 is a positive regulator of autophagy and has been shown to

deacetylate the products of several autophagy genes (ATG5, ATG7, and ATG8/LC3) in HeLa cells, it is possible that the downregulation of SIRT1 we observe in $CD8^{+}CD28^{-}$ T cells (Figures 3-1A, 3-1B) contributes to this autophagy defect *(129)*. What role, if any, does the interaction between SIRT1 and SQSTM1 play in T cell autophagy remains to be determined. SQSTM1 is a multifunctional LC3-binding protein that selectively delivers ubiquitinated proteins into the autophagosome. Interestingly, it was recently reported that SIRT1 activator, resveratrol, induces autophagy in leukemia cells in an SQSTM1-dependent manner *(130)*. Furthermore, other interventions demonstrated to improve organismal lifespan, such as caloric restriction, also activate both SIRT1 and autophagy activity, thus underscoring the rationale to better understand the interactions between SIRT1 and autophagy-associated factors *(131)*.

Finally, the newly identified interactor telomeric repeat binding factor 2 (TERF2 or TRF2) connects SIRT1 with telomere maintenance in T cells. Composed of tandem repeats of the TTAGGG sequence, telomeres are extended by the DNA polymerase telomerase and serve as protective structures on the ends of chromosomes. Defects in telomere length and telomere-associated proteins are linked to several age-related diseases and pre-mature aging syndromes *(132)*. Notably, $CD28^{-}$ T cells are characterized by shortened telomere length, a likely consequence of their extensive replicative history and inability to upregulate telomerase activity in response to various activation signals *(25)*. TERF2 is a telomere-binding protein important for the formation of larger telomeric complexes, as well as facilitating proper DNA repair mechanisms. Recently, p300-mediated acetylation of TERF2 at position K293 was demonstrated to regulate its stability and DNA-binding activity *(133)*. Whether SIRT1 serves as the deacetylase for TERF2, and is connected to the telomeric defects observed in $CD8^{+}CD28^{-}$ T cells, will require further investigation.

In conclusion, we identify metabolic and cytotoxic reprogramming in $CD8^+CD28^-$ T cells, driven by dysregulation of the SIRT1-FoxO1 pathway. A better understanding of the molecular pathways within $CD8^+CD28^-$ T cells will not only provide greater insight into their role during aging and various disease states, but may also afford novel therapeutic opportunities to manipulate their accumulation and function. We hope such studies will serve to delay or even reverse the immunological defects observed in the elderly, thus strengthening a fundamental component of healthy aging.

Table 5-1. SIRT1 Interactome MiST Scores

Gene Name	Entrez ID	MiST Score	Gene Name	Entrez ID	MiST Score
SDF2L1	23753	0.993	ACTA2	59	0.867
TUBB8	347688	0.993	ACTG2	72	0.867
TUBB4	10382	0.991	SS18	6760	0.867
SQSTM1	8878	0.984	TNR6C	57690	0.865
KIAA1967	57805	0.984	HNRNPC	3183	0.864
SIRT1	23411	0.981	GNAI3	2773	0.862
RPL5	6125	0.974	MYO1B	4430	0.862
TAL1	6886	0.969	LRRFIP2	9209	0.856
MYL12B	103910	0.943	EEF1D	1936	0.856
CORO1C	23603	0.933	MYO18A	399687	0.856
VCP	7415	0.923	KPNB1	3837	0.853
TRIM28	10155	0.889	PAICS	10606	0.853
ABI1	10006	0.880	ALDOA	226	0.853
NCBP1	4686	0.880	PURA	5813	0.834
MYL6B	140465	0.880	HSPD1	3329	0.823
RFTN1	23180	0.880	SPTBN1	6711	0.823
RNMT	8731	0.879	CCT8	10694	0.823
TERF2	7014	0.879	CCT2	10576	0.823
GAPR1	152007	0.879	TUBB2C	10383	0.820
RAP2C	57826	0.879	LCK	3932	0.813
ATP5B	506	0.879	MYL6	4637	0.809
EIF4B	1975	0.879	RPS4Y2	140032	0.806
ACTR3	10096	0.879	MYH9	4627	0.788
DBN1	1627	0.879	RPL34	6164	0.785
LMO7	4008	0.879	GNAI2	2771	0.783
GNB1	2782	0.879	MYO1G	64005	0.771
HADHA	3030	0.878	GNB2	2783	0.769
DLST	1743	0.871	HNRNPF	3185	0.768
KARS	3735	0.871	ACTA1	58	0.767
EEF1G	1937	0.871	ACTC1	70	0.767
ATP6V1G1	9550	0.871	CORO1A	11151	0.759
ENO1	2023	0.871	HSP90AA1	3320	0.756
SART1	9092	0.870	DLD	1738	0.754
FLII	2314	0.869			

List of significant SIRT1 interactors ordered by decreasing MiST score.

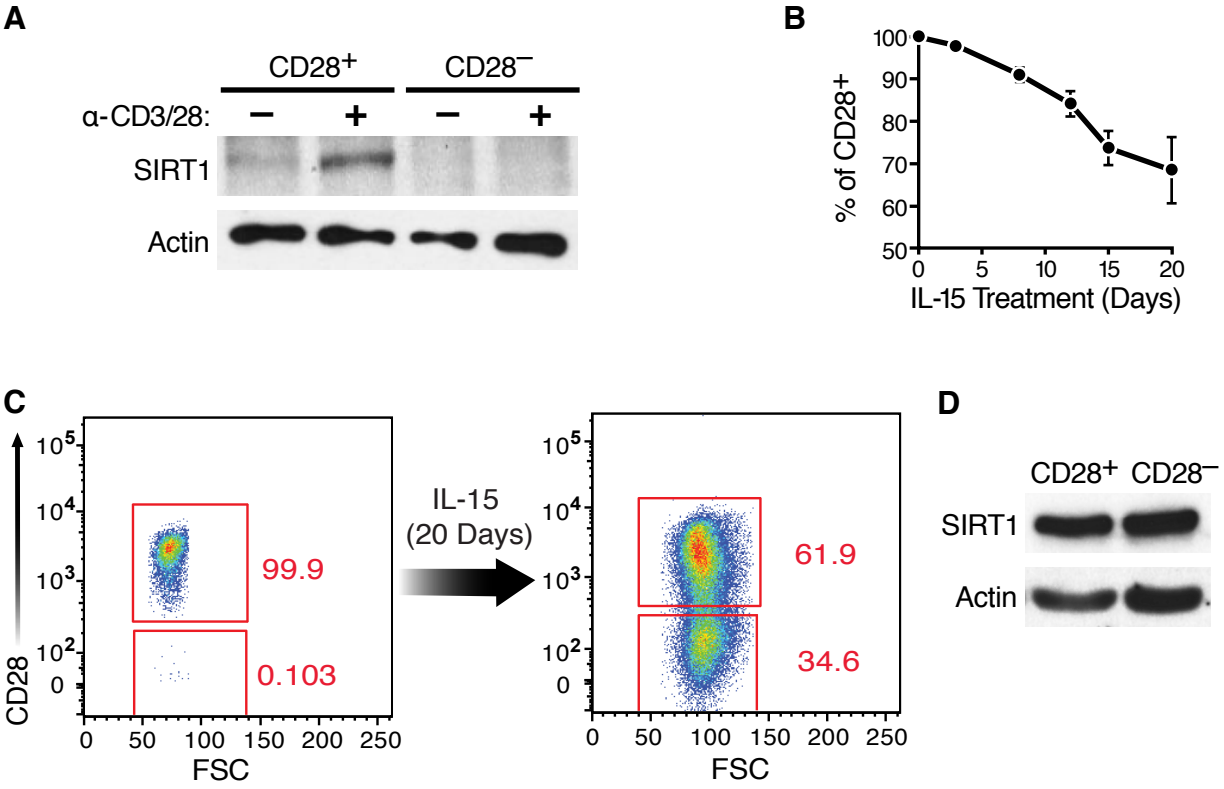

Figure 5-1. Loss of SIRT1 expression is independent of CD28 downregulation in CD8⁺ T cells.

(A) SIRT1 protein expression assessed by immunoblot from sorted human T cells activated with αCD3/28 for 48 hours. **(B-D)** *Ex-vivo* $CD8^{+}CD28^{-}$ T cells were generated from a long-term treatment of sorted $CD8^{+}CD28^{+}$ T cells (99.9% purity). **(B)** CD28 surface expression was monitored by flow cytometry during long-term IL-15 treatment. **(C)** Representative FACs showing purity of primary and secondary sort. **(D)** SIRT1 protein expression was measured after sorting *ex vivo*–generated $CD28^{+}$ and $CD28^{-}$ T cells (Day 20 of IL-15 treatment). Data are mean ± SEM of **(B)** 3 donors/condition. **(A,B,D)** Representative data (≥ 2 independent donors).

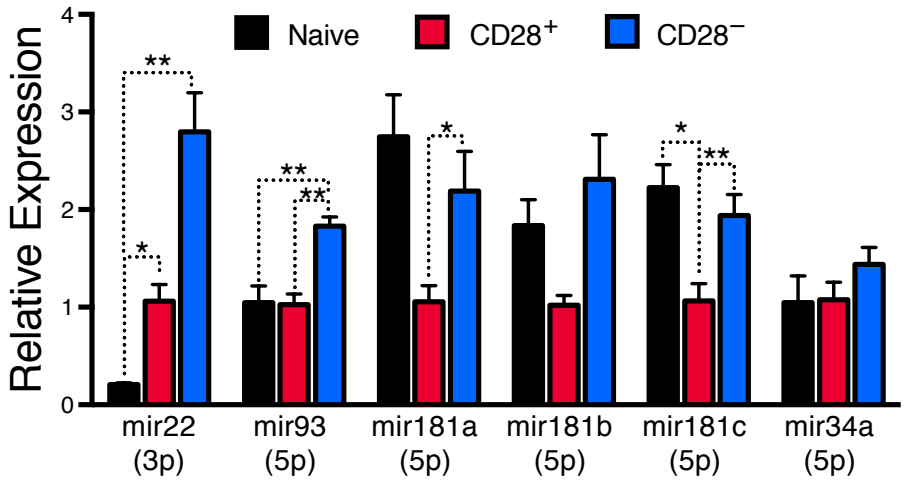

Figure 5-2. Expression of candidate SIRT1-targeting miRNAs in CD8⁺ T cell subsets. Expression of selected miRNAs were assessed by qRT-PCR in sorted T cell subsets, normalized to U6 expression. Data are mean ± SEM of 5 donors. *P<0.05, **P<0.01; paired two-tailed student's t-test.

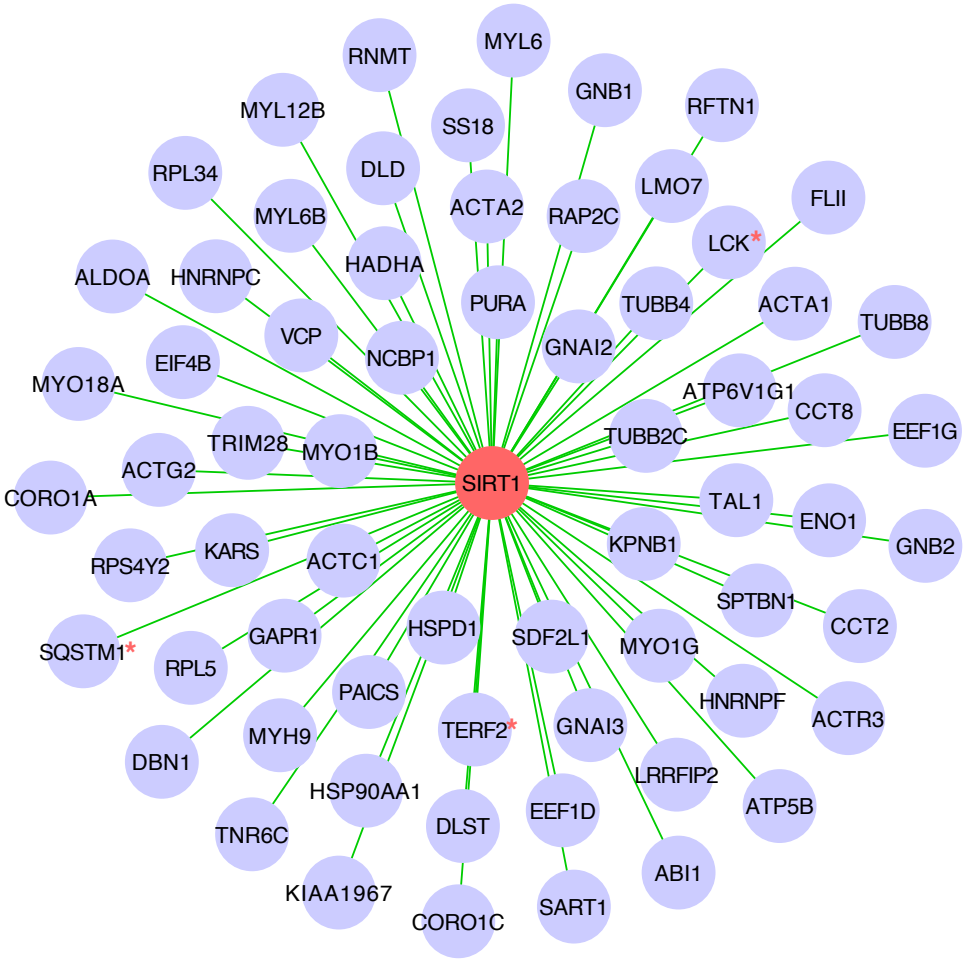

Figure 5-3. SIRT1 interactome in Jurkat T cells.
Affinity mass-spectrometry was performed on SIRT1 overexpressing Jurkat T cell lines. Top SIRT1-interactors above 0.75 cut-off from MIST from 3 independent experiments. *Selected SIRT1-interactors associated with immune aging.

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