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Dedifferentiation Of Cytotoxic Lymphocytes Into Central Memory Cd8+ T Cells: Lessons From Antiviral T Stem Cells On The Architecture Of Aging & Immunotherapy

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Abstract
Startling advances in biological gerontology coupled with the ongoing, global demographic transition to older populations have intensified humankind's ancient quest to understand the nature of aging. This dissertation explores mechanisms of mortality through the prism of immunology. To effect defense against pathogens both exogenous and endogenous, the adaptive immune system leverages the tremendous proliferative capacity with which it is endowed to generate terminally differentiated lymphocytes that potently eliminate or suppress threats to organismal health. This proliferative capacity derives from the integrity of genomically encoded information, and, while considerable, is not infinite: it can be depleted with age and following repeated rounds of antigen-driven proliferation, for example, when driven by recurring or persisting microbial infections, or cancer. In the studies detailed herein, genome integrity homeostasis was probed during the immune response to viral infection. CD8+ T cells were observed to experience significant DNA damage in the course of their attempts to control viral replication. Multiparametric flow cytometry identified a rare population of antigen-specific T stem cells that might represent an evolutionary strategy to minimize genotoxicity. The programming, metabolic profile, anatomic localization, proliferative capacity, and ontogeny of these T stem cells were analyzed in relation to previously established effector, memory precursor, and central memory populations. Developmentally, T stem cells were able to self-renew and both give rise to abundant cytotoxic effector CD8+ T cells in the presence of antigen as well as contribute to the pool of long-term central memory CD8+ T cells following antigen clearance. Remarkably, partially-differentiated transit-amplifying effector CD8+ T cells also contributed to long-term central memory, in a process of apparent dedifferentiation. Programmatically, T stem cells were endowed with superior expression of multiple genome maintenance and repair activities, including heightened responsiveness to DNA strand breaks, telomerase expression, and suppression of potentially mutagenic transposition by the ancient LINE-1 retrotransposon. Together, these features suggest that this characteristic of amplified genome integrity surveillance may be a fundamental feature of somatic stem cells broadly, and important for the long-term maintenance of antigen-experienced T cell populations in particular. Additionally, despite relative proliferative quiescence, T stem cells unexpectedly manifested intense signaling flux. This was associated with robust expression of a panoply of both stimulatory and inhibitory cell surface receptors, including PD-1. Intensified signaling consequent to genetic ablation of inhibitory receptors compromised T stem cell viability during acutely-resolving viral infections. These findings hold significant implications for basic understanding of pathogen-driven peripheral T cell differentiation, formation of long-term immunological memory, and aging; and for the design of therapeutic, prophylactic, and diagnostic applications intended to further the cause of maximizing healthy human lifespan.

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LESSONS FROM ANTIVIRAL T STEM CELLS ON THE ARCHITECTURE OF AGING & IMMUNOTHERAPY

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My scientific career formally commenced in a quirky *Drosophila* genetics laboratory with a Socratic bang, delivered by way of a prompt I shall never forget, intoned in an inimitable Swiss-German accent impossible not to notice: "*tell-you-me-this*." Thusly – with questions instead of answers – did Adrian Rothenfluh return -- often frustratingly, always fruitfully -- my many adolescent queries about the nature of biology and research, and thereby prompt me to begin thinking critically and independently. To him, then himself a graduate student and now a Professor, I owe a deep debt of gratitude.

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ABSTRACT

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Startling advances in biological gerontology coupled with the ongoing, global demographic transition to older populations have intensified humankind's ancient quest to understand the nature of aging. This dissertation explores mechanisms of mortality through the prism of immunology. To effect defense against pathogens both exogenous and endogenous, the adaptive immune system leverages the tremendous proliferative capacity with which it is endowed to generate terminally differentiated lymphocytes that potently eliminate or suppress threats to organismal health. This proliferative capacity derives from the integrity of genomically encoded information, and, while considerable, is not infinite: it can be depleted with age and following repeated rounds of antigen-driven proliferation, for example, when driven by recurring or persisting microbial infections, or cancer. In the studies detailed herein, genome integrity homeostasis was probed during the immune response to viral infection. $CD8^+$ T cells were observed to experience significant DNA damage in the course of their attempts to control viral replication. Multiparametric flow cytometry identified a rare population of antigen-specific T stem cells that might represent an evolutionary strategy to minimize genotoxicity. The programming, metabolic profile, anatomic localization, proliferative capacity, and ontogeny of these T stem cells were analyzed in relation to previously established effector, memory precursor, and central memory populations. Developmentally, T stem cells were able to self-renew and both give rise to abundant cytotoxic effector $CD8^+$ T cells in the presence of antigen as well as contribute to the pool of long-term central memory $CD8^+$ T cells following antigen clearance. Remarkably, partially-differentiated transit-amplifying effector $CD8^+$ T cells also contributed to long-term central memory, in a process of apparent dedifferentiation. Programmatically, T stem cells were endowed with superior expression of multiple genome maintenance and repair activities, including

heightened responsiveness to DNA strand breaks, telomerase expression, and suppression of potentially mutagenic transposition by the ancient LINE-1 retrotransposon. Together, these features suggest that this characteristic of amplified genome integrity surveillance may be a fundamental feature of somatic stem cells broadly, and important for the long-term maintenance of antigen-experienced T cell populations in particular. Additionally, despite relative proliferative quiescence, T stem cells unexpectedly manifested intense signaling flux. This was associated with robust expression of a panoply of both stimulatory and inhibitory cell surface receptors, including PD-1. Intensified signaling consequent to genetic ablation of inhibitory receptors compromised T stem cell viability during acutely-resolving viral infections. These findings hold significant implications for basic understanding of pathogen-driven peripheral T cell differentiation, formation of long-term immunological memory, and aging; and for the design of therapeutic, prophylactic, and diagnostic applications intended to further the cause of maximizing healthy human lifespan.

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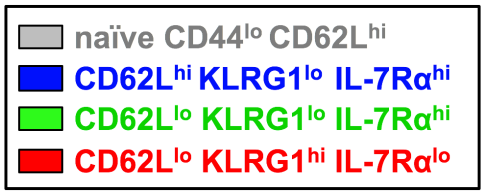
ABBREVIATIONS AND LEGEND

MFI	mean fluorescence intensity
pi	post infection
LCMV	Lymphocytic Choriomeningitis Virus
PBMC	peripheral blood mononuclear cell
TCR	T cell receptor
iPSC	induced pluripotent stem cell
GFP	green fluorescent protein
Ag	antigen

General legend for most figures, except where otherwise indicated: gray depicts naïve, antigen-inexperienced $CD8^+$ T cells. Blue, green, and red denote subsets of LCMV-specific $CD8^+$ T cells, as defined by differential expression of the cardinal markers of memory and effector T cell differentiation CD62L, IL-7Rα, and KLRG1.



.:: CHAPTER 1 ::.
AGING, IMMUNOLOGICAL MEMORY, AND THE GENOME INTEGRITY PARADOX OF T CELL CLONAL EXPANSION

1.1 Aging is of ancient and central concern to the human condition

"Our new Constitution is now established, and has an appearance that promises permanency; but in this world nothing can be said to be certain, except death and taxes."

- Benjamin Franklin, 1789

Writing in a period of transition and intense uncertainty – shortly before his own death, and on the occasion of the birth of a new nation – Benjamin Franklin opined on the few constants that figure in life. While the subject of taxation lies beyond the scope of inquiry in this work, death lies at its center. Indeed, mortality and the universal process of aging that inevitably precipitates it have invited curiosity and concern throughout the history of humankind. The very birth of literature some four thousand years ago – in the Sumerian epic of Gilgamesh – centered upon the pain of death and a quest for immortality. In the present day, ongoing demographic transitions in industrially developed and developing nations have heightened attention to the issue of graying populations (Olshansky et al., 1990; Vaupel, 2010; Olshansky, 2016). At the beginning of the present decade, 13-22% of European, American, and Chinese populations exceeded 60 years of age, and this figure is projected to rise to 27-34% by 2050. Globally, there will be more than two billion people 60 years of age or older by 2050, by which point this class of older individuals will outnumber the population of children (0-14 years of age) for the first time in human history (United Nations

Department of Economic and Social Affairs Population Division, 2012). These trends pose tremendous challenges at every level of social organization, including the maintenance of health and care for the ill. Beyond any particular physiological, lifestyle, or environmental parameter, age itself is the single greatest risk factor for the pathologies that inflict the largest burden of morbidity and mortality, including cardiovascular disease, cancer, and neurodegenerative conditions (Marrero et al., 2012; Kohanski, 2016). This begs the question of whether the aging process can be sufficiently understood in order to enable interventions that might potentiate healthier and perhaps longer life trajectories.

1.2 History and universality of mortality

While the question of aging has been mooted for millennia, extensively explored by titans of literature, and even empirically pursued by notables ranging from ancient Chinese emperors to Isaac Newton, only since the birth of cell theory in the mid 19th century has it become experimentally tractable. Building on microscopic observations from Theodor Schwann and Robert Remak, the pioneering physician, pathologist, and public health visionary Rudolph Virchow famously declared that "*omnis cellula e cellula"* (Virchow, 1855) – all cells are derived by way of genesis from pre-existing cells, not begotten from some amorphous, inanimate source. This signal insight in turn opened the door for aging to be examined and potentially understood for the first time in terms of an observable, tangible, quantity – cells.

The dawn of the modern study of aging had to await the ability to study live, actively metabolizing cells as opposed to chemically preserved specimens. This moment arrived a half century later with the advent of *ex vivo* tissue culture. In the 19-teens, just a few months before he was awarded the Nobel prize that cemented his outsized public stature and further amplified the authority of his biomedical pronouncements, Alexis Carrel of the Rockefeller Institute initiated an elaborate, decades-long series of experiments involving culture of chicken cells. He put fresh cells into culture flasks, and as long as the nutrient medium was regularly replenished, he observed that the cells appeared to persist, metabolize, and divide without end. Carrel, his colleagues, and an enthralled public interpreted this seemingly ceaseless vitality as being consistent with biological immortality (Shay and Wright, 2000). At the cellular level at least, they believed, death need not be foreordained. Though some in the scientific community doubted this conclusion and the strength of the data that supported it, this interpretation reigned as dogma for the better part of the next 50 years.

Not more than several dozen paces from where these words are being written, Leonard Hayflick and his colleagues turned this notion on its head starting in the early 1960's in Philadelphia. In careful culture experiments with primary human fibroblasts, they observed that non-transformed cells consistently ceased replicating after 40-50 divisions (Hayflick and Moorhead, 1961). With the notable exception of genetically unstable cancer cells, physiologically normal cells in culture appeared to be possessed not of unbounded but rather finite replicative potential. This observation, later termed replicative senescence or the

"Hayflick limit" by the immunologist Macfarlane Burnet (Burnet, 1974), is thought to represent a cellular model of organismal aging, as mutations in many genes that accelerate its onset also cause premature aging in animal models and humans (Lombard et al., 2005). The paradigm of senescence has been extended both *in vitro* to dozens of additional cell types and *in vivo* across multiple organisms (Herbig et al., 2006; Baker et al., 2011), even including prokaryotes (Ackermann et al., 2003; Stewart et al., 2005), which until recently were believed to represent a key deviation from Hayflick's paradigm (Charlesworth, 2000). Though there may be an intriguing exception in the case of hydra (Boehm et al., 2012), Hayflick's fundamental insight into the lives of cells remains a pillar of modern biological inquiry – in short, the lease on life for any given cells and the organism they comprise is definite; everything must die.

1.3 A central role for the immune system in systemic aging

If death is inevitable, what then is the mechanism of mortality? In the late 19th century the German developmental biologist August Weismann speculated that "death takes place because a worn-out tissue cannot forever renew itself" (Weismann, 1889). Since then numerous observations and mechanistic explanations have emerged, with most amounting to a time-dependent accumulation of various types of cellular damage, including especially to the genome and the epigenome (Lopez-Otin et al., 2013; Vijg, 2014), and regulation of lifespan by genetic modulation of the net rate of damage accumulation (Burnet, 1973; Gladyshev, 2012). However, how indeterminate deficiencies at

the cellular level manifest in the organism-scale loss of functionality and fecundity that define aging remains incompletely understood, and is currently the subject of exceptionally vigorous investigation across multiple disciplines. Not long after Hayflick's revelation, and building on earlier work by Burnet concerning somatic mutation rates in the immune system and age-dependent autoimmunity (Burnet, 1959), the gerontologist Roy Walford proposed that of all the separate and complex tissues that together comprise the mammalian body plan, the interlocking assemblage of leukocytes commonly referred to as the "immune system" might be particularly central to overall organismal aging (Walford, 1969). Developed extensively over the course of the next several decades, this notion that systemic aging is a function of pathological immune processes has been borne out by several complementary lines of evidence.

First among these are multiplying observations that many of the most salient infirmities of aging are accompanied by dysregulated immune function, for example in the cases of atherosclerosis, wherein senescent macrophages accumulate in subendothelial spaces and drive atheroma formation (Childs et al., 2016); in neurocognitive degeneration, wherein T cell deficiency has been shown to diminish the adult hippocampal neurogenesis (Huang et al., 2010) thought to underlie substantial aspects of learning, memory, and emotional regulation (Eriksson et al., 1998; Spalding et al., 2013); and perhaps most intriguingly in cancer, wherein benign tumors may exploit age-diminished adaptive immunosurveillance to embark on the transformation to clinically-evident

malignancy (Cheney and Walford, 1974; Miller, 1996; Castro et al., 2009; Pawelec et al., 2009; Malaguarnera et al., 2010; Chou and Effros, 2013).

Even more saliently than associations with chronic ailments however, immune function in the elderly is often linked to substantially decreased functionality with respect to its most familiar role, that of pathogen defense. This nearly universal time-acquired immunodeficiency syndrome limits the protections of vaccination and engenders susceptibility to a panoply of communicable diseases, including most prominently influenza, herpes zoster, urinary tract infections, infective endocartitis, bacterial meningitis, and nosocomial infections generally (Gavazzi and Krause, 2002). Importantly, infectious diseases in the aged are both more frequent and more severe, resulting in increased demand for clinical services and increased mortality (Gavazzi and Krause, 2002).

While age-associated immunodeficiency can clearly herald death by infection, an inverse correlation has also been documented, in that more robust immune status characterizes those who survive into the ninth, tenth, or even eleventh decade of life (Strindhall et al., 2007; Sansoni et al., 2008; Derhovanessian et al., 2010). Longevity associated with increased resistance to infection has also been documented in mutants of the roundworm *Caenorhabditis elegans* (Garsin et al., 2003), suggesting the possibility of an evolutionarily-conserved relationship between immunity and organismal aging.

1.4 History of immunity

If declining immune function at least in part precipitates overall, organismal aging, what is it that drives senescence in the immune system in the first place, and why might that process proceed briskly in some individuals and more conservatively others? This dissertation attempts to provide a biological framework within which to address these vital questions and discuss their implications.

A reasonable point at which to start dissecting the terminus of immunity is the beginning of its understanding, which coincidentally corresponds to the birth of modern history 2500 years ago in the form of an Athenian general's unprecedented chronicle of the three decades' civil war that lead to the disintegration of an empire and the collapse of humanity's first experiment in democracy. In his sweeping *History of the Peloponnesian War,* Thucydides described the social and psychological stresses of repeated sieges, and documented the infectious disease outbreaks that terrorized the densely-packed citizens of Athens even more so than sightings of advancing Spartan phalanxes. Himself a survivor of the devastating 430 BC Plague of Athens that claimed at least a full third the population, Thucydides recounted the thoroughly indiscriminate nature of the "pestilential disorder" that afflicted commoners and elites alike, causing all those affected to "drop like sheep." Importantly, while Athenian society as a whole was devastated by contagion, some individuals recovered after having been stricken. Indeed, Thucydides recorded that

> *"though many lay unburied, birds and beasts would not touch them, or died after tasting them… The bodies of dying men lay one upon the other… [But] those who had recovered from the disease… had now no fear for themselves; for the same man was never attacked twice – at least never fatally."* (Thucydides, History of the Peloponnesian War, 2.51).

Today, the concept of immunological memory recognized as the hallmark of the adaptive immune system can be traced to that original observation of acquired immunity (from the Latin *immunitas,* for "exemption") to subsequent microbial infection (Masopust et al., 2007). Starting 1500 years later in India and China and up until the 18th century in Europe, and prompted by the emergence of smallpox as a frequent, major killer in urban centers, deliberate transfer of pustular fluids from smallpox scabs into the skin of uninfected persons (so called "variolation") was practiced in the hope that a slightly milder yet protective infection might result. Apparently prompted by bucolic folklore, the English empiricist Edward Jenner is credited with formalizing the notion that inoculation not with live smallpox but rather with the related agent cowpox (or *Variolae vaccinae* as he called it, from the Latin *vacca* for cow) could offer protection just as well but without the certain virulence and mortal risk of a full-blown smallpox infection (Baxby, 1999).

Thereafter, the practice and later the science of "vaccination" (so dubbed by Louis Pasteur, in recognition of Jenner's contribution) advanced in earnest, especially with the advent of germ theory and Pasteur's development of reproducible attenuation techniques that for the first time allowed rationally converting a virulent microbe into a candidate vaccine that could be empirically tested in animal and human test subjects for the ability to confer protective

immunity. Successful rabies and anthrax vaccines ensued, followed in the mid 20th century by effective vaccines for yellow fever (contributed by Max Theiler) and polio (Jonas Salk). In prodigious feats of biomedical creativity unlikely to be witnessed again, uber-vaccinologist Maurice Hilleman developed 8 of the 14 vaccines in current vaccine schedules, including those for measles, mumps, hepatitis A, hepatitis B, chickenpox, meningitis, pneumonia and *Haemophilus influenza.* Because of vaccines, the ancient scourge of smallpox has been eradicated (Foege, 2001), and polio – which once paralyzed half a million annually (Kew et al., 2005) – as well as measles and tetanus have been restricted from large swaths of the globe. Lately, newly-introduced vaccines against rotavirus and HPV, respectively, have substantially reduced the incidence of severe pediatric gastroenteritis (Parashar et al., 2016) and infection with the virus responsible for the vast majority of cervical and anogenital cancers (Markowitz et al., 2016). Most recently, a vaccine against Ebola has been developed (Henao-Restrepo et al., 2017), and a candidate vaccine against Zika has demonstrated efficacy in mouse and non-human primate model systems (Pardi et al., 2017).

1.5 Nature of immunological memory

Underlying all of these collectively stupendous achievements in biomedical and public health engineering is the capacity of the adaptive immune system to remember a primary or “priming” infection and exploit the imprint of this experience to respond with greater force and superior efficiency to any

subsequent encounters with the pathogenic agent in question, whether that be a virus, bacterium, parasite, tumor, or artificially engineered antigen (i.e., a vaccine). However, though the capacity for remembrance of pathogens past is a defining feature of all 65,000 extant vertebrate species, and may even have enabled the Cambrian explosion 500 million years ago (Pancer and Cooper, 2006), the nature of the physiology that underlies it remains incompletely understood. In particular, while so-called "memory" lymphocytes are recognized as the repository for immunological experience, and thus the source of recall ability, their developmental origins are the subject of lively controversy (Ahmed et al., 2009; Restifo and Gattinoni, 2013) and a longstanding holy grail of biomedical research (Lefrançois and Masopust, 2009).

Multifarious are the subtypes of lymphocytes thought to mediate immunological memory, including natural killer cells (Sun et al., 2009), B cells, and subtypes of T cells (so called because they develop in the thymus) defined by expression of either the CD4 or CD8 molecules (Ahmed and Gray, 1996). Of these, T cells are particularly important for the control of tumors, viruses, and intracellular bacteria, among other pathogens. This insight emerged from multiple observations of increased susceptibility to infection or tumorigenesis and more severe pathogenesis in animals (including non-human and human primates, as well as mice) with congenital or acquired deficiencies in T cells or genetic factors vital for their function (Kagi et al., 1994; Jin et al., 1999; Stepp et al., 1999; Koebel et al., 2007).

Much of the substantial knowledge accumulated about T cell biology has come from studies with lymphocytic choriomeningitis virus (LCMV), a human pathogen maintained in a rodent host reservoir that was discovered in 1933 by Charles Armstrong by way of an encephalitis epidemic in St. Louis (Zhou et al., 2012). LCMV is a single-stranded RNA virus and archetypal member of the Arenaviridae family. Its genome consists of two ambisense RNA segments that encode four proteins. Viral entry is mediated by binding of LCMV's glycoprotein to α-dystroglycan, a widely-expressed receptor on host cells that enables an unusually broad tropism for LCMV and thus multi-organ dissemination (Cao et al., 1998). Outcomes of infection range from subclinical illness in healthy individuals to thrombocytopenia and life-threatening encephalitis in immunocompromised individuals, and severe congenital defects (Fischer et al., 2006). Infection elicits a vigorous and reproducible immune response in both rodents and humans (Kotturi et al., 2011) whose resolution is dependent on $CD8^+$ T cells (Byrne and Oldstone, 1984; Moskophidis et al., 1987). After initial exposure to viral antigen, a naïve $CD8^+$ T cell specific for the antigen becomes activated and is caused to initiate a phase of rapid proliferation and differentiation that spawns abundant clonal descendants over the course of several days or weeks (Murali-Krishna et al., 1998). To maximize organismal survival and fitness, most of these progeny cells are robustly endowed with a variety of potent molecular effector mechanisms that conduce to purging virus from infected cells, or whole infected cells from the host. For example, a particularly prominent cytotoxic effector mechanism revolves around the perforin-granzyme pathway

(Kagi et al., 1994), in which perforin and granzyme proteins are exocytosed from specialized secretory lysosomes into the cleft formed between the abutting killer and virally-infected cell membranes. Perforin, a 67-kilodalton protein with an N-terminal membrane-attack-complex domain similar to that of cytolysins in $Gram^+$ bacteria, oligomerizes into 19-24mers to form pores of roughly 150 Å in the target cell's plasma membrane (Law et al., 2010). These transiently perturb calcium flux and inflict osmotic stress, thereby triggering a stereotypical wounded-membrane repair response that in turn enables granzymes to access the target cell's cytosol via an endocytic pathway (Law et al., 2010; Thiery et al., 2011). Once within the target cell, granzymes, a family of pro-apoptotic serine proteases, cleave a series of substrates in the cytosol, mitochondria, and nucleus to effect regulated auto-destruction of the cell (as opposed to the disorderly and inflammatory alternative of necrosis), thereby efficiently removing it as a threat to the overall health of the host organism (Thiery and Lieberman, 2014). Following clearance of virus, the vast majority of the cells generated from the founding, antigen-specific naïve $CD8^+$ T cell are no longer needed and commit to apoptosis in a process termed contraction (Lau et al., 1994). However, a small percentage of cells escapes this culling and persists long-term in order to mediate defense should the pathogen ever be re-encountered (Lau et al., 1994; Kaech and Wherry, 2007). This population is what are known as memory T cells.

1.6 Provenance of immunological memory: one burning question and three clues

While the existence of memory T cells following the resolution of a pathogenic perturbation is firmly established, and their properties – including lifelong persistence and vast proliferative capacity upon pathogen re-encounter – have been examined extensively, the precise sequence of developmental elaborations that transform an inert, naïve lymphocyte into a hardened, durable, protective memory T cell is hotly contested (discussed in depth in Chapter 2). In essence, that which is in question has been termed the "molecular biography" of the cell (Shaywitz and Melton, 2005): *whence memory?* Though even a semblance of consensus on this matter has remained elusive, several crucial clues to memory T cell provenance have emerged.

1.6.1 T cell proliferative potential is finite

The first clue concerns finitude: a single, activated naïve $CD8^+$ T cell can divide repeatedly to generate up to 100,000 progeny cells following antigenic stimulation (Rocha et al.,1989). Following resolution, memory cells derived from the original cell can execute a similarly expansive feat of multiplication upon pathogen re-exposure, as can secondary and tertiary memory cells that emerge following repeated rounds of infection and resolution. Clearly, the proliferative capacity of T cells is tremendous. However, it is apparently not unbounded. While some of the early experiments in *ex vivo* culture appeared to indicate that human T cells were possessed of unlimited replicative capacity (Morgan et al., 1976), subsequent analyses revealed these initial interpretations were

confounded by the presence of pathologically immortalized, transformed cells (Effros, 2004). Instead, clonal populations of untransformed T cells invariably ceased proliferating in culture after repeated rounds of mitogenic stimulation (Effros, 2004). Subsequently, this paradigm was extended to the behavior of T cells *in vivo*, wherein clonally-derived T cells were caused to undergo repeated rounds of infection-driven expansion and contraction in the context of a serial transplantation scenario that maximized the potential for replicative stress. While robust during the first three rounds of antigen-driven proliferation, T cell expansion all but ceased when cells were activated by a fourth infection (Masopust et al., 2006). Further *in vivo* studies have noted a strictly inverse relationship between replicative history and future potential for proliferation and/or conferring protection from stringent pathogenic challenges (Hinrichs et al., 2009; Nolz and Harty, 2011; Martin et al., 2012; Fraser et al., 2013, Van Braeckel-Budimir N et al., 2017).

That T cells can undergo extensive yet ultimately finite rounds of replication both *in vitro* and *in vivo* is highly reminiscent of Hayflick's seminal observations of fibroblast senescence. In fact, though quantitating cell division *in vivo* is challenging, based on estimates of ~15-20 divisions per round of antigen-driven expansion (Murali-Krishna et al., 1998; Kaech et al., 2002; Masopust et al., 2007), it seems likely that the T cells subjected to the replicative stress of serial expansion (Masopust et al., 2006) likely underwent a total of 45-60 divisions before exhausting proliferative capacity. This corresponds closely to the range originally reported for fibroblast senescence (Hayflick, 1965).

What then might be basis for this vital finitude? In a broader context, this amounts to a timeless question: *what is the biological substrate of aging?* In the case of Hayflickian senescence, decades of inquiry have indicated that proliferative potential is circumscribed by the accumulation of genomic and epigenomic damage. One example of this is manifested in the progressive, division-dependent attrition of telomeres, the nucleoprotein complexes that cap chromosome ends and thereby preserve genome integrity (discussed in depth in Chapter 3). While there exists a striking paucity of understanding concerning genome integrity homeostasis in leukocytes, a preponderance of evidence indicates that T cell telomeres shorten with progressive proliferation and/or age (Sallusto et al., 1999; Hodes et al., 2002; Chou et al., 2013). Beyond telomeres, other forms of genomic insults increase, including chromosomal rearrangements (Ramsey et al., 1995). Thus, T cell proliferative potential is potent yet limited, apparently like that of any other cell type, and this finitude corresponds with decreasing genomic integrity.

1.6.2 Memory T cells are long-lived

A second feature of the biology of memory T cells of relevance to their provenance is cellular longevity. Unlike their naive T cell progenitors, whose lifespans are limited, at least in rodents (Tsukamoto et al., 2009; Sprent and Surh, 2011), memory T cells have the capacity to persist for life (Homann et al., 2001; Hammarlund et al., 2003; Lang and Nikolich-Zugich, 2011). Remarkably, in experimental conditions of serial transplantation into successive young recipient

mice, memory T cells have even been observed to exhibit lifespans that exceed those of the original mice in which they were generated (Lau et al., 1994). What is more, while naïve T cells protected from replicative stress nevertheless unmistakably exhibit age-related decline in functionality (Decman et al, 2010), similarly unstressed memory T cells retain functionality regardless of the passage of time (Haynes et al., PNAS 2003; V. Decman and E.J. Wherry, unpublished observations). That memory T cells can persist apparently indefinitely as well as retain functionality – in stark contrast to naïve T cells and indeed most other mammalian cell types – suggests that they must be endowed with protective mechanisms that confer these extraordinary properties.

1.6.3 Pathogen-driven T cell expansion: a genotoxic maelstrom of mitosis

A third clue to memory T cell origins traces to the first and arguably most important phase of adaptive immune responses. Before any given naïve T cell encounters a pathogen for which it is specific, it exists in a state of metabolic and proliferative quiescence that is actively maintained by various transcriptional programs (Jones and Thompson, 2007; Feng et al., 2011). Following activation, naïve T cells initiate a concerted phase of metabolic exertion, proliferation, and differentiation in order to generate pathogen-specific effector T cells that are sufficiently numerous to mediate organismal defense. Importantly, proliferation of T cells during this period can be intense: in contrast to the staid cell cycles of roughly 20 hours that characterize archetypal mammalian cell types (Posakony et al., 1977; Baserga, 1985), pathogen-specific T cells can divide extremely rapidly (Murali-Krishna et al., 1998; J. Yewdell, personal communication). In one

notable study, virus-specific naïve T cells labeled with a proliferation-tracking dye were exposed to viral antigen *in vivo*, and subsequent division was monitored every 24 hours for several days afterwards. Strikingly, cell division durations as short as 3-4 hours were observed **(Figure 1.1)**. Such extraordinarily swift amplification is understandable in the context of the existential threat of an interloping (and itself rapidly multiplying) microbe, though the molecular mechanisms that underpin it are unknown. It is very likely however to have important, probably deleterious consequences for genome integrity, as cell division even in the most salutary of circumstances is inherently genotoxic. For example, cells of every type are constitutively subjected to DNA damage, including: nucleophlic attack by water leading to depurination and depyrimidation, which occur at an estimated 10,000 bases/day/cell (Friedberg et al., 2005); cytosine deamination (100 bases/day/cell); and oxidative damage from hydroxyl radicals generated consequent to quotidian mitochondrial metabolism. Importantly, repair of each of these genomic insults is facilitated by the fact they occur on one strand of the double-helix, leaving the opposing strand as a template for accurate repair (Ruzankina et al., 2008). During cell division however, the double-stranded nature of DNA is suspended. This disruption dramatically escalates the chance that any given error, minor and readily reversible in most situations, might instead yield a mutation via misrepair or neglect. Worse, un- or mis-processed damaged bases can interfere with or even precipitate collapse of progressing replication forks, leading to potentially catastrophic double-strand breaks (Ruzankina et al., 2008). In addition to these

elevated risks from commonplace DNA damage, the incomplete fidelity of DNA polymerases ensures that at least a handful of mutations will be generated for every round of genome replication (Loeb and Monnat, 2008).

In short, even in the most leisurely of contexts, cell division is a risky proposition. However, proliferating, pathogen-specific T cells cycle to an extreme extent that is rarely observed in adult mammalian physiology. Given indications that accelerated proliferation may be associated with threats to genome integrity even beyond those inherent in standard, unhurried cell division (Cairns, 1975; Wu et al., 2005; Martin et al., 2011; Schoppy et al., 2012), the genotoxic risk for activated T cells responding to pathogens is likely to be enormous. Because the cells in which immunological memory resides must transit this mitotic maelstrom to survive and mediate potentially life-long defense against further pathogen encounters, any explanation for the origins of memory T cells must take into account this fundamental problem of information homeostasis.

1.6.4 Memory T cell origins and the paradox of genome integrity homeostasis

While memory T cell origins remain a matter of debate, the several clues discussed above help delineate the contours of possibility that must be considered. First, T cells can divide extensively but not without limitation, and proliferative history correlates with accumulation of (epi)genomic damage. Second, at least in the absence of replicative stress, memory T cells can persist for tremendously long periods, including without any apparent loss of functionality. Given that almost every other known cell type exhibits an age-

related decline in quantity, quality, or both, these observations suggest that memory T cells are endowed with protective mechanisms that enable these characteristics of extraordinary longevity. Third, despite the fact that cell division under any circumstances is inherently genotoxic, T cell proliferation is tremendous in both scope and rate when responding to a pathogenic perturbation. That memory T cells both survive this enormously stressful process and also retain a capacity for further extensive and rapid division is an additional indication suggesting the presence of mechanisms that minimize genotoxicity.

In sum, to effectively address the ever-present multiplicity of threats posed by pathogens of all sorts, T cells need to be able to proliferate extensively, do so extremely rapidly, and persist potentially for the duration of a lifespan. Each of these individual stipulations constitutes a substantial threat of compromised genome integrity and premature senescence. How these necessary yet apparently mutually exclusive imperatives are reconciled amounts to a major paradox in modern immunobiological inquiry: on one hand, extensive proliferation and prolonged survival are required to generate and sustain immunity, while on the other lies the existential pressure to avoid excessive division as well as the inevitable genotoxic ravages of time so as not to prematurely exhaust finite replicative potential. **Simply put, how does the immune system manage mortality?** This essential question is the animating force for the research presented in this dissertation, and it is suggested that the cradle of immunological memory may be found in its answer.

1.7 Stem cells as a solution to the genome integrity paradox

A possible explanation for this modern immunological conundrum concerns stem cells. The evidentiary basis for this fundamental concept in developmental biology began to emerge in the years before and shortly after the second world war.

1.7.1 Four-dimensional anatomy: the history of tissue turnover

In 1937, the technique of autoradiography had just recently been pioneered by Antoine Lacassagne in Paris, and the *Institut du Radium* at which he labored was abuzz with excitement following the award of the 1935 Nobel prize in chemistry to faculty members Frederic and Irene Joliot-Curie. This recognition was for the discovery of methods to synthesize radioactive elements at will, which in turn came about in the wake of the two Nobel prizes awarded in 1903 and 1911 to Marie and Pierre Curie, Irene's parents, for their discovery and isolation of elemental radium and further, groundbreaking studies on the nature of radioactivity. This string of scientific triumphs and the manifest potential of radiolabeled molecules as tools in biological research attracted the substantial funding necessary to construct a cyclotron at the *Institut* (Laurie, 2007). In this well-resourced environment of intellectual ferment, post-doctoral trainee Charles Leblond joined Lacassagne's research group and mastered autoradiography. Early observations demonstrated that metabolism of phosphorous and other minerals in adult animal models was unexpectedly rapid. Importantly, these findings began to undermine the then-prevailing paradigm of "cellular stability", in

which the principal physiological function of mitosis was held to be for growth toward the end of achieving sexual maturity (Leblond, 1995); with minor exceptions, the tissues of the human body, like the cogs and wheels of an automobile, were thought to stable, permanent structures (Leblond, 1991). However, addressing this matter definitively was precluded by technological constraints, as the radiographical techniques of the time could visualize tissues but not individual cells. World war interrupted this thread of inquiry, and prompted Leblond to flee to Montreal. When research resumed at McGill University, a fruitful collaboration with physicists coupled with exploitation of the newly-available radioisotope tritium lead to a methodological breakthrough in the form of a hundred-fold improvement in autoradiography resolution, and the consequent ability to visualize single cells (Belanger and Leblond, 1946). For the first time, the fourth dimension – that of time itself – was amenable for the study of anatomy and physiology *in vivo.*

Using these newly refined autoradiography techniques, Leblond and colleagues thereafter reported a conceptual breakthrough. By carefully observing the kinetics of radionuclide labelling in the intestines of fully grown rodents, they observed not rare but rather frequent mitoses in epithelial cells, and calculated that the entire epithelium not only turned over regularly, but did so in a mere matter of days (Leblond and Stevens, 1948; Leblond et al., 1948). This notion was such a dramatic departure from the accepted model of post-growth cellular stability that it was roundly dismissed, and even mocked by critics (Bennett and Bergeron, 2007). Nevertheless, continued research in Leblond's

group and corroborating reports from others' eventually established the modern consensus that while many tissues in model organisms are indeed comprised of mostly or even entirely quiescent cells, several – most prominently including bone marrow, alimentary epithelia, epidermis, and the spermatogenic compartment – rapidly turn over and continually renew themselves (Messier and Leblond, 1960; Leblond, 1995). Recent developments in accelerator mass spectrometry and biomedical adaptation of radiocarbon dating technology commonly used in archeology have extended this paradigm to previously inaccessible human tissues (Spalding et al., 2005).

The understanding that the lives of cells in multiple, critical tissues are ephemeral rather than perpetual occasioned a profound rethinking of metazoan physiology and raised numerous important questions. Perhaps foremost among them: if multitudes of cells are being birthed and replaced on a regular and often rapid basis, from where do new cells derive – what is the mechanism of self-renewal (Dor and Melton, 2004)?

1.7.2 Discovery of somatic stem cells as the source of continuously generated tissue effector cells

More than a century of independent, discursive threads of inquiry in developmental biology and evolutionary theory culminated in a solution to this problem. In a word: "stammzelle", or stem cells, a concept that the eminent German biologist Ernst Haeckel proposed in 1877 as he pondered how a single cell – the fertilized egg – might undergo the wondrous transformations that eventually give rise to the multitudes of cells and cell types that comprise the

mature organism (Ramalho-Santos and Willenbring, 2007). Contemporaneous investigations in embryology paralleled burgeoning research in hematology, which sought to elucidate the relationships between and origins of the multiple cell types that were coming to be appreciated in blood. As early as 1896, the hematologist Artur Pappenheim hypothesized the existence of a single progenitor cell that could give rise to both red and white blood cells (Pappenheim, 1896). Later, as the various lineages of the hematopoietic system came into focus following the promulgation of the staining techniques developed by Paul Ehrlich in the 1870's, he proposed a remarkably prescient genealogy of hematopoiesis rooted in a "polyvalent stem cell" that resembles the accepted modern paradigm (Ramalho-Santos and Willenbring, 2007).

Investigation into the nature of hematopoiesis continued in the following decades, often on the basis of observation, supposition and deduction, as opposed to direct demonstration. Following the atomic bombings of Hiroshima and Nagasaki however, interest in and funding for the field intensified dramatically when it was appreciated that blood was uniquely implicated in the pathogenesis of radiation poisoning (Na Nakorn et al., 2002; Weissman, 2014). A major goal in the field, inspired by the incipient Cold War and fears of impending nuclear conflict, was to discover treatments for ameliorating or even reversing this ghastly, greatly feared condition. Early experiments uncovered that lead shielding of spleen or bones (but, for example, not kidneys) *during* exposure to irradiation prevented mortality (Jacobson et al., 1951), and that pre-treatment with certain substances, for example high dose cysteine, could

attenuate subsequent radio-injury (Patt et al., 1949). However, cysteine administration *after* irradiation was entirely ineffectual (Patt et al., 1949). The first demonstrations of a *post-hoc* treatment arrived only when it was shown that transplantation of spleens or bone marrow could rescue mortality in mice and guinea pigs previously exposed to an otherwise-lethal dose of ionizing radiation (Lorenz et al., 1951; Jacobson et al., 1951).

What was the mechanism of this hotly-anticipated cure for radiation poisoning? Initial thinking in the field favored the hypothesis that humoral substances present in the transplanted tissue somehow effected repair of radiation lesions in the recipient hosts. This notion was discarded after a series of elegant experiments exploiting novel xenochimaeric and chromosomal marker strategies showed that the freshly-generated blood cells circulating in transplant recipients after recovery were unambiguously derived from the donor and not the recipient (Ford et al., 1956; Nowell et al., 1956), thus establishing the presence of cells with tissue reconstituting activity in adult bone marrow (Weissman, 2014; Eaves, 2015). Several years later, the dynamic Canadian duo of Ernest McCulloch and James Till devised methodology to quantify this activity for the first time, initially by way of a cumbersome limiting-dilution assay (McCulloch and Till, 1960), and then substantially more tractably following the serendipitous observation that otherwise-lethally irradiated mice whose hematopoietic systems had been reconstituted manifested macroscopic nodules, or colonies on their spleens approximately a week post-transplantation. Colony abundance corresponded linearly to the number of bone marrow cells that had been

transplanted, thus offering the ability to readily and rapidly (if retrospectively) measure progenitor cell number (Till and McCulloch, 1961). Importantly, after noting that the splenic colonies were comprised of multiple cell types including erythrocytes, megakaryotcytes, granulocytes, macrophages, and lymphocytes, the McCulloch and Till team then devised a clever method of uniquely chromosomally marking single hematopoietic cells using low-dose irradiation. Using this technique, they observed that the heterogeneous colonies were clonal: that is, all cells in a given colony, irrespective of cell type, bore the same, unique chromosomal marker, indicating descent from a common progenitor (Becker et al., 1963; Wu et al., 1968). Shortly thereafter, demonstration that serial transplantation of donor-derived colonies into secondary irradiated recipient mice could themselves form new, multi-lineage colonies and also still rescue otherwise-lethal radiation poisoning established the critical principal of autopoiesis (Siminovitch et al., 1963) – the ability of a system to recreate itself, often referred to somewhat less precisely as self-renewal.

Thus, the torrid, postwar quarter century of experimentation on irradiation pathogenesis and blood formation birthed the theoretical and experimental foundations upon which the modern concept of somatic stem cells is rooted. These potent cells constitutively maintain, and upon injury can reconstitute their tissue of origin by way of the capacities for extensive proliferation, (often but not necessarily multi-lineage) differentiation, and self-renewal (Siminovitch et al., 1963; Eaves, 2015). A preponderance of less direct, yet nevertheless compelling experimentation synergized with these concepts in other tissues and

developmental contexts, including epidermis (Storey et Leblond, 1951), spermatogenesis (Clermont and 1953), and gastrointestinal epithelia (Cheng and Leblond, 1974), thereby indicating that stemness is the biological force underlying the continuous renewal of a panoply of both solid and liquid tissues, especially those tending towards rapid turnover dynamics.

1.7.3 Genome integrity maintenance – the source of stemness?

Of course, at least within the tissues for which a clear role for stem cells had been established, the great question then became: what is the nature of stemness? What characteristics distinguish such cells from non-stem cells, and in particular, what processes conduce to their remarkable, life-long generative properties?

Clues to this puzzle began emerging piecemeal decades ago, but weren't consolidated into a coherent theoretical framework until a seminal publication situated stem cells within the evolutionary dynamics of carcinogenesis (Cairns, 1975). From these considerations emerged a simple, critical question: given the inherent genotoxicity of mitosis, how does rapid, ongoing cellular turnover in multiple tissues *not* precipitate an untimely collapse of genome integrity and consequent burden of cancer so overwhelming as to utterly squelch reproductive fitness (much less survival into old age)? To reconcile this paradox, Cairns posited that cellular and molecular mechanisms must exist that 1) minimize genotoxicity in stem cells and 2) minimize the possible damage to organismal homeostasis should accumulated mutations transform any given stem cell into a

tumor (Cairns, 1975). Though some of Cairns' specific predictions have not been borne out, the overall framework he proposed to undergird the growing evidentiary basis for emerging stem cell theory remains a touchstone in contemporary developmental biology. Specifically, Cairns anticipated that key stem cell characteristics would include: rarity, to restrict the overall size of the long-lived compartment in which transforming mutations might accumulate; relative proliferative quiescence, to indirectly minimize the accumulation of mutations arising from cell division; and endowment with various molecular mechanisms that actively promote the conservation of genome integrity (Cairns, 1975).

To varying extents, preceding and subsequently-published data have substantiated each of these claims. A major finding that emerged from early hematopoietic transplantation studies in the 1960's was the fact that less than 1% of cells in the bone marrow are possessed of reconstitution potential (Fuchs, 2009), and stem cell populations that have been identified in other tissues are almost uniformly tiny fractions of the total cellularity whose homeostasis they support. With respect to cycling dynamics, during early histological examinations of spermatogenesis in seminiferous epithelia, Leblond and colleagues deduced the presence of spermatogonial stem cells and noted early on that they appeared to be proliferatively quiescent relative to differentiating spermatocytes (Clermont and Leblond, 1953). A decade later, early characterizations of hematopoietic stem cells recorded resistance to radiation exposure and chemotherapeutic drugs known to preferentially target dividing cells, thus suggesting that stem cells

in liquid tissue appeared to also be marked by quiescence (Becker et al., 1965; Bruce et al., 1966). More recent studies using pharmacological and genetic label-retaining strategies have documented deep proliferative quiescence in multiple somatic stem cell compartments (Cotsarelis et al., 1990; Wilson et al., 2008; Tian et al., 2011; Bernitz et al., 2016), and established the general principle that cycling frequency correlates inversely with (re)generative activity – essentially that proliferative potential is a function of proliferative past (Fuchs, 2009). Cairns' remaining prediction, that stem cells might be programmatically specialized to actively forestall genomic disintegration, so far remains the least supported. Briefly however, the activity of at least one important pathway of genome integrity homeostasis appears to be substantially enriched in multiple somatic stem cell compartments – that of telomerase and the maintenance of telomere length (Vaziri et al., 1994; Flores et al., 2008). The possibility that additional genome integrity conservation mechanisms might be at play in stem cells is the subject of Chapter 3 in this work.

Thus, though minimally differentiated with respect to tissue effector functions (e.g. oxygen ferrying or hemostatic capacity in blood, action potential generation in brain, barrier function in the skin, or nutrient absorption activity in the intestine) stem cells may in fact be highly specialized for the maintenance of genome integrity through activation of multiple, independent pathways that comprise both passive (e.g. proliferative quiescence) and active modalities (e.g. DNA repair). Failure or exhaustion of these mechanisms accelerates senescence and the loss of stemness (Allsopp et al., 2003; Rossi et al., 2007;

Bernitz et al., 2016). If self-renewal is the single most defining characteristic of stem cells, a strong possibility is that genome integrity maintenance is the essence of self-renewal (Rando, 2006).

1.8 Conclusions

In summary, a developmental hierarchy rooted in a rare yet potent stem cell population that can repeatedly give rise to abundant effector cells appears to be a fundamental, conserved strategy of renewal in multiple tissues and metazoan species (Figure 1.2). This strategy is deployed to rapidly generate – or, upon injury, to regenerate – cellularity in a manner that minimizes the inevitable loss of (epi)genomic integrity consequent to cell division which would eventually lead to either malignant transformation or loss of function and degeneration (i.e. aging).

Though the proliferative dynamics of tissues have been pondered since the advent of cell theory almost a century and a half ago, and empirically explored in increasing depth during the modern, postwar period, the full extent of the role of stem cell hierarchies in somatic homeostasis remains unsettled. In unprecedented detail, a spate of recent reports has elucidated surprising turnover kinetics in tissues including brain, heart, and adipose tissue (Eriksson et al., 1998; Spalding et al., 2008; Spalding et al., 2013; Bergmann et al., 2015), rekindling in fresh contexts the long-standing question of the mechanism of life-long cell renewal (Dor and Melton, 2004). In these newly-scrutinized tissues, in others such as liver, pancreas, and skeleton, and even in well-studied tissues like blood and intestinal epithelium, multiple, sometimes contrasting reports have

emerged questioning, supporting, or substantially revising the role of stem cell hierarchies (Tian et al., 2011; Smart et al., 2011; Ali et al., 2014; Sun et al., 2014; Yanger et al., 2014; Chan et al., 2015, Kopp et al., 2016). Comprehensively understanding this fundamental matter in each of the complement of tissues that comprise the mammalian body plan remains of unsurpassed importance in biomedical research given the tremendous implications for basic understanding of physiological and pathological processes, as well as for the design of interventional prophylactic and therapeutic applications to preserve or potentiate human health.

Curiously, given the field of Immunology's deep roots in basic principles of developmental biology and the recognition of increasingly broad roles for lymphocytes in organismal homeostasis, the intertwined matters of stemness and genome integrity maintenance in the adaptive immune system have only recently begun to receive serious attention. Broadly, this dissertation probes the (re)generative nature of the adaptive immune system. Chapter 2 approaches this from a cellular perspective, and presents the isolation and characterization of rare, antigen-specific stem cells in the T lymphocyte compartment. These T stem cells are shown to underwrite the extraordinarily rapid generation of cytotoxic cells that effect defense against microbial infections. Surprisingly, following the resolution of infection-injury, some differentiated, cytotoxic effector T cells appear to de-differentiate and re-acquire stemness attributes, thus challenging the cardinal rule of irreversible differentiation that has attended the overall fields of hematology and immunology for decades. The broader context

and implications of dedifferentiation for developmental biology and the aging process are discussed. Chapter 3 probes the maintenance of genome integrity at a molecular level, first with respect to two well-known pathways, those of telomerase and double-strand break repair. The chapter concludes with a presentation of preliminary evidence suggesting the existence of an entirely new genome integrity maintenance mechanism involving endogenous transposons. Chapter 4 asks how T stem cells manage intense mitogenic signaling pressure to balance the opposing needs for active proliferation and differentiation to combat an invading pathogen versus the imperative of quiescence to conserve future proliferative potential. Observations are presented that T stem cells robustly express PD-1 and multiple other signal-attenuating receptors, and do so in order to prevent hyperactivation-induced depletion. The implications for immunological memory formation and immunotherapy applications are discussed.

Figure 1.1

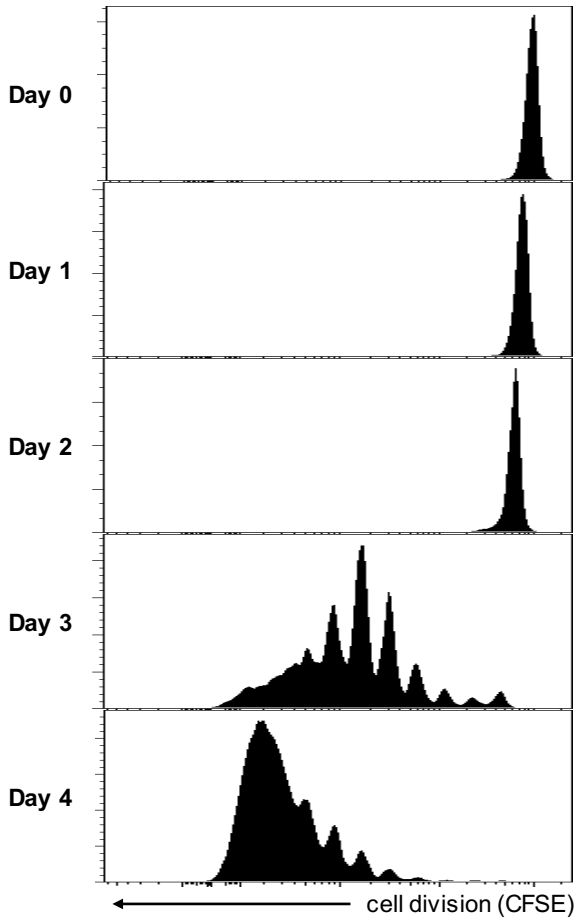


Figure 1.1: Precipitous proliferative dynamics of antigen-specific CD8$^+$ T cells responding to viral infection. LCMV-reactive TCR-transgenic P14 CD8$^+$ T cells were labeled with the proliferation-tracking dye CFSE and transplanted into naïve mice, which were subsequently infected with LCMV-Armstrong. Proliferation – by way of CFSE dilution – was monitored over the following 96 hours. After the adaptive immunological reaction commences (~48 hours pi), CD8$^+$ T lymphocyte division is rapid, with cell cycle durations as short as ~4 hours. Adapted from Kurachi et al., 2014.

Figure 1.2

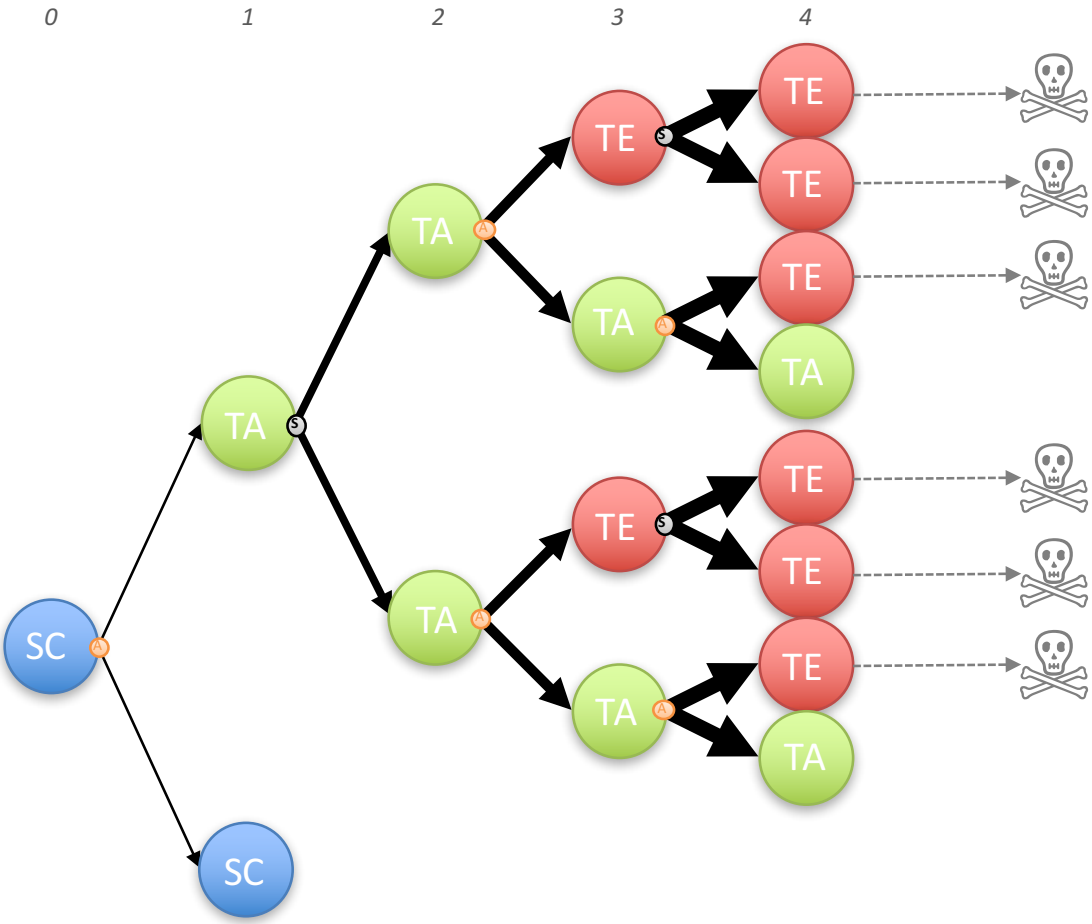


Figure 1.2: Generic proliferative hierarchy rooted in a stem cell population. Stem cell proliferative hierarchies in multiple vertebrate tissues sustain rapid, continuous generation of abundant, terminally differentiated tissue effector cells. Asymmetric division (depicted with a small, orange badge) and restrained progenitor proliferation minimizes the loss of genomic integrity inherent to mitosis, thereby conserving proliferative capacity to meet future physiological demand. Stem cells are depicted in blue; transit amplifying cells in green; terminally-differentiated effector cells in red. Number of divisions relative to the founding stem cell are indicated above. Adapted from Clermont and Leblond, 1953; and Cairns, 1975.

.:: CHAPTER 2 ::.
ISOLATION OF T STEM CELLS, AND DEDIFFERENTIATION OF CYTOTOXIC LYMPHOCYTES INTO $CD8^+$ CENTRAL MEMORY T CELLS

Understanding how development of immunological memory is orchestrated has extensive implications for vaccines, cancer immunotherapies, autoimmunity, and possibly the aging process. The first chapter discussed this issue in the context of the paramount requirement for the maintenance of genome integrity, and suggested that the existence of a stem cell subpopulation might enable pathogen-specific T lymphocytes to reconcile the apparently mutually-exclusive goals of extensive and extremely rapid proliferation with avoidance of premature exhaustion of finite proliferative potential.

2.1 Efflux activity (the side population phenotype) as an unbiased technique for isolating novel somatic stem cell populations

Probing the hypothesis that somatic stem cells might underlie immunological memory is conceptually straightforward yet technically challenging. A major, long-standing issue in developmental biology is identifying stem cells for a tissue of interest in the first place. Tissue stem cells were first imagined in the mid 19^{th} century, formally demonstrated to exist in the 1960's, and only molecularly defined and prospectively isolated in the late 1980's following decades of concerted inquiry in experimental hematology entailing laborious fractionation and transplantation experiments (Spangrude et al., 1988). By evolutionary design and biological definition (as discussed in Chapter 1) stem cells are

exceedingly rare, and this fact has complicated and to this day continues to complicate investigations into the nature of (re)generativity in any given tissue.

One practical method of circumventing much of this investigational complexity arose in the late 1990's based on observations originating in the context of cancer pharmacology three decades previously. During the dawn of anti-neoplastic chemotherapy, it was observed in treatment of both patients and transformed cell lines *in vitro* that resistance arising against one drug often engendered cross-resistance to a range of other drugs with disparate chemical structures and biological modes of action (Kessel et al., 1968). This phenomenon became known as multidrug resistance and is one of the most frequent forms of chemotherapy resistance (Muller et al., 1994). Mechanistic dissection of this process led first to the biochemical (Juliano and Ling, 1976) and ultimately the genetic isolation (Gros et al., 1986; Cole et al., 1992; Doyle et al., 1998) of the molecular factors involved, which turned out to be a subset of the ABC superfamily of transporters with promiscuous substrate specificities. These ATP-dependent enzymes are embedded in the plasma membrane and extrude a broad range of structurally diverse, xenobiotic substrates (including chemotherapeutic agents) from the cell against a concentration gradient (Schinkel and Jonker, 2003). This activity decreases intracellular drug concentration and thus confers resistance.

Separately, ongoing characterization of hematopoiesis coupled with advances in flow cytometry technology generated the intriguing insight that

hematopoietic stem cells (HSC) efficiently extrude certain fluorescent dyes such as Rhodamine-123 and Hoechst-33342, and that this property could be used to crudely isolate rare HSC from the complex and far more abundant mixture of non-stem-cell bone marrow cells that readily take-up and retain dyes (Pallavicini et al., 1985; Spangrude and Johnson, 1990). Using newly-generated monoclonal antibodies, a subsequent study elucidated the mechanistic basis for this: although broad, expression of MDR-related ABC proteins was observed to be particularly high on HSC, and furthermore correlated with fluorescent dye efflux activity (Chaudhary and Roninson, 1991). A methodological refinement several years later led to the observation of a so-called "side population" phenotype in which dye-stained bone marrow cells evaluated flow cytometrically exhibited strong, dual-emission peak fluorescence throughout, except for a visually distinct, quantitatively minor fraction of cells at the lower end of the spectrum of staining intensity. This fraction was isolated and shown in transplantation experiments to contain the vast majority of HSC activity (Goodell et al., 1996). Further studies genetically potentiating or abrogating expression of the MDR-related ABC genes ABCB1 and ABCG2 directly confirmed that these were principally responsible for the side population phenotype, and revealed that hematopoietic cells genetically deficient in efflux capacity were hypersensitive to chemotherapeutic drugs and other xenobiotics (Bunting et al., 2000; Zhou et al., 2001; Zhou et al., 2002). Perhaps most importantly from the broader perspective of developmental biology, the efflux / side population staining technique perfected by Goodell et al. enabled HSC to be isolated with remarkable ease and purity on the basis of a

defined physiological capacity, i.e. without any other *a priori* knowledge of HSC biology (e.g. unique proteomic or transcriptomic signatures). Together with Chaudhary and Roninson's earlier observation, this indicated the possibility that heightened efflux capacity might be an important aspect of stem cell biology generally, and offered the unprecedented opportunity to explore this notion in a methodologically tractable manner. Indeed, wide deployment of the side population technique subsequently uncovered confirmed or candidate stem cell populations in multiple tissues, including muscle, intestinal epithelium, endometrium, breast, testis, brain, solid tumors, and liquid tumors (Lassalle et al., 2004; Challen and Little, 2006; Golebiewska et al., 2011; Maruyama et al., 2013; von Furstenberg et al., 2014) and even across multiple species (Goodell et al., 1997). Thus, the "side population" technique of isolating candidate stem cell populations on the basis of a physiological profile of uniquely efficient dye efflux capacity can be a broadly effective strategy for discerning the presence of and initially isolating a putative stem cell population with an unknown proteomic signature (i.e. marker profile) in any given tissue. In short, the side population technique can sidestep much (though not necessarily all) of the laborious, iterative fractionation associated with historical searches for stem cells.

2.2 Efflux activity in antiviral CD8$^+$ T cells identifies CD62L as a candidate marker of T stemness

Given its demonstrable utility in identifying or clarifying progenitor cells in a multitude of other contexts, the side population technique seemed an opportune approach to begin to address the theoretical possibility that a rare population of

stem cells might exist within the sizeable pool of T lymphocytes that respond to invading pathogens. Since it is likely that the greatest threat to T cell genome integrity arises consequent to the hyper-proliferation, intense metabolism, and elaborate epigenetic transformations that attend the expansion phase of the adaptive immune response to pathogen invasion (as discussed in Chapter 1), and since stem cells are perhaps most likely to be physiologically required or at least experimentally evident in this context, analysis was focused primarily on this initial, priming period. As such, mice (C57Bl/6 strain) were infected intraperitoneally with the acutely-resolving, Armstrong strain of LCMV. A single cell suspension of splenocytes was prepared from spleens harvested during the expansion phase of the infection (d6 pi), incubated with Hoechst 33342 dye, and counter-stained with both fluorochrome-conjugated antibodies against a panel of surface proteins and a peptide-MHC tetramer to distinguish virus-specific T cells. When H33342 fluorescence was visualized simultaneously at 450 nm and 675 nm, a quantitatively minor side population was plainly evident in $CD8^+$ T cells **(Figure 2.1, left)**. This was intriguing, and suggested the possibility that a subpopulation of $CD8^+$ T cells with stem cell characteristics might actually exist.

To facilitate investigation of the cells in this side population, it was desirable to identify cell surface antigens that could distinguish them. Prior literature has established that side populations and non-side populations are antigenically distinct in multiple tissues (Challen and Little, 2005). Therefore, expression of a panel of markers of known relevance to memory $CD8^+$ T cell differentiation was screened in the side population versus the non-side

population of $CD8^+$ T cells during the expansion phase of the response to viral infection. From this it emerged that the protein CD62L was expressed to a greater extent in the side population, rather substantially in the total population of $CD8^+$ T cells **(Figure 2.1, right)** and less dramatically in the LCMV GP_{33-41}-specific subpopulation thereof (discussed later in the chapter).

2.3 Scrutiny of cellular heterogeneity during the antiviral expansion phase and re-evaluation of effector/memory T cell differentiation models prompted by unexpected expression of CD62L

The selectin CD62L is a cell adhesion molecule that interacts with specialized endothelial cells in lymphoid tissues and thereby plays an important role in the homing of lymphocytes (Gallatin et al., 1983). That CD62L was expressed preferentially in the side population of antigen-specific $CD8^+$ T cells suggested the possibility that this readily-measurable antigen might possibly be a marker for a hypothesized T stem cell subpopulation. However, that CD62L was expressed at all on antigen-specific T cells during the antigen-driven expansion phase of robust LCMV infection was unexpected, given the predominant consensus that all $CD8^+$ T cells during the expansion phase are firmly engaged with and committed to the effector fate; that such effector cells are marked by the *absence* of CD62L protein expression, and that any expression of CD62L during antiviral T cell expansion is negligible or at most irrelevant; and that instead CD62L expression marks preceding naïve T cells and succeeding T cells that emerge from effector T cell progenitors and subsequently commit to the memory fate weeks after antigen clearance and resolution of an acute infection (Kaech et al.,

2002; Wherry et al., 2003; Masopust, Murali-Krishna, et al., 2007; Pauken and Wherry, 2015). This surprising observation prompted closer scrutiny of heterogeneity in $CD8^+$ T cells in the expansion phase of the immunological response to viral infection, and a re-assessment of the principal models for memory T cell differentiation.

2.3.1 Contrasting developmental models for memory T cell formation

Despite the numerous mechanistic insights into the origins of T cell memory that have been gained in recent years, considerable debate remains concerning how long-lived memory $CD8^+$ T cells form (Ahmed et al., 2009; Kaech and Cui, 2012; Restifo and Gattinoni, 2013). Two distinct models have been proposed to explain this process. First, the post-effector model proposes that long-lived, self-renewing memory $CD8^+$ T cells are the progeny of cytotoxic effector $CD8^+$ T cells that emerge during the expansion phase. This model is supported by lineage tracing experiments that defined the developmental relationships of effector and memory T cells (Jacob and Baltimore, 1999; Opferman et al., 1999; Kaech et al., 2003; Wherry et al., 2003; Joshi et al., 2007; Sarkar et al., 2007; Harrington et al., 2008; Bannard et al., 2009). Functional studies also failed to observe $CD8^+$ T cells with key memory properties during the expansion phase, supporting the notion that memory $CD8^+$ T cells differentiate from surviving effector $CD8^+$ T cells following the clearance of antigen (Kaech et al., 2002). An alternative model proposes that memory T cells arise prior to effector T cell formation, and that effector T cells are generated from the progressive differentiation of memory T cells (Sallusto and Lanzavecchia, 2011; Restifo and Gattinoni, 2013; Fuertes

Marraco et al., 2015). Recent observations of phenotypic heterogeneity arising as early as the first division of activated naive cells (Chang et al., 2007; Metz et al., 2016) are consistent with a model in which the emergence of memory T cells does not require transit through an effector stage. Thus, the literature is tangled in a classic chicken-and-egg problem of ontogeny, with dueling models proposing either that cytotoxic effector differentiation exclusively precedes memory differentiation, or alternatively, the converse. Discerning which (if either) of these models most accurately reflects biological reality has been a long-standing goal in immunology and has tremendous implications for basic principles of developmental biology as well as numerous applications with relevance to human health. Here, this challenge is addressed directly. Data are presented that elucidate classical concepts of stem cell biology in danger-driven peripheral cytotoxic T lymphopoiesis, and also identify an unexpected and hematopoietically-unprecedented pattern of effector T cell de-differentiation in the post-antigen phase that plays an important yet non-essential role in memory T cell formation. Together, these findings support both pre- and post-effector models of $CD8^+$ T cell memory formation and conduce to a unified framework for memory T cell ontogeny.

2.4 Characterization of a $CD8^+$ T cell subset present during the expansion phase that expresses CD62L

To further investigate the surprising result of the side population assay – in which a sub-population of $CD8^+$ T cells expressing CD62L despite the presence of antigen was revealed – the heterogeneity of antigen-specific $CD8^+$ T cells was

scrutinized during the antigen-driven clonal expansion phase of acutely resolving viral infection. Previous work has identified IL-7Rα and KLRG1 as markers that define so-called "memory precursor" and terminally differentiated effector $CD8^+$ T cell subsets, respectively (Kaech et al., 2003; Huster et al., 2004; Joshi et al., 2007). While the great majority of cells within the splenic $KLRG1^{lo}$ IL-$7R\alpha^{hi}$ subset lacked robust expression of CD62L protein, clearly evident was a distinct subpopulation of LCMV GP_{33-41}-specific, TCR-transgenic P14 $CD8^+$ T cells with pronounced CD62L expression at d8 pi with LCMV-Armstrong (**Figure 2.2)**. The presence of this $KLRG1^{lo}$ IL-$7R\alpha^{hi}$ $CD62L^{hi}$ subset in the expansion phase was not unique to P14 TCR-transgenic $CD8^+$ T cells or LCMV and was observed in $CD8^+$ T cell responses to bacterial and other viral infections (**Figure 2.3)**, as well as in endogenous, non-TCR-transgenic responses to LCMV (**Figure 2.4)**. CD62L-expressing cells were also evident in other sub-populations defined by KLRG1 and IL-7Rα, but were most frequent and most consistently present across different infection types in the $KLRG1^{lo}$ IL-$7R\alpha^{hi}$ population (**Figures 2.2 and 2.3)**.

$CD8^+$ T cells expressing CD62L despite the presence of antigen have been observed previously (Wherry et al., 2003; Wherry et al., 2004; Huster et al., 2004; Marzo et al., 2005; Sarkar et al., 2007, Lin et al., 2016), but detailed examination of the properties of such cells is lacking, especially in contexts not complicated by supraphysiological manipulations (e.g. the use of TCR-transgenic naïve precursor T cells spiked in at extremely high frequencies) that greatly ease experimentation yet substantially compromise interpretability. Thus, to dissect

the properties of CD62L-expressing cells to the greatest extent possible, the expression of additional phenotypes and markers of effector and memory $CD8^+$ T cell differentiation was analyzed in the $CD62L^{hi}$ $KLRG1^{lo}$ $IL\text{-}7R\alpha^{hi}$ subset (depicted in blue; hereafter called the $CD62L^{hi}$ subset for simplicity). While these cells retained expression of CD95, Bcl-2, and the transcription factor TCF-1 at levels similar to those of naïve $CD8^+$ T cells, this subset also robustly upregulated expression of numerous markers of activation and differentiation, including CD44, CD38, CXCR3, CD122, IL-18R, Sca, CD27, and CD28 (**Figures 2.5A and 2.5D)**. The transcription factor Eomes is required for optimal central memory $CD8^+$ T cell formation following acutely-resolving infection (Banerjee et al., 2010; Zhou et al., 2010; Paley et al., 2013). Eomes was highly and preferentially upregulated in the $CD62L^{hi}$ subset at d8 pi compared to naïve $CD8^+$ T cells (depicted in grey) or compared to the $CD62L^{lo}$ $KLRG1^{lo}$ $IL\text{-}7R\alpha^{hi}$ (depicted in green; hereafter called the $IL\text{-}7R\alpha^{hi}$ subset) or the $CD62L^{lo}$ $KLRG1^{hi}$ $IL\text{-}7R\alpha^{lo}$ (depicted in red; hereafter called the $KLRG1^{hi}$ subset) effector subsets (**Figure 2.5A)**. In contrast, T-bet and Blimp-1, transcription factors that coordinately specify effector $CD8^+$ T cell differentiation (Intlekofer et al., 2005; Rutishauser et al., 2009; Xin et al., 2016), were modestly upregulated in the $CD62L^{hi}$ subset relative to naïve precursors, but to a markedly lesser extent compared to the $IL\text{-}7R\alpha^{hi}$ or $KLRG1^{hi}$ effector subsets (**Figure 2.5A)**. As expected given initial results indicating greater expression of CD62L in the $CD8^+$ side population, efflux activity was markedly enriched in the $CD62L^{hi}$ subset relative to the $IL\text{-}7R\alpha^{hi}$ and $KLRG1^{hi}$ subsets **(Figure 2.6)**.

The phenotypic characteristics of this expansion phase CD62L^{hi} subset shared some features with previously described putative memory stem cells (T_{SCM}) in terms of CD95, CXCR3, and Sca expression (Restifo and Gattinoni, 2013). However, these previous studies also suggested that T_{SCM} are more akin to naïve CD8^{+} T cells, including, in some cases, low expression of CD44 (Zhang et al., 2005; Gattinoni et al., 2009), which differs from the CD62L^{hi} subset identified here that are clearly distinct from naïve CD8^{+} T cells and are CD44^{hi} **(Figures 2.5A and 2.5D)**. Moreover, relative to naïve CD8^{+} T cells, the effector-associated molecules Granzyme A and Granzyme B were modestly upregulated in the CD62L^{hi} subset **(Figures 2.5C and 2.5D),** though expression of these molecules was higher in the IL-7Rα^{hi} or KLRG1^{hi} subsets. This CD62L^{hi} subset also readily expressed the cytokines IFNγ and TNF upon antigenic restimulation *ex vivo* **(Figure 2.5B)**, but was the only expansion-phase population that efficiently produced IL-2 **(Figure 2.5B)**. Thus, during the expansion phase of LCMV infection the CD62L^{hi} subset exhibits phenotypic and functional features of CD8^{+} T cell memory as well as limited effector differentiation.

One possibility was that these CD62L^{hi} cells simply were not fully activated following antigen encounter. However, vital-dye-mediated proliferative history tracking using physiological numbers of LCMV GP33-specific TCR-transgenic P14 CD8^{+} T cells demonstrated that the vast majority of cells in the CD62L^{hi} subset had undergone at least 8 divisions by the peak of expansion **(Figure 2.7)**. These findings unambiguously preclude the possible interpretation that the

CD62L^{hi} population might be merely an artifact of incomplete recruitment (i.e., aberrant or aborted activation) of naïve CD8^{+} T cells into the immune response, as has been observed in some experimental scenarios (Kaech and Ahmed, 2001).

Interestingly, the proliferative history data for all three antigen-specific populations indicated that the CD62L^{hi} subset divided fewer times than the IL-7Rα^{hi} and KLRG1^{hi} subsets. This pattern was modestly evident in contexts of physiological numbers of precursor naïve P14 T cells, and substantially more so when supraphysiological numbers were used **(Figures 2.8A and 2.8B)**. Thus, it appeared that CD62L^{hi} cells were distinguished not only functionally and phenotypically, but also by unique proliferative dynamics. To probe this matter in greater depth, multiple independent methods were used to monitor cell division in the CD62L^{hi}, IL-7Rα^{hi}, and KLRG1^{hi} subsets towards the end of the expansion phase. Short-term (24 hr) BrdU pulsing from d7-8 pi, Ki-67 expression at d8 pi, and TCR-transgenic P14 CD8^{+} T cells on a Fucci-transgenic cell cycle reporter background (Sakaue-Sawano et al., 2008) all indicated less cell cycling in the CD62L^{hi} subset compared to the other two subsets between days 7-8 pi **(Figure 2.8C)**. This hierarchy of proliferation was reflected in the absolute abundance of cells in each subset that had accumulated in the spleen at the peak of the expansion phase **(Figure 2.8D)**. As such, the CD62L^{hi} subset of CD8^{+} T cells identified in the expansion phase exhibits a distinctive pattern of differentiation including robust expression of key factors associated with T cell memory and minimal expression of effector-associated molecules. Moreover, these cells had

proliferated extensively, indicating they were not simply weakly activated, and yet nevertheless exhibited a degree of proliferative quiescence relative to more the more rapidly-cycling IL-7Rα^{hi}, and KLRG1^{hi} subsets. Together, these observations suggested that CD62L^{hi} cells may have unique characteristics among the pool of CD8^{+} T cells responding to infection *in vivo*, and raise the question of what their developmental role might be.

2.5 Self-renewal of the CD62L^{hi} subset and ontogenetic relationship to effector and memory lineages

The functional, phenotypic, and proliferative characteristics of the CD62L^{hi} subset in the expansion phase suggested possible connections both to emerging concepts (that is, within the field of immunology) of stem cell biology as well as well-established T cell memory. However, the developmental relationship between this subset and the two known CD62L^{lo} effector CD8^{+} T cell subsets as well as long-term memory CD8^{+} T cells was not clear. To address these questions, it was important to delineate the lineage relationships between the CD62L^{hi}, IL-7Rα^{hi}, and KLRG1^{hi} subsets *in vivo*, and define the contribution of the CD62L^{hi} subset to long-term memory. Thus, the three subsets were flow cytometrically isolated at d6 pi and transplanted into separate, congenically-disparate recipients at d4 pi **(Figure 2.10A)**. All donor populations were labeled with a proliferation tracking dye. The numerical recovery, proliferation, and developmental potential of each subset was then assessed at the peak of the expansion phase of the infected recipient animals. At d8 pi the CD62L^{hi} subset had given rise to the most abundant population of virus-specific progeny, the vast

majority of which had extensively proliferated as indicated by full proliferation dye dilution **(Figure 2.10B, 4C)**. The IL-7Rα^{hi} subset also gave rise to many dye-diluted cells, but the numerical recovery was lower than for the CD62L^{hi} subset. In contrast, the KLRG1^{hi} subset gave rise to few recovered cells and many of these cells had not extensively divided. These observations are consistent with the terminal nature of the KLRG1^{hi} subset of effector CD8^{+} T cells. These data also identify the CD62L^{hi} subset as a population with superior ability to generate progeny during antigen-driven clonal expansion.

The three different donor subsets also differed in their capacity to give rise to progeny cells in each of the other CD8^{+} T cell subsets. The terminally-differentiated KLRG1^{hi} subset gave rise almost exclusively to progeny with an identical phenotype **(Figures 2.10D and 2.10E)**. IL-7Rα^{hi} effector CD8^{+} T cells gave rise to both IL-7Rα^{hi} and KLRG1^{hi} progeny, but did not appreciably produce CD62L^{hi} cells in this expansion-phase assay (Figs. 4D and 4E). By contrast, CD62L^{hi} cells generated all three subsets including additional CD62L^{hi} cells **(Figures 2.10D and 2.10E)**. This latter observation, together with the CTV dilution data demonstrating that nearly all cells of the CD62L^{hi} donor subset divided **(Figure 2.10C)**, is consistent with self-renewal of the CD62L^{hi} subset in this setting of antigen-driven proliferation. Thus, a positive association was revealed between developmental potential and proliferative output: the CD62L^{hi} KLRG1^{lo} IL-7Rα^{hi} subset is endowed with self-renewal capacity and the broadest developmental potential. Consistent with established principles of stem cell

biology, this subset also appeared to possess the greatest proliferative reserve and capacity to generate abundant new progeny.

It was next of interest to probe the relationship of this CD62L^{hi} progenitor subset to well-defined CD8^{+} T cell memory present following the clearance of antigen. A cardinal attribute of CD8^{+} T cell memory is the capacity to persist in the absence of antigen and mount a proliferative recall response to antigenic re-encounter (Kaech and Wherry, 2007). Thus, each of the three CD8^{+} T cell subsets were sort-purified from the peak of the expansion phase of viral infection and transplanted in equal numbers to separate, naive recipient mice. A month later, these recipient mice were challenged with the persisting, clone 13 strain of LCMV and the expansion and phenotype of cells derived from each of the donor subsets was examined. One week following rechallenge, robust recall **(Figure 2.12A)** and the presence of CD62L^{hi} cells **(Figure 2.12B)** were evident in the progeny of the CD62L^{hi} progenitor subset, indicating the capacity to persist, mount a vigorous proliferative response upon antigen re-encounter, and self-renew. The IL-7Rα^{hi} subset also manifested recall capacity, though to a lesser extent than the CD62L^{hi} subset, raising the possibility that this IL-7Rα^{hi} subset had acquired at least some attributes of the memory fate despite substantial effector differentiation during the expansion phase **(Figure 2.5)**. The KLRG1^{hi} subset, as expected (Joshi et al., 2007; Sarkar et al., 2008), gave rise to few cells capable of mediating effective recall responses. In a related experiment, the three CD8^{+} T cell subsets were sort-purified from the peak of the expansion phase of viral infection, transplanted into separate naïve recipient mice, and

challenged with LCMV clone 13 immediately (as opposed to one month later) **(Figure 2.13A)**. Approximately three weeks post challenge a comparable hierarchy of donor-derived cell abundance was evident, with the progeny of the CD62L^{hi} subset clearly advantaged over those of the two CD62L^{lo} effector subsets **(Figure 2.13A)**. To ensure that these observations weren't skewed by any differences in environmental variables including viral load, this experiment was repeated but modified to co-transplant congenically disparate CD62L^{hi} and IL-7Rα^{hi} subsets into the same recipient mice, followed by immediate challenge with LCMV clone 13. Nine days post challenge the progeny of the CD62L^{hi} subset substantially outnumbered those of the IL-7Rα^{hi} subset **(Figure 2.13B)**. Thus, the CD62L^{hi} subset appears to be endowed with robust recall capacity that is superior to that of cells lacking CD62L expression; it also appears to be markedly advantaged with respect to proliferative capacity and/or survival ability in the context of immediate and enduring replicative stress.

These observations prompted further probing of the phenotypic and functional attributes of cells derived from antigen-specific, expansion-phase subsets adoptively transferred into antigen-free recipient mice. Thus, the CD62L^{hi} or IL-7Rα^{hi} subsets isolated at d8 pi and transplanted into naive recipient mice to examine antigen-independent maintenance differentiation status with respect to cardinal attributes of central memory. The KLRG1^{hi} effector subset persisted poorly under these conditions (data not shown), consistent with data above **(Figures 2.10, 2.12, 2.13)** and published observations (Joshi et al., 2007; Sarkar et al., 2008). Following transplantation of the CD62L^{hi} subset, the donor

cells recovered were mostly CD62L^{hi} six months **(Figure 2.11A-B)** or two weeks later **(Figure 2.11C-D)**. Moreover, cells in the CD62L^{hi} subset were able to undergo homeostatic proliferation between days 8-21 pi more efficiently than cells that lacked CD62L expression or cotransferred, control naïve OT-1 cells **(Figure 2.11E)**. These characteristics align with the well-described properties of central memory (Sallusto et al., 1999; Wherry et al., 2003), and suggest that the pool of CD62L^{hi} memory CD8^{+} T cells in the post-antigen phase draws substantially from the CD62L^{hi} subset in the expansion phase. Indeed, phenotypic and functional examination of the CD62L^{hi} subset during the expansion and the post-antigen phases revealed both core similarities (e.g. heightened anticipatory DNA damage surveillance) as well as differences (e.g. extent of CD43 and CD62L expression) suggesting that fundamental memory properties are engendered during the antigen-driven expansion phase, and perhaps further evolve following antigen clearance **(Figure 2.14)**.

Because the entire IL-7Rα^{hi} subset (i.e. independent of CD62L expression) has previously been shown to be the source of long-lived memory CD8^{+} T cells (Kaech et al., 2003), it was important to determine whether this central memory developmental potential could be fully explained by the expansion phase CD62L^{hi} subset. Thus, the IL-7Rα^{hi} subset (i.e. IL-7Rα^{hi} cells depleted of CD62L^{hi} cells; >99% of sorted cells were CD62L^{lo}) isolated at d8 pi was transplanted to antigen-free mice. Six months **(Figure 2.11A-B)** and two weeks **(Figure 2.11C-D)** post-transfer a substantial fraction of cells derived from IL-7Rα^{hi} CD62L^{lo} donor cells now expressed CD62L as well as CXCR3, an additional phenotypic feature of

central memory. IL-7Rα^{hi} CD62L^{lo}-derived cells also displayed evidence of homeostatic proliferation **(Figure 2.11E)** consistent with long-term, self-renewing memory CD8^{+} T cells arising from CD62L^{lo} IL-7Rα^{hi} effector CD8^{+} T cells. Thus, while the expansion phase CD62L^{hi} subset examined here is endowed with multiple stemness characteristics and contributes to the pool of long-term memory CD8^{+} T cells, the IL-7Rα^{hi} effector subset, partially differentiated consequent to intense antigenic and inflammatory signaling, surprisingly also appears possessed of memory potential, albeit at a markedly lower efficiency than the CD62L^{hi} subset. This observation of unanticipated developmental plasticity is consistent with the notion of dedifferentiation, a phenomenon that has been recently appreciated in other mammalian tissues but that is unprecedented in hematopoietic lineages **(Figure 2.15)**.

2.6 Discussion

There has been considerable debate concerning the ontogeny of long-lived memory T cells. Here, a subpopulation of antigen-specific CD8^{+} T cells is identified during antigen-driven expansion that in fact robustly expresses CD62L. This CD62L^{hi} population of CD8^{+} T cells manifests a unique constellation of properties – including multiple stem cell-like characteristics – that distinguishes it from other CD8^{+} T cell subpopulations identifiable in the presence of antigen. Examination of this rare CD62L^{hi} population has provided a fresh perspective on the formation of long-lived memory T cells.

2.6.1 CD62L^{hi} cells constitute a distinct subpopulation of expansion-phase CD8^{+} T cells

The data presented suggest that the CD62L^{hi} subset makes a robust contribution to immunological memory. These cells have an immunophenotype (e.g. KLRG1^{lo} IL-7Rα^{hi} CD122^{hi} CD27^{hi} CD44^{hi}) suggesting that they are more closely related to previously described memory precursor cells (Kaech et al., 2003; Huster et al., 2004) than to terminally differentiated, cytotoxic effector cells. However, in both form and function, the rare CD62L^{hi} subset is clearly distinct from the more abundant CD62L^{lo} subset of KLRG1^{lo} IL-7Rα^{hi} cells classically referred to as "memory precursors." The CD62L^{hi} subset expresses more CXCR3 and less CX_3CR1, and, unique among antigen-specific cells in the expansion phase, highly expresses a multitude of molecules implicated in progenitor biology including Sca, a marker of hematopoietic stem cells in the bone marrow and a determinant of their developmental potential (Spangrude et al., 1988; Ito et al., 2003); CD47, a molecule on hematopoietic stem cells that interacts with SIRPα on macrophages to provide protection from errant phagocytosis in inflammatory milieux (Jaiswal et al., 2009); and TCF-1, a critical executor of the of the ancient Wnt signaling pathway that animates self-renewal activity in numerous embryonic, somatic, and cancer stem cell populations (Reya et al., 2003; Reya and Clevers, 2005). As detailed in chapters 3 and 4, additional progenitor-associated molecules expressed by the CD62L^{hi} subset include telomerase (Morrison et al., 1996), and CXCR5 (Wu et al., 2015; He et al., 2016; Im et al., 2016; Leong et al., 2016). Notably, the activities of many of these progenitor-associated factors have also been implicated in memory fate commitment in both

$CD8^+$ and $CD4^+$ T cells (Kaech et al., 2002; Gattinoni et al., 2009; Van et al., 2012; Lin et al., 2016).

In addition to progenitor-associated molecules, expansion-phase $CD62L^{hi}$ $CD8^+$ T cells exhibited the lowest expression of the effector-fate-promoting transcription factors T-bet and Blimp-1, and were unique in their high expression of the memory-fate-promoting transcription factor Eomes and ability to synthesize the memory-associated cytokine IL-2. Despite their proliferative quiescence relative to the $CD62L^{lo}$ effector subsets, these cells nevertheless had divided at least 7-10 times by the peak of the expansion phase and had acquired the ability synthesize the effector-associated cytokines IFNγ and TNFα. Moreover, these cells retained robust proliferative capacity and could give rise to all effector subsets while simultaneously regenerating themselves. Together, these data describe a set of properties that identify this $CD62L^{hi}$ subset of antigen-specific $CD8^+$ T cells arising in the expansion phase as a distinct cell type with substantial relevance both for generating abundant cytotoxic effector cells in the presence of antigen signaling, and for contributing to long-term T cell memory following infection resolution and antigen clearance.

2.6.2 Implications of the Side Population / efflux phenotype

An intriguing aspect of the differentiation state of the $CD62L^{hi}$ population was its preferential efflux activity (i.e. relative to the two $CD62L^{lo}$ effector populations), as identified in the side population assay. In these studies, CD62L as a marker of progenitor T cell populations first came to light in the context of a quest using this

assay for long-hypothesized T stem cells. In retrospect, this association between CD62L expression and efflux activity is not surprising, given that the transcription factor FoxO1 is a positive upstream regulator of the ABC-family pump Mdr1 (Kang et al., 2010), and preliminary indications that FoxO1 is highly expressed in the $CD62L^{hi}$ population (data not shown). Another ABC-family pump strongly implicated in multi-drug resistance and efflux activity is Bcrp. This factor is known to be upregulated on hematopoietic progenitor cells by hypoxia (Krishnamurthy et al., 2004). Of note, lymphoid tissue is often hypoxic, with oxygen tension ranging from 1-5% (Caldwell et al., 2001), and the $CD62L^{hi}$ population is preferentially localized thereto (discussed in Chapter 4). Thus, expression of FoxO1 and localization to a hypoxic microenvironment are likely factors involved in regulating the efflux activity of the $CD62L^{hi}$ population.

Matters of regulation notwithstanding, the precise role of efflux in antigen-specific $CD8^{+}$ T cells remains unclear. Since mice (and particularly their stem cells) genetically deficient in ABC-family efflux pumps are highly susceptible to the cytotoxic effects of ABC-family efflux substrates such as chemotherapeutic drugs or endogenous porphyrins generated as a byproduct of quotidian metabolism (Jonker et al., 2002; Zhou et al., 2002; Krishnamurthy et al., 2004), a likely possibility is that efflux activity minimizes exposure to cytotoxic substances and thus potentiates long-term survival of progenitor populations. An additional, non-exclusive possibility is that ABC-family pump substrates include endocrine or paracrine pro-differentiation factors. Thus, efflux capacity in cell populations with stemness characteristics may assist in the maintenance of the undifferentiated

(with respect to effector function) state — a fundamental aspect of self-renewal. There is some precedent for this in other systems (Challen and Little, 2005; Lin and Goodell, 2006). Future studies should address this possibility directly in the $CD8^+$ T cell progenitor compartment, possibly beginning with a biochemical survey of substrates associated with ABC-family efflux pumps expressed therein. Additionally, mice genetically deficient for ABC-family factors (e.g. Bcrp and Mdr1) and upstream regulators thereof (e.g. FoxO1) are available, and can be used to directly address some of the many questions raised by the efflux activity of $CD8^+$ T cells during pathogen infection: is efflux activity at all diminished consequent to FoxO1 deficiency or relative oxygen abundance? Is the differentiation or maintenance of the $CD62L^{hi}$ progenitor population impaired in the absence of ABC family members, both in unmanipulated conditions and those of deliberate exposure to cytotoxic substances (e.g. chemotherapy treatment)?

2.6.3 Transcriptional circuitry and fate determination

The transcription factor expression pattern of the expansion phase $CD62L^{hi}$ subset – TCF-1^{hi} $Eomes^{hi}$ and T-bet^{lo} Blimp-1^{lo} – would be expected to promote the memory fate and suggests a distinct inductive event for these cells. One possibility is that TCF-1 expression is sustained or induced in the $CD62L^{hi}$ subset via preferential Wnt signaling, as has been suggested using models of *in vitro* priming in the presence of Wnt3a (Gattinoni et al., 2009), or via asymmetric partitioning of Wnt signaling cascade factors (Lin et al., 2016). Additionally, or alternatively, the expression pattern of key chemokine receptors (e.g. CX_3CR1^{lo}

CXCR3^{hi} CXCR4^{hi} CXCR5^{hi}) and lymphoid localization bias *in vivo* suggest these cells may have preferential access to signals that induce early commitment to the memory fate (discussed in Chapter 4). Whether this reflects instructive signals from antigen presenting cells as might occur during asymmetric division or whether preferential access to cytokine or growth and differentiation factors influences these decisions is unclear. Regardless, it will be of considerable interest in the future to determine how these cells acquire their unique differentiation state.

2.6.4 Reconciliation via dedifferentiation: toward a unified model of T cell memory formation

"In science, ideology tends to corrupt; absolute ideology [corrupts] absolutely"
- Robert Nisbet

The data presented herein concerning the existence of a proliferative hierarchy and the lineage relationships between CD62L^{hi} progenitor cells and CD62L^{lo} effector cells bear obvious implications for models about the formation of immunological memory. One model of memory CD8$^+$ T cell formation posits that memory fate commitment occurs early after initial activation such that memory and effector CD8$^+$ T cells co-exist during the expansion phase (Kaech and Cui, 2012). Support for this model comes from studies of stem cell-like memory T cells (T_{SCM}) in which cells with phenotypic and functional properties of memory arise directly from naïve T cells upon stimulation (Restifo and Gattinoni, 2013). Observations of cells with apparent memory properties arising *in vivo* after early asymmetric division (Chang et al., 2007) or *in vitro* in the presence of IL-15 (Manjunath et al., 2001) or Wnt signaling agonists (Gattinoni et al., 2009)

reinforce the notion that the genesis of T cell memory does not depend upon transit through an effector phase, and in turn that effector differentiation is inimical to memory potential. Alternatively, a second model holds that memory $CD8^+$ T cells are the progeny of cells that have differentiated into cytotoxic effector $CD8^+$ T cells and subsequently further differentiate to acquire memory T cell properties following antigen clearance (Kaech and Cui, 2012). The potential for effector cells to give rise to memory is diminished by increased or prolonged TCR stimulation (Sarkar et al., 2008), but in any case, memory fate commitment occurs well *after* effector differentiation and the resolution of infection. Lineage tracing experiments where cells with an effector phenotype or expressing genes reporting effector differentiation can develop into memory T cells (Jacob and Baltimore, 1999; Opferman et al., 1999; Kaech et al., 2003; Wherry et al., 2003; Joshi et al., 2007; Sarkar et al., 2007; Harrington et al., 2008; Bannard et al., 2009) provide support for this model, as do functional studies that failed to observe $CD8^+$ T cells during the expansion phase with cardinal memory properties of robust proliferative capacity in response to antigenic or homeostatic signals (Kaech et al., 2002).

How to reconcile these opposing models, each with extensive support, has been a longstanding challenge, and fundamental questions thus remain. How is the pool of developing effector T cells maintained, and what population or populations of cells have the potential to continue to generate new progeny during robust infections? The data presented here may resolve some of these questions in two ways.

First, a highly activated yet relatively proliferatively quiescent subpopulation of cells present early in the expansion phase is identified that gives rise to effector $CD8^+$ T cells. This CD62L-expressing population is phenotypically and functionally endowed with multiple manifestations of both stemness and memory T cell biology. While previous analyses of the expansion phase have identified cellular heterogeneity in CD62L and KLRG1 expression and have inferred lineage dynamics between these subpopulations based on mathematical modeling (Buchholz et al., 2013), herein direct evidence is presented that $CD62L^{hi}$ $CD8^+$ T cells in the expansion phase can give rise to the full spectrum of effector subsets upon antigenic stimulation. Moreover, these $CD62L^{hi}$ $CD8^+$ T cells are demonstrated to self-renew during the expansion phase. This may explain why antigen-specific populations are not rapidly depleted during vigorous expansion, and suggests a mechanism for the generation and maintenance of responses during scenarios of persisting antigenic stimulation, such as chronic infections or tumorigenesis.

Second, through lineage tracing experiments, the data presented here show that the post-antigen phase pool of memory cells is seeded in part from the stem-cell-like $CD62L^{hi}$ subset present during the expansion phase, consistent with the pre-effector model. However, in support of the post-effector model, IL-$7R\alpha^{hi}$ $CD8^+$ T cells depleted of $CD62L^{hi}$ cells also appear to contribute to the pool of long-lived central memory $CD8^+$ T cells, which recent evidence has unambiguously indicated to be (or be substantially comprised of) stem cells

(Graef et al., 2014). Thus, somewhat surprisingly, this observation suggests that long-lived, self-renewing $CD8^+$ T cell memory can be derived not only from developmentally and proliferatively potent $CD62L^{hi}$ cells that emerge soon after (if not at the moment of) initial activation at the inception of an adaptive immune response, but also from $CD62L^{lo}$ cells that have proliferated more extensively and undergone at least partial, antigen-driven effector differentiation, but then in a process of apparent dedifferentiation, reacquired memory-progenitor properties following clearance of antigen and waning inflammation.

Until quite recently, the possibility of physiological dedifferentiation was believed to be strictly impossible in mammals. This thinking stemmed largely from interpretations of theoretical work developed in the mid 20^{th} century by Conrad Waddington, the British embryologist-philosopher. Most notably, Waddington proposed a conceptual framework that sought to explain several fundamental observations in embryology (Waddington, 1957). Among these were the remarkable overall consistency of development from zygote to well-apportioned neonate, despite nearly unfathomable complexity with respect to prodigious proliferation and intertwined processes of differentiation and morphogenesis. Despite so many trillions of possibilities for fate choices to go spectacularly awry, they usually didn't. How was this possible?

A related curiosity concerned apparent cellular stability: if an amorphous, utterly undifferentiated progenitor cell could undergo the tremendous transformations in form and function necessary to become any of the diverse

array of highly specialized cell types that are necessary to sustain the physiology of the mature organism – say, a rhythmically contracting cardiomyocyte or a neuron transducing action potentials – what was to stop this transformational process from proceeding in reverse? Surely, Waddington reasoned, there must be some inhibitory mechanism, as chaos would undoubtedly ensue if spontaneous changes in differentiation state were to lead cardiomyocytes to stop pulsating or neurons to cease generating electrical impulses (Merrell and Stanger, 2016).

To account for these phenomena, Waddington conceptualized differentiation as a series of successive branching decisions embedded in a three-dimensional epigenetic landscape whose contours are sculpted by the activity of genes **(Figure 2.15A)**. Importantly, fate commitments in development proceeded much like a marble poised to roll down a hill to the point of lowest local elevation. Both the consistency and irreversibility of differentiation were functions of the increasingly steep ridges arising between the valleys of potential that the marbles (cells and incipient tissues) traversed; when a cell reached its position at the bottom of the hill, there was no going back uphill (Mills and Sansom, 2015). Though first conceived as early as 1940, thus even before the recognition of DNA as the molecular basis for heredity, Waddington's metaphor has become a useful and highly cited construct for thinking about the nature of cell type identity and the formative processes that conduce to it during development (Rajagopal and Stanger, 2016).

As originally envisioned, Waddington's topography exerted an inexorable pull on developing cells and tissues – as it were, an unavoidable epigenetic gravity. Even at the outset however, this was not interpreted in the most absolute sense of precluding reverse differentiation in all possible scenarios. For example, contemporaneous experiments demonstrated that mature salamander cells could dedifferentiate and redifferentiate to regenerate amputated limbs (Hay, 1958), and that the fate of a terminally differentiated frog genome could be reverted to the ancestral, pluripotent state of its embryonic progenitor in the extreme experimental scenario of nuclear transplantation (Gurdon, 1962). Nevertheless, this observation was generally regarded as a curious exception, one perhaps idiosyncratic to relatively short-lived amphibians. Moreover, any conceivable dedifferentiation events in mammals, arguably more complex and dependent upon structural consistency, were believed to be of universally pathological import. Indeed, dating from the 19th century hypotheses of Virchow and his student Julius Conheim, tumorigenesis has long been imagined as errant embryogenesis (Conheim, 1875), and modern investigation has substantiated this notion by way of the observation of cancer initiation precipitated by dedifferentiation of lineage-committed cells into a stem-like state (Krivtsov et al., 2006).

Given these many considerations, it was thus a significant shock when mammalian dedifferentiation was dramatically and unambiguously reported, first purely phenomenologically in the case of cloned sheep (Wilmut et al., 1997), and then with mechanistic precision by way of pluripotent stem cells induced *in vitro*

by defined factors (Takahashi and Yamanaka, 2006). Thus, contrary to long-standing dogma, it turns out that dedifferentiation as a general matter *is* possible, including in mammals: epigenetic gravity, as envisioned in Waddington's topography, can be defied **(Figure 2.15C)**. Importantly, while these extreme, experimentally-induced cases exemplify complete loss of lineage identity and consequent resumption of developmental pluripotency, dedifferentiation *per se* need not entail traversing such drastic epigenetic distances. A formal definition holds that "dedifferentiation refers to the reversion of a committed or differentiated cell (defined by the expression of differentiation markers or other features of a specialized cell) into a cell with greater developmental potential" (Merrell and Stanger, 2016). Thus, in *Drosophila* gonads, both female and male germ cells can regain stem cell identity after initiation of differentiation (Brawley and Matunis, 2004; Kai and Spradling, 2004), while in rodents, lineage-committed B lymphocytes can dedifferentiate into progenitor cells following genetic manipulation *in vivo*, yet still retain hematopoietic character (Cobaleda et al., 2007).

Despite these important conceptual advances, questions remain concerning the relevance of mammalian dedifferentiation in physiological circumstances. Recently, dedifferentiation *in vivo* has been reported in the contexts of murine spermatogenesis (Nakagawa et al., 2010), intestinal epithelia (van Es et al., 2012), pulmonary epithelia (Tata et al., 2013), and hepatocytes (Tarlow et al., 2014). Together, these observations suggest that dedifferentiation may play a major role in the physiology of multiple mammalian tissues and cell

types. However, to date there have been no reports of physiological dedifferentiation in cells of the immuno-hematopoietic lineage, though speculation on this matter – including particularly in $CD8^+$ T cells – has been published (Joshi and Kaech, 2008). Furthermore, all of the aforementioned observations source from experimentally contrived scenarios of genetic, chemical, or physical injury. It remains unknown whether dedifferentiation can occur spontaneously in mammals.

Thus, the observations presented in the studies herein that intermediately-differentiated IL-7Rα^{hi} effector T cells (which lack robust CD62L expression) can reacquire phenotypic (e.g. CD62L expression) and functional manifestations of the CD62L^{hi} progenitor cells that developmentally precede them, are a notable indication that dedifferentiation may play a role not only in mammalian germinal, epithelial, and hepatic lineages, but also in immuno-hematopoietic tissue as well. Moreover, these data provide evidence for multiple developmental origins of memory $CD8^+$ T cells, and thereby help reconcile the longstanding dichotomy of the pre- and post- effector models for memory formation. Because the CD62L-depleted IL-7Rα^{hi} effector T cells gave rise to central memory $CD8^+$ T cells slightly less efficiently, these data further support a linear model of effector differentiation with decreasing potential for memory formation. Expansion phase CD62L^{hi} $CD8^+$ T cells with minimal effector differentiation have the most per-cell memory potential, followed by intermediately-differentiated IL-7Rα^{hi} effector $CD8^+$ T cells lacking CD62L expression, with the fully effector-differentiated KLRG1^{hi} subset contributing little to the long-term, self-renewing memory pool. Thus,

these data indicate a parsimonious model in which long-term central memory sources during the expansion phase from not one but rather multiple clonal subsets of differing extents (i.e. minimal versus extensive) of effector differentiation **(Figure 2.16)**.

2.7 Conclusions

Interrogating the CD62L^{hi} subset of CD8^{+} T cells during the expansion-phase of the adaptive immune response to viral infection uncovered novel biological features that enrich and extend our understanding of the molecular programming and developmental sequencing underpinning the generation of memory and effector T cells.

An abiding paradox in the field centers on the question of how activated T cells can divide extensively and extraordinarily rapidly **(Figure 1.1)** – putatively with toxic consequences for the integrity of their genomes – yet nevertheless retain long-term viability and the potential to extensively (and rapidly) proliferate upon subsequent encounters with pathogen-derived antigens, or upon continued antigen presence and sustained replicative stress (e.g. in the context of chronic microbial infections or cancers). In other tissues with intense turnover kinetics, for example the gastrointestinal epithelium, stem cells present a solution to a similar problem by asymmetrically dividing and thereby unequally apportioning the burden of proliferation to downstream, transit-amplifying cells **(Figure 1.2)**. However the existence of stem cells and their possible relationship with the memory T cell fate have been controversial. The data presented in this chapter

identify a rare subset of antigen-specific $CD8^+$ T cells during the expansion phase that resemble somatic stem cells in other tissues, including with respect to immunophenotype, transcriptional programming, and relative proliferative quiescence. These features include heightened expression of protective molecules that confer resistance to apoptosis (e.g. Bcl-2), resistance to errant phagocytosis (e.g. CD47), and xenobiotic efflux activity. Additionally, this $CD62L^{hi}$ subset highly expressed molecules that potentiate self-renewal, including Sca-1 and executors of the primordial Wnt signaling pathway. Collectively, and along with the genome integrity surveillance and signal calibration activities that are the subjects of chapters 3 and 4, respectively, these features render $CD62L^{hi}$ cells uniquely capable of withstanding the intense, multifaceted stresses inherent in the expansion phase of adaptive immune responses to pathogenic perturbations. In turn, these $CD62L^{hi}$ cells are equipped to subserve the evolutionarily ancient imperative of immunological memory: long-term preservation of clonal integrity to avoid selective deficiency in the repertoire of antigen-specific lymphocytes; and to maintain the capacity for further responsiveness to renewed or sustained encounter with antigen (Intlekofer et al., 2006).

An additional, long-standing question in immunobiology concerns the nature of the developmental pathways which naïve, antigen-inexperienced T cells must traverse in order to acquire the cardinal properties of memory T cells. To a large extent, enduring controversy about this matter has amounted to a chicken-and-egg problem (Ahmed et al., 2009; Restifo and Gattinoni, 2013): do

effector T cells precede memory T cells, as was first assumed by many, or do stem-like memory T cells spawn effectors, as has more recently been suggested? Multiple observations herein indicate support for the second view. In particular, $CD8^+$ T cell clonal expansion is structured according to a classical proliferative hierarchy rooted in a rare stem/progenitor population. This $CD62L^{hi}$ population bears multiple phenotypic and functional manifestations of classically-defined central memory, and efficiently gives rise to $CD62L^{lo}$ effector $CD8^+$ T cells. This linear, memory-to-effector differentiation pathway has been recently corroborated by varied, largely inferential reports in the literature that developmentally situate the memory fate prior to the effector fate on the basis of mathematical modeling (Buchholz et al., 2013), transcriptional analyses (Roychoudhuri et al., 2015), and epigenetic surveys (Pauken et al., 2016; Scott-Browne et al., 2016). However, the data herein also support a prominent contribution to (at least primary) long-term cell central memory from the partially-differentiated IL-7Rα^{hi} subset of effector $CD8^+$ T cells. This observation was surprising given traditional interpretations of Waddington's epigenetic landscape (Waddington, 1957) holding that effector differentiation from a stem cell progenitor is strictly unidirectional. However, reacquisition of progenitor-fate properties is consistent with the emerging appreciation of substantial mammalian developmental plasticity. In the present context, $CD62L^{lo}$ effector T cells whose fate is arrived at after that of $CD62L^{hi}$ stem-memory-like T cells in the presence of antigen appear to dedifferentiate into $CD62L^{hi}$ long-term memory cells following the cessation of antigen and/or inflammatory signaling. Thus, instead of

dichotomous views maintaining that the memory fate exclusively precedes the effector fate, or the converse, it appears that memory-like cells differentiate into effector cells, which in turn can give rise to memory cells. As such, a subset of effector cells (those highly expressing IL-7Rα) are simultaneously the egg and the chicken.

Important directions of future investigation will entail substantiating this reconciliatory ontogeny, given the caveat that developmental state in these studies was distinguished on the basis of a single cell surface marker, i.e. CD62L. Comprehensive transcriptional evaluation of the CD62L^{hi} and IL-7Rα^{hi} subsets – an important biological aim in its own right – may uncover additional differential protein expression that can be used to flow cytometrically distinguish these two subsets for more precise lineage tracing experiments. Global epigenetic profiling can also help to define cell states and the full extent of developmental distance between the CD62L^{hi} and IL-7Rα^{hi} subsets, both in the presence of antigen and after it has been cleared.

Beyond, a major question raised by these observations concerns any potential differences between long-term stem/central memory T cells that derive from cells that always manifested a stem/progenitor fate (e.g. the CD62L^{hi} subset) versus those that arise consequent to dedifferentiation from partially differentiated IL-7Rα^{hi} effector cells **(Figure 2.16)**. As one prominent *Drosophila* biologist has put it, is there any difference between counterfeit and original stem cells (Spradling and Fan, 2010)? Given the very different histories of proliferation

and deployment of various protective programs between these two expansion phase T cell subsets, one might indeed expect substantial differences (Mills and Sansom, 2015), which in turn could be of functional consequence for the overall capacity of long-term T cell memory. Consistent with this expectation is the observation that IL-7Rα^{hi} cells, in stark contrast to CD62L^{hi} cells, were almost bereft of ability to persist in conditions of enduring replicative stress (Figure 2.13). Nevertheless, future work will need to probe this matter empirically, as well as the related matter of these two populations' relative contributions to both primary and secondary long-term (i.e. "central") memory formation. At present, the studies presented herein clarify the developmental origins of CD8^{+} T cell memory and point to novel opportunities to further dissect the molecular basis of immunological memory and progenitor cell biology.

Figure 2.1

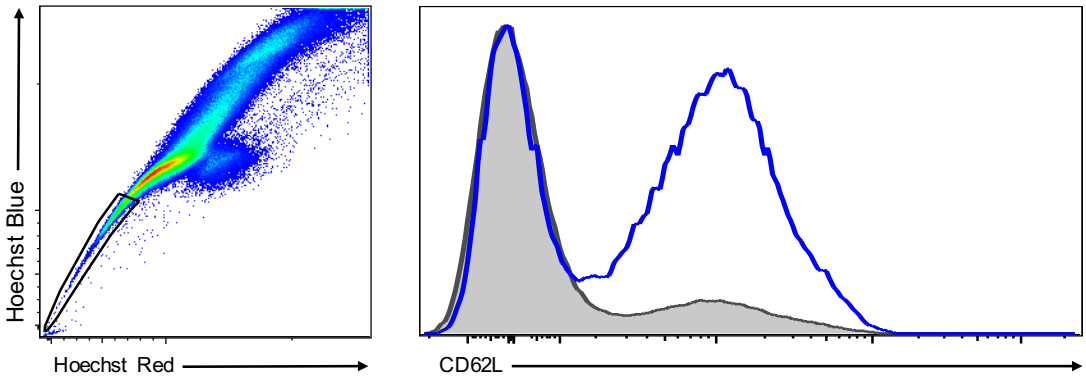


Figure 2.1: Efflux activity during the CD8$^+$ T cell response to viral infection enriches for expression of CD62L. Splenocytes from mice d6 pi LCMV-Armstrong were incubated in Hoechst 33342 for 90 minutes at 37° C and then counter-stained with fluorescently-labeled antibodies against surface markers. Simultaneous, dual-wavelength visualization of Hoechst 33342 fluorescence in CD8$^+$ T cells revealed an actively effluxing, so-called “side population” (left). Analysis of CD62L expression (right) in the side population (blue histogram) versus all remaining, non-effluxing cells (gray histogram) revealed a marked enrichment in the former.

Figure 2.2

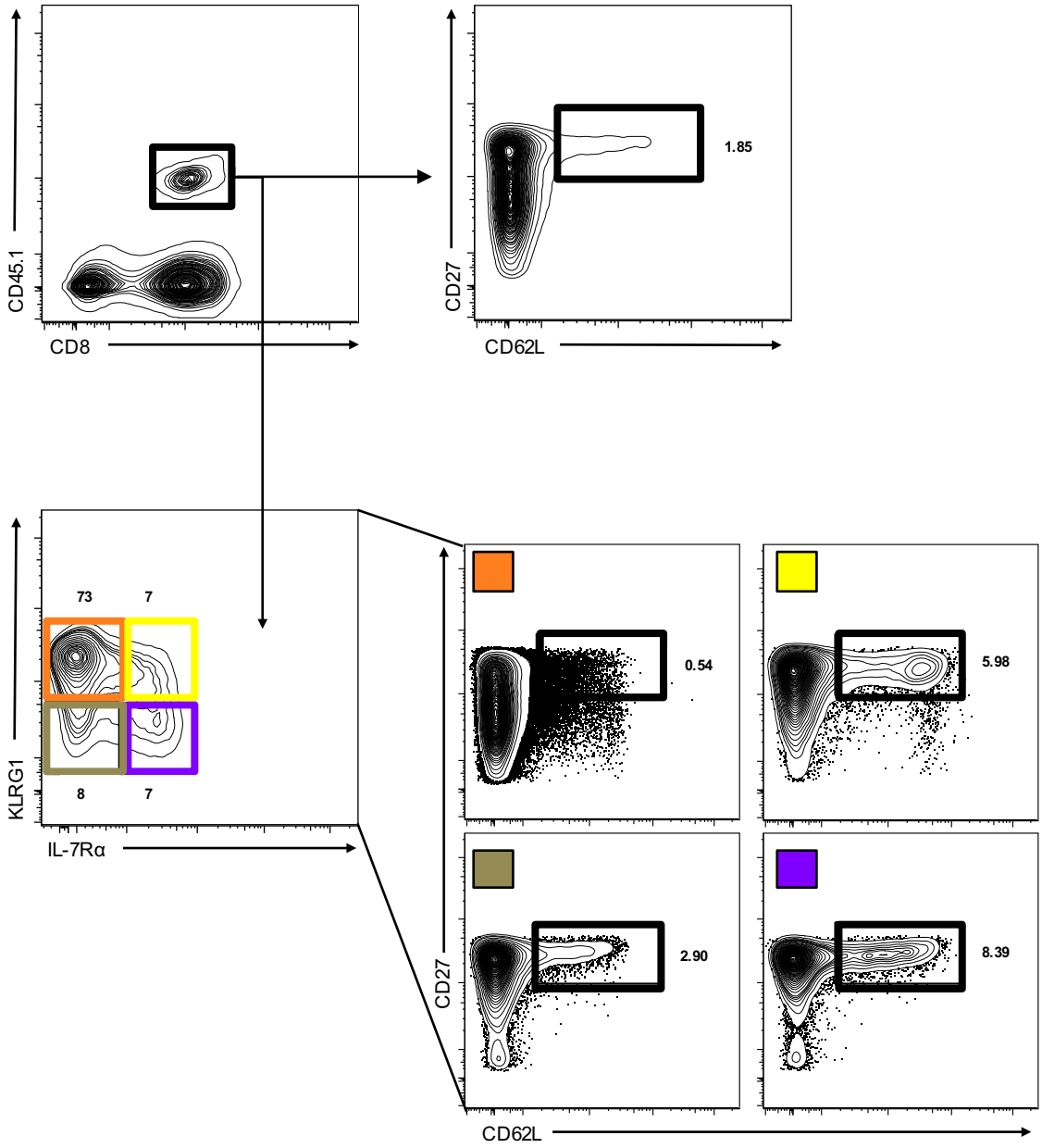


Figure 2.2: An antigen-specific, central memory-like CD62L^{hi} CD27^{hi} subpopulation exists during the antiviral expansion phase and is enriched within the KLRG1^{lo} IL-7Rα^{hi} population. Bivariate plots of flow cytometric data depict the immunophenotypes of splenocytes harvested at d8 pi LCMV-Armstrong from CD45.2^{+} C57Bl/6 mice that had been transplanted prior to infection with CD45.1^{+} LCMV-specific P14 TCR-transgenic CD8^{+} T cells. The CD45.1^{+} P14 population of antigen specific cells is identified at the top-left, and then sub-fractionated by various markers. Of particular note is the KLRG1^{lo} IL-7Rα^{hi} CD62L^{hi} CD27^{hi} sub-population (bottom right). Numbers denote frequency within the gated population.

Figure 2.3

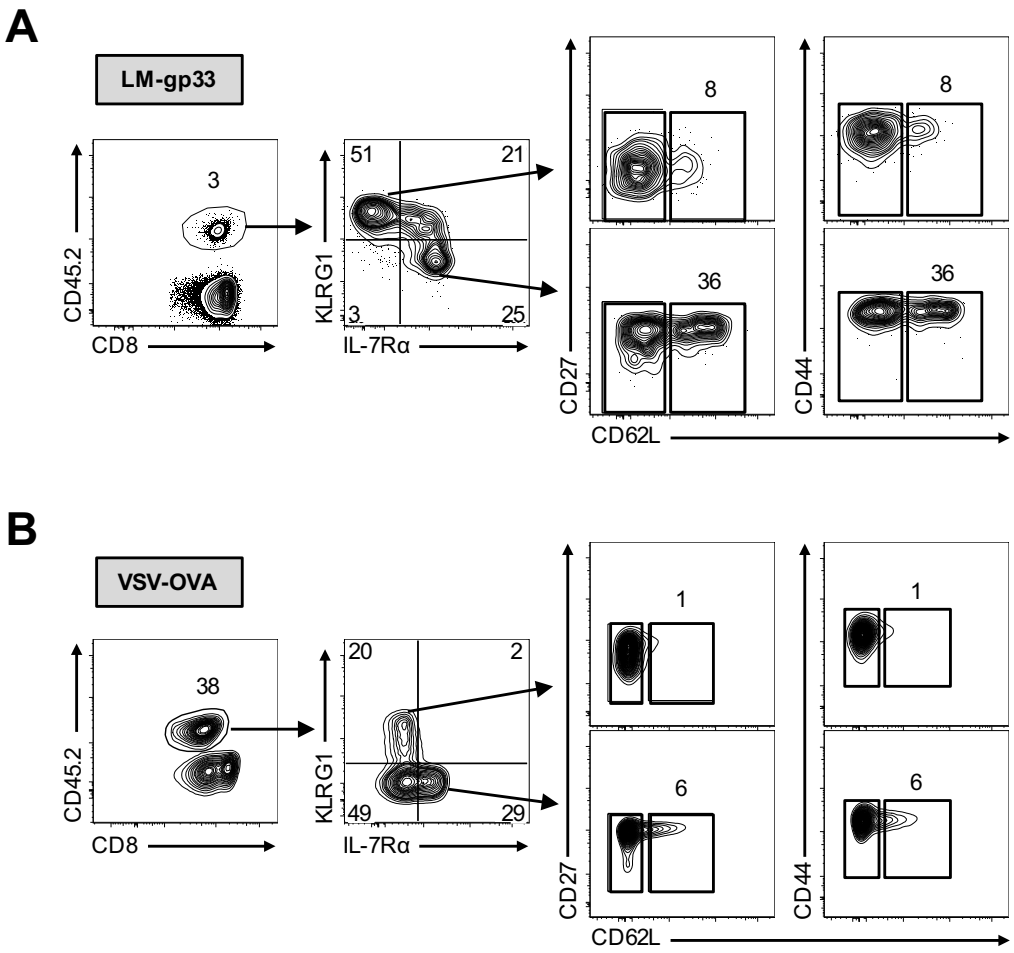


Figure 2.3: Presence of a CD62L^{hi} KLRG1^{lo} IL-7Rα^{hi} subset in antigen-specific CD8^{+} T cells during the expansion phase of bacterial and viral infections. A) C57Bl/6 mice received 5,000 congenically disparate P14 TCR-transgenic CD8^{+} T cells and then were infected intravenously with *Listeria monocytogenes* expressing the LCMV gp33 epitope (LM-gp33). The presence of the three expansion phase subsets of CD8^{+} T cells was determined for responding P14 cells by flow cytometry at d8 pi. **B)** C57Bl/6 mice received 50,000 congenically disparate OT-I TCR-transgenic CD8^{+} T cells and then were infected intravenously with vesicular stomatitis virus (VSV) expressing OVA (VSV-OVA). The presence of expansion phase subsets of CD8^{+} T cells was determined for responding OT-1 cells by flow cytometry at d8 pi.

Figure 2.4

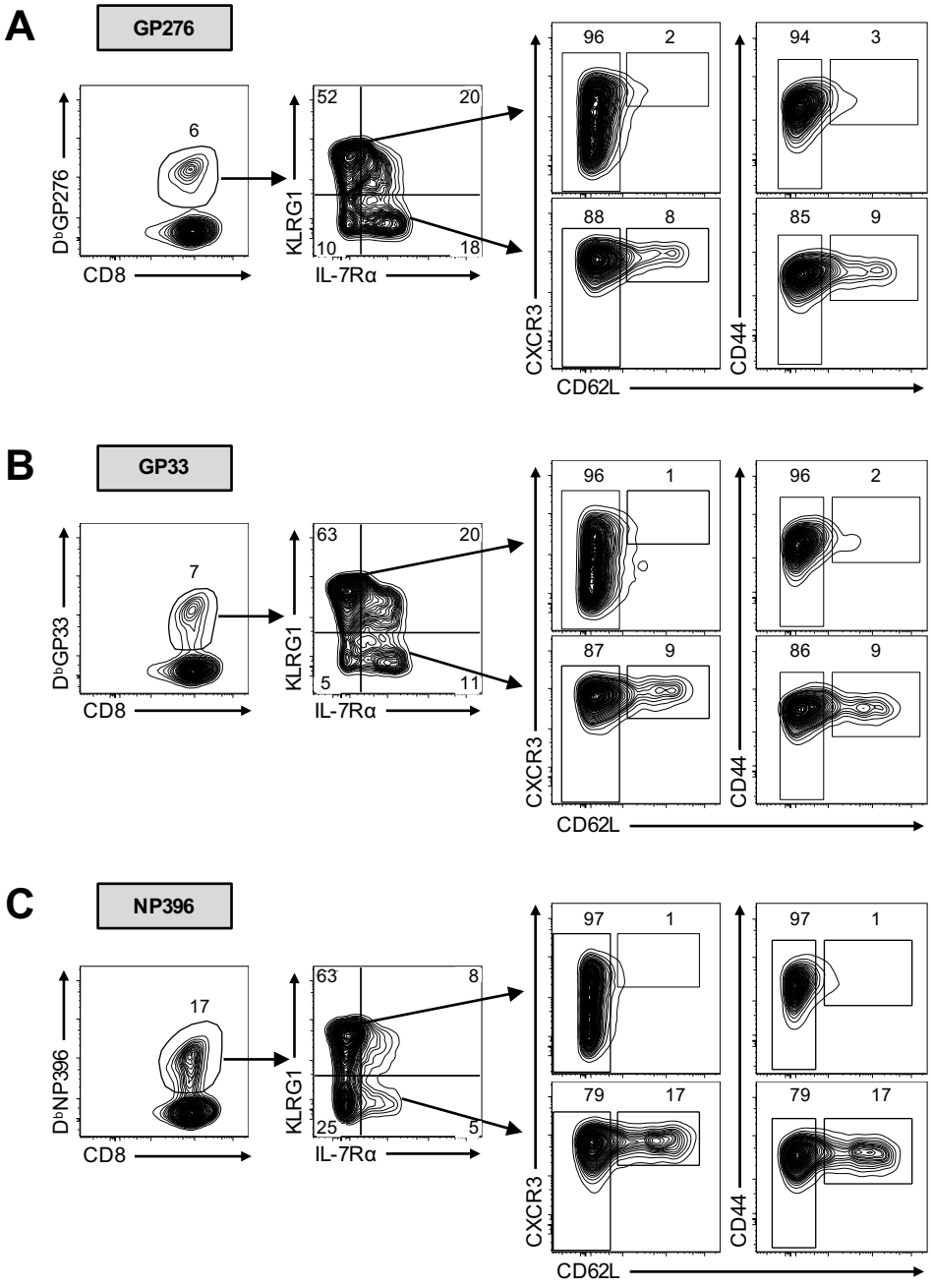


Figure 2.4: Presence of a CD62L^{hi} KLRG1^{lo} IL-7Rα^{hi} subset in endogenous, antigen-specific CD8^{+} T cells during the expansion phase of the adaptive immune response to LCMV infection. C57Bl/6 mice were infected with LCMV-Armstrong. At d8 pi, splenocytes were stained with peptide-MHC tetramers to detect CD8^{+} T cell populations specific for the subdominant D^{b}GP276 **(A)** and D^{b}GP33 **(B)** epitopes, and the dominant D^{b}NP396 epitope **(C)**.

Figure 2.5

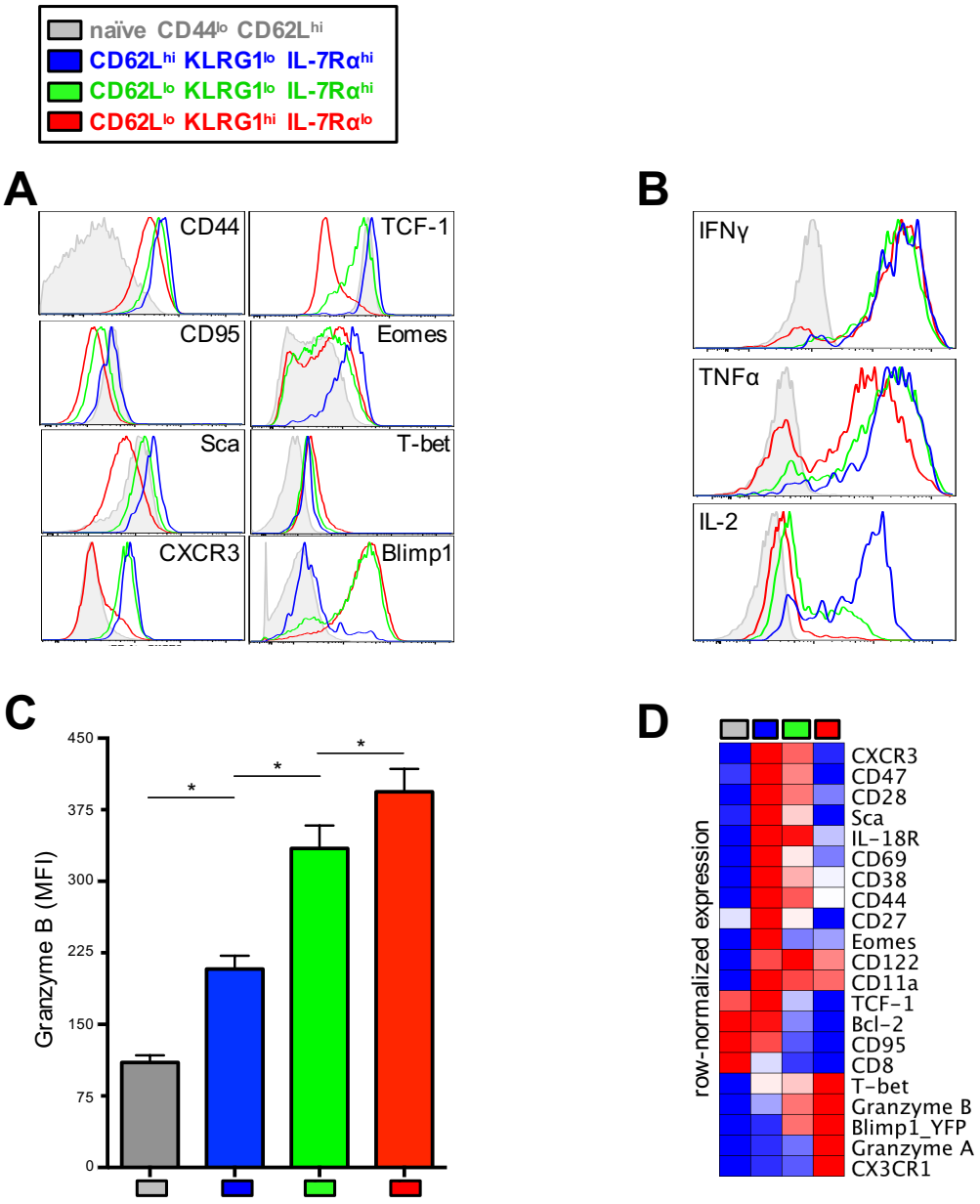


Figure 2.5: Immunophenotypic characterization of the antigen-specific CD62L^{hi} population during the expansion phase of acute viral infection. Subsets of GP33-specific splenocytes were flow-cytometrically evaluated at d8 pi LCMV-Armstrong for expression of the factors implicated in the activation and differentiation of CD8^{+} T cells. **A)** Histograms depicting expression of markers implicated in lymphocyte stemness (left), and of transcription factors associated with memory vs. effector fate decisions. **B)** Expression of cytokines after *ex vivo* restimulation with GP33 peptide in the presence of 100 μM TAPI-2 in LCMV-specific CD8^{+} T cell subsets (indicated in blue, green, and red) and polyclonal naïve CD8^{+} T cells (indicated in grey). **C)** Expression of Granzyme B in LCMV-specific CD8^{+} T cell subsets and polyclonal naïve cells. **D)** Heatmap depicting relative protein expression determined by flow cytometry for indicated markers; red indicates higher expression, while blue indicates lower expression.

Figure 2.6

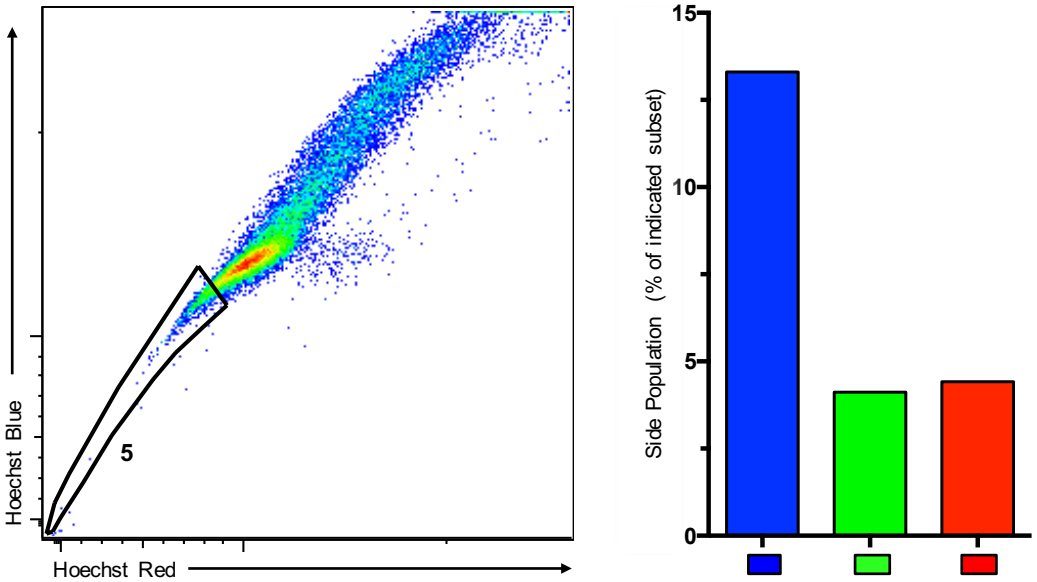


Figure 2.6: Preferential efflux activity in antigen-specific CD62L^{hi} CD8^{+} T cells. Splenocytes from mice d8 pi LCMV-Armstrong were incubated in Hoechst 33342 for 90 minutes at 37° C and then counter-stained with D^{b}GP33 tetramer and antibodies against surface markers. A side population of actively effluxing cells was evident in antigen-specific CD8^{+} T cells (left), and in subsets thereof (right). The extent of effluxing was observed to be greatest in the CD62L^{hi} compartment.

Figure 2.7

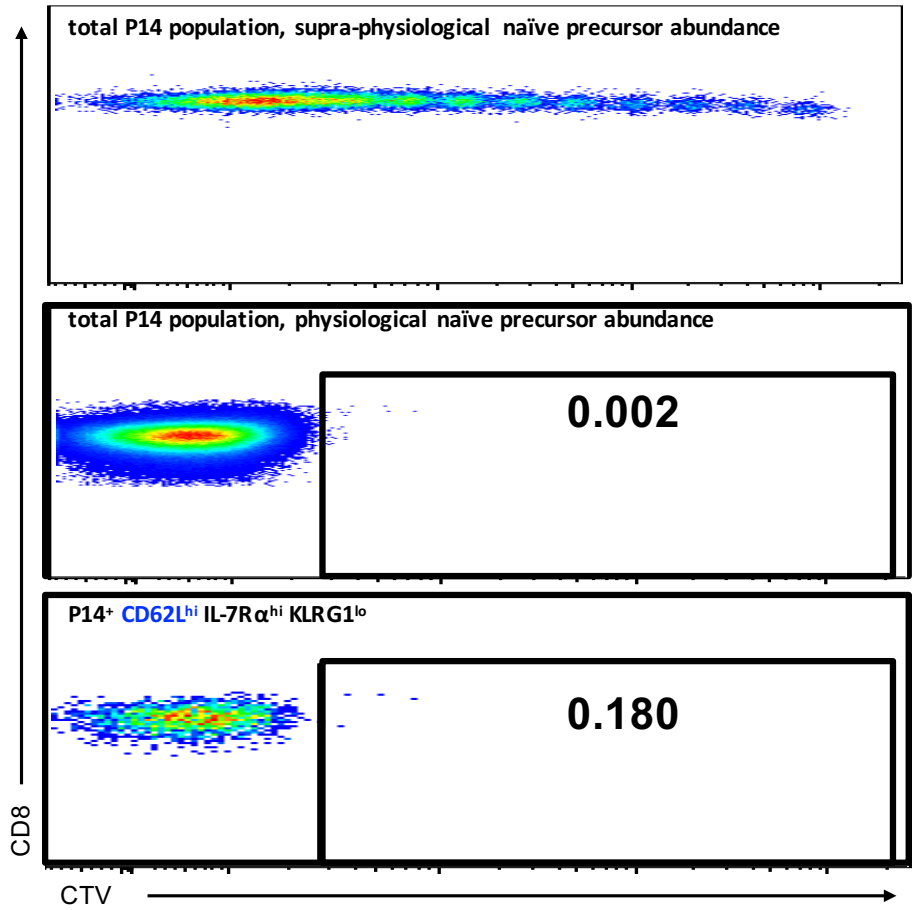


Figure 2.7: Extensive proliferation in the antigen-specific CD62L^{hi} population precludes the possible interpretation of incomplete recruitment. Naïve TCR-transgenic P14 CD8^{+} T cells were labeled with the proliferation-tracking dye CTV and transplanted into naïve recipient mice. These were subsequently infected with LCMV-Armstrong. At d8 pi splenocytes were harvested and analyzed flow cytometrically to evaluate CTV dilution in the unfractionated P14 population (middle panel) and the CD62L^{hi} subset thereof (bottom panel). The top panel depicts CTV dilution in P14 cells transplanted in supra-physiological quantities, which, owing to the skewed ratio of antigen-specific T cells to antigen presenting cells, might be expected to manifest a pattern of limited proliferation consistent incomplete recruitment or aberrant activation.

Figure 2.8

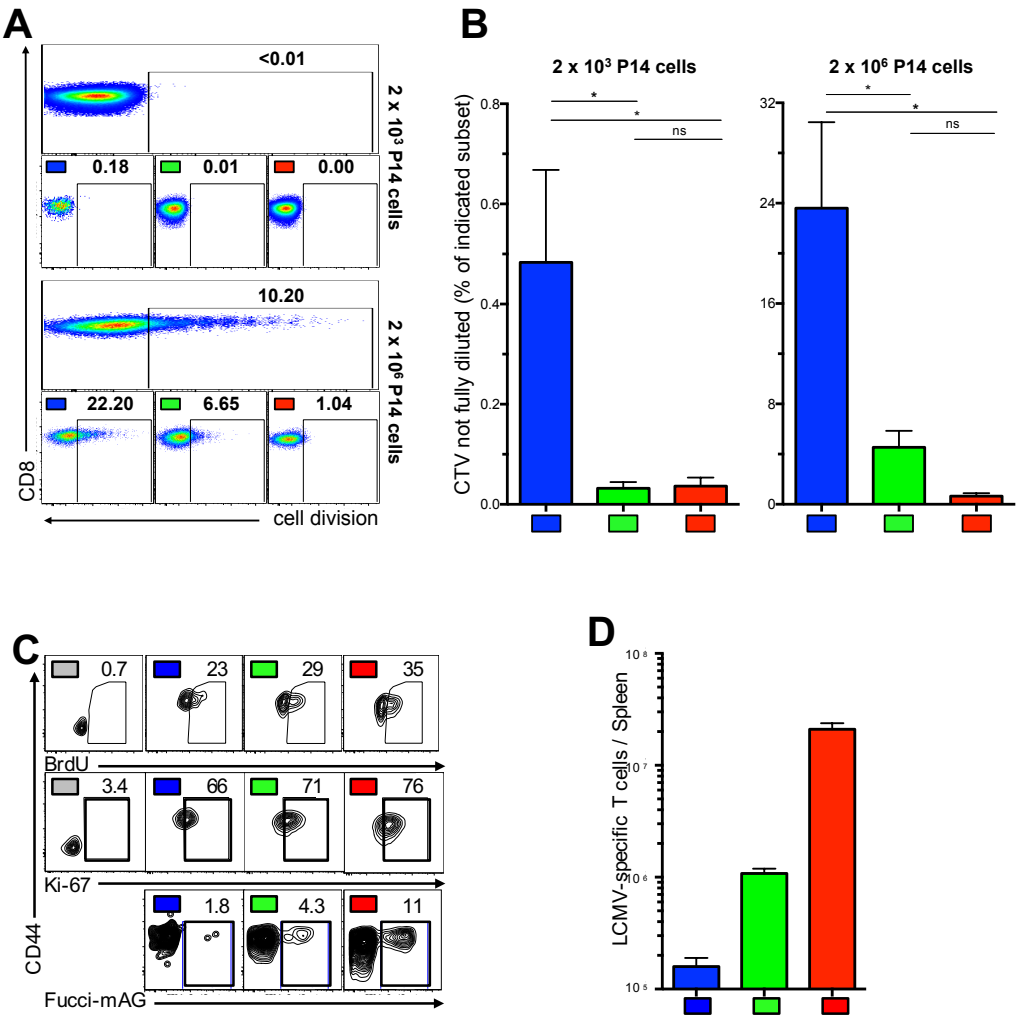


Figure 2.8: Proliferative index, proliferative history, and absolute abundance suggest expansion phase, antigen-specific CD8$^+$ T lymphocyte subsets may be structured in a proliferative hierarchy. A-B: Proliferative history of P14 CD8$^+$ T cells and subsets thereof under conditions of physiological and supraphysiological naïve precursor numbers. **A)** C57Bl/6 mice received physiological (2 x 10^3, top panels) or supraphysiological (2 x 10^6, bottom panels) numbers of proliferation-dye-labeled P14 cells, and were then infected with LCMV-Armstrong. At d8 pi, proliferation dye dilution was measured by flow cytometry in donor P14 cells in the spleen. Dye dilution is depicted in total P14 populations (large, upper panels) and in the CD62L^{hi}, IL-7Rα^{hi} and KILRG1^{hi} subsets (smaller, lower panels). **B)** Statistical summary of the data in part A. Asterisks indicate statistical significance ($p < 0.05$) determined by one-way analysis of variance (ANOVA). Error bars depict mean ± SEM. **C)** Proliferative index analyzed at the end of the expansion phase. Top: Mice containing P14 cells were pulsed with BrdU at d7 pi and BrdU incorporation in the three LCMV-specific CD8$^+$ T cell subsets (and naïve CD8 T cells) was assessed at d8 pi. Middle: Ki-67 expression was examined at d8 pi in the three subsets and in naïve CD8$^+$ T cells. Bottom: Expression of the cell cycle reporter Fucci-mAG was assessed in the three subsets indicated. **D)** Abundance of cells in each of the three LCMV-specific subsets at d8 pi in the spleen.

Figure 2.9

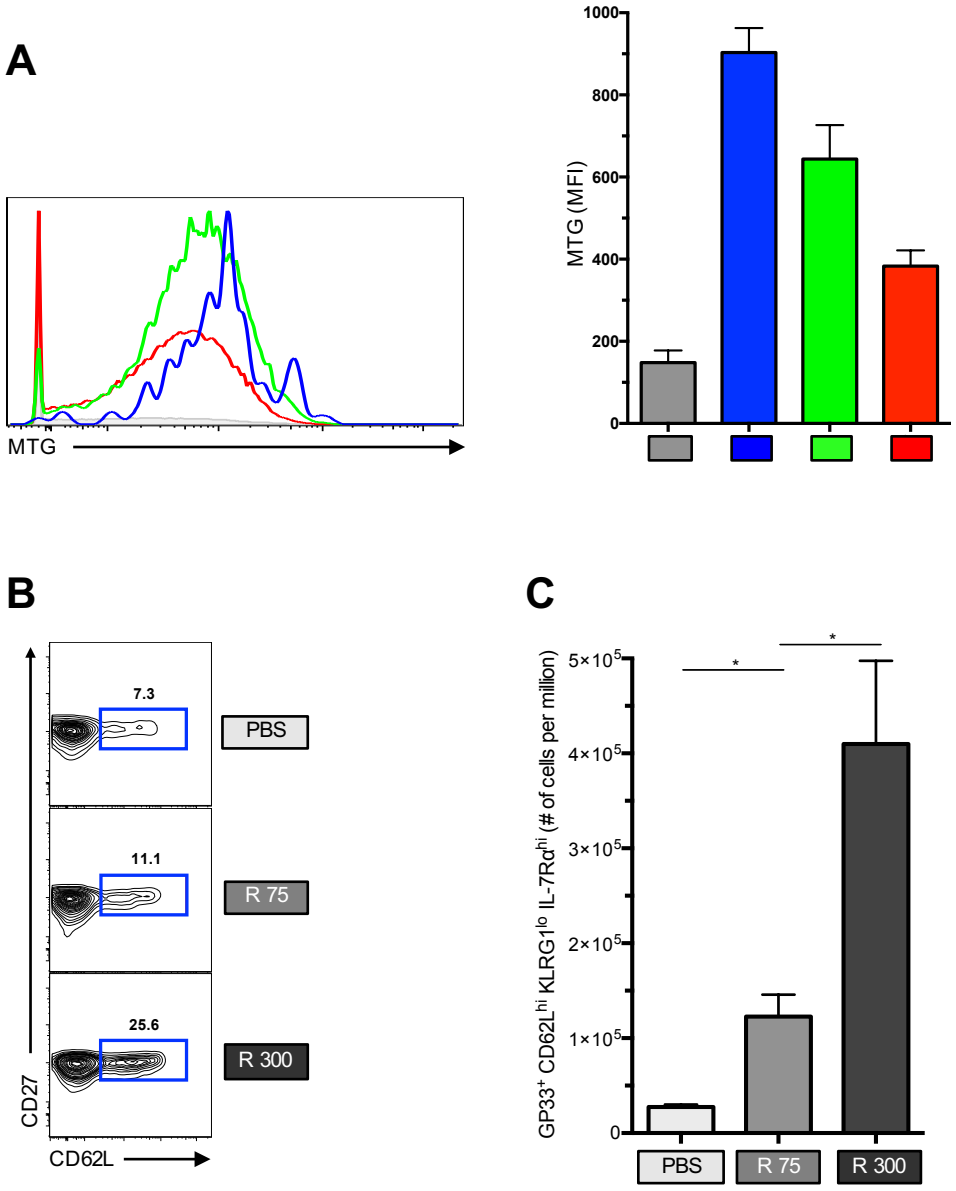


Figure 2.9: Mitochondrial-biased metabolism characterizes and drives abundance of the CD62L^{hi} subset. A) Measurement of mitochondrial mass by way of MTG (mitotracker-green) fluorescence in naïve CD8^{+} T cells and subsets of P14 splenocytes isolated at d8 pi LCMV-Armstrong. B-C: Treatment with increasing doses of Rapamycin progressively increases the relative and absolute abundance of the CD62L^{hi} subset. In this experiment, mice were treated with daily injections of PBS or Rapamycin at 75 µg/kg or 300 µg/kg following infection with LCMV-Armstrong. At d8 pi, PBMC were evaluated for the abundance of antigen-specific subsets. Flow cytometry plots in **B)** depict CD62L and CD27 expression within the GP33^{+} KLRG1^{lo} IL-7Rα^{hi} compartment; **C)** statistically depicts the abundance of GP33^{+} CD62L^{hi} KLRG1^{lo} IL-7Rα^{hi} cells (i.e. the CD62L^{hi} subset).

Figure 2.10

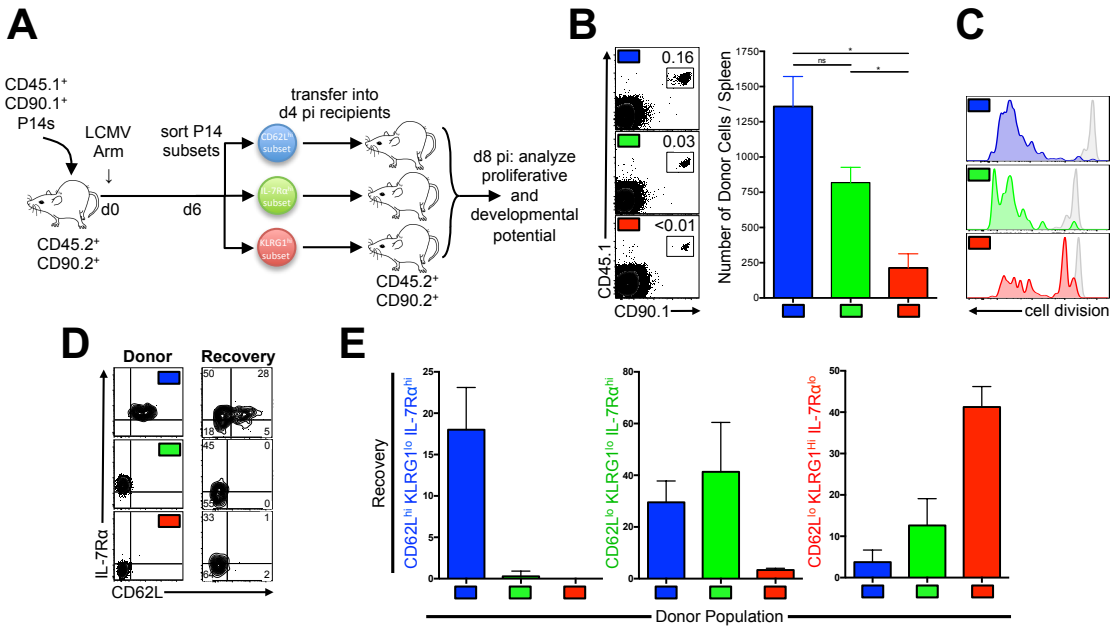


Figure 2.10: Self-renewal and lineage relationships of expansion phase LCMV-specific CD8$^+$ T cell subsets. A) Schematic of experimental design to determine lineage relationships and proliferative capacity. **B)** Expansion potential of the CD62L^{hi}, IL-7Rα^{hi} and KLRG1^{hi} LCMV-specific CD8$^+$ T cell subsets isolated and transplanted to infected mice as indicated in part A. Representative flow cytometric plots from recipient mice (n=5 per group; left) and quantification of absolute number of recovered donor P14 cells for the indicated subsets (middle). **C)** Proliferation dye dilution in donor-derived, LCMV specific CD8$^+$ T cell subsets from part B, relative to control, co-transplanted population of unrelated OT-I cells (grey). **D)** Phenotype of cells recovered from each donor subsets from part B. Left: phenotype of donor cell with respect to IL-7Rα and CD62L expression (note, lower "red" population was 99.97% KLRG1^{hi}). Right: phenotype of P14 cells recovered after transplant of the indicated populations on the left. **E)** Summary of phenotypes of P14 cells recovered after transplant as outlined in part A.

Figure 2.11

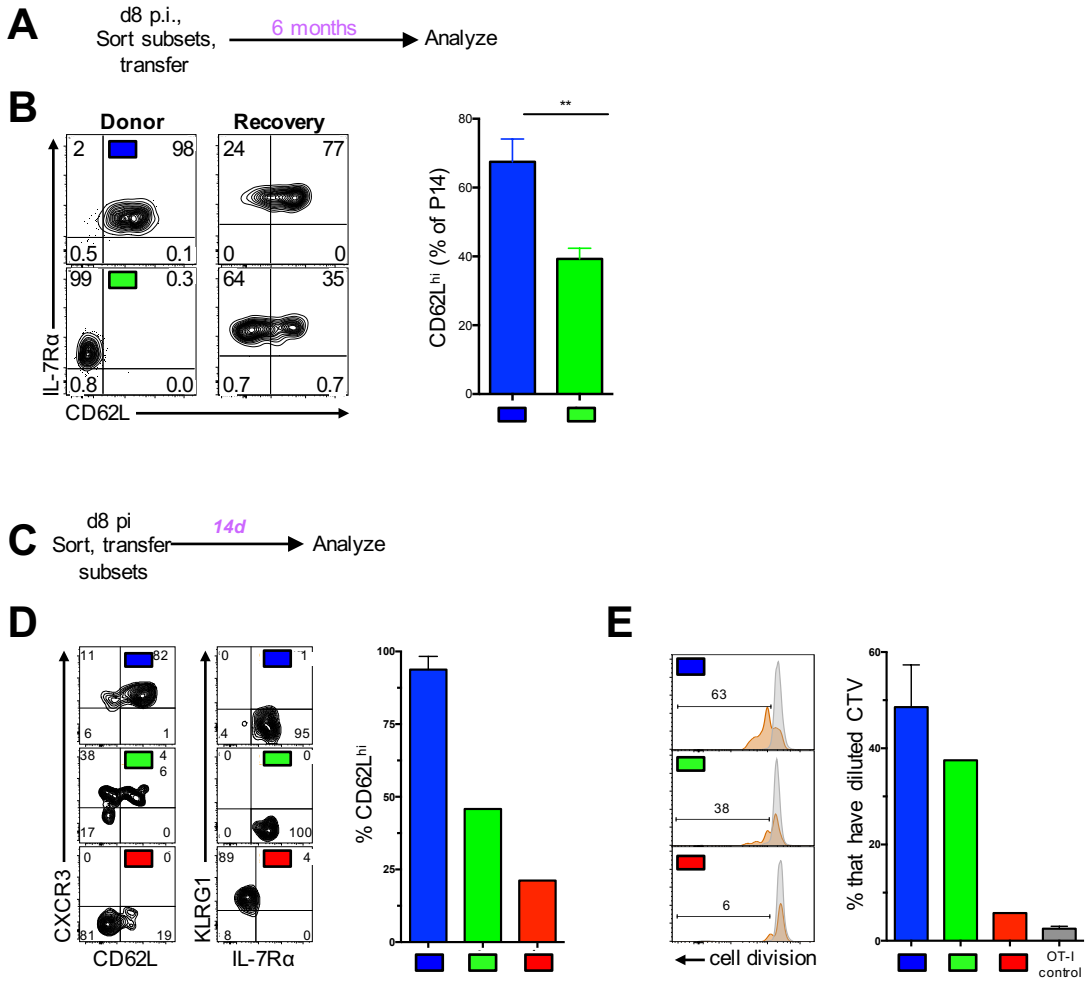


Figure 2.11: Lineage relationships between expansion-phase and post-antigen-phase subsets. A) Experimental design: splenic P14 subsets were sorted at d8 pi **(B, left)** and transplanted into congenically disparate naïve recipient mice. After 6 months, the frequency of donor cells with a CD62L^{hi} "central memory" phenotype was determined in recipient spleens **(B, right flow cytometry plot and bar graph)**. **C)** Experimental design: CD62L^{hi}, IL-7Rα^{hi} and KLRG1^{hi} P14 subsets were sorted at d8 pi LCMV and proliferation dye-labeled. After mixing with congenically disparate, proliferation dye-labeled OT-I naïve CD8^{+} T cells as a control, sorted P14 cells of each subset were then transplanted into congenically disparate, naïve recipient mice. Two weeks later the immunophenotype **(D)** and extent of proliferation dye dilution **(E)** of donor cells were evaluated in recipient mouse spleens (orange histograms denote P14 cells, grey histograms denote control, co-transplanted naïve OT-I cells).

Figure 2.12

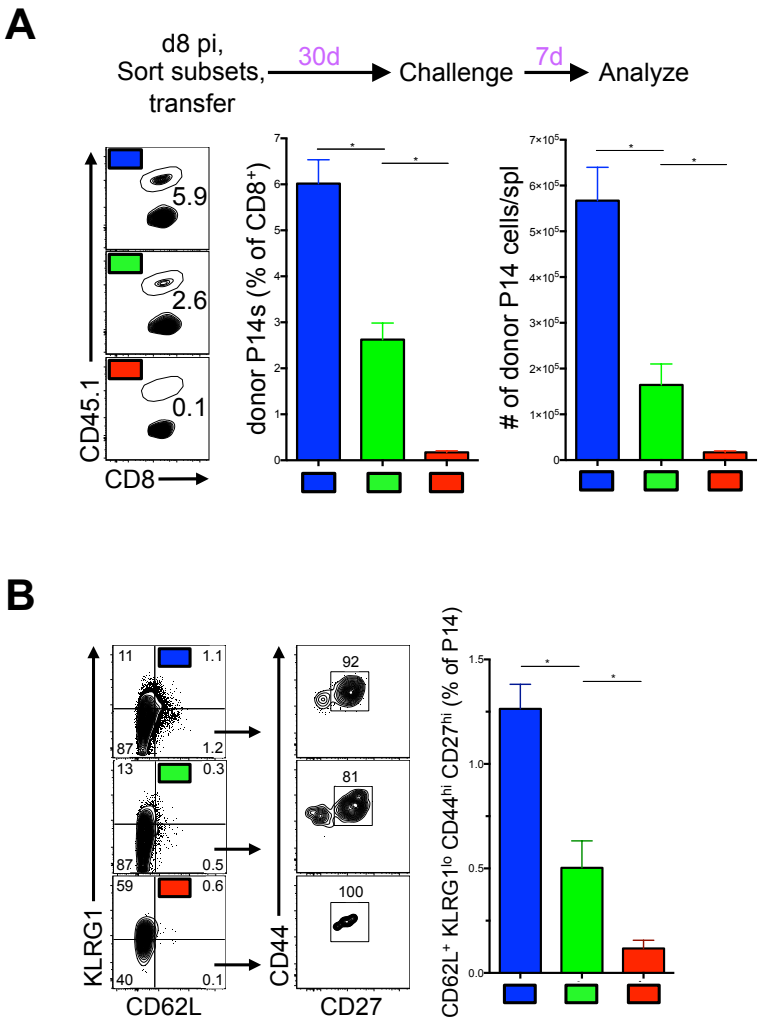


Figure 2.12: Superior memory-recall capacity of the antigen-specific CD62L^{hi} population. Experimental design: splenic P14 subsets were sorted at d8 pi and transplanted to congenically disparate naïve recipient mice. **A)** One month later, recipient mice were challenged with LCMV clone 13 and the abundance of donor P14 cells was assessed in spleens on d7 pi. **B)** The immunophenotype of donor-derived cells was evaluated (left), and the frequency of regenerated CD62L^{hi} subset cells was determined (right).

Figure 2.13

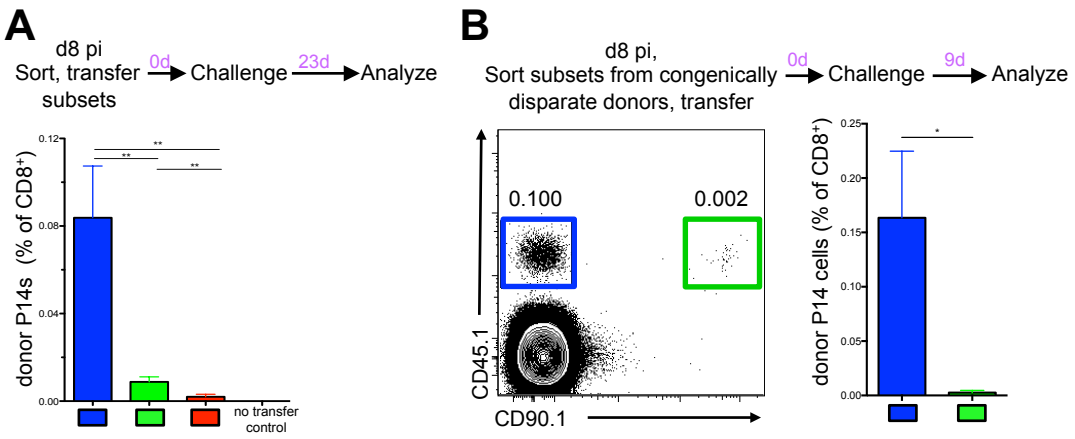


Figure 2.13: Superior potential of the CD62L^{hi} population to persist under conditions of immediate and enduring replicative stress. A) Top: experimental design: CD62L^{hi}, IL-7Rα^{hi} and KLRG1^{hi} P14 subsets were sorted at d8 pi LCMV and equal numbers were transplanted into congenically disparate naïve recipient mice. These were immediately challenged with LCMV clone 13, and the abundance of donor cells was evaluated in PBMC at 23 dpi (bar graph, bottom). **B)** Top: experimental design: CD62L^{hi} and IL-7Rα^{hi} P14 subsets were sorted at d8 pi LCMV from congenically disparate donor mice and equal numbers were co-transplanted into naïve recipient mice. These were immediately challenged with LCMV clone 13, and the abundance of donor cells was evaluated in spleens at d9 pi (bottom).

Figure 2.14

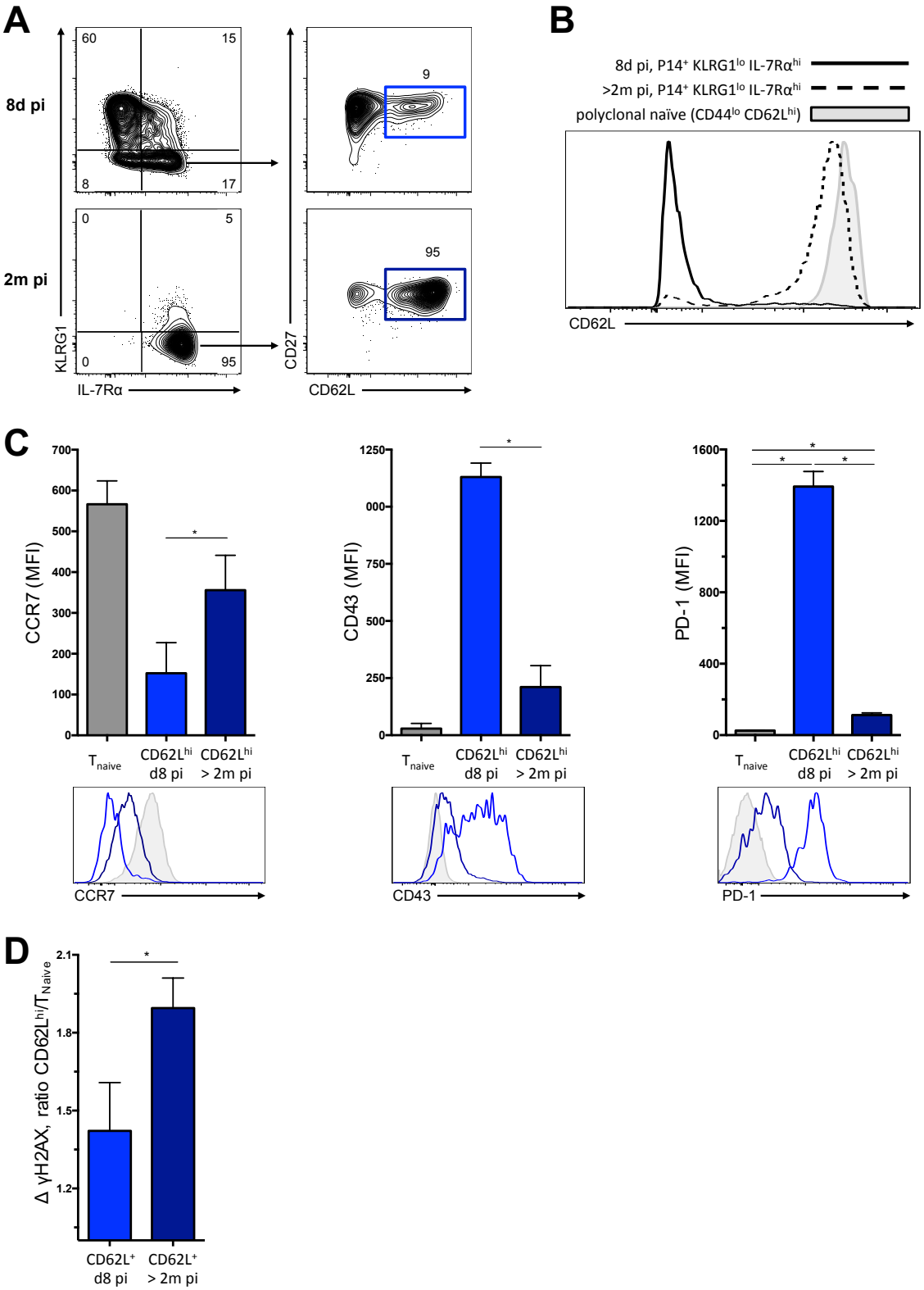

A
8d pi
2m pi
60
15
8
17
0
5
0
95
9
95
KLRG1
IL-7Rα
CD27
CD62L
B
8d pi, P14+ KLRG1lo IL-7Rαhi
>2m pi, P14+ KLRG1lo IL-7Rαhi
polyclonal naïve (CD44lo CD62Lhi)
CD62L
C
CCR7 (MFI)
CD43 (MFI)
PD-1 (MFI)
Tnaive
CD62Lhi d8 pi
CD62Lhi > 2m pi
CCR7
CD43
PD-1
D
Δ γH2AX, ratio CD62Lhi/TNaive
CD62L+ d8 pi
CD62L+ > 2m pi

Figure 2.14: Phenotypic and functional differences between CD62L^{hi} subsets during the antigen-driven expansion phase and the post-antigen phase. A-C) PBMC were isolated from mice at d8 pi and > 2 months pi LCMV-Arm and stained separately with anti-CD8α conjugated to distinct fluorophors. Following CD8α staining, samples were combined and stained together for CCR7, CD43, congenic marker (to identify P14 cells), and lineage markers. CD62L expression was examined within the KLRG1^{lo} IL-7Rα^{hi} progenitor compartment (gated on LCMV-specific cells) at 8 days and > 2 months pi, and depicted as bivariate plots in **(A)** and as overlaid histograms in **(B)**. **C)** Expression of CCR7 (left), CD43 (middle), and PD-1 (right) on polyclonal naïve cells (grey), and LCMV-specific CD62L^{hi} KLRG1^{lo} IL-7Rα^{hi} cells at 8 days pi (light blue) and > 2 months pi (dark blue). **D)** Phosphorylation of H2AX following ex vivo irradiation was assessed as in Fig 3C, and is here depicted as the capacity of LCMV-specific CD62L^{hi} KLRG1^{lo} IL-7Rα^{hi} cells to phosphorylate H2AX relative to that of polyclonal naïve cells.

Figure 2.15

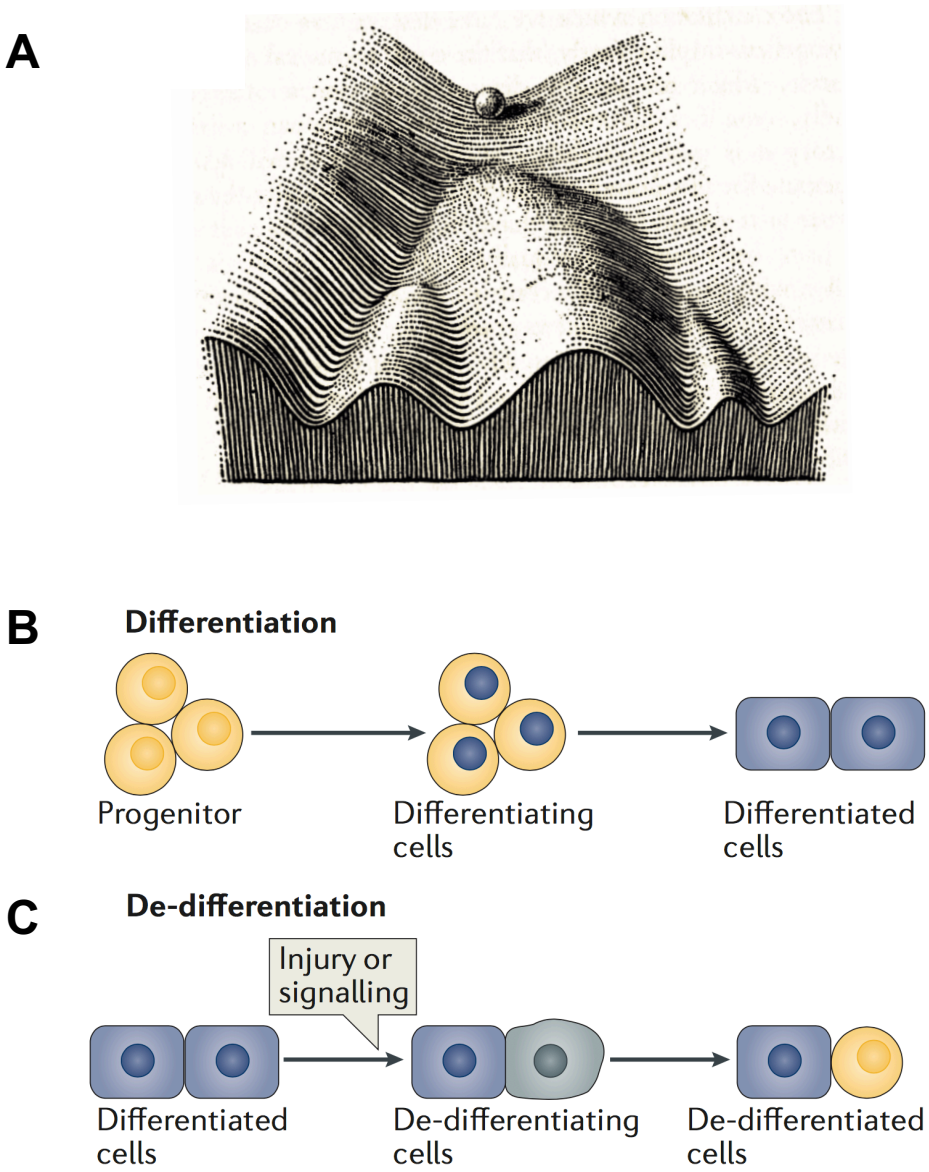


Figure 2.15: Differentiation and Dedifferentiation. A-B) Differentiation entails traversing an epigenetic landscape, as originally conceptualized by Conrad Waddington, with concomitant acquisition of specialized form and function. In this schematic "progenitor" cells correspond to the CD62L^{hi} subset of antigen-specific CD8^{+} T cells; "differentiating cells" correspond to the IL-7Rα^{hi} subset; and "differentiated cells" correspond to the KLRG1^{hi} subset discussed in the text. **C)** Though cellular specialization was believed to be unidirectional, convincing data in amphibians and more recently in mammals has demonstrated that differentiated cells can partially or entirely revert to or assume the undifferentiated state of their progenitors. Adapted from Waddington, C.H. The Strategy of the Genes: a Discussion of Some Aspects of Theoretical Biology. London: Allen & Unwin, 1957. Reproduced with permission from Merrell AJ and Stanger BZ. Adult cell plasticity in vivo: de-differentiation and transdifferentiation are back in style. Nat Rev Mol Cell Biol. 2016 Jul; 17(7):413-25.

Figure 2.16

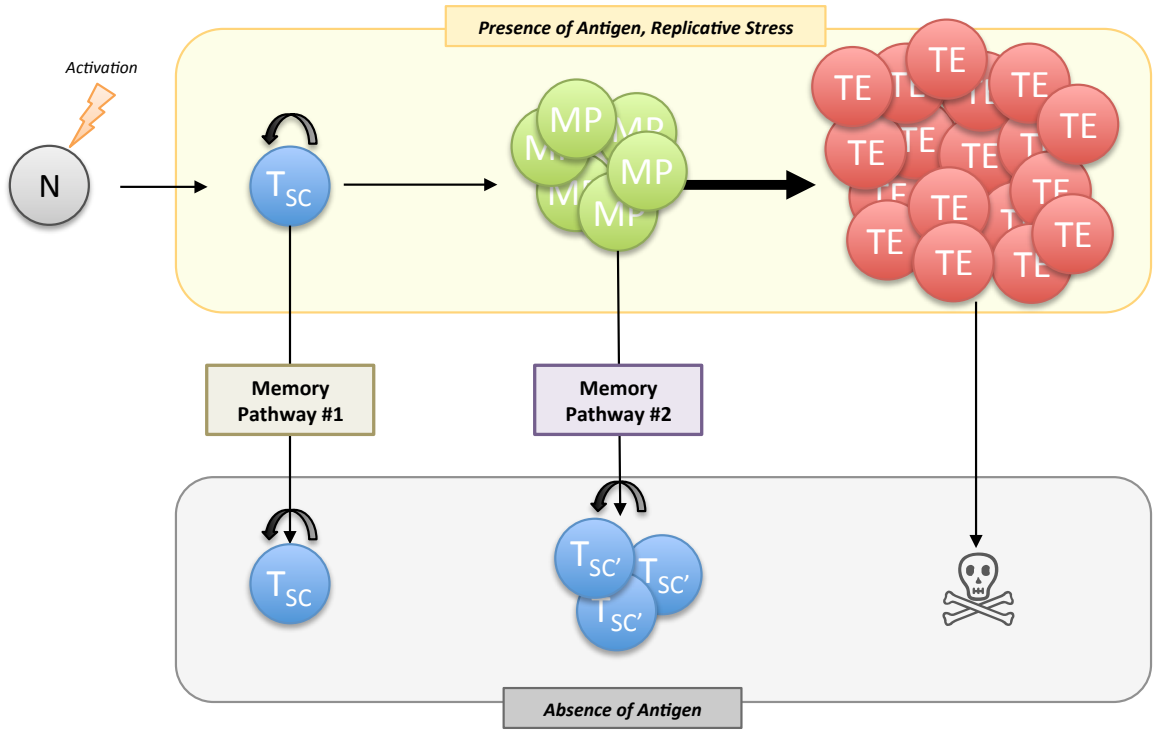


Figure 2.16: Model for stem-cell-driven effector T cell differentiation in the presence of antigen and subsequent dedifferentiation into memory T cells in the absence of antigen. A naïve T cell is activated by encounter with cognate antigen in the context of immunological danger and gives rise to a clonal stem cell hierarchy marked by increasing proliferation, and progressive cytotoxic effector differentiation. Following antigen elimination and the resolution of acute infection, terminally-differentiated effector cells (red) largely commit to apoptosis, whereas intermediately effector-differentiated memory precursor cells (green) gradually reacquire the stemness of their progenitors (blue). In the post-antigen phase, the pool of memory T cells, composed of T_{SC} and T_{SC}' populations, is heterogeneous with respect to proliferative past and history of effector differentiation.

.:: CHAPTER 3 ::.
SPECIALIZED FUNCTION FOR T STEM CELLS IN CONSERVING GENOME INTEGRITY; IMPLICATIONS FOR AGING CLOCKS

3.1 Genome integrity maintenance and the stem cell theory of aging

DNA damage is an ongoing, daily occurrence with which all cells must contend. For example, it's been estimated that the average cell experiences roughly 100,000 spontaneous DNA lesions per day (Lindahl, 1993). While damage to any of the structures that constitute the cell — e.g. lipid membranes, RNA species, proteins, cytoskeletal components, various macromolecular machines and organelles, — can threaten viability and as such must be addressed, if repair is not practical, outright replacement is always a possibility, assuming energy and raw building blocks are available to support *de novo* synthesis. In contrast, because chromatin is the ultimate repository for the information that templates all other cellular structures, any injuries thereto can be uniquely pernicious. In parallel to cosmological considerations about the nature of black holes, mere matter can always be retrieved, recycled, or rebuilt, as necessary; however, once lost, information is gone forever. As such all nucleated cells in all known species deploy multitudinous, complementary surveillance and repair mechanisms to minimize genomic damage (Blanpain et al., 2011). The fidelity of maintenance and repair activities is incomplete, however, and numerous datasets across multiple species indicate that errors occur, accumulate, and gradually diminish genome integrity over time (Herbig et al., 2006; Lopez-Otin et al., 2013).

The idea that declining genome integrity might be involved in the aging process has been pondered for decades. But is this merely one of the many curious features of aging, or is it actually important? Though cause and effect relationships concerning information maintenance can be exceedingly difficult to discern, there is growing support for the notion that declining genome integrity not only accompanies the aging process at the cellular and organismal levels, it also drives it. This is perhaps most dramatically evident in the progeroid syndromes that manifest in both model organism systems and humans consequent to mutations in factors involved in genome maintenance (de Boer et al., 2002; Kujoth et al., 2005; Burtner and Kennedy, 2010). Moreover, while inactivation of genome maintenance factors abbreviates lifespan in multiple species with broadly varying lifespans, genetic analyses have indicated that a major determinant of the lifespan differences *between* species is the extent or nature of the activity of DNA damage surveillance and repair factors (Lorenzini et al., 2009; Ma et al., 2016). Thus, with respect to the aging process, genomic information is vitally important: corruption thereof causally leads either to loss-of-function or hyperplastic gain-of-function, and almost invariably to decreased fitness and an earlier ultimate demise.

However, developmental biologists have long appreciated that most of the cells that constitute a metazoan soma over a lifetime have a finite, often very limited lifespan, and are replenished from self-renewing stem cell compartments, either continuously or in injury-regeneration scenarios. As such, while all nucleated cells must monitor and sustain chromatin fidelity to some extent, an

extensive body of theoretical writing and more limited empirical evidence suggest that stem cells may be particularly important for and invested in the genome maintenance and repair activities that ultimately are most proximally related to organismal aging (Cairns, 1975; Blanpain et al., 2011). Together, these notions constitute the so-called stem cell theory of aging, which holds that stem cells have a prominent, perhaps predominant role in conserving overall, organismal genome integrity; and that the accumulation of genomic and epigenomic mutations that nevertheless inevitably accumulate in stem cells ultimately diminishes their function and thereby hastens organismal death (Krishnamurthy and Sharpless, 2007; Brunauer et al., 2015). In this chapter, this hypothesis is probed with respect to T stem cells. Is genomic integrity relevant to the functions of anti-pathogen T cells? Do T stem cells resemble other stem cell populations in terms of specialized functions with respect to genomic integrity conservation? Can analysis of genome integrity in T cells, which replicate uniquely rapidly, in turn inform understanding of stem cell biology more generally?

3.2 Superior surveillance of genome integrity in T stem cells

The observations presented in the previous chapter revealed the existence of a quantitatively minor subset of cells in the anti-viral T cell expansion phase with unique developmental features, including robust yet restrained proliferation, substantial efflux activity, and a protein expression profile that intersects with that of stem and progenitor cells in other developmental contexts. In many biological systems there is a known (if little understood) association between progenitor

cells in proliferative hierarchies and the maintenance of genomic integrity (Flores et al., 2008). Analysis of global gene expression in unfractionated, antigen-specific $CD8^+$ T cells isolated at various time points before, during, and after an acutely-resolving viral infection indicated that the cellular DNA damage response machinery is highly dynamically regulated **(Figure 3.1)**. This offered an initial indication that DNA damage and/or the response thereto is likely to be important in the lives of $CD8^+$ T cells responding to pathogenic perturbations, and provided the impetus to specifically interrogate this issue in the three expansion phase subsets of $CD8^+$ T cells first described in the prior chapter.

To begin to assess DNA damage in the three subsets, the alkaline comet assay (Olive and Banáth, 2006) was employed to directly measure DNA breaks. This approach clearly revealed increased tail moments indicative of DNA damage in the $KLRG1^{hi}$ subset, while both the IL-$7R\alpha^{hi}$ and $CD62L^{hi}$ subsets exhibited lower tail moments **(Figure 3.2)**. While these data suggested that terminal differentiation may be associated with loss of genome integrity, the resolution of this assay was insufficient to discern whether differences existed in the IL-$7R\alpha^{hi}$ and $CD62L^{hi}$ subsets. An alternative approach was called for. A major mechanism by which cells monitor DNA damage operates through phosphorylation of the variant histone H2AX, which in turn leads to the recruitment of DNA repair machinery (Bonner et al., 2008). Therefore H2AX phosphorylation was measured directly *ex vivo* during the effector phase. The IL-$7R\alpha^{hi}$ subset of LCMV-specific $CD8^+$ T cells displayed slightly lower pH2AX relative to naïve $CD8^+$ T cells, while pH2AX was even lower in the $KLRG1^{hi}$

subset, suggesting diminished efficiency of DNA strand break surveillance in this terminally-differentiated subset. In contrast, H2AX phosphorylation was markedly elevated in the LCMV-specific CD62L^{hi} subset, with *ex vivo* pH2AX expression 24% greater than naïve CD8^{+} T cells and 41% greater than observed in the IL-7Rα^{hi} subset **(Figure 3.3A)**. These data suggested the CD62L^{hi} subset was characterized by either greater DNA damage or enhanced surveillance of genome integrity. To test whether these differences reflected the ability of cells to respond to experimentally induced DNA strand breaks, each subset and control naïve CD8^{+} T cells were exposed to equal amounts of DNA damage using controlled ionizing irradiation *ex vivo* at a dose known to affect progenitor cell biology (Mohrin et al., 2010). While all subsets upregulated H2AX phosphorylation following irradiation, the extent of induction in IL-7Rα^{hi} and KLRG1^{hi} effector subsets was lower than that of naïve CD8^{+} T cells **(Figure 3.3B)**. In contrast, the CD62L^{hi} subset was substantially more efficient in initiating the H2AX-mediated DNA damage response pathway compared to naïve CD8^{+} T cells or compared to either of the two CD62L^{lo} effector CD8^{+} T cell subsets **(Figure 3.3B)**; the CD62L^{hi} subset was ~33-100% more efficient than the other subsets of CD8^{+} T cells examined **(Figure 3.3B, right panel)**. These results suggested that the CD62L^{hi} subset may be preferentially endowed with the ability to surveil and respond to DNA damage, while the IL-7Rα^{hi} and KLRG1^{hi} effector subsets may be disadvantaged in this respect.

Interestingly, the Wnt signaling pathway has been implicated in the regulation of genome maintenance activities (Kendziorra et al., 2011; Hoffmeyer

et al., 2012), and the data reported in the prior chapter indicated that TCF-1, a principal transcriptional effector of transduced Wnt signals, is preferentially expressed in the CD62L^{hi} subset relative to the CD62L^{lo} subsets **(Figures 2.5A and 2.5D)**. To probe whether a causal relationship existed between TCF-1 expression and heightened responsiveness to DNA damage, TCR-transgenic P14 CD8^{+} T cells conditionally deficient for TCF-1 (by virtue of CD4-Cre expression in thymocyte precursors driving excision of a floxed TCF-1 allele) were generated, transplanted into naïve recipient mice, and assessed for their ability to phosphorylate H2AX 8 days pi LCMV. Relative to WT controls, cells in the TCF-1-deficient CD62L^{hi} subset (but not the IL-7Rα^{hi} or KLRG1^{hi} subsets) exhibited diminished basal pH2AX expression **(Figure 3.4)**. Unexpectedly, no difference was observed in the ability of cKO vs. WT CD62L^{hi} CD8^{+} T cells to upregulate H2AX phosphorylation following *ex vivo* irradiation. This may be due to contamination of the putative cKO fraction by cells bearing the Cre and floxed target allele genotype that nevertheless escaped phenotypic excision and deletion of TCF-1 as a consequence of the known incomplete efficiency of the CD4-Cre driver utilized in these experiments. In any event, there does appear to be a causal relationship between TCF-1 expression and H2AX phosphorylation, to some extent. The full import of this should be explored in future experiments that exploit more efficient Cre driver alleles or fluorescent-reporter strategies to exclude false-positive cells that have not completely excised their TCF-1 alleles.

To further interrogate pathways of genome maintenance, telomerase activity was examined in antigen-specific CD8^{+} T cell subsets during the

expansion phase. After sort-purifying CD62L^{hi}, IL-7Rα^{hi}, and KLRG1^{hi} subsets, telomerase holoenzyme activity was assessed. The CD62L^{hi} subset exhibited markedly higher telomerase activity than either naïve CD8^{+} T cells or the CD62L^{lo} effector subsets **(Figure 3.5)**. This elevated telomerase activity suggests that the CD62L^{hi} subset acquires multiple genome surveillance and maintenance activities following initial activation of naïve precursors.

The data above suggested that the CD62L^{hi} subset was endowed with greater capacity to withstand and/or respond to DNA damage. To directly interrogate this question *in vivo*, a genome integrity disruption assay was designed. Mice were infected with LCMV and exposed to the DNA damaging agent doxorubicin during the expansion phase at a time point – d6 pi – when all three LCMV-specific subsets are robustly dividing. Doxorubicin exposure dampened the expansion of responding LCMV-specific CD8^{+} T cells in a dose-dependent manner **(Figure 3.6A, bottom)**, and quantitatively impacted all three subsets. The KLRG1^{hi} subset was markedly diminished and the IL-7Rα^{hi} subset was modestly enriched **(Figure 3.6B)**. In contrast, the CD62L^{hi} subset was preferentially resistant to doxorubicin exposure **(Figure 3.6B)**. Though the precise mechanism is unclear, this result is consistent with the restrained proliferation of the CD62L^{hi} subset, its substantial efflux activity, and its superior capacity to respond to DNA damage induced by ionizing irradiation *ex vivo*. Together these observations point to genome integrity surveillance and maintenance as key properties necessary for a robust CD8^{+} T cell response to infection, and particularly for the generation or survival of the CD62L^{hi} subset.

3.3 Retrotransposon dynamics during T stem cell and T effector differentiation

First discovered by Barbara McClintock in the 1940's, jumping genes or transposable/mobile elements are important players in evolution (McClintock, 1950) and comprise an enormous proportion of mammalian genomes – around 40%, depending on species (Bodak et al., 2014). Among transposable elements, LINE-1's (long interspersed nuclear element 1, hereafter L1) are particularly abundant and the most well-studied. L1 loci span 6 kilobases and contain a 5' untranslated region with internal promoter activity, and two non-overlapping ORFs encoding a chaperone / RNA binding protein and an endonuclease / reverse transcriptase. Roughly 500,000 L1 copies stud the (human) genome, comprising about 17% of its total, while 600,000 L1 copies amount to 19% of the mouse genome (Bodak et al., 2014). The passage of time since the initial infection and integration into the germline millions of years ago has mutated the vast majority beyond any semblance of retrotranspositional competence; however roughly 0.02-0.5% of L1 loci have retained the capacity for expression and quasi-random genomic reintegration, and are termed "hot". Copy-and-paste retrotransposition can be highly genotoxic if integration events disrupt function at a critical locus or introduce gain-of-function mutations in an oncogene, and indeed seminal studies have recently implicated L1 activity in the aging process (Li et al., 2013) and a substantial percentage of human cancers (Lee et al., 2012; Shukla et al., 2013; Ewing et al., 2015). Because of these deleterious consequences, with rare exceptions (Kano et al., 2009), mammalian tissues

normally suppress hot L1 loci. However, recent observations indicate that transient derepression may occur consequent to cell division (Abyzov et al., 2013) or epigenetic remodeling (Klawitter et al., 2016). Given that activated $CD8^+$ T cells divide rapidly and extensively (cf. Figure 1.1), and undergo substantial epigenetic transformations during effector differentiation (Pauken et al., 2016; Scott-Browne et al., 2016), it seemed reasonable to wonder whether L1 expression, normally tightly controlled, might be perturbed in these cells.

3.3.1 Mobilome matters: effector T cell differentiation is associated with derepression of the ancient retrotransposon LINE-1

LINE-1 RNA abundance was measured in naive $CD8^+$ T cells and in the three antigen-specific $CD8^+$ T cell subpopulations present during the expansion phase of the adaptive immune system's response to viral infection. Naive cells manifested only background LINE-1 levels. In contrast, activated, antigen-specific cells exhibited upregulation of LINE-1, with modest levels in minimally-differentiated T stem cells, intermediate quantities in intermediately-differentiated effector cells, and heightened levels in terminally-differentiated effector cells **(Figure 3.7, right)**. Interestingly, preliminary experiments indicated that this pattern of LINE-1 re-expression correlating with effector T cell differentiation manifested at both the RNA and DNA levels, suggesting that the dormant L1 transposon is not only re-expressed at the RNA level, but also continues its lifecycle to make protein and reverse transcribe its RNA genome into DNA. Given that reverse transcription is usually known to occur in the nucleus via a process termed target-primed retrotransposition (Gorbunova et al., 2014), it is

very likely that at least some of this DNA is being integrated into the nuclear genome. Irrespective of future experiments to test this hypothesis directly, this finding of effector T cell differentiation associated with transposon re-expression has implications for basic principles of genome integrity maintenance and developmental biology. Additionally, these observations may also serve as platform for several useful experimental and clinical applications including highly tractable lineage tracing *in vivo* and an irreversible, granular biological clock to report physiological as opposed to chronological age.

3.3.2 Heightened expression of the antiretroviral cytidine deaminase Apobec3 in T stem cells

To preserve genome integrity against the existential threat of mutagenic transposition, mammalian cells mount a variety of defenses to suppress expression of jumping genes, perhaps most prominently the retroelement restriction factor Apobec3 (Esnault et al., 2005; Muckenfuss et al., 2006). Given that T stem cells preferentially express multiple genome integrity maintenance activities including telomerase and heightened DSB surveillance, and considering the genotoxic threat posed by endogenous retroelements, it is reasonable to hypothesize that expression of Apobec3 might be an additional mechanism by which $CD62L^{hi}$ T stem cells maintain genome integrity. Thus, expression was evaluated at the RNA level in naive $CD8^+$ T cells and in the three principal antigen-specific $CD8^+$ T cell subpopulations present during the expansion phase of the adaptive immune system's response to viral infection. Apobec3 expression was negligible in naive $CD8^+$ T cells but markedly upregulated in T

stem cells, and sharply diminished in differentiated, CD62L^{lo} effector CD8^{+} T cells **(Figure 3.7, left)**. It is notable that within the three principal antigen-specific CD8^{+} T cell subpopulations, this expression pattern of Apobec3 is exactly the inverse of that of LINE-1 (described above). While a role for Apobec3 in restricting LINE-1 expression has been published *in vitro*, to date no evidence for this has been uncovered *in vivo*. Together, these observations of Apobec3 and L1 expression lead to the hypothesis, testable in future work making use of Apobec3-deficient T cells, that the former has a causal role in regulating the latter *in vivo*, quite possibly in the service of preventing L1 retrotransposition and consequent diminished genome integrity. Additional experimentation should address whether there are any consequences to CD8^{+} T cell function arising from unrestrained retrotransposition.

3.4 Conclusions

One of the more distinguishing features of these expansion phase CD62L^{hi} CD8^{+} T cells was the stark upregulation of genome maintenance pathways, as evidenced by a superior anticipatory response to irradiation-induced DNA damage, a marked increase in telomerase activity, and elevated expression of the retrotransposon restriction factor Apobec3a. One suggestion about stem-like memory T cells arising early after stimulation is that such cells simply remain more naïve-like and by remaining *less* differentiated thereby maintain better memory potential. While the CD62L^{hi} expansion-phase CD8^{+} T cells studied here are indeed more proliferatively quiescent and restrained with respect to the

elaboration of a full effector gene expression program, upregulation of genome integrity surveillance is a major change distinguishing these cells from naïve or subsets of CD62L^{lo} effector CD8^{+} T cells. Thus, this CD62L^{hi} subset does not simply remain less differentiated. Instead, this population acquires a markedly different pattern of differentiation compared to naïve, IL-7Rα^{hi} or KLRG1^{hi} cells. Proliferative hierarchies subserve tissue homeostasis in many settings of rapid cellular turnover including hematopoiesis and epithelial barrier maintenance (Weissman, 2000; Simons and Clevers, 2011) and such proliferative hierarchies have been previously implicated in effector CD8^{+} T cell generation (Sarkar et al., 2008; Buchholz et al., 2013; Gerlach et al, 2013). The data presented here now provide mechanistic understanding for a key feature of this proliferative hierarchy in CD8^{+} T cells in the induction of a genome integrity maintenance circuit. With antigen-driven CD8^{+} T cells dividing as fast as once every 4 hours during the expansion phase, it is likely that much of the genomic damage that occurs is due to replication fork errors and/or chromosomal segregation. It is interesting that in other cell types slowing cell division time by as little as 20 minutes can be sufficient to allow repair of genomic instability and damage (Sansregret et al., 2017). In this respect, while the CD62L^{hi} subset has divided extensively, its slower cell division rate may be an additional feature of the genome integrity-protective module deployed by these cells. Future studies should evaluate the implications of different types of genomic stress on these cells. A model is presented in which the lineage relationships elucidated in the prior chapter are

correlated with the genome integrity status inferred from the data presented in the present chapter **(Figure 3.8)**.

Figure 3.1

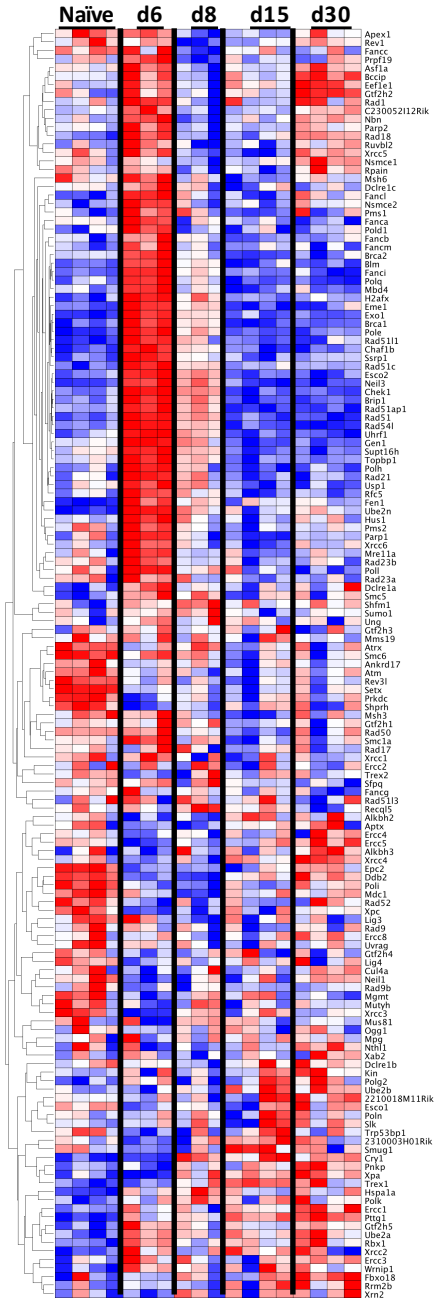


Figure 3.1: Dynamic regulation of DNA damage response circuitry during antiviral CD8$^+$ T cell proliferation & differentiation. The gene ontology term "DNA damage" was used to construct a set of genes associated with DNA damage; transcript levels of these genes, as measured by oligonucleotide microarrays, were then clustered according to the similarity of their transcriptional regulation in antigen-specific CD8$^+$ T cells responding to acute viral infection. Splenic T cells were profiled in naïve mice, and days 6, 8, 15, and 30 post infection with LCMV-Armstrong (as detailed in Crawford et al., 2014). Each row represents a transcript, and each column represents data from a single microarray; there are three or four replicates per time point. It is evident that the transcriptional response to DNA damage is dynamically regulated in CD8$^+$ T cells over the course of their response to infection.

Figure 3.2

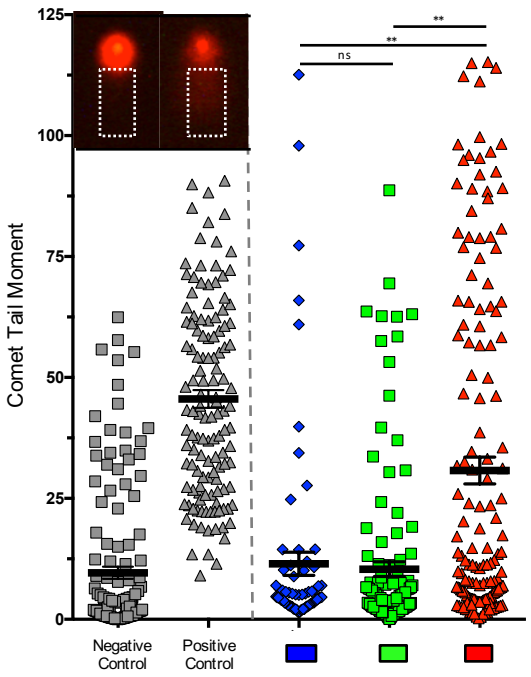


Figure 3.2: Elevated DNA damage in terminally-differentiated, antigen-specific CD8$^+$ T cells. Alkaline comet assays indicating extent of chromatin disintegration were performed for sorted subsets of splenic, antigen-specific CD8$^+$ T cells isolated at d8 pi LCMV-Armstrong. Naïve CD8$^+$ T cells were used as a negative control; naïve CD8$^+$ T cells exposed *ex vivo* to 250 rads of ionizing irradiation served as a positive control. Representative microscopic images of negative control and positive control cells are inlaid.

Figure 3.3

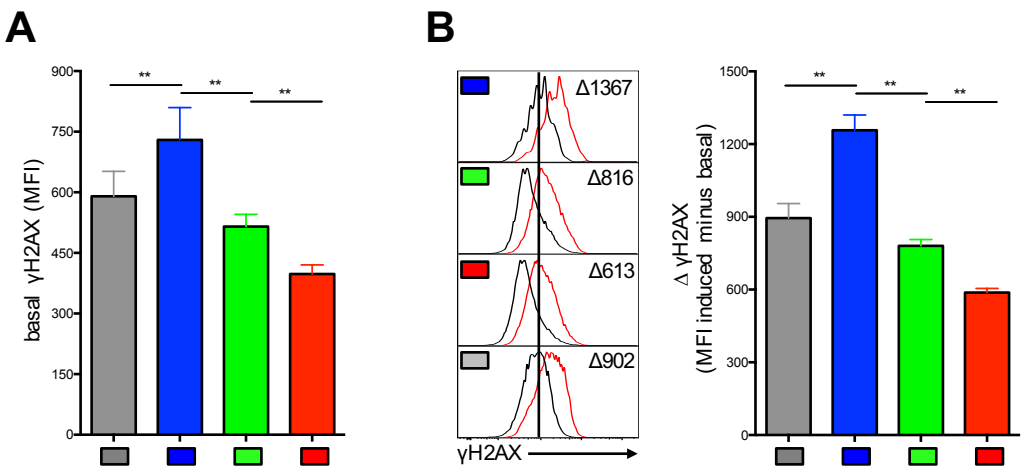


Figure 3.3: Elevated basal and DNA damage-inducible H2AX phosphorylation in antigen-specific T stem cells. A) Basal phosphorylation of H2AX measured directly ex vivo in the indicated LCMV-specific $CD8^+$ T cell subsets isolated at d8 pi LCMV-Armstrong or naïve $CD8^+$ T cells. **C)** Damage-induced phosphorylation of H2AX in the indicated LCMV-specific $CD8^+$ T cell subsets isolated at d8 pi LCMV-Armstrong or naïve $CD8^+$ T cells. Cells were exposed to 250 rads of ionizing radiation ex vivo and then incubated at 37°C for 90 minutes. Histograms (left) depict the extent of basal (black lines) and irradiation-induced (red lines) pH2AX. Bar graph depicts the inducibility of pH2AX, calculated as the difference between the basal and irradiation-induced values.

Figure 3.4

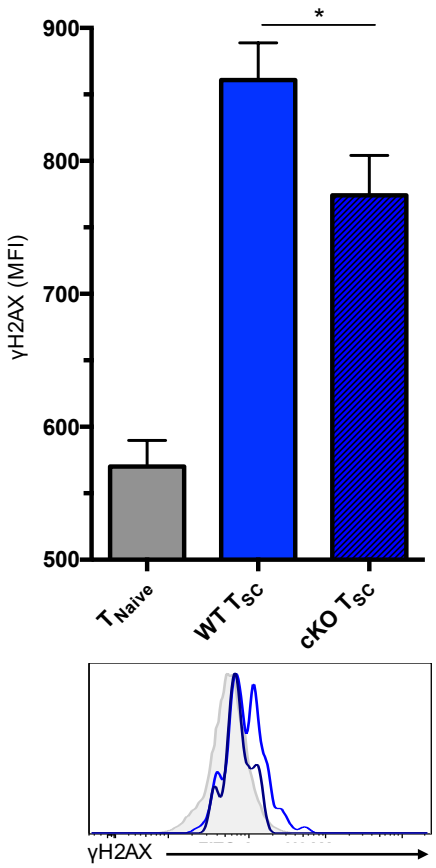


Figure 3.4: Diminished basal H2AX phosphorylation in TCF1-deficient T stem cells. Differentially congenic WT P14 $CD8^+$ T cells and P14 $CD8^+$ T cells conditionally homozygously deficient for TCF-1 were mixed in a 1:1 ratio and co-transplanted into naïve recipient mice. Following infection with LCMV-Armstrong, splenocytes were obtained at d8 pi and evaluated flow cytometrically for phosphorylation of the variant histone H2AX on antigen-specific WT and cKO $CD62L^{hi}$ subset cells.

Figure 3.5

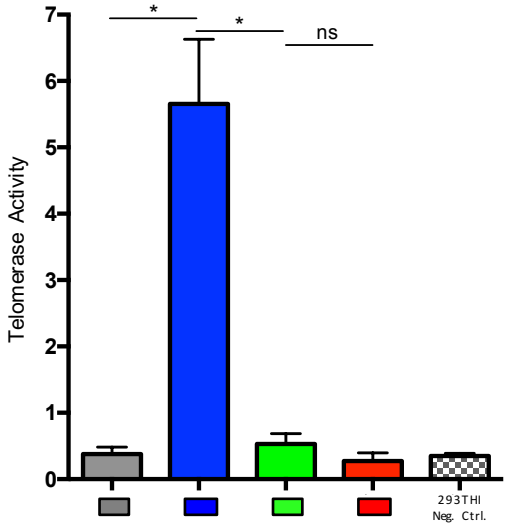


Figure 3.5: Elevated telomerase activity in antigen-specific T stem cells during viral infection. Telomerase activity was assessed by QTRAP assay (see methods) for the indicated splenic LCMV-specific $CD8^+$ T cell subsets isolated at d8 pi LCMV-Armstrong or naïve $CD8^+$ T cells. Heat-inactivated 293T cells were used as a negative control.

Figure 3.6

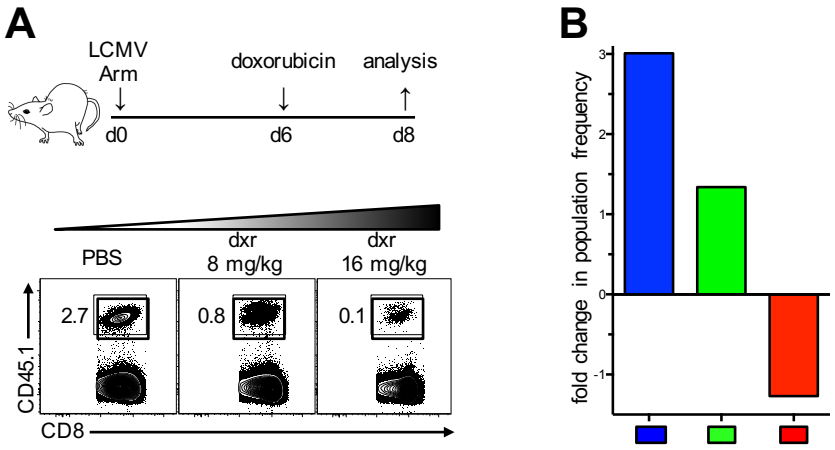


Figure 3.6: Antigen-specific T stem cells preferentially survive genotoxic insult *in vivo*. **A**) Top: experimental design for *in vivo* doxorubicin (dxr) treatment. Bottom: the frequency of donor P14 cells ($CD45.1^{+}$) with as a function of dxr dose at d8 pi LCMV Arm. **B)** Fold enrichment or reduction of the frequency of each of the three LCMV-specific $CD8^{+}$ T cell subsets of total P14 cells at d8 pi with LCMV Arm in mice treated with dxr (8 mg/kg) vs. PBS. treated mice.

Figure 3.7

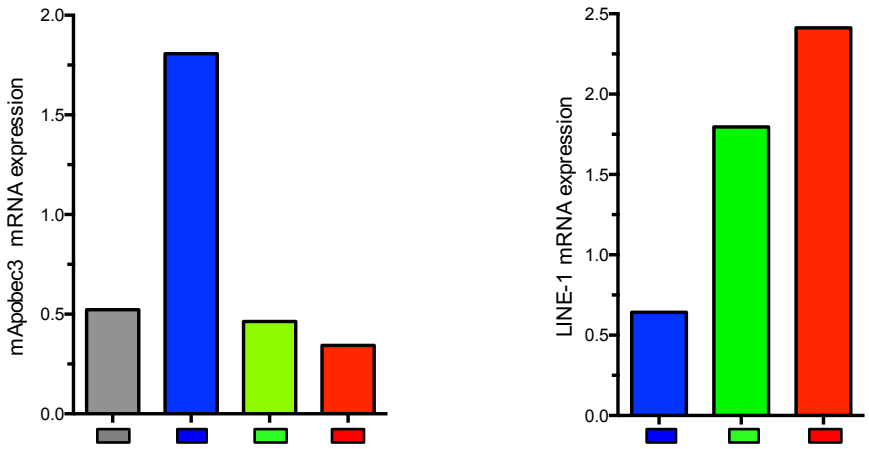


Figure 3.7: Elevated expression of the retroelement restriction factor Apobec3 in antigen-specific T stem cells correlates with relaxed suppression of the ancient retrotransposon LINE-1 during antiviral CD8$^+$ T cell differentiation. RNA isolated from subsets of P14 splenocytes sorted at 8 pi LCMV-Armstrong was used for qPCR analysis of the abundance of Apobec3 and LINE-1 mRNA.

Figure 3.8

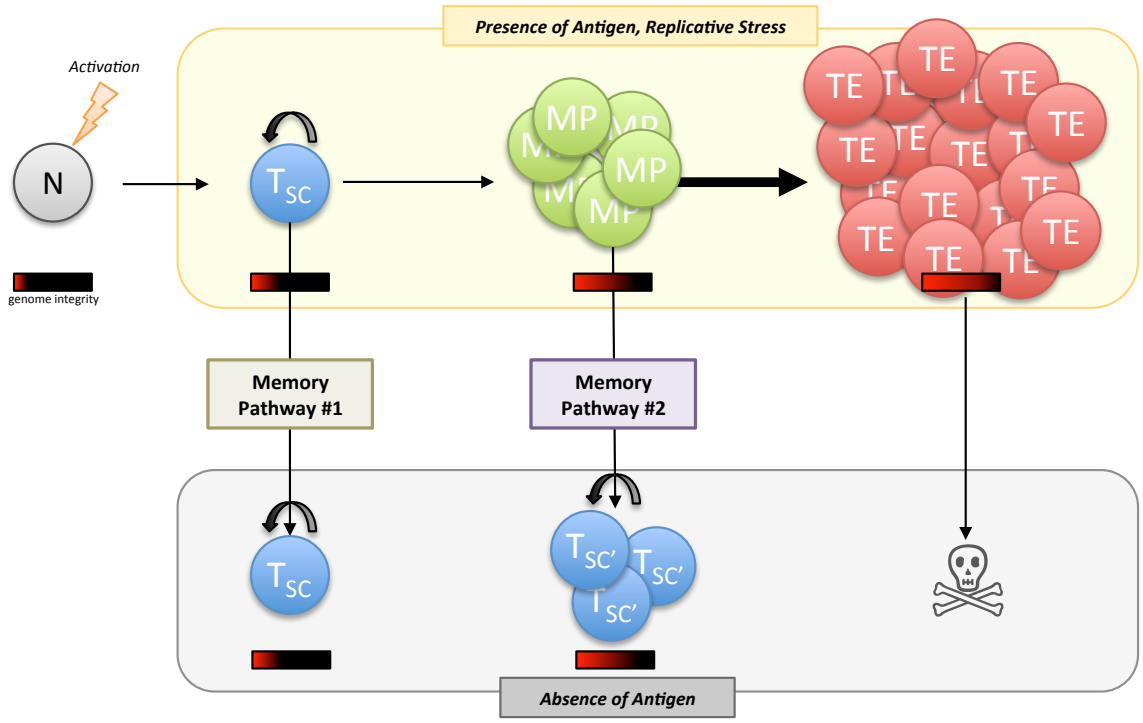


Figure 3.8: Model for effector and memory T cell differentiation and dedifferentiation including genome integrity. *N.b. the schematic above is an elaboration of Figure 2.16; here, the parameter of genome integrity is considered.* A naïve T cell is activated by encounter with cognate antigen in the context of immunological danger and gives rise to a clonal stem cell hierarchy marked by increasing proliferation, progressive cytotoxic effector differentiation, and diminishing genome integrity (underlying "power bars" in red and black; these refer to multiple aspects of genome integrity, including telomere length, unresolved or misrepaired double strand breaks, proliferation-induced point mutations, and putative activation-induced retrotransposon retro-integration events). Following antigen elimination and the resolution of acute infection, terminally-differentiated effector cells (red) largely commit to apoptosis, whereas intermediately effector-differentiated transient-amplifying / memory precursor cells (green) gradually reacquire the stemness of their progenitors (blue). In the post-antigen phase, the pool of memory T cells, composed of T_{SC} and T_{SC}' populations, is heterogeneous with respect to proliferative past and history of effector differentiation, and putatively as well in terms of genome integrity (which corresponds to a multitude of potential parameters, including accumulated mutations, retrotransposion reintegrations, single and double strand breaks, and telomere length).

.:: CHAPTER 4 ::.
SIGNAL FLUX IN AND SIGNALING-DEPENDENT ABUNDANCE OF T STEM CELLS; IMPLICATIONS FOR IMMUNOTHERAPY

4.1 Command and control: multifactorial regulation of T cell function

That T cells are equipped with tremendously potent effector mechanisms with the potential to profoundly remodel organismal physiology is perhaps most saliently manifest in recent reports of cancer immunotherapies, wherein therapeutic manipulation of T cell function in human patients has been shown to precipitate dramatic and durable remissions of even large or disseminated tumors in a highly specific manner and with minimal off-target pathophysiology (**Figure 4.1**). Conversely, the destructive potential of T cells is equally apparent, as evidenced by the profound burden of suffering borne by those afflicted with autoimmune disorders, many of which are T cell-dependent (Bluestone et al., 2010). Accordingly, 500 million years of evolution since the dawn of adaptive immunity have conspired to construct an elaborate apparatus regulating T cell functionality such that these cells' considerable physiological force maximally subserves organismal fitness and minimizes the potential for mischief. An overarching research aim for decades has been to decipher this regulatory regime, both to better understand basic immunological phenomena as well to develop the ability to rationally manipulate immune function for prophylactic and therapeutic ends.

A leading model to explain the regulation of lymphocyte function posits that they integrate both positive and negative micro-environmental signals that as a whole conduce to either activation (initial or continued) or inhibition. This notion originated in the so-called "two signal" hypothesis (Bretscher and Cohn,

1970; Lafferty et al., 1974) that was subsequently elaborated by Janeway and Matzinger, and which held that in order to spring forth from dormancy into concerted action, lymphocytes required not only recognition of their cognate antigen, but also receipt of a second, “costimulatory” signal to provide contextual information, such as whether the antigen in question was initially encountered in a pathological or physiological scenario (e.g., respectively, a potentially dangerous viral infection, or a foreign yet nevertheless innocuous food substance in the gastrointestinal tract). Since then a variety of additional classes of signals have been recognized that complexify the original model, such that not just two but rather multiple external signals are sensed and interpreted by lymphocytes in a manner that enables a nuanced, highly calibrated response to prevailing environmental conditions (Smith-Garvin et al., 2009). The molecular nature of these signals and their mechanisms of transduction has been elucidated in increasing detail in recent years (Attanasio and Wherry, 2016). However, from a cellular perspective, it remains very much unclear the extent to which any given signal is sensed by and directs the function of the many subsets of lymphocytes known to exist. For example, CD28 is a prominent receptor of costimulatory signals that enhance TCR- (i.e., antigen) induced proliferation of T cells. However, since its identification in the early 1980’s, it’s become clear that CD28 is not uniformly expressed on T cells. Instead, there can be a broad range of expression on different T cell subsets, suggesting that signals transduced by CD28 and a rapidly growing list of related molecules may differentially impact cells depending on T cell subtype.

Here, these matters are explored with respect to the CD62L^{hi} T stem cell population whose isolation and initial characterization was detailed in previous chapters. Therein, this rare population of cells was shown to be uniquely potent, for example with respect to the capacity to generate legions of cytotoxic effector cells. How is this power engaged, directed, and regulated? Data are presented in this chapter indicating that T stem cells highly express a panoply of sensors for multiple classes of signals, both activating and inhibitory. Far from being inert with respect to signaling, as originally envisioned given their relative proliferative quiescence, T stem cells instead appear to be remarkably sensitive to and interconnected with their environments. Unexpectedly, this quantitatively minor subset may in fact reside at an cellular signaling nexus that can powerfully initiate immune responses *de novo*, or potentiate or terminate existing ones.

4.2 Anatomic localization of T stem cells

While environmental signals and the intracellular interpretation thereof are important for all cells, they appear to be particularly pertinent for stem/progenitor cells whose decisions regarding state and fate commitments impact not only themselves but also many multitudes of downstream progeny cells and all the various cells and tissues with which *those* cells interact. Moreover, perhaps due to their unique requirements for life-long persistence and generative output, stem cells rely considerably on external inputs from neighboring cells, for example in terms of signals and nutrients that minimize cellular stress and promote survival (Mendez-Ferrer et al., 2010). This notion that stem cells function not strictly

autonomously in an amorphous void but rather exist in and depend upon a biologically-defined niche was originally proposed in an attempt to explain discrepancies between predicted and observed behavior in hematopoietic stem cells isolated from different anatomic locations (Schofield, 1978). Decades later, demonstration and cellular delineation of stem cell niches – first in *Drosophila* ovaries (Xie and Spradling, 2000) and subsequently in multiple rodent tissues (Lander et al., 2012) – substantiated this hypothesis and cemented the modern concept that niches are micro-environments with specific compositions of supporting cell types, hormones, nutrients, mechanical forces, and gas tensions (Simon and Keith, 2008; Mendez-Ferrer et al., 2010; Sun et al., 2012). Together, this surrounding ensemble sustains stem cell populations and helps modulate their delicate balance between self-renewal and terminal differentiation. Therefore, understanding stemness includes but extends well beyond merely isolating and studying stem cells themselves. In short, anatomy matters.

To begin to understand the environmental circumstances in which T stem cells exist and that might bear on understanding mechanisms by which they are regulated, a gross anatomical survey of the abundance of $CD62L^{hi}$ T stem cells was undertaken. The $CD62L^{hi}$ population was rare but evident in all tissues examined 8 days pi with LCMV, and present at the highest frequency in lymph nodes **(Figure 4.2A).** Beyond gross anatomy, the micro-anatomical residence of the three virus-specific $CD8^{+}$ T cells subsets was analyzed in the spleen using intravascular antibody labeling, a technique that indicates localization in the blood-accessible splenic red pulp relative to the blood-inaccessible, lymphoid

white pulp (Galkina et al., 2005; Anderson et al. 2014). Evident was a clear a hierarchy of labeling **(Figure 4.2B)**. As a reference point, naïve $CD8^+$ T cells exhibited the lowest extent of intravascular labeling, reflecting the known residence of these cells in the white pulp **(Figure 4.2B)**. The two $CD62L^{lo}$ effector $CD8^+$ T cell subsets were highly labeled, indicating homing to the red pulp and consistent with previous observations (Jung et al., 2010; Seo et al., 2016). In contrast, the $CD62L^{hi}$ subset exhibited intermediate intravascular antibody labeling, indicating preferential localization to the lymphoid white pulp among the three antigen-specific $CD8^+$ T cell subsets **(Figure 4.2B)**. This is consistent with the preferential lymph node localization of the $CD62L^{hi}$ subset **(Figure 4.2A)**, and suggests the possibility that effector and memory $CD8^+$ T cell differentiation might correlate with anatomical positioning. Indeed, recent studies have indicated that T cells localized to the white pulp during the expansion phase have greater potential to contribute to immunological memory than those in the red pulp (Seo et al., 2016). Moreover, these gross- and micro-anatomical patterns of lymphoid-biased residence evident in the $CD62L^{hi}$ subset were paralleled by pronounced expression of CXCR3 and CXCR5 **(Figure 4.3)**, localization molecules associated with memory and progenitor T cell biology, respectively (Sung et al., 2012; Im et al., 2016). Simultaneously, the $CD62L^{hi}$ subset exhibited very low expression of CX_3CR1 **(Figure 4.3)**, a localization factor associated with effector T cell differentiation (Gerlach et al., 2016). The $CD62L^{hi}$ subset also highly expressed CD69 **(Figure 4.3)**, a molecule that strongly promotes lymphoid localization (Mackay et al., 2015), together with

CXCR5. Thus, the expansion phase CD62L^{hi} subset resides preferentially in specific gross- and micro-anatomical regions, namely those with lymphoid character. Coincidentally, this geography is matched by expression of molecules that drive lymphoid homing. Together, these anatomical and molecular features distinguish the CD62L^{hi} T stem cell subset from the two CD62L^{lo} subsets as well as from precursor naïve T cells, and suggest that it is distributed not randomly but instead in a defined niche. This is likely to be important for committing to and sustaining memory and progenitor fate decisions.

4.3 T stem cells' TCR signaling flux and sensory apparatus

That T stem cells exhibited unique patterns of both localization (**Figure 4.2**) and proliferation (**Figure 2.8**) suggested possible differences in signal reception and/or transduction. To assess whether CD62L^{hi} T stem cells exhibited evidence of differential stimulation during the expansion phase relative to the other subsets, Nur77-GFP reporter mice were exploited. In this construct, GFP expression reflects recent TCR stimulation (Moran et al., 2011). GFP expression was readily apparent, yet similar, in the two LCMV-specific CD62L^{lo} effector CD8^{+} T cell subsets at d5 pi (**Figure 4.4**). In contrast, CD62L^{hi} T stem cells expressed considerably higher GFP than either of the two CD62L^{lo} subsets, suggesting stronger and/or more recent TCR signal transduction **(Figure 4.4)**. In point of fact, this indication of stronger stimulation was reflected in elevated expression on the CD62L^{hi} subset of CD3 and CD8 **(Figure 4.5)**, essential elements of the TCR signal transduction complex. This was also the case for

CD28 **(Figure 4.6)**, the stereotypical immunoglobulin (Ig) superfamily receptor for costimulatory signals that plays critical roles in both the initiation of T cell responses as well as in the therapeutic potentiation thereof in the context of immunotherapies for cancers and chronic viral infections (Hui et al., 2017; Kamphorst et al., 2017). The preferential expression by $CD62L^{hi}$ cells of CD28 was matched by a similar pattern of expression of a bevy of additional costimulatory receptors, including CD27, ICOS, and GITR **(Figure 4.6)**. What is more, $CD62L^{hi}$ cells also robustly expressed receptors for multiple additional classes of stimulatory signals, including those denoting inflammatory state (aka signal 3, **Figure 4.7**) and licensing help from kindred lymphocyte populations (e.g. $CD4^+$ T cells and/or B cells; **Figure 4.8**). Together, these observations indicate that T stem cells, relative to their downstream $CD62L^{lo}$ progeny, are both exceptionally sensitive to their environmental surroundings and also robustly transduce signals therefrom.

The simultaneous observations of heightened receptivity to stimulatory signals in tandem with elevated expression of the downstream Nur77 transcription factor appeared paradoxical given the relatively attenuated proliferation of the $CD62L^{hi}$ subset **(Figure 2.8A-C)**. Recent studies have demonstrated that expansion-phase $CD8^+$ T cells in the white pulp exhibit lower two-dimensional effective affinity relative to $CD8^+$ T cells in the red pulp (Seo et al., 2016). The $CD62L^{hi}$ T stem cell subset was enriched in the white pulp, but appeared to receive stronger stimulation, rather than the weaker stimulation that would be predicted based on lower effective affinity unless these cells possessed

a mechanism to limit the effects of TCR stimulation. One way by which T cells modulate their sensitivity to antigen signaling is by deploying expression of inhibitory receptors that attenuate stimulatory signals. For example, one prominent such receptor is CTLA-4, first established as a negative regulator of T cell function more than two decades ago (Walunas et al., 1994; Krummel and Alison, 1995). CTLA-4, absent on naïve T cells, is rapidly expressed on the surface of T cells following activation and is upregulated by ligation of CD28. Engagement of CTLA-4 can dampen the amplitude of T cell responses by disrupting AKT signaling and/or by outcompeting CD28 for its activating ligands, in either case restraining costimulation and overall T cell functionality (Parry et al., 2005). The essentiality of this restraint in maintaining self-tolerance and limiting collateral tissue damage during immune responses is manifest in the severe autoimmunity induced by pharmacological blockade of CTLA-4 in the context of human anti-cancer immunotherapies, and in the lymphoproliferation and lethal, multiorgan immunopathology that rapidly emerge in mice genetically deficient for CTLA-4 (Tivol et al., 1995).

Given that CD62L^{hi} cells exhibit such a strongly activated phenotype, and since CTLA-4 is such an important regulator of T cell function, its expression was evaluated during the expansion phase of viral infection. A hierarchy of expression existed in LCMV-specific CD8^{+} T cell subsets: interestingly, the CD62L^{hi} subset expressed the highest amount of CTLA-4, whereas progressive effector differentiation in the two CD62L^{lo} subsets was associated with lower CTLA-4 abundance **(Figure 4.9)**. This pattern of expression was evident for multiple

additional inhibitory receptors, including PD-1 and LAG-3, which act as rheostats to tune antigen-responsiveness in peripheral tissues (Okazaki et al., 2013; Honda et al., 2014), and CD5, an additional negative regulator of T cell signaling whose expression has been shown to be proportional to TCR signaling strength, at least in the thymus (Azzam et al. 1998) **(Figure 4.9)**.

The elevated expression of PD-1 on the $CD62L^{hi}$ subset was particularly remarkable, given the rich biological and clinical context in which this molecule is situated. A member of the CD28 Ig receptor superfamily, PD-1 shares a number of inhibitory features with CTLA-4, yet differs from it in several structural and mechanistic respects (Parry et al., 2005). Functionally, PD-1 attenuates T cell function both by antagonizing TCR signaling (Okazaki et al., 2013) and by disrupting CD28-mediated costimulatory signals (Hui et al., 2017, Kamphorst et al., 2017). PD-1 is expressed primarily on leukocyte populations, while its ligand PD-L1 can be upregulated by IFNγ signaling in multiple cellular lineages, including both hematopoietic and epithelial cells. Notably, pharmacological blockade of the PD-L1—PD-1 signaling axis can dramatically potentiate suppressed T cell responses to persisting pathogens in the context of chronic microbial infections (Barber et al., 2006) and cancers (Topalian et al., 2015).

Intriguingly, when PD-L1 expression was measured in subsets of antigen-specific $CD8^{+}$ T cells during the expansion phase of viral infection, a pattern similar to that of PD-1 was detected **(Figure 4.10A)**. Specifically, among antigen-experienced T cells, the $CD62L^{hi}$ subset expressed the greatest amount of PD-

L1, whereas PD-L1 expression was diminished in CD62L^{lo} subsets. When the CD62L^{hi} population was itself subsetted based on its heterogeneous expression of PD-1 **(Figure 4.9)**, higher PD-L1 expression was observed on the subpopulation of CD62L^{hi} cells that expressed more PD-1, indicating that both the PD-1 inhibitory receptor and its ligand are simultaneously expressed by CD62L^{hi} T stem cells **(Figure 4.10B)**.

In summary, attenuated proliferation during the expansion phase and constrained effector differentiation of the CD62L^{hi} subset despite elevated TCR signaling (as evidenced by Nur77 expression) and activation status (expression of CD69, as well as additional markers such as CD11a and CD38 – cf. **Figure 2.5**) is associated with heightened expression of a constellation of inhibitory receptors known to restrain T cell activation and terminal differentiation. In at least one instance, this robust inhibitory receptor expression is paralleled by similarly pronounced expression of its own ligand, underscoring the depths to which inhibition and restraint may be fundamentally important themes in the lives of CD62L^{hi} T stem cells.

4.4 Sensitivity to Strength of Stimulus

Given the multiple indications that CD62L^{hi} T stem cells appeared to be a focal point for the sensing of environmental cues, it was important to explore this notion further and ask whether varying the strength of signaling inputs might have functional consequences. For example, at least at the level of bulk (i.e. unsubsetted) antigen-specific CD8^{+} T cell populations, it has been established

that strength of TCR signaling impacts the balance of effector vs. memory fate commitment decisions (Marzo et al., 2005; Sarkar et al., 2007; Sarkar et al., 2008). Thus it was of interest to ask whether the abundance of the CD62L^{hi} subset varied in populations of endogenous, epitope-specific CD8^{+} T cell populations that are known to receive different amounts of stimulation (Sarkar et al., 2007). Consistent with the notion that subdominant responses receive less stimulation, a higher frequency of the CD62L^{hi} subset was evident in the subdominant GP276-specific response compared to the more dominant GP33- or NP396-specific responses **(Figure 4.11A)**. To examine the relationship between the CD62L^{hi} subset and strength of TCR stimulation in more detail, graded doses of naïve P14 CD8^{+} T cells were transplanted into naïve recipient mice, and these chimaeras were then infected with LCMV. Supra-physiological numbers of TCR-transgenic CD8^{+} T cells are known to increase competition between responding cells for stimulatory signals, thereby decreasing the average stimulation available to any given T cell (Butz and Bevan, 1998; Marzo et al., 2005). Indeed, with increasing dose of transferred naïve P14 cells, a higher frequency of the CD62L^{hi} subset was observed at d8 pi **(Figure 4.11B)**, consistent with previous studies (Wherry et al., 2003; Marzo et al., 2005; Sarkar et al., 2007). This pattern was paralleled by increased expression of CD62L itself within CD62L^{hi} cells **(Figure 4.11B)**, as well as decreased expression within the overall antigen-specific P14 "tissue" of the effector-differentiation-promoting transcription factor Blimp-1 **(Figure 4.11C)**. Additionally, the enhanced emergence of the CD62L^{hi} subset in the context of attenuated strength of stimulation also corresponded to an

increase in the frequency of cells that had proliferated to a lesser extent, as indicated by incomplete CTV dilution **(Figures 2.8A, bottom, and 2.8B, right).** Thus, CD62L^{hi} T stem cells are characterized by superlative signal flux, and this matters functionally: attenuated strength of external antigen signals impacted the proliferation and differentiation state of CD62L^{hi} cells, and increased their frequency amongst total populations of epitope-specific CD8^{+} T cells.

4.5 Compromise of T stem cell compartment consequent to genetic deficiency of inhibitory receptors

Given that attenuating signal strength potentiated T stem cells, it was of interest to determine whether intensifying signal strength might have the opposite effect. That unperturbed T stem cells naturally experience high signal flux **(Figure 4.4)** even while robustly expressing a formidable array of signal-attenuating factors **(Figures 4.9 and 4.10)** renders this question particularly salient – could increased strength of signaling deleteriously impact T stem cells? To address this possibility, P14 CD8^{+} T cells were generated to be genetically deficient for the inhibitory receptor PD-1. Naïve WT and PD-1-deficient P14 cells were mixed together in a 1:1 ratio and co-transplanted into recipient mice, and these chimaeras were subsequently exposed to viruses that generate acutely-resolving infections **(Figure 4.12A)**. In the settings of Flu and LCMV-Armstrong infections administered intranasally, the splenic PD-1-deficient CD62L^{hi} T stem cell population was diminished at d8 pi, consistent with the hypothesis; however, this phenotype was not observed following intraperitoneal infection with LCMV-Armstrong (data not shown). Though the explanation for the dependence of the

phenotype upon route of infection is unclear, intranasal but not intraperitoneal infection can engender presentation of processed viral antigen that persists for months after replication-competent virus has been cleared, resulting in long-lasting T cell stimulation (Zammit et al., 2006; Takamura et al., 2010). These considerations are consistent with the notion that signal strength may be toxic to T stem cells above a certain threshold, and that this threshold was exceeded by the overall integration of the strength and duration of signaling in intranasally- but not intraperitoneally-initiated infections. To probe this hypothesis, a strategy was devised to further intensify signal strength within the context of an intraperitoneally-initiated infection by ablating expression of an additional signal-attenuating factor. Accordingly, P14 $CD8^+$ T cells were generated to be simultaneously deficient for PD-1 and LAG-3, a CD4-homologous inhibitory receptor with a known role in restraining the proliferation of activated T cells during viral infections, both *per se* (Workman et al., 2004) and in synergistic cooperation with PD-1 (Blackburn et al., 2009). Hence, the aforementioned experiment was repeated using doubly-IR-deficient P14 cells and LCMV infection initiated via the intraperitoneal route. In this setting, doubly-IR-deficient $CD8^+$ T cells expanded to a greater extent than did WT cells; however, the IR-deficient $CD62L^{hi}$ T stem cell compartment was markedly diminished **(Figure 4.12B).** Thus, T stem cells appear to be programmatically equipped to sense and transduce strong environmental signals, including at the high levels that attend the mitotic maelstrom of the expansion phase of microbial infections. However, intensifying the strength of this signaling eventually overwhelms the buffering

mechanisms that T stem cells deploy to maintain a careful balance between self-renewal, terminal differentiation, and death: in the absence of a single inhibitory receptor – PD-1 – T stem cell viability is compromised in some (intranasally-introduced infections) but not all scenarios. When signal attenuation capacity is further undermined by combined inhibitory receptor deficiency, T stem cells appear invariably diminished.

4.6 Conclusions and Future Directions

By way of heightened expression of multiple classes of signal receptors, and to a greater extent than other subsets, $CD62L^{hi}$ T stem cells were observed to be highly sensitive to environmental cues. The full import of this observation remains undefined; however, given $CD62L^{hi}$ cells' role as progenitors that spawn abundant downstream progeny upon demand, exquisite environmental sensitivity on the part of these cells makes sense in terms of carefully calibrating T lymphopoietic output with organismal requirements. I.e., to either activate or amplify, or alternatively to attenuate or terminate T cell responses to pathogenic perturbations, it is not difficult to envision multiple signals conveying either peril or calm efficiently converging on T stem cells, which in turn respond by escalating or diminishing proliferative output.

In the studies herein, the expression of a broad range of signal receptors (and various regulators thereof) has been measured. Future work should undertake a comprehensive transcriptomic and proteomic survey to grasp the full breadth of T stem cells' signaling sensitivity. This is important for both basic

understanding of T cell immunobiology, as well as for the design of immunomodulatory therapeutics and prophylactics. For example, T stem cells robustly express the receptor for IL-18, a a potent proinflammatory cytokine. Leukocytes and other cells utilize multiple additional factors to signal inflammatory status, including IL-12, IFNα, and IFNγ. Do T stem cells express the apparatus necessary to sense and interpret these signals? If so, how do each of these cells influence T stem cell fate and state, especially with respect to balancing proliferation versus quiescence, or self-renewal versus differentiation? Of note, T stem cells robustly express Sca-1 **(Figure 2.5)**, which is known to play an important role in the transduction of IFNα signaling (Essers et al., 2009). Furthermore, hematopoietic stem cells, from which T stem cells ultimately derive, and with which they very likely overlap transcriptionally (Luckey et al., 2006; Im et al., 2016), are highly sensitive to both IFNα and IFNg and modulate their proliferative output in response to those signals (Essers et al., 2009; Baldrige et al., 2010). Additional signals known to be important in shaping memory and effector T cell fate commitment decisions include the anti-inflammatory molecule IL-10 (Laidlaw et al., 2015) and the alarmin IL-33 (Bonilla et al., 2012). It is likely that T stem cells express receptors for these signals, perhaps quite robustly; this possibility should be empirically determined.

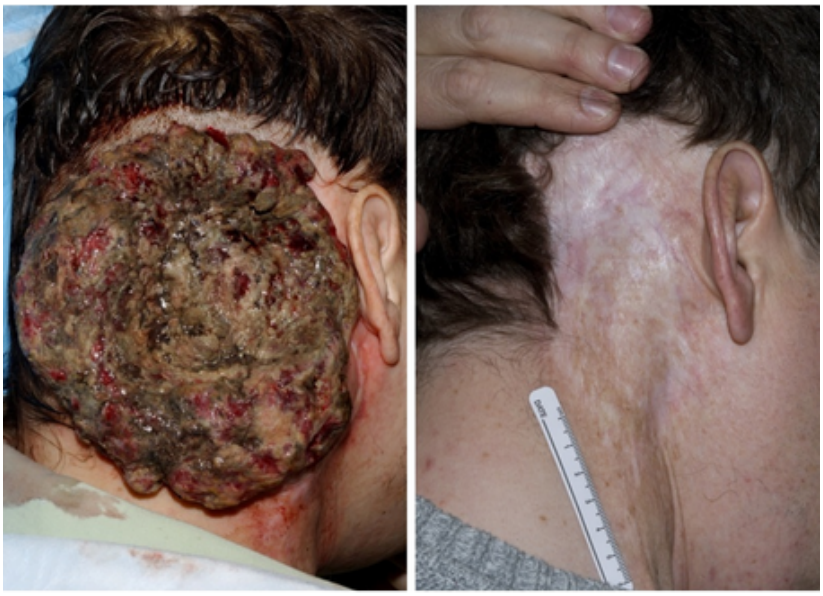
Beyond local agents, organism-wide parameters and behaviors have been associated with various immunological outcomes of interest, and separately with specific signaling events. For example, emerging evidence suggests that moderate (but not exhaustive) exercise can enhance responsiveness to

vaccination against a variety of viral pathogens, including varicella zoster virus (Irwin et al., 2007) and influenza (Kohut et al., 2004; Hallam and Kohut 2013. Intriguingly, musculature has recently been appreciated as a facultative endocrine tissue that – exclusively in the context of physical exertion – secretes a variety of hormones (called "myokines") that can potently remodel organismal physiology (Rao et al., 2014; Schnyder and Handschin, 2015). It is worth noting that one such myokine is IL-15 (Rinnov et al., 2015); that IL-15 is critical for the homeostatic proliferation and long-term maintenance of $CD8^+$ T cell memory (Goldrath et al., 2002); and furthermore that $CD62L^{hi}$ T stem cells very highly express CD122 **(Figure 2.5)**, a key component of the IL-15 receptor complex. Future work should empirically probe whether these coincidences arise from mechanistic intersections. Overall, given the desirability of understanding the interplay between unfolding adaptive immune responses and both proximal and broader environmental cues, and of enhancing immune responses to both prophylactic and therapeutic modulations, it will be important to achieve a comprehensive grasp of the signaling sensitivity of T stem cells.

Irrespective of breath of environmental sensitivity, it is readily apparent that $CD62L^{hi}$ T stem cells are acutely responsive to external signals. They may therefore deploy inhibitory receptors to prevent over-stimulation and consequent depletion (via excessive terminal differentiation and/or death). This notion is borne out in the compromised T stem cell compartment that is observed in the genetic absence of inhibitory receptors. The longer-term functional implications of this result for immunological memory formation remain to be established;

however, given that stem cells are essential for long-term tissue homeostasis, especially in the context of constitutive or on-demand rapid turnover (cf. Chapter 1), it is challenging to envision constructive outcomes with respect to overall organismal homeostasis and immunocompetence. What is more, clinical anti-cancer treatment strategies are currently being contemplated or developed that entail permanent incapacitation of PD-1 function, and/or transient suppression of not just one but multiple signal-attenuation factors in T cells (Wolchock et al., 2013; Liu et al., 2015; Nolan et al., 2017). These efforts may conceivably conduce to control of tumors refractory to other treatment modalities and are worthy of consideration; however, the possibilities of T stem cell toxicity and likely consequent accelerated immunosenescence and immunodeficiency must not be discounted, and should be carefully monitored and weighted in the strategic cost-benefit calculus. This is important both for the treatment of primary tumors, and for engendering the immunological memory that may be necessary to prevent future cancer recurrence.

Figure 4.1



2005 **2010**

Figure 4.1: Proof-of-principle of curative immunotherapy for advanced cancer. A patient was treated with immunotherapy for a bulky, ulcerating, metastatic tumor. The therapy elicited a complete regression that endured for at least five years. Reproduced with permission from: Crompton JG, Sukumar M, and Restifo NP. Uncoupling T cell expansion from effector differentiation in cell-based immunotherapy. *Immunol Rev*. 2014 Jan; 257(1):264-76.

Figure 4.2

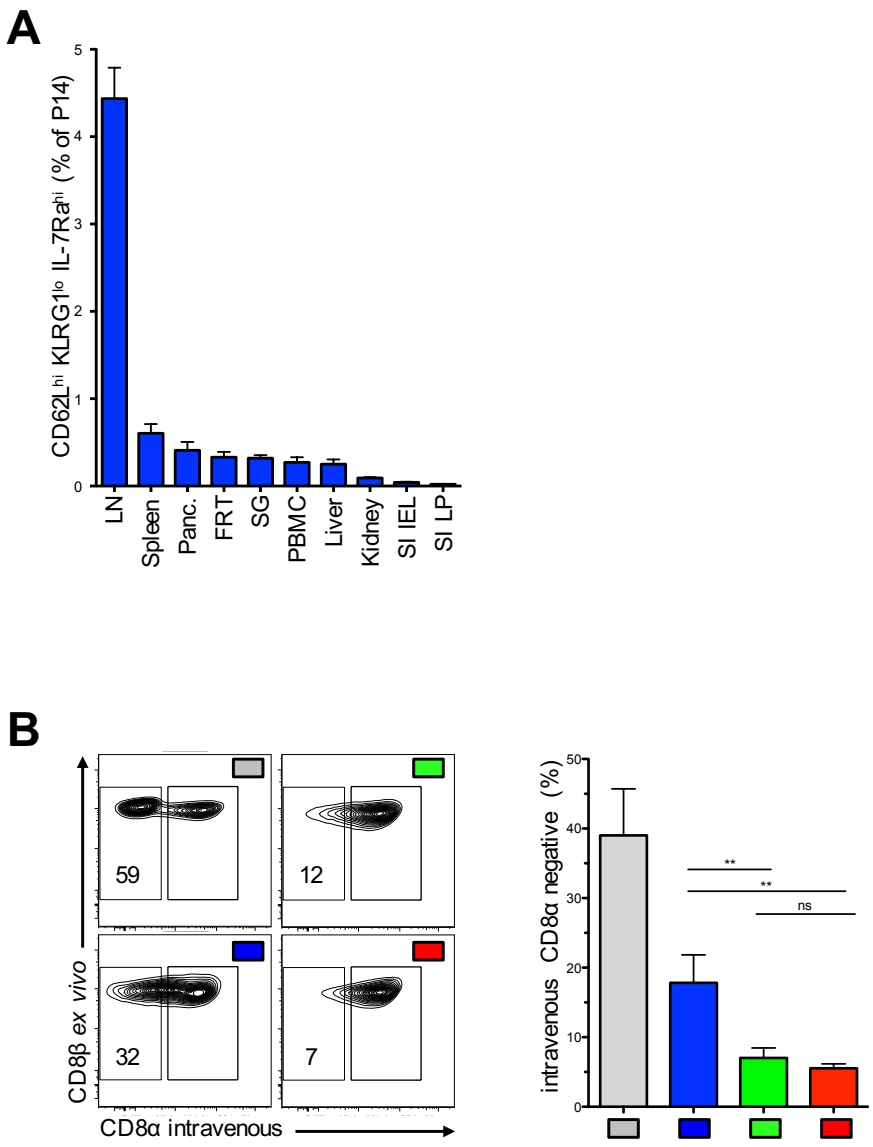


Figure 4.2: Gross- and micro-anatomical analysis reveals preferential lymphoid localization of antigen-specific CD62L^{hi} T stem cells during the CD8^{+} T cell expansion phase of viral infection. A) Frequency of the CD62L^{hi} subset of CD8^{+} T cells (among total P14 cells) in various tissues at d8 pi LCMV-Armstrong. **B)** Accessibility to splenic blood compartment (i.e. red pulp) of the different LCMV-specific CD8^{+} T cell subsets by intravascular antibody staining at d8 pi LCMV.

Figure 4.3

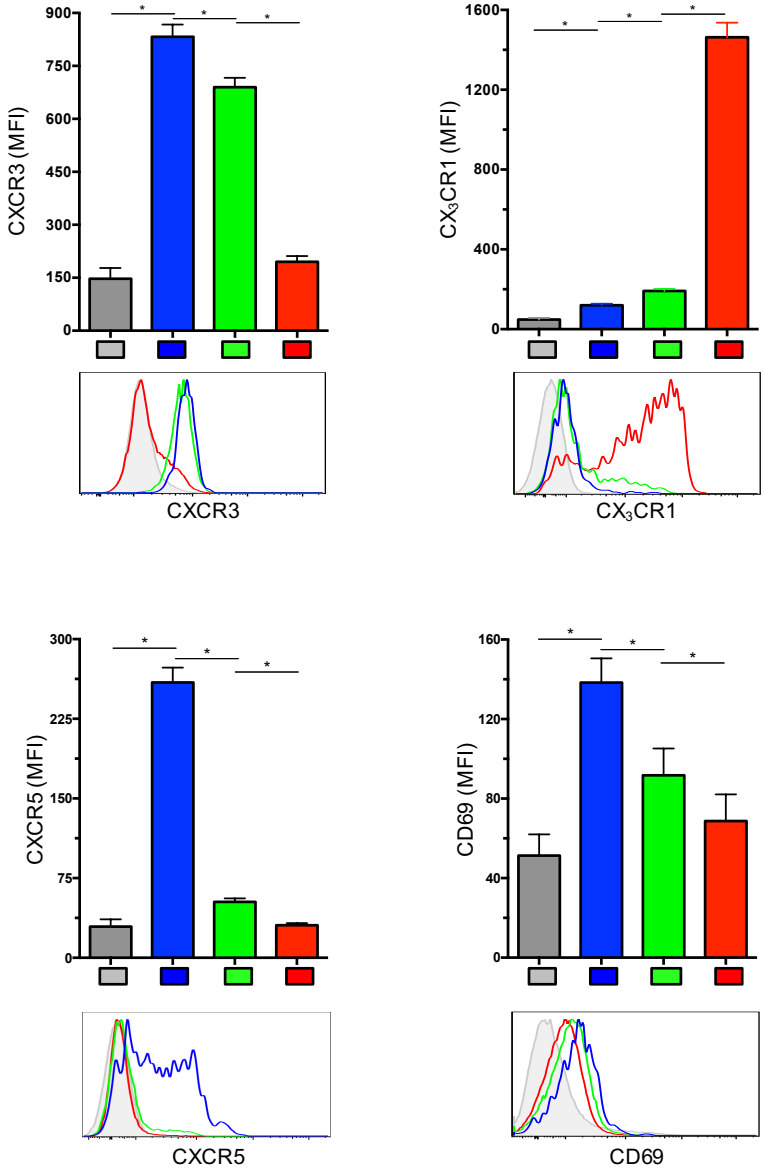


Figure 4.3: Expression of localization molecules on antigen-specific CD8$^+$ T cell subsets during the expansion phase of the CD8$^+$ T cell response to viral infection. Along with naïve CD8$^+$ T cells, subsets of splenic, GP33-specific CD8$^+$ T cells were evaluated at d8 pi LCMV-Armstrong by flow cytometry for expression of CXCR3, CX_3CR1, CXCR5, and CD69.

Figure 4.4

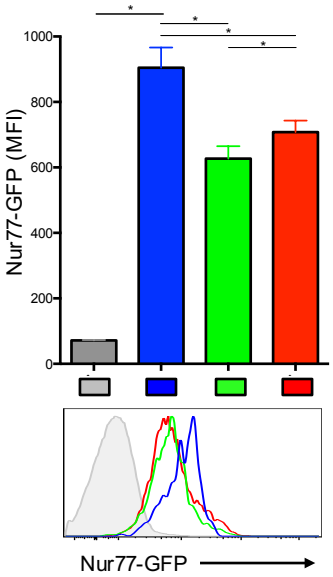


Figure 4.4: Expression of the signal-strength reporter Nur77^{GFP} in subsets of antigen-specific CD8^{+} T cells. P14 CD8^{+} T cells transgenic for a GFP reporter of the TCR-signaling strength-responsive locus Nur77 were transplanted into naïve recipient mice. Following infection with LCMV-Armstrong, subsets of P14 splenocytes were flow-cytometrically evaluated for GFP expression at d5 pi.

Figure 4.5

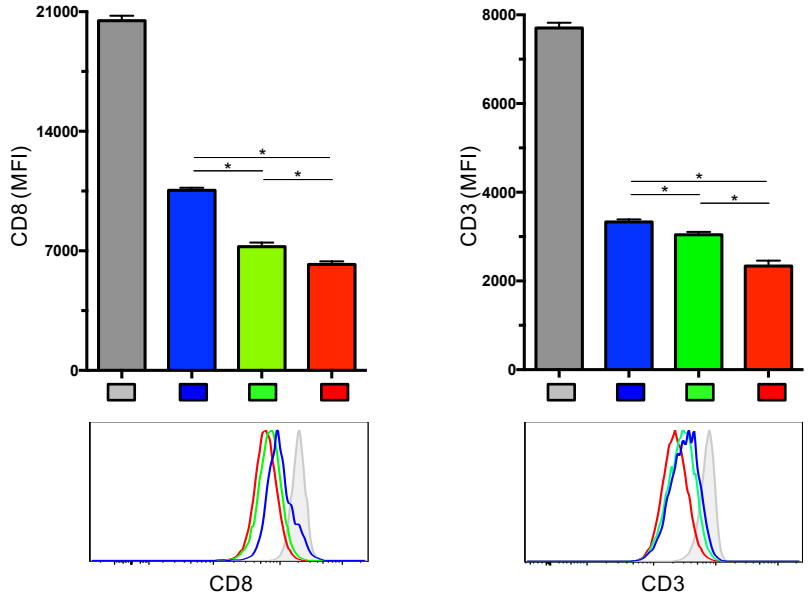


Figure 4.5: Signal 1 -- Robust expression of antigen receptor factors on antigen-specific T stem and progenitor cells during the expansion phase of the CD8$^+$ T cell response to viral infection. Subsets of GP33-specific splenocytes were flow-cytometrically evaluated at d8 pi LCMV-Armstrong for expression of the antigen co-receptor CD8, and CD3, the signal-transducing constituent of the antigen receptor.

Figure 4.6

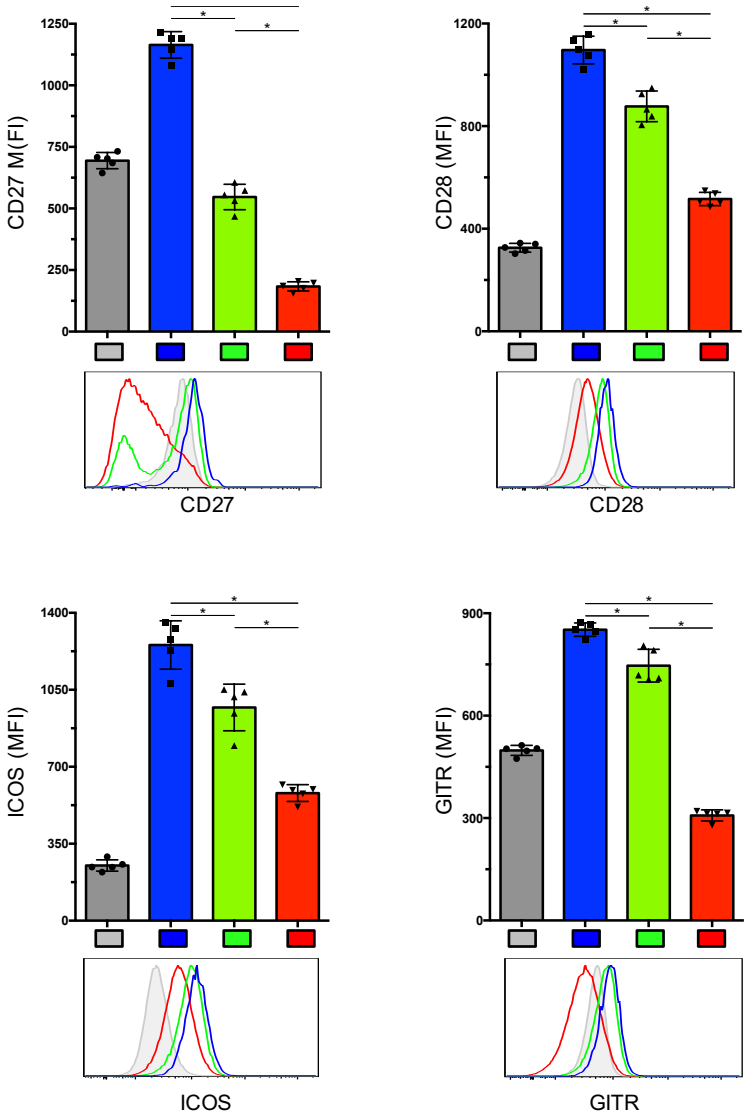


Figure 4.6: Signal 2 -- Robust expression of multiple costimulatory receptors on antigen-specific T stem and progenitor cells during the expansion phase of the CD8$^+$ T cell response to viral infection. Subsets of GP33-specific splenocytes were flow-cytometrically evaluated at d8 pi LCMV-Armstrong for expression of the co-stimulatory receptors CD27, CD28, ICOS, and GITR.

Figure 4.7

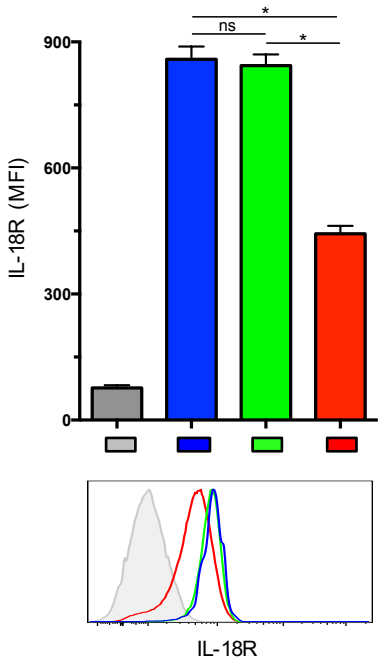


Figure 4.7: Signal 3 -- Elevated expression of IL-18R on antigen-specific T stem and progenitor cells during the expansion phase of the CD8$^+$ T cell response to viral infection. Subsets of GP33-specific splenocytes were flow-cytometrically evaluated at d8 pi LCMV-Armstrong for expression of the receptor for the inflammatory cytokine IL-18.

Figure 4.8

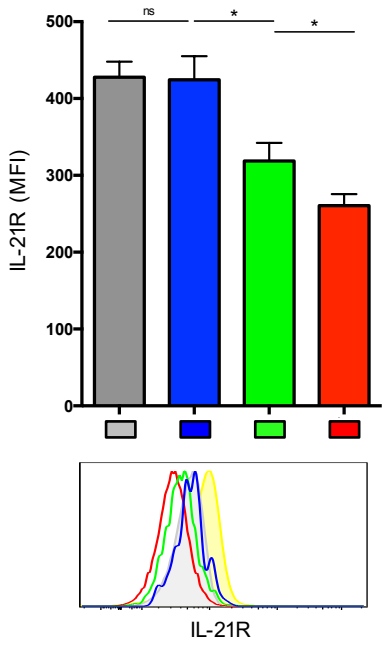


Figure 4.8: Signal 4 -- Elevated expression of IL-21R on antigen-specific T stem and progenitor cells during the expansion phase of the CD8$^+$ T cell response to viral infection. Subsets of GP33-specific splenocytes were flow-cytometrically evaluated at d8 pi LCMV-Armstrong for expression of the IL-21 receptor. As an internal positive control, expression of IL-21R on B cells is depicted in the histogram overlaid in yellow.

Figure 4.9

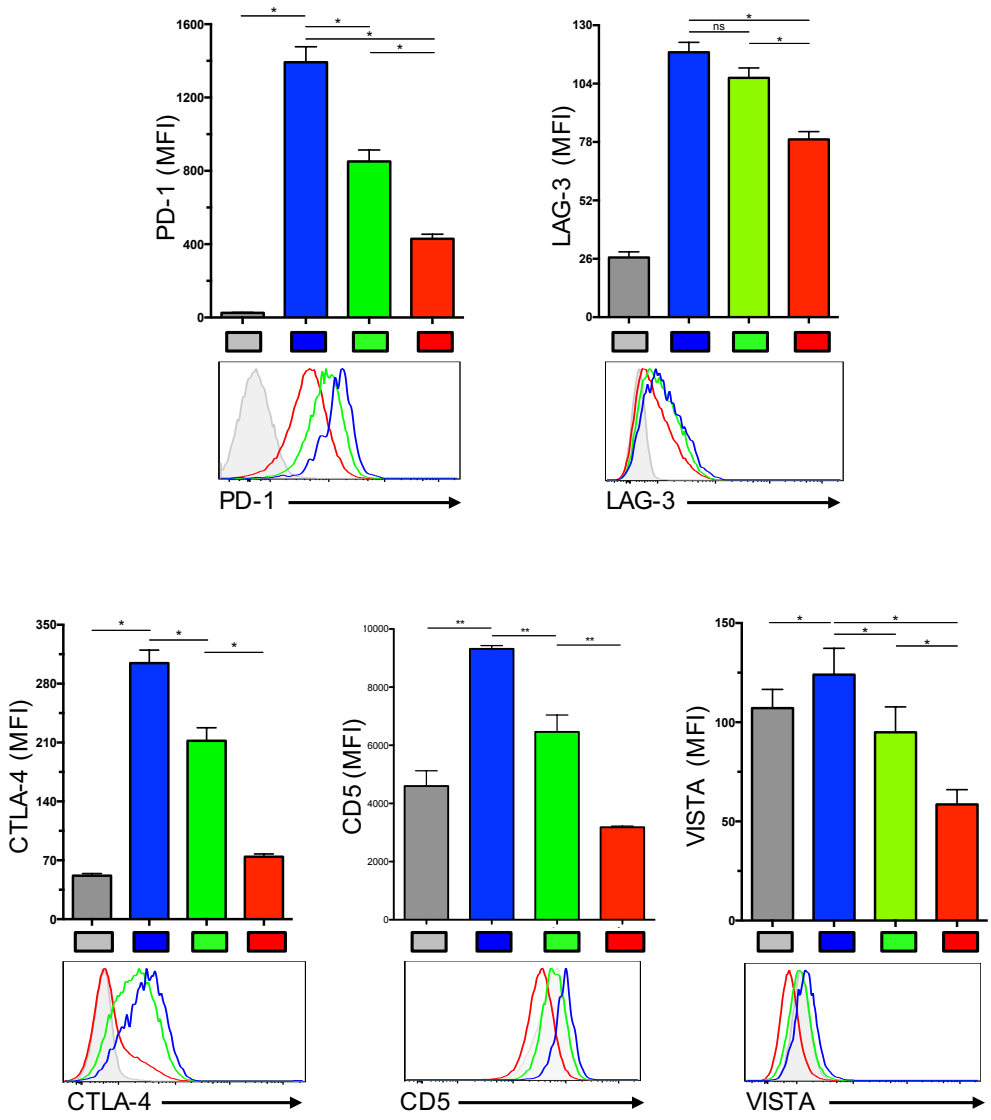


Figure 4.9: Heightened expression of multiple inhibitory receptors on antigen-specific T stem and progenitor cells during the expansion phase of the CD8$^+$ T cell response to viral infection. Subsets of GP33-specific splenocytes were flow-cytometrically evaluated at d8 pi LCMV-Armstrong for expression of the signal-attenuating inhibitory receptors PD-1, LAG-3, CTLA-4, CD5, and VISTA.

Figure 4.10

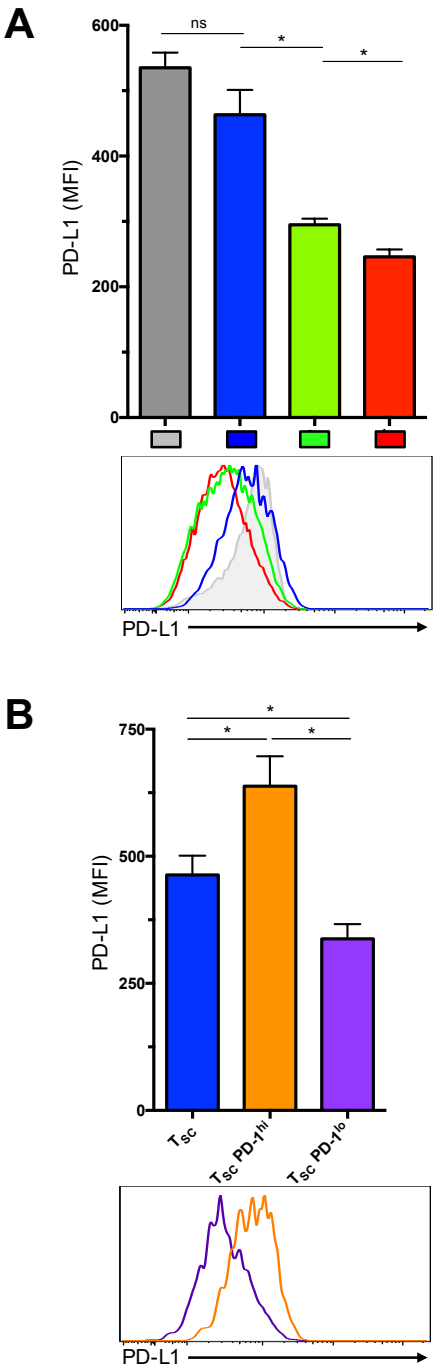


Figure 4.10: Heightened expression of PD-L1 on expansion-phase antigen-specific T stem cells and coordinate regulation of the PD-1/PD-L1 receptor-ligand pair therein. A) Naïve $CD8^+$ T cells and subsets of GP33-specific splenocytes were flow-cytometrically evaluated at d5 pi LCMV-Armstrong for expression of PD-L1, the ligand of the inhibitory receptor PD-1. **B)** The $CD62L^{hi}$ T stem cell population (blue bar), heterogeneous with respect to PD-1 expression, was subsetted based on high (orange bar and histogram) and low (purple bar and histogram) expression of PD-1. PD-L1 was then examined in these $PD\text{-}1^{high/low}$ subsets.

Figure 4.11

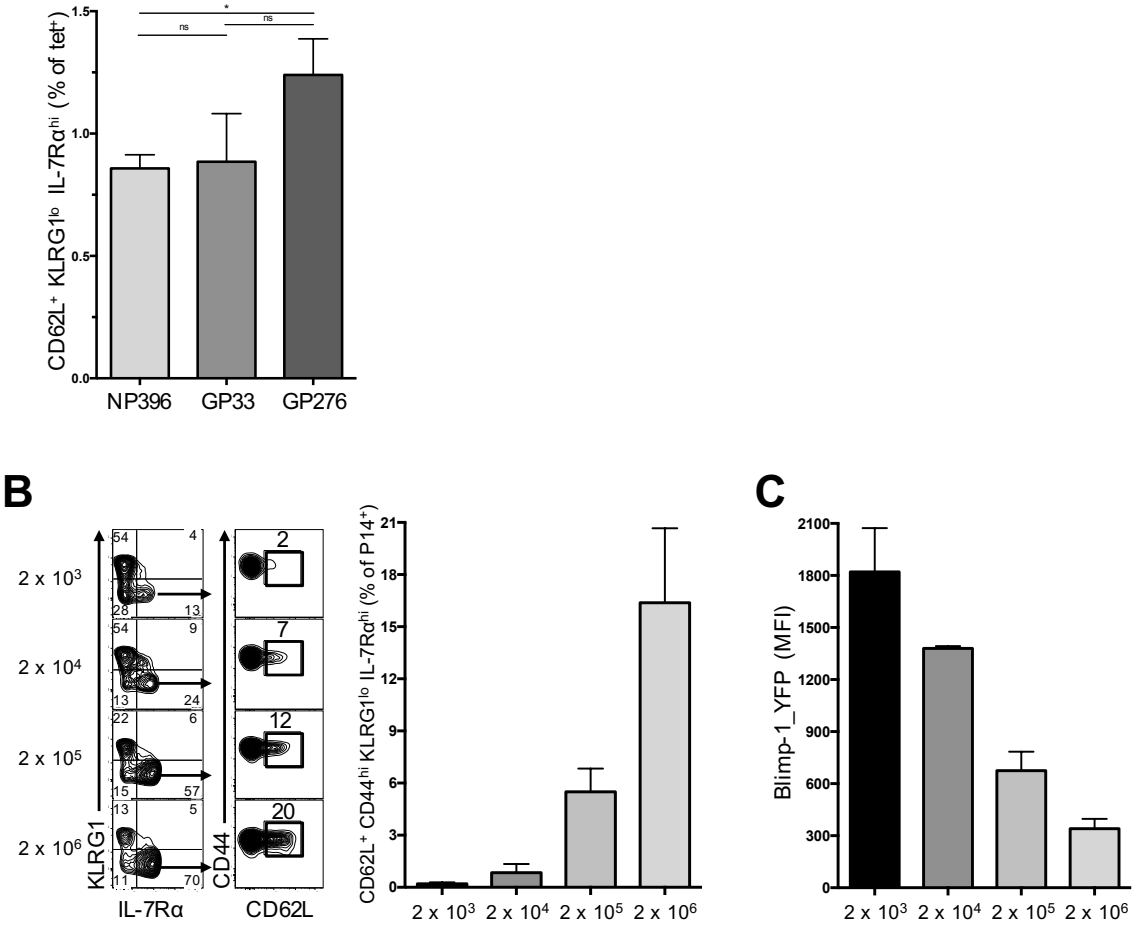


Figure 4.11: Attenuation of signaling strength diminishes antiviral CD8$^+$ T cell effector differentiation and augments T stem and progenitor cell abundance. A) Frequency of the splenic CD62L^{hi} subset at d8 pi for endogenous, non-TCR transgenic, virus-specific CD8$^+$ T cell populations specific for LCMV D^{b}NP$_{396-404}$, D^{b}GP$_{33-41}$, D^{b}GP$_{276-286}$. **B-C)** Naïve P14 CD8$^+$ T cells of increasing numbers (from 2 x 10^3 up to 2 x 10^6) were transplanted into groups of recipient mice. Following infection with LCMV-Armstrong, splenocytes were obtained at d8 pi and evaluated flow cytometrically. **B)** Expression of cardinal markers of effector and memory differentiation (left), and frequency of the CD62L^{hi} subset as a function of the abundance of naïve precursor P14 cells transplanted prior to viral infection. **C)** Expression in the total P14 population of a YFP reporter of Blimp-1 expression as a function of naïve P14 precursor abundance.

Figure 4.12

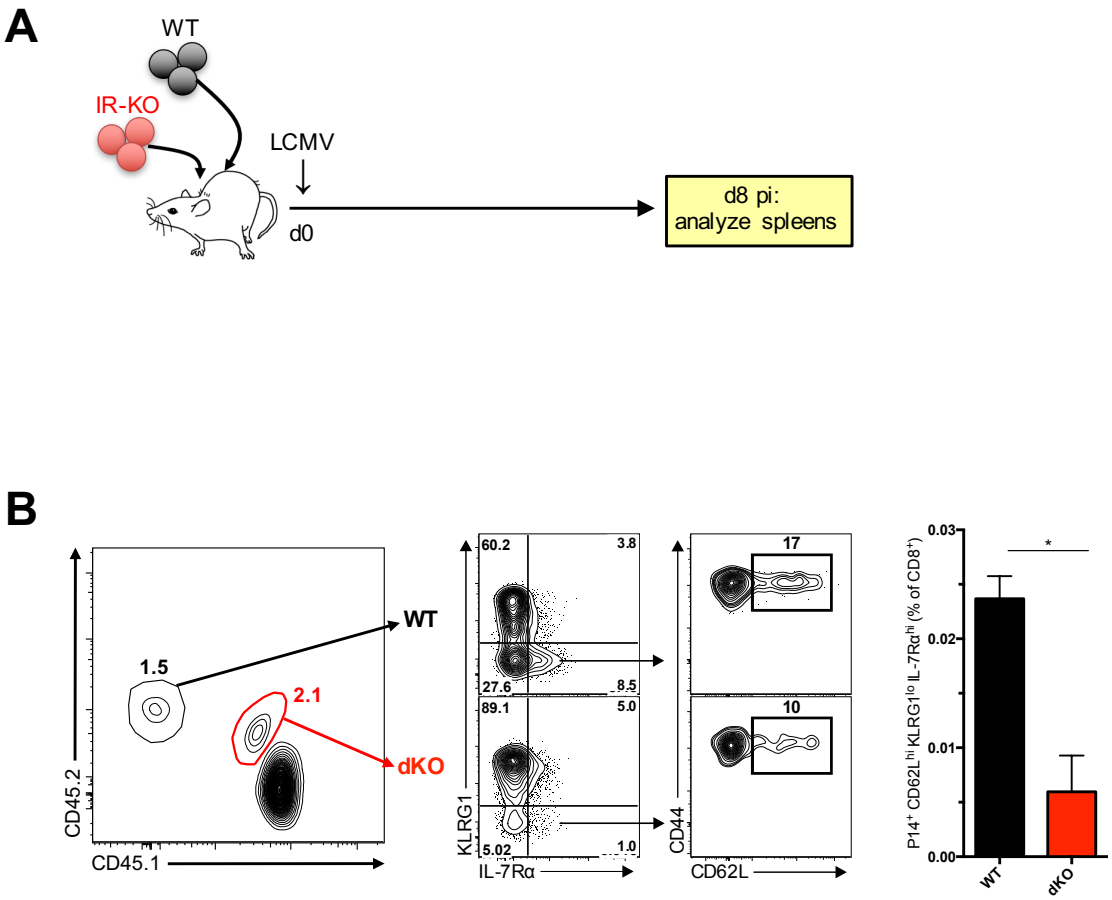


Figure 4.12: Diminished antigen-specific T stem/progenitor compartments in acute viral infection consequent to deficiency of inhibitory receptors. A) Experimental design: differentially congenic WT P14 $CD8^+$ T cells and P14 $CD8^+$ T cells genetically deficient for inhibitory receptors were mixed in a 1:1 ratio and co-transplanted into naïve recipient mice. **B)** Following intraperitoneal infection with LCMV-Armstrong, PBMC were obtained at d8 pi and evaluated flow cytometrically for the subset composition of WT P14 cells, and P14 cells doubly-deficient for PD-1 and LAG-3 ("dKO").

.:: CHAPTER 5 ::.
PERSPECTIVE

5.1 Summation

In recent years substantial progress in basic understanding of adaptive immunity has been matched by an explosion of popular interest in immunological science. Serious infectious disease, once dismissed in industrially developed nations as a mostly vanquished problem, has resurged following recent outbreaks of, to take but two recent examples, measles and Ebola. The former has served to re-introduce a complacent public to the importance of vaccination (Foege, 2001; Orenstein and Ahmdied, 2017), while the latter has underscored the enduring urgency of further development in basic and applied vaccinology. Meanwhile, after nearly a century of dormancy, the notion of harnessing the adaptive immune system's unparalleled potency and specificity to combat cancer is finally beginning to flourish. Advances in cancer immunology are regularly reported in the literature, and even given prominence in the popular press. Furthermore, given the growing appreciation of the immune system as a critical, cell-extrinsic tumor suppressor (Pawelec et al., 2009; Schreiber et al., 2011), interest is emerging in potentiating adaptive immunity not only therapeutically against established cancers but also prophylactically to prevent their occurrence — or reoccurrence, following remission of primary cancers — in the first place.

Ultimate success in all of these avenues of inquiry — both prophylactic and therapeutic manipulation of immunity to counter infection as well as malignancy — depends upon the generation of memory lymphocytes, which can

simultaneously initiate novel immune responses against re-encountered antigens (e.g. in the context of pathogens or tumors) while maintaining the capacity to spawn additional or sustain long-persisting immune responses upon subsequent encounters.

However, despite decades of research and notwithstanding recent major advances in the field, understanding of the developmental origins of memory lymphocytes has remained confounded by dramatically conflicting literatures and glaring gaps in knowledge (Ahmed et al., 2009; Restifo and Gattinoni, 2013).

Empirically, it is straightforward to start with an antigenically inexperienced vertebrate animal, immunize it with it with a defined antigen and a strong adjuvant, and be able to readily identify and isolate memory lymphocytes several weeks later. However, the process of transformation from a naive, transient lymphocyte into a durable, functionally robust memory lymphocyte is largely a black box. What signals and imprints define and potentiate this process? From what developmental origins do memory lymphocytes issue, and when and where during the awesomely complex dynamics of adaptive immune responses do they emerge?

In particular, ever since the seminal publication late last century of an initial developmental structure of memory lymphopoiesis (Sallusto et al., 1999), a fundamental question has concerned the potential existence of lymphocyte stem cells. Given that adaptive immune responses by their very nature entail prodigious proliferation causing the generation and regeneration of lymphocytic

tissue, thinkers in the field noted early on the strong parallels of this process to stem cell-mediated physiology in other tissues with rapid tissue turnover kinetics such as blood, epidermis, intestinal epithelium and sperm (Fearon et al., 2001; Lanzavecchia and Sallusto, 2002; Luckey et al., 2006). If stem cells therein are ultimately responsible for the generation and continual regeneration of mature erythrocytes, epithelial cells, *et cetera*, might a hypothetically equally rare and equally potent lymphocyte stem cell mediate similar functions in the adaptive immune compartment, and perhaps represent the long-sought root origin of memory lymphocytes? If so, could understanding the nature of these agents of regeneration enrich our understanding of both physiology and pathology, and offer novel opportunities for prophylactic and therapeutic interventions?

These considerations have animated the research presented here from its inception, and have culminated in the following findings:

1. **Developmental dynamics of anti-pathogen T cell proliferation**: A classical proliferative hierarchy rooted in a rare yet potent stem cell population drives anti-pathogen $CD8^+$ T lymphopoiesis.

2. **Elucidation of extraordinary developmental plasticity in T lymphopoiesis**: A salvage pathway of $T_{effector}$ to T_{memory} cell dedifferentiation maximizes proliferative reserve available for future immunity and reveals dual cellular pathways to immunological memory.

3. **A new frontier of T cell retrotransposition dynamics**: T cell activation triggers reawakening of an ancient DNA parasite, with implications for an irreversible molecular clock embedded in T stem cells.

4. **Organismal and cellular perspectives on getting old**: Effector T cell differentiation as a microcosm of organismal aging, and a specialized role for stem cells as guardians of genome integrity.

5. **Insight into mechanisms of immunosenescence & immunotherapy**: Heightened expression of inhibitory receptors on and hyperactivation sensitivity of T stem cells.

5.1.1 A classical proliferative hierarchy rooted in a rare yet potent stem cell population drives anti-pathogen $CD8^+$ T lymphopoiesis.

Antigen-driven T cell expansion is structured according to a classical proliferative hierarchy entailing a linear differentiation pathway anchored in a rare yet potent stem cell population that progressively gives rise to increasingly specialized cytotoxic effector T cells. This finding greatly clarifies longstanding questions concerning the developmental nature of danger-driven lymphopoiesis, and provides an answer to the mystery of how antigen-specific effector T cells can be generated with such tremendous dispatch and abundance while nevertheless avoiding severe degradation of the underlying genome integrity needed to preserve the clonal lineage. In short, following activation by encounter with antigen coupled with danger signaling, T stem cells are ontogenetically set-aside and programmatically endowed with a panoply of defense mechanisms and genome-protective activities that *in toto* conduce to maintaining a vital reserve of self-renewing generative capacity. This in turn underwrites immunocompetence against future antigen encounters.

5.1.2 A salvage pathway of $T_{effector}$ to T_{memory} cell dedifferentiation maximizes proliferative reserve available for future immunity and reveals dual cellular pathways to immunological memory.

Chapter 2 presented data indicating that intermediately-differentiated effector $CD8^+$ T cells, having arisen from T stem cells by way of antigen-driven proliferation, can convert into self-renewing $CD8^+$ T stem cells (also called "central memory") following the resolution of infection and the termination of antigen signaling. This process of a cell reacquiring stemness characteristics that it had lost consequent to differentiation is consistent with dedifferentiation, a phenomenon well-known in amphibian biology but poorly understood in mammalian development. Once thought impossible in higher organisms, pivotal research in the 1990's (Wilmut et al., 1997) and in the 2000's (Takahashi and Yamanaka, 2006) upended the traditional, Waddington-esque understanding of unyielding epigenetic gravity by elucidating the existence and extraordinary potential of mammalian dedifferentiation (e.g. organismal cloning and the *in vitro* induction of developmental pluripotency in fully differentiated cells). Though these initial achievements occurred exclusively in highly contrived, experimental circumstances, they nevertheless fomented further inquiry and lead to recognition by the Nobel Prize for Physiology or Medicine in 2012. More recent findings have extended these groundbreaking observations *in vivo*, including during the regeneration of spermatogenesis (Nakagawa et al., 2010) and epithelial differentiation following injury (Takeda et al., 2011; Tata et al., 2013), and in the pathological context of tumorigenesis (Krivtsov et al., 2006). Nevertheless, to date there have not been any reports of physiological dedifferentiation in the immuno-hematopoietic system.

Thus, the observation of potential physiological dedifferentiation in the adaptive immune system is novel and may bear substantial implications for basic principles in developmental biology, genome integrity maintenance and the architecture of aging, and mechanisms of malignant transformation of non-stem progenitor cells into cancer stem cells.

5.1.3 T cell activation triggers reawakening of an ancient DNA parasite, with implications for an irreversible molecular clock embedded in T stem cells.

The coupled observations during memory and effector T cell differentiation of the inversely correlated expression of the LINE-1 retrotransposon and one of its principle restriction factors, Apobec3, may represent a new frontier in immunology and developmental biology more generally. First discovered during the dawn of modern eukaryotic whole genome study (Adams et al.,1980), L1 transposons for decades have often been considered genetic detritus whose extensive repeat sequences principally served to confound early genome sequencing efforts. Recently however, L1 transposons have emerged as major players in physiology, pathology, aging, and evolution (Gorbunova et al., 2014) – as foretold by Barbara McClintock's trailblazing research on "jumping genes" (McClintock, 1950). Retrotransposition into quasi-random locations throughout the genome and can be substantially genotoxic, by way of either insertional mutagenesis or the introduction of double strand breaks. Accordingly, cells have evolved to expend considerable efforts to suppress L1 expression.

The data presented in Chapter 3 indicate that L1 repression is disrupted

by antiviral T cell activation. L1 expression closely correlated with extent of effector differentiation, with the greatest expression in terminally differentiated effector cells, and the least in minimally differentiated CD62L^{hi} T stem cells. This phenomenon may have several major implications, including:

- L1 copy number may appreciably increase as a function of extent of differentiation; conversely, overall genome integrity may diminish consequent to L1-mediated insertional mutagenesis and/or double strand breaks.

- Though T stem cells robustly express the L1 restriction factor Apobec3, a modest level of L1 expression nevertheless escapes this putative suppression. Thus, at a much slower rate – relative to differentiated effector T cells – L1 copy number may increase in T stem cells, especially following repeated rounds of activation and/or proliferation. Since T stem cells are self-renewing and apparently long-lived, L1 abundance therein may represent an irreversible molecular clock that can be measured in a variety of experimental and possibly even clinical/diagnostic applications. It is worth noting that all present molecular clocks (e.g. telomere length) are known to be reversible in at least some cases, and may therefore be incompletely reliable.

- A notable corollary of these notions is the possibility of ubiquitous genotypic mosaicism in antigen-experienced T cells. That is, given activation-induced L1 derepression and random retrotransposition, it is highly possible that every single descendent of an activated progenitor T cell bears a unique genome characterized by distinct L1 insertion sites.

- A further corollary is that shared and unique L1 insertion sites can be used to reconstruct developmental phylogenies and thereby retrospectively delineate lineage relationships *in vivo*, potentially in both model organism systems and humans, and in the absence of transgenic manipulations that can confound similar approaches that rely on genetic engineering or transduction with exogenous retroviruses.

- The research herein on effector and memory T cell differentiation has been significantly informed by the rich literature of progenitor cell biology that has matured since Charles Leblond's pioneering research on mammalian tissue turnover *in vivo* using tritiated thymidine (Leblond et al., 1959). In turn it may be possible that the lessons concerning T stemness and the dynamics of effector differentiation gained in this dissertation research can inform novel, general principles in developmental biology. In particular, if differentiation of effector T cells from a relatively quiescent, geno-protective T stem cell source entails derepression of LINE-1 retrotransposons, it is reasonable to hypothesize that a similar phenomenon might characterize classical proliferative hierarchies in other tissues, for example those that subserve ongoing hematopoiesis, epitheliogenesis, neurogenesis, and spermatogenesis. Are terminally differentiated hematopoietic cells (e.g. granulocytes), neurons, and epithelial cells characterized by the scars of accrued L1 retrotransposition? Does L1 copy number slowly increase in other stem cell populations in blood, gut, skin, brain, etc.?

In addition to these implications, the phenomenon of L1 derepression consequent to T cell activation prompts several questions, including:

- Is LINE-1 re-expression related to – either casually or consequentially – the substantial epigenetic remodeling entailed in the transitions from a quiescent naïve lymphocyte through to a blasting, cytotoxic effector T cell? There are some indications in the literature that this may be the case – for example, iPSC reprogramming is known to precipitate retrotransposon derepression (Klawitter et al., 2016), and epigenetic therapy of lung cancers (e.g. with HDAC or DNMT inhibitors) has been reported to relax suppression of dormant endogenous retroviruses in an interferon-dependent manner (Chiappinelli et al., 2015). Given that genetic parasites comprise not just a fraction but rather the majority of our genome (de Koning et al., 2011), a reshuffling of these elements may be an inescapable intermediate in major and/or rapid transformations of epigenetic states.

- While L1 transposons comprise nearly a fifth of the genome, they are in fact a minority of the total complement of endogenous replicating elements. This prompts the question of whether other known DNA parasites (for example so-called Alu transposons, or endogenous retroviruses) also are derepressed by T cell activation and effector differentiation, and if they might also pose a risk of insertional mutagenesis.

5.1.4 Effector T cell differentiation as a microcosm of organismal aging, and a specialized role for stem cells as guardians of genome integrity.

While stem cells are commonly regarded as residing in an amorphous, undifferentiated state, for some time thinkers have proposed that instead they represent a highly specialized cell type that can efficiently conserve genome integrity in order to retard malignant transformation or aging (Cairns, 1975; Schofield, 1978). Nevertheless, evidentiary support for this fundamental proposition in stem cell biology is remarkably limited. In this light, the data in Chapters 2 and 3 substantially enrich this idea, revealing that multiple independent pathways that can regulate genomic integrity are enriched in T stem cells, and conversely, relatively suppressed in terminally differentiated effector cells. These include:

- telomerase activity
- responsiveness to DNA damage via H2AX phosphorylation
- expression of the endogenous retroelement restriction factor Apobec3
- cell surface membrane efflux of environmental toxins via ABC transporters

These observations concerning geno-protective activities are complemented by data on genome integrity itself. Specifically, comet assay measurements indicated that chromatin integrity is significantly diminished in terminally differentiated effector cells a week after initial activation of precursor naïve T cells. This suggests the possibility that antiviral T cell differentiation may represent a microcosm of organismal aging, which is also characterized – both causally and consequentially – by accumulation of senescent cells and declining genome integrity. That these processes which unfold over a lifespan occur in the

context of infection within just a week is biologically interesting in its own right, and additionally may offer opportunities for dissecting mechanisms of aging in a tractable, short-term experimental system. For example, rapamycin was the first drug discovered to increase mammalian lifespan (Harrison et al., 2009). However, this advance came only by way of years-long, resource-intensive experimentation. In contrast, during the critical first week of antiviral T cell differentiation, *in vivo* treatment with rapamycin increases the abundance of $CD62L^{hi}$ T stem cells **(Figure 2.9)**, and thereby putatively enhances the genome integrity of the entire antigen-specific "tissue". Anti-pathogen T cell differentiation may thus be a useful context in which to rapidly screen various pharmacological or other interventions for potential anti-aging effects.

5.1.5 Heightened expression of environmental signaling receptors on and hyperactivation sensitivity of T stem cells

In the first decade of the 20^{th} century Paul Ehrlich proposed the deeply controversial idea that the immune system might play a central physiological role not only in defense against infectious disease but also susceptibility to cancer (Ehrlich, 1909). Despite promising early experimental and clinical explorations of this concept by Ehrlich and Robert Koch in Europe and William Coley in New York, further development of immunotherapy suffered from a feeble theoretical framework and fell out of favor as the post-war vogues of radiotherapy and chemotherapy captured medical attention to the exclusion of alternative approaches. Half a century later, Lewis Thomas and Macfarlane Burnet re-energized the notion on firmer theoretical grounds (Thomas, 1959; Burnet, 1967),

and Burnet helpfully coined the term “immunosurveillance”. Evidentiary support trickled in for several decades and finally exploded in recent years as two different immunotherapy modalities began generating dramatic clinical results, including complete and durable regression of even disseminated, metastatic tumors: autologous transplantation of T cells reprogrammed *ex vivo*, and pharmacological blockade of inhibitory / checkpoint receptors.

Despite these gains, meaningful clinical response to immunotherapies is achieved in only a minority of patients. Investigators globally are now focused on understanding this heterogeneity in efficacy in order to realize the full potential of Ehrlich’s century-old idea. In this vein, important observations have revealed that the success of anti-tumor T cell transplantation therapy is a function of self-renewal capacity (Gattinoni et al., 2005; Gattinoni et al., 2011). In parallel, seminal research from multiple groups around the globe has shown that expression of inhibitory receptors including PD-1 is a major impediment to anti-tumor T cell efficacy, and that pharmacological blockade of PD-1 signaling can overcome this obstacle to unleash anti-tumor immunity (Wherry and Kurachi, 2015; Chen and Mellman, 2017).

Thus the central issues facing the field include the separate but related matters of 1) unfettering and 2) sustaining T cell proliferation that can mediate anti-tumor immunity. What is the nature of proliferative capacity in antigen-specific T cell populations, and how can it be maximized? What are the mechanisms of inhibitory receptor blockade, particularly with respect to

differential sensitivity of and impact upon subsets of antigen-specific T cells – which in turn are endowed with differing self-renewal capacities?

The characterizations of T stem cell biology here offer substantial potential to elucidate both of these intertwined issues. In particular, T stem cells are a principal locus of proliferative reserve from which legions of effector T cells are spawned. They exist at a nexus of intense environmental signaling and therefore robustly express multiple inhibitory receptors, including PD-1, and at least in the contexts so far examined, to an extent greater than other T cell subpopulations. The data detailed in Chapter 4 suggests that prolonged stimulation of T stem cells genetically lacking PD-1 braking function results in depletion of this quantitatively minor yet physiologically central subpopulation, perhaps conducing to accelerated immunosenescence. Both from the perspectives of maximizing proliferative potential and avoiding immunosenescence, and understanding the biology of inhibitory receptors like PD-1 and the mechanisms and consequences of blockade thereof, further understanding of the nature of T stem cells will be important.

APPENDIX A: MATERIALS AND METHODS

Mice and infections

All animals were purchased from Jackson Laboratories or Charles River Laboratories, or bred at the University of Pennsylvania (Philadelphia, PA). Experiments were performed in accordance with protocols approved by the University of Pennsylvania Institutional Animal Care and Use Committee. BAC-transgenic Blimp-1-YFP reporter mice were from E. Meffre (Yale University, New Haven, CT), Fucci reporter mice (Sakaue-Sawano et al., 2008) were from Yongwon Choi (University of Pennsylvania, Philadelphia, PA). Mice deficient for TCF-1, PD-1, and LAG-3 were gifts of Avinash Bhandoola (National Cancer Institute, Bethesda, MD), Arlene Sharpe (Harvard Medical School, Boston, MA), and Dario Vignali (University of Pittsburgh School of Medicine, Pittsburgh, PA), respectively. Nur77^{GFP} reporter mice and CD90.1^{+} mice were obtained from Jackson Laboratories. Deficiency, reporter, and congenic alleles were all bred on to a P14 TCR-transgenic (Pircher et al., 1989) C57Bl/6 background.

Mice were infected with 2 × 10^5 plaque-forming units (PFUs) of lymphocytic choriomeningitis virus (LCMV) Armstrong strain intraperitoneally or 2-4 ×10^6 PFU LCMV clone 13 strain intravenously, as previously described (Wherry et al., 2003). Viruses were grown and titered as previously described (Wherry et al., 2003).

Adoptive transfers and lymphocyte isolation

At indicated days pi, spleens and lymph nodes were harvested from CD90.2^{+} donor mice. CD90.1^{+} P14 CD8^{+} T cells were negatively purified by way of staining with biotinylated monoclonal antibodies (CD4 #RM4-5, CD19 #1D3, CD90.2 #53-2.1, TER-119, NK1.1 # PK136, Ly-6G #1A8) followed by counter-staining with streptavidin-conjugated magnetic beads (EasySep Streptavidin RapidSpheres, from STEMCELL Technologies of Vancouver, Canada) and magnetic separation. Subsequently sorted cell populations were adoptively

transferred intravenously into recipient mice. In some experiments, before transfer cells were labeled with 10 μM of the proliferation tracking vital dye CellTrace Violet ("CTV", from Thermo Fisher Scientific of Waltham, MA) for 10 minutes in the dark at 24°C.

BrdU treatment and detection

Animals were treated with 2 mg of BrdU (Sigma-Aldrich of St. Louis, MO) i.p. 24 hours prior to tissue harvest and analysis. BrdU incorporation was assessed by the BrdU Flow Kit per manufacturer's instructions (BD Biosciences of Franklin Lakes, NJ).

Flow cytometry

Cells were stained with LIVE/DEAD Fixable Dead Cell Stain (Thermo Fisher Scientific) to discern cell viability. Surface staining was performed at 4° in the dark for 45 minutes in RPMI-1640 medium supplemented with 5 mM HEPES. Intracellular staining was performed using the Foxp3 / Transcription Factor Staining Buffer Set (Affymetrix, of San Diego, CA) or Cytofix/Cytoperm reagents (BD Biosciences) per manufacturer's instructions. Monoclonal antibodies were purchased from BD Biosciences; BioLegend of San Diego, CA; Affymetrix; Cell Signaling Technology of Danvers, MA; EMD Millipore of Billerica, MA; and SouthernBiotech of Birmingham, AL; and included: CD8α #53-6.7, CD8β #YTS156.7.7, CD16/32 #93, CD27 #LG.7F9, CD43 #1B11, CD44 #IM7, CD45.1 #A20, CD62L #MEL-14, CD90.1 #OX-7, CCR7 #4B12, IL-7Rα #A7R34, PD-1 #RMP1-30, LAG-3 #C9B7W, CTLA-4 #UC10-4F10-11, CD5 #53-7.3, CXCR5 #L138D7, KLRG1 #2F1, CX_3CR1 #SA011F11, IFN-γ #XMG1.2, TNF-α #MP6-XT22, IL-2 #JES6-SH4, T-bet #4B10, Eomes #Dan11mag, TCF-1 #C63D9, pSer139-H2AX #JBW301, Ki-67. For intracellular cytokine staining, single-cell suspensions were pre-treated for 1 hour with 0.1 mM TAPI-2 (Jabbari and Harty, 2006) and then incubated for 5 hours at 37°C in the presence of brefeldin A (BD Biosciences), with or without 1 μM GP_{33-41} peptide. MHC class I peptide tetramers were made and used as described previously (Wherry et al., 2003).

Data were acquired on a LSR II Flow Cytometer (BD Biosciences) and analyzed with FlowJo software (FlowJo LLC of Ashland, OR). Cell sorting was performed using a FACSAria II (BD Biosciences).

Ex vivo irradiation and pSer139-H2AX inducibility assay

Single cell suspensions of splenocytes were exposed to 0 or 250 rads of gamma irradiation and incubated for 90 minutes at 37°C in RPMI supplemented with 10% FBS. Cells were then stained for surface antigens and intracellular pSer139-H2AX and acquired flow cytometrically.

Doxorubicin treatment

At the indicated time point post infection, mice were treated intravenously with PBS or doxorubicin at 8 mg/kg or 16 mg/kg.

Comet assay

The alkaline comet assay was performed as described (Olive and Banáth, 2006), with minor modifications. Briefly, a single-cell suspension of splenocytes was embedded in molten agarose on CometSlides (Trevigen of Gaithersburg, MD) and subjected to alkaline lysis for 1 hr. Following electrophoresis cells were stained with ethidium bromide and 100-300 cells per slide were imaged microscopically. Tail moments were evaluated using CASP software (Końca et al., 2003).

Telomerase activity assay

The Telomeric Repeat Amplification protocol was performed as described (Herbert et al., 2006). Heat-inactivated 293T cells were used as a negative control.

Side population assay

The side population assay was performed as described (Lin and Goodell., 2006), with minor adaptations. Briefly, a single cell suspension was stained with

Hoechst 33342 and incubated for 90 minutes at 37°C. Cells were washed, stained with surface antibodies and 7-AAD for viability discrimination at 4°C, and finally resuspended and evaluated using an LSR II equipped with a 355 nm ultraviolet laser.

Statistical Analysis

Student's t test (paired and unpaired), and ANOVA tests were performed using Prism software (Graphpad, La Jolla, CA). Asterisks indicate statistical significance ($p < 0.05$). Error bars depict mean ± SEM.

APPENDIX B: BIBLIOGRAPHY

Ackermann M, Stearns SC, Jenal U. Senescence in a bacterium with asymmetric division. Science. 2003 Jun 20;300(5627):1920.

Adams JW, Kaufman RE, Kretschmer PJ, Harrison M, Nienhuis AW. A family of long reiterated DNA sequences, one copy of which is next to the human beta globin gene. Nucleic Acids Res. 1980 Dec 20;8(24):6113-28.

Ahmed R, Bevan MJ, Reiner SL, Fearon DT. The precursors of memory: models and controversies. Nat Rev Immunol. 2009 Sep;9(9):662-8.

Ahmed R, Gray D. Immunological memory and protective immunity: understanding their relation. Science. 1996 Apr 5;272(5258):54-60.

Ali SR, Hippenmeyer S, Saadat LV, Luo L, Weissman IL, Ardehali R. Existing cardiomyocytes generate cardiomyocytes at a low rate after birth in mice. Proc Natl Acad Sci U S A. 2014 Jun 17;111(24):8850-5.

Allsopp RC, Morin GB, DePinho R, Harley CB, Weissman IL. Telomerase is required to slow telomere shortening and extend replicative lifespan of HSCs during serial transplantation. Blood. 2003 Jul 15;102(2):517-20.

Altman, J.D., Moss, P., Goulder, P., Barouch, D.H., McHeyzer, W.M., Bell, J.I., McMichael, A.J., and Davis, M.M. (1996). Phenotypic analysis of antigen-specific T lymphocytes. Science 274, 94–96.

Anderson KG, Mayer-Barber K, Sung H, Beura L, James BR, Taylor JJ, Qunaj L, Griffith TS, Vezys V, Barber DL, Masopust D. Intravascular staining for discrimination of vascular and tissue leukocytes. Nat Protoc. 2014 Jan;9(1):209-22.

Attanasio J, Wherry EJ. Costimulatory and Coinhibitory Receptor Pathways in Infectious Disease. Immunity. 2016 May 17;44(5):1052-68.

Azzam HS, Grinberg A, Lui K, Shen H, Shores EW, Love PE. CD5 expression is developmentally regulated by T cell receptor (TCR) signals and TCR avidity. J Exp Med. 1998 Dec 21;188(12):2301-11.

Baker DJ, Wijshake T, Tchkonia T, LeBrasseur NK, Childs BG, van de Sluis B, Kirkland JL, van Deursen JM. Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. Nature. 2011 Nov 2;479(7372):232-6.

Baldridge MT, King KY, Boles NC, Weksberg DC, Goodell MA. Quiescent haematopoietic stem cells are activated by IFN-gamma in response to chronic infection. Nature. 2010 Jun 10;465(7299):793-7.

Banerjee A, Gordon SM, Intlekofer AM, Paley MA, Mooney EC, Lindsten T, Wherry EJ, Reiner SL. Cutting edge: The transcription factor eomesodermin enables CD8+ T cells to compete for the memory cell niche. J Immunol. 2010 Nov 1;185(9):4988-92.

Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, Freeman GJ, Ahmed R. Restoring function in exhausted CD8 T cells during chronic viral infection. Nature. 2006 Feb 9;439(7077):682-7.

Baserga, R. The Biology of Cell Reproduction, Harvard University Press, 1985

Baxby D. Edward Jenner's Inquiry; a bicentenary analysis. Vaccine. 1999 Jan 28;17(4):301-7.

Becker AJ, McCulloch EA, Siminovitch L, Till JE. The effect of differing demands for blood cell production on DNA synthesis by hemopoietic colony-forming cells of mice. Blood. 1965 Sep;26:296-308.

Becker AJ, McCulloch EA, Till JE. Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. Nature. 1963 Feb 2;197:452-4.

Belanger LF, Leblond CP. A method for locating radioactive elements in tissues by covering histological sections with a photographic emulsion. Endocrinology. 1946 Jul;39:8-13

Bennett G, Bergeron J. Charles Leblond 1910-2007. Nat Cell Biol. 2007 Jul;9(7):723.

Bergmann O, Zdunek S, Felker A, Salehpour M, Alkass K, Bernard S, Sjostrom SL, Szewczykowska M, Jackowska T, Dos Remedios C, Malm T, Andrä M, Jashari R, Nyengaard JR, Possnert G, Jovinge S, Druid H, Frisén J. Dynamics of Cell Generation and Turnover in the Human Heart. Cell. 2015 Jun 18;161(7):1566-75.

Bernitz JM, Kim HS, MacArthur B, Sieburg H, Moore K. Hematopoietic Stem Cells Count and Remember Self-Renewal Divisions. Cell. 2016 Nov 17;167(5):1296-1309.e10.

Blackburn SD, Shin H, Haining WN, Zou T, Workman CJ, Polley A, Betts MR, Freeman GJ, Vignali DA, Wherry EJ. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. Nat Immunol. 2009 Jan;10(1):29-37.

Blanpain C, Mohrin M, Sotiropoulou PA, Passegue E. DNA-damage response in tissue-specific and cancer stem cells.

Cell Stem Cell. 2011 Jan 7;8(1):16-29.

Bluestone JA, Herold K, Eisenbarth G. Genetics, pathogenesis and clinical interventions in type 1 diabetes. Nature. 2010 Apr 29;464(7293):1293-300.

Bodak M, Yu J, Ciaudo C. Regulation of LINE-1 in mammals. Biomol Concepts. 2014 Oct;5(5):409-28.

Boehm AM, Khalturin K, Anton-Erxleben F, Hemmrich G, Klostermeier UC, Lopez-Quintero JA, Oberg HH, Puchert M, Rosenstiel P, Wittlieb J, Bosch TC.

FoxO is a critical regulator of stem cell maintenance in immortal Hydra. Proc Natl Acad Sci U S A. 2012 Nov 27;109(48):19697-702.

Bonilla WV, Fröhlich A, Senn K, Kallert S, Fernandez M, Johnson S, Kreutzfeldt M, Hegazy AN, Schrick C, Fallon PG, Klemenz R, Nakae S, Adler H, Merkler D, Löhning M, Pinschewer DD. The alarmin interleukin-33 drives protective antiviral $CD8^+$ T cell responses. Science. 2012 Feb 24;335(6071):984-9.

Bonner WM, Redon CE, Dickey JS, Nakamura AJ, Sedelnikova OA, Solier S, Pommier Y. GammaH2AX and cancer. Nat Rev Cancer. 2008 Dec;8(12):957-67.

Brawley C, Matunis E. Regeneration of male germline stem cells by spermatogonial dedifferentiation in vivo. Science. 2004 May 28;304(5675):1331-4.

Bretscher P, Cohn M. A theory of self-nonself discrimination. Science. 1970 Sep 11;169(3950):1042-9.

Bruce WR, Meeker BE, Valeriote FA. Comparison of the sensitivity of normal hematopoietic and transplanted lymphoma colony-forming cells to chemotherapeutic agents administered in vivo. J Natl Cancer Inst. 1966 Aug;37(2):233-45.

Brunauer R, Kennedy BK. Progeria accelerates adult stem cell aging. Science. 2015 Jun 5;348(6239):1093-4.

Buchholz VR, Flossdorf M, Hensel I, Kretschmer L, Weissbrich B, Gräf P, Verschoor A, Schiemann M, Höfer T, Busch DH. Disparate individual fates compose robust CD8+ T cell immunity. Science. 2013 May 3;340(6132):630-5.

Bunting KD, Zhou S, Lu T, Sorrentino BP. Enforced P-glycoprotein pump function in murine bone marrow cells results in expansion of side population stem cells in vitro and repopulating cells in vivo. Blood. 2000 Aug 1;96(3):902-9.

Burnet FM. A genetic interpretation of ageing. Lancet. 1973 Sep 1;2(7827):480-3.

Burnet FM. Auto-immune disease. II. Pathology of the immune response. Br Med J. 1959 Oct 17;2(5154):720-5.

Burnet FM. Immunological aspects of malignant disease. Lancet. 1967 Jun 3;1(7501):1171-4.

Burnet FM. Intrinsic mutagenesis: a genetic basis of ageing. Pathology. 1974 Jan;6(1):1-11.

Burnet FM. The concept of immunological surveillance. Prog Exp Tumor Res. 1970;13:1-27.

Burtner CR, Kennedy BK. Progeria syndromes and ageing: what is the connection? Nat Rev Mol Cell Biol. 2010 Aug;11(8):567-78.

Butz EA, Bevan MJ. Massive expansion of antigen-specific CD8+ T cells during an acute virus infection. Immunity. 1998 Feb;8(2):167-75.

Byrne JA, Oldstone MB. Biology of cloned cytotoxic T lymphocytes specific for lymphocytic choriomeningitis virus: clearance of virus in vivo. J Virol. 1984 Sep;51(3):682-6.

Cairns J. Mutation selection and the natural history of cancer. Nature. 1975 May 15;255(5505):197-200.

Caldwell CC, Kojima H, Lukashev D, Armstrong J, Farber M, Apasov SG, Sitkovsky MV. Differential effects of physiologically relevant hypoxic conditions on T lymphocyte development and effector functions. J Immunol. 2001 Dec 1;167(11):6140-9.

Canela A, Sridharan S, Sciascia N, Tubbs A, Meltzer P, Sleckman BP, Nussenzweig A. DNA Breaks and End Resection Measured Genome-wide by End Sequencing. Mol Cell. 2016 Sep 1;63(5):898-911.

Cao W, Henry MD, Borrow P, Yamada H, Elder JH, Ravkov EV, Nichol ST, Compans RW, Campbell KP, Oldstone MB. Identification of alpha-dystroglycan as a receptor for lymphocytic choriomeningitis virus and Lassa fever virus. Science. 1998 Dec 11;282(5396):2079-81.

Castro F, Leal B, Denny A, Bahar R, Lampkin S, Reddick R, Lu S, Gravekamp C. Vaccination with Mage-b DNA induces CD8 T-cell responses at young but not old age in mice with metastatic breast cancer. Br J Cancer. 2009 Oct 20;101(8):1329-37.

Challen GA, Little MH. A side order of stem cells: the SP phenotype. Stem Cells. 2006 Jan;24(1):3-12.

Chan CK, Seo EY, Chen JY, Lo D, McArdle A, Sinha R, Tevlin R, Seita J, Vincent-Tompkins J, Wearda T, Lu WJ, Senarath-Yapa K, Chung MT, Marecic O, Tran M, Yan KS, Upton R, Walmsley GG, Lee AS, Sahoo D, Kuo CJ, Weissman IL, Longaker MT. Identification and specification of the mouse skeletal stem cell. Cell. 2015 Jan 15;160(1-2):285-98.

Chang JT, Wherry EJ, Goldrath AW. Molecular regulation of effector and memory T cell differentiation. Nat Immunol. 2014 Dec;15(12):1104-15.

Charlesworth B. Fisher, Medawar, Hamilton and the evolution of aging. Genetics. 2000 Nov;156(3):927-31.

Chaudhary PM, Roninson IB. Expression and activity of P-glycoprotein, a multidrug efflux pump, in human hematopoietic stem cells. Cell. 1991 Jul 12;66(1):85-94.

Chen DS, Mellman I. Elements of cancer immunity and the cancer-immune set point. Nature. 2017 Jan 18;541(7637):321-330.

Cheney KE, Walford RL. Immune function and dysfunction in relation to aging. Life Sci. 1974 Jun 1;14(11):2075-84.

Cheng H, Leblond CP. Origin, differentiation and renewal of the four main

epithelial cell types in the mouse small intestine. V. Unitarian Theory of the origin of the four epithelial cell types. Am J Anat. 1974 Dec;141(4):537-61.

Chiappinelli KB, Strissel PL, Desrichard A, Li H, Henke C, Akman B, Hein A, Rote NS, Cope LM, Snyder A, Makarov V, Budhu S, Slamon DJ, Wolchok JD, Pardoll DM, Beckmann MW, Zahnow CA, Merghoub T, Chan TA, Baylin SB, Strick R. Inhibiting DNA Methylation Causes an Interferon Response in Cancer via dsRNA Including Endogenous Retroviruses. Cell. 2015 Aug 27;162(5):974-86.

Childs BG, Baker DJ, Wijshake T, Conover CA, Campisi J, van Deursen JM. Senescent intimal foam cells are deleterious at all stages of atherosclerosis. Science. 2016 Oct 28;354(6311):472-477.

Chou JP, Effros RB. T cell replicative senescence in human aging. Curr Pharm Des. 2013;19(9):1680-98.

Clermont Y, Leblond CP. Renewal of spermatogonia in the rat. Am J Anat. 1953 Nov;93(3):475-501.

Cobaleda C, Jochum W, Busslinger M. Conversion of mature B cells into T cells by dedifferentiation to uncommitted progenitors. Nature. 2007 Sep 27;449(7161):473-7.

Cole SP, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurz EU, Duncan AM, Deeley RG. Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. Science. 1992 Dec 4;258(5088):1650-4

Conheim J. Congenitales, quergestreiftes muskelsarkon der nireren. Virchows Arch 1875, 65:64

Conselice, C. J., Wilkinson, A., Duncan, K. & Mortlock, A. The Evolution of Galaxy Number Density at Z<8 and its Implications. Astrophysical Journal. 2016.

Cotsarelis G, Sun TT, Lavker RM. Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. Cell. 1990 Jun 29;61(7):1329-37.

Crawford A, Angelosanto JM, Kao C, Doering TA, Odorizzi PM, Barnett BE, Wherry EJ. Molecular and transcriptional basis of $CD4^+$ T cell dysfunction during chronic infection. Immunity. 2014 Feb 20;40(2):289-302.

Crompton JG, Sukumar M, Restifo NP. Uncoupling T-cell expansion from effector differentiation in cell-based immunotherapy. Immunol Rev. 2014 Jan;257(1):264-76.

Crompton JG, Sukumar M, Restifo NP. Uncoupling T-cell expansion from effector differentiation in cell-based immunotherapy. Immunol Rev. 2014 Jan;257(1):264-76.

de Boer J, Andressoo JO, de Wit J, Huijmans J, Beems RB, van Steeg H, Weeda

G, van der Horst GT, van Leeuwen W, Themmen AP, Meradji M, Hoeijmakers JH. Premature aging in mice deficient in DNA repair and transcription. Science. 2002 May 17;296(5571):1276-9.

de Koning AP, Gu W, Castoe TA, Batzer MA, Pollock DD. Repetitive elements may comprise over two-thirds of the human genome. PLoS Genet. 2011 Dec;7(12):e1002384.

Decman V, Laidlaw BJ, Dimenna LJ, Abdulla S, Mozdzanowska K, Erikson J, Ertl HC, Wherry EJ. Cell-intrinsic defects in the proliferative response of antiviral memory CD8 T cells in aged mice upon secondary infection. J Immunol. 2010 May 1;184(9):5151-9.

Derhovanessian E, Maier AB, Beck R, Jahn G, Hähnel K, Slagboom PE, de Craen AJ, Westendorp RG, Pawelec G. Hallmark features of immunosenescence are absent in familial longevity. J Immunol. 2010 Oct 15;185(8):4618-24.

Dor Y, Melton DA. How important are adult stem cells for tissue maintenance? Cell Cycle. 2004 Sep;3(9):1104-6.

Doyle LA, Yang W, Abruzzo LV, Krogmann T, Gao Y, Rishi AK, Ross DD. A multidrug resistance transporter from human MCF-7 breast cancer cells. Proc Natl Acad Sci U S A. 1998 Dec 22;95(26):15665-70.

Eaves CJ. Hematopoietic stem cells: concepts, definitions, and the new reality. Blood. 2015 Apr 23;125(17):2605-13.

Effros RB. From Hayflick to Walford: the role of T cell replicative senescence in human aging. Exp Gerontol. 2004 Jun;39(6):885-90.

Ehrlich P. Über den jetzigen Stand der Karzinomforschung. Ned. Tijdschr. Geneeskd. 1909; 5: 273–90.

Eriksson PS, Perfilieva E, Björk-Eriksson T, Alborn AM, Nordborg C, Peterson DA, Gage FH. Neurogenesis in the adult human hippocampus. Nat Med. 1998 Nov;4(11):1313-7.

Esnault C, Heidmann O, Delebecque F, Dewannieux M, Ribet D, Hance AJ, Heidmann T, Schwartz O. APOBEC3G cytidine deaminase inhibits retrotransposition of endogenous retroviruses. Nature. 2005 Jan 27;433(7024):430-3.

Essers MA, Offner S, Blanco-Bose WE, Waibler Z, Kalinke U, Duchosal MA, Trumpp A. IFNalpha activates dormant haematopoietic stem cells in vivo. Nature. 2009 Apr 16;458(7240):904-8.

Ewing AD, Gacita A, Wood LD, Ma F, Xing D, Kim MS, Manda SS, Abril G, Pereira G, Makohon-Moore A, Looijenga LH, Gillis AJ, Hruban RH, Anders RA, Romans KE, Pandey A, Iacobuzio-Donahue CA, Vogelstein B, Kinzler KW, Kazazian HH Jr, Solyom S. Widespread somatic L1 retrotransposition occurs early during gastrointestinal cancer evolution. Genome Res. 2015 Oct;25(10):1536-45.

Fearon DT, Manders P, Wagner SD. Arrested differentiation, the self-renewing memory lymphocyte, and vaccination. Science. 2001 Jul 13;293(5528):248-50.

Feng X, Wang H, Takata H, Day TJ, Willen J, Hu H. Transcription factor Foxp1 exerts essential cell-intrinsic regulation of the quiescence of naive T cells. Nat Immunol. 2011 Jun;12(6):544-50.

Fischer SA, Graham MB, Kuehnert MJ, Kotton CN, Srinivasan A, Marty FM, Comer JA, Guarner J, Paddock CD, DeMeo DL, Shieh WJ, Erickson BR, Bandy U, DeMaria A Jr, Davis JP, Delmonico FL, Pavlin B, Likos A, Vincent MJ, Sealy TK, Goldsmith CS, Jernigan DB, Rollin PE, Packard MM, Patel M, Rowland C, Helfand RF, Nichol ST, Fishman JA, Ksiazek T, Zaki SR; LCMV in Transplant Recipients Investigation Team. Transmission of lymphocytic choriomeningitis virus by organ transplantation. N Engl J Med. 2006 May 25;354(21):2235-49.

Flores I, Canela A, Vera E, Tejera A, Cotsarelis G, Blasco MA. The longest telomeres: a general signature of adult stem cell compartments. Genes Dev. 2008 Mar 1;22(5):654-67.

Foege WH. The wonder that is global health. Nat Med. 2001 Oct;7(10):1095-6.

Ford CE, Hamerton JL, Barnes DW, Loutit JF. Cytological identification of radiation-chimaeras. Nature. 1956 Mar 10;177(4506):452-4.

Fraser KA, Schenkel JM, Jameson SC, Vezys V, Masopust D. Preexisting high frequencies of memory CD8+ T cells favor rapid memory differentiation and preservation of proliferative potential upon boosting. Immunity. 2013 Jul 25;39(1):171-83.

Friedberg EC, Walker GC, Siede W, Wood RD, Schultz RA, Ellenberger T. 2005. DNA Repair and Mutagenesis. ASM Press, Washington, DC.

Fuchs E. The tortoise and the hair: slow-cycling cells in the stem cell race. Cell. 2009 May 29;137(5):811-9.

Fuertes Marraco SA, Soneson C, Cagnon L, Gannon PO, Allard M, Abed Maillard S, Montandon N, Rufer N, Waldvogel S, Delorenzi M, Speiser DE. Long-lasting stem cell-like memory CD8+ T cells with a naïve-like profile upon yellow fever vaccination. Sci Transl Med. 2015 Apr 8;7(282):282ra48.

Galkina E, Thatte J, Dabak V, Williams MB, Ley K, Braciale TJ. Preferential migration of effector CD8+ T cells into the interstitium of the normal lung. J Clin Invest. 2005 Dec;115(12).

Gallatin WM, Weissman IL, Butcher EC. A cell-surface molecule involved in organ-specific homing of lymphocytes. Nature. 1983 Jul 7-13;304(5921):30-4.

Garsin DA, Villanueva JM, Begun J, Kim DH, Sifri CD, Calderwood SB, Ruvkun G, Ausubel FM. Long-lived C. elegans daf-2 mutants are resistant to bacterial pathogens. Science. 2003 Jun 20;300(5627):1921.

Gattinoni L, Klebanoff CA, Palmer DC, Wrzesinski C, Kerstann K, Yu Z,

Finkelstein SE, Theoret MR, Rosenberg SA, Restifo NP. Acquisition of full effector function in vitro paradoxically impairs the in vivo antitumor efficacy of adoptively transferred CD8+ T cells. J Clin Invest. 2005 Jun;115(6):1616-26

Gattinoni L, Lugli E, Ji Y, Pos Z, Paulos CM, Quigley MF, Almeida JR, Gostick E, Yu Z, Carpenito C, Wang E, Douek DC, Price DA, June CH, Marincola FM, Roederer M, Restifo NP. A human memory T cell subset with stem cell-like properties. Nat Med. 2011 Sep 18;17(10):1290-7.

Gattinoni L, Zhong XS, Palmer DC, Ji Y, Hinrichs CS, Yu Z, Wrzesinski C, Boni A, Cassard L, Garvin LM, Paulos CM, Muranski P, Restifo NP. Wnt signaling arrests effector T cell differentiation and generates CD8+ memory stem cells. Nat Med. 2009 Jul;15(7):808-13.

Gavazzi G, Krause KH. Ageing and infection. Lancet Infect Dis. 2002 Nov;2(11):659-66.

Gerlach C, Moseman EA, Loughhead SM, Alvarez D, Zwijnenburg AJ, Waanders L, Garg R, de la Torre JC, von Andrian UH. The Chemokine Receptor CX3CR1 Defines Three Antigen-Experienced CD8 T Cell Subsets with Distinct Roles in Immune Surveillance and Homeostasis. Immunity. 2016 Dec 20;45(6):1270-1284.

Gerlach C, Rohr JC, Perié L, van Rooij N, van Heijst JW, Velds A, Urbanus J, Naik SH, Jacobs H, Beltman JB, de Boer RJ, Schumacher TN. Heterogeneous differentiation patterns of individual CD8+ T cells. Science. 2013 May 3;340(6132):635-9. doi: 10.1126/science.1235487.

Gladyshev VN. On the cause of aging and control of lifespan: heterogeneity leads to inevitable damage accumulation, causing aging; control of damage composition and rate of accumulation define lifespan. Bioessays. 2012 Nov;34(11):925-9.

Goldrath AW, Sivakumar PV, Glaccum M, Kennedy MK, Bevan MJ, Benoist C, Mathis D, Butz EA. Cytokine requirements for acute and Basal homeostatic proliferation of naive and memory CD8+ T cells. J Exp Med. 2002 Jun 17;195(12):1515-22.

Golebiewska A, Brons NH, Bjerkvig R, Niclou SP. Critical appraisal of the side population assay in stem cell and cancer stem cell research. Cell Stem Cell. 2011 Feb 4;8(2):136-47.

Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med. 1996 Apr 1;183(4):1797-806.

Goodell MA, Rosenzweig M, Kim H, Marks DF, DeMaria M, Paradis G, Grupp SA, Sieff CA, Mulligan RC, Johnson RP. Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. Nat Med. 1997 Dec;3(12):1337-45.

Gorbunova V, Boeke JD, Helfand SL, Sedivy JM. Human Genomics: Sleeping dogs of the genome. Science. 2014 Dec 5;346(6214):1187-8.

Graef P, Buchholz VR, Stemberger C, Flossdorf M, Henkel L, Schiemann M, Drexler I, Höfer T, Riddell SR, Busch DH. Serial transfer of single-cell-derived immunocompetence reveals stemness of CD8+ central memory T cells. Immunity. 2014 Jul 17;41(1):116-26.

Gros P, Ben Neriah YB, Croop JM, Housman DE. Isolation and expression of a complementary DNA that confers multidrug resistance. Nature. 1986 Oct 23-29;323(6090):728-31.

Gurdon JB. Adult frogs derived from the nuclei of single somatic cells. Dev Biol. 1962 Apr;4:256-73.

Hallam J. Exercise-mediated changes in plasmacytoid dendritic cell production of IFNα and the effects of moderate intensity exercise on immune response to influenza infection and influenza vaccine. Graduate Theses and Dissertations. 2013; 13618.

Hammarlund E, Lewis MW, Hansen SG, Strelow LI, Nelson JA, Sexton GJ, Hanifin JM, Slifka MK. Nat Med. 2003 Sep;9(9):1131-7. Duration of antiviral immunity after smallpox vaccination.

Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, Nadon NL, Wilkinson JE, Frenkel K, Carter CS, Pahor M, Javors MA, Fernandez E, Miller RA. Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. Nature. 2009 Jul 16;460(7253):392-5.

Hay ED. The fine structure of blastema cells and differentiating cartilage cells in regenerating limbs of Amblystoma larvae. J Biophys Biochem Cytol. 1958 Sep 25;4(5):583-91.

Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. Exp Cell Res. 1961 Dec;25:585-621.

Hayflick L. The limited in vitro lifetime of human diploid cell strains. Exp Cell Res. 1965 Mar;37:614-36.

Haynes L, Eaton SM, Burns EM, Randall TD, Swain SL. CD4 T cell memory derived from young naive cells functions well into old age, but memory generated from aged naive cells functions poorly. Proc Natl Acad Sci U S A. 2003 Dec 9;100(25):15053-8.

He R, Hou S, Liu C, Zhang A, Bai Q, Han M, Yang Y, Wei G, Shen T, Yang X, Xu L, Chen X, Hao Y, Wang P, Zhu C, Ou J, Liang H, Ni T, Zhang X, Zhou X, Deng K, Chen Y, Luo Y, Xu J, Qi H, Wu Y, Ye L. Follicular CXCR5-expressing CD8+ T cells curtail chronic viral infection. Nature. 2016 Aug 2;537(7620):412-428.

Henao-Restrepo AM, Camacho A, Longini IM, Watson CH, Edmunds WJ, Egger M, Carroll MW, Dean NE, Diatta I, Doumbia M, Draguez B, Duraffour S, Enwere

G, Grais R, Gunther S, Gsell PS, Hossmann S, Watle SV, Kondé MK, Kéïta S, Kone S, Kuisma E, Levine MM, Mandal S, Mauget T, Norheim G, Riveros X, Soumah A, Trelle S, Vicari AS, Røttingen JA, Kieny MP. Efficacy and effectiveness of an rVSV-vectored vaccine in preventing Ebola virus disease: final results from the Guinea ring vaccination, open-label, cluster-randomised trial (Ebola Ça Suffit!). Lancet. 2017 Feb 4;389(10068):505-518.

Herbert BS, Hochreiter AE, Wright WE, Shay JW. Nonradioactive detection of telomerase activity using the telomeric repeat amplification protocol. Nat Protoc. 2006; 1(3):1583-90.

Herbig U, Ferreira M, Condel L, Carey D, Sedivy JM. Cellular senescence in aging primates. Science. 2006 Mar 3;311(5765):1257.

Hinrichs CS, Borman ZA, Cassard L, Gattinoni L, Spolski R, Yu Z, Sanchez-Perez L, Muranski P, Kern SJ, Logun C, Palmer DC, Ji Y, Reger RN, Leonard WJ, Danner RL, Rosenberg SA, Restifo NP. Adoptively transferred effector cells derived from naive rather than central memory CD8+ T cells mediate superior antitumor immunity. Proc Natl Acad Sci U S A. 2009 Oct 13;106(41):17469-74.

Hodes RJ, Hathcock KS, Weng NP. Telomeres in T and B cells. Nat Rev Immunol. 2002 Sep;2(9):699-706.

Hoffmeyer K, Raggioli A, Rudloff S, Anton R, Hierholzer A, Del Valle I, Hein K, Vogt R, Kemler R. Wnt/β-catenin signaling regulates telomerase in stem cells and cancer cells. Science. 2012 Jun 22;336(6088):1549-54.

Homann D, Teyton L, Oldstone MB. Differential regulation of antiviral T-cell immunity results in stable CD8+ but declining CD4+ T-cell memory. Nat Med. 2001 Aug;7(8):913-9.

Honda T, Egen JG, Lämmermann T, Kastenmüller W, Torabi-Parizi P, Germain RN. Tuning of antigen sensitivity by T cell receptor-dependent negative feedback controls T cell effector function in inflamed tissues. Immunity. 2014 Feb 20;40(2):235-47.

Huang GJ, Smith AL, Gray DH, Cosgrove C, Singer BH, Edwards A, Sims S, Parent JM, Johnsen A, Mott R, Mathis D, Klenerman P, Benoist C, Flint J. A genetic and functional relationship between T cells and cellular proliferation in the adult hippocampus. PLoS Biol. 2010 Dec 14;8(12):e1000561.

Hui E, Cheung J, Zhu J, Su X, Taylor MJ, Wallweber HA, Sasmal DK, Huang J, Kim JM, Mellman I, Vale RD. T cell costimulatory receptor CD28 is a primary target for PD-1-mediated inhibition. Science. 2017 Mar 31;355(6332):1428-1433.

Huster KM, Busch V, Schiemann M, Linkemann K, Kerksiek KM, Wagner H, Busch DH. Selective expression of IL-7 receptor on memory T cells identifies early CD40L-dependent generation of distinct CD8+ memory T cell subsets. Proc Natl Acad Sci U S A. 2004 Apr 13;101(15):5610-5.

Im SJ, Hashimoto M, Gerner MY, Lee J, Kissick HT, Burger MC, Shan Q, Hale

JS, Lee J, Nasti TH, Sharpe AH, Freeman GJ, Germain RN, Nakaya HI, Xue HH, Ahmed R. Defining CD8+ T cells that provide the proliferative burst after PD-1 therapy. Nature. 2016 Sep 15;537(7620):417-421.

Intlekofer AM, Takemoto N, Wherry EJ, Longworth SA, Northrup JT, Palanivel VR, Mullen AC, Gasink CR, Kaech SM, Miller JD, Gapin L, Ryan K, Russ AP, Lindsten T, Orange JS, Goldrath AW, Ahmed R, Reiner SL. Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin. Nat Immunol. 2005 Dec;6(12):1236-44.

Intlekofer AM, Wherry EJ, Reiner SL. Not-so-great expectations: re-assessing the essence of T-cell memory. Immunol Rev. 2006 Jun;211:203-13.

Ito CY, Li CY, Bernstein A, Dick JE, Stanford WL. Hematopoietic stem cell and progenitor defects in Sca-1/Ly-6A-null mice. Blood. 2003 Jan 15;101(2):517-23. Epub 2002 Aug 29.

Jabbari A, Harty JT. Simultaneous assessment of antigen-stimulated cytokine production and memory subset composition of memory CD8 T cells. J Immunol Methods. 2006 Jun 30; 313(1-2):161-8.

Jacob J, Baltimore D. Modelling T-cell memory by genetic marking of memory T cells in vivo. Nature. 1999 Jun 10;399(6736):593-7.

Jacobson LO, Simmons EL, Marks EK, Eldredge JH. Recovery from radiation injury. Science. 1951 May 4;113(2940):510-11.

Jaiswal S, Jamieson CH, Pang WW, Park CY, Chao MP, Majeti R, Traver D, van Rooijen N, Weissman IL. CD47 is upregulated on circulating hematopoietic stem cells and leukemia cells to avoid phagocytosis. Cell. 2009 Jul 23;138(2):271-85.

Jin X, Bauer DE, Tuttleton SE, Lewin S, Gettie A, Blanchard J, Irwin CE, Safrit JT, Mittler J, Weinberger L, Kostrikis LG, Zhang L, Perelson AS, Ho DD. Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques. J Exp Med. 1999 Mar 15;189(6):991-8.

Jones RG, Thompson CB. Revving the engine: signal transduction fuels T cell activation. Immunity. 2007 Aug;27(2):173-8.

Jonker JW, Buitelaar M, Wagenaar E, Van Der Valk MA, Scheffer GL, Scheper RJ, Plosch T, Kuipers F, Elferink RP, Rosing H, Beijnen JH, Schinkel AH. The breast cancer resistance protein protects against a major chlorophyll-derived dietary phototoxin and protoporphyria. Proc Natl Acad Sci U S A. 2002 Nov 26;99(24):15649-54.

Joshi NS, Cui W, Chandele A, Lee HK, Urso DR, Hagman J, Gapin L, Kaech SM. Inflammation directs memory precursor and short-lived effector CD8+ T cell fates via the graded expression of T-bet transcription factor. Immunity. 2007 Aug 27(2):281-95.

Joshi NS, Kaech SM. Effector CD8 T cell development: a balancing act between

memory cell potential and terminal differentiation. J Immunol. 2008 Feb 1;180(3):1309-15.

Juliano RL, Ling V. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. Biochim Biophys Acta. 1976 Nov 11;455(1):152-62.

Jung YW, Rutishauser RL, Joshi NS, Haberman AM, Kaech SM. Differential localization of effector and memory CD8 T cell subsets in lymphoid organs during acute viral infection. J Immunol. 2010 Nov 1;185(9):5315-25.

Kaech SM, Ahmed R. Memory CD8+ T cell differentiation: initial antigen encounter triggers a developmental program in naïve cells. Nat Immunol. 2001 May;2(5):415-22.

Kaech SM, Cui W. Transcriptional control of effector and memory CD8+ T cell differentiation. Nat Rev Immunol. 2012 Nov;12(11):749-61.

Kaech SM, Wherry EJ, Ahmed R. Effector and memory T-cell differentiation: implications for vaccine development. Nat Rev Immunol. 2002 Apr;2(4):251-62.

Kaech SM, Wherry EJ. Heterogeneity and cell-fate decisions in effector and memory CD8+ T cell differentiation during viral infection. Immunity. 2007 Sep;27(3):393-405.

Kagi D, Ledermann B, Bürki K, Seiler P, Odermatt B, Olsen KJ, Podack ER, Zinkernagel RM, Hengartner H. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. Nature. 1994 May 5;369(6475):31-7.

Kai T, Spradling A. Differentiating germ cells can revert into functional stem cells in Drosophila melanogaster ovaries. Nature. 2004 Apr 1;428(6982):564-9.

Kamphorst AO, Wieland A, Nasti T, Yang S, Zhang R, Barber DL, Konieczny BT, Daugherty CZ, Koenig L, Yu K, Sica GL, Sharpe AH, Freeman GJ, Blazar BR, Turka LA, Owonikoko TK, Pillai RN, Ramalingam SS, Araki K, Ahmed R. Rescue of exhausted CD8 T cells by PD-1-targeted therapies is CD28-dependent. Science. 2017 Mar 31;355(6332):1423-1427.

Kano H, Godoy I, Courtney C, Vetter MR, Gerton GL, Ostertag EM, Kazazian HH Jr. L1 retrotransposition occurs mainly in embryogenesis and creates somatic mosaicism. Genes Dev. 2009 Jun 1;23(11):1303-12.

Kendziorra E, Ahlborn K, Spitzner M, Rave-Fränk M, Emons G, Gaedcke J, Kramer F, Wolff HA, Becker H, Beissbarth T, Ebner R, Ghadimi BM, Pukrop T, Ried T, Grade M. Silencing of the Wnt transcription factor TCF4 sensitizes colorectal cancer cells to (chemo-) radiotherapy. Carcinogenesis. 2011 Dec;32(12):1824-31.

Kessel D, Botterill V, Wodinsky I. Uptake and retention of daunomycin by mouse leukemic cells as factors in drug response. Cancer Res. 1968 May;28(5):938-41.

Kew OM, Sutter RW, de Gourville EM, Dowdle WR, Pallansch MA. Vaccine-derived polioviruses and the endgame strategy for global polio eradication. Annu Rev Microbiol. 2005;59:587-635.

Klawitter S, Fuchs NV, Upton KR, Muñoz-Lopez M, Shukla R, Wang J, Garcia-Cañadas M, Lopez-Ruiz C, Gerhardt DJ, Sebe A, Grabundzija I, Merkert S, Gerdes P, Pulgarin JA, Bock A, Held U, Witthuhn A, Haase A, Sarkadi B, Löwer J, Wolvetang EJ, Martin U, Ivics Z, Izsvák Z, Garcia-Perez JL, Faulkner GJ, Schumann GG. Reprogramming triggers endogenous L1 and Alu retrotransposition in human induced pluripotent stem cells. Nat Commun. 2016 Jan 8;7:10286.

Koebel CM, Vermi W, Swann JB, Zerafa N, Rodig SJ, Old LJ, Smyth MJ, Schreiber RD. Adaptive immunity maintains occult cancer in an equilibrium state. Nature. 2007 Dec 6;450(7171):903-7.

Kohanski RA, Deeks SG, Gravekamp C, Halter JB, High K, Hurria A, Fuldner R, Green P, Huebner R, Macchiarini F, Sierra F. Reverse geroscience: how does exposure to early diseases accelerate the age-related decline in health? Ann N Y Acad Sci. 2016 Dec;1386(1):30-44.

Kohut ML, Arntson BA, Lee W, Rozeboom K, Yoon KJ, Cunnick JE, McElhaney J. Moderate exercise improves antibody response to influenza immunization in older adults. Vaccine. 2004 Jun 2;22(17-18):2298-306.

Konca K, Lankoff A, Banasik A, Lisowska H, Kuszewski T, Góźdź S, Koza Z, Wojcik A. A cross-platform public domain PC image-analysis program for the comet assay. Mutat Res. 2003 Jan 10; 534(1-2):15-20.

Kopp JL, Grompe M, Sander M. Stem cells versus plasticity in liver and pancreas regeneration. Nat Cell Biol. 2016 Mar;18(3):238-45.

Kotturi MF, Swann JA, Peters B, Arlehamn CL, Sidney J, Kolla RV, James EA, Akondy RS, Ahmed R, Kwok WW, Buchmeier MJ, Sette A. Human $CD8^+$ and $CD4^+$ T cell memory to lymphocytic choriomeningitis virus infection. J Virol. 2011 Nov;85(22):11770-80.

Krishnamurthy J, Sharpless NE. Stem cells and the rate of living. Cell Stem Cell. 2007 Jun 7;1(1):9-11.

Krishnamurthy P, Ross DD, Nakanishi T, Bailey-Dell K, Zhou S, Mercer KE, Sarkadi B, Sorrentino BP, Schuetz JD. The stem cell marker Bcrp/ABCG2 enhances hypoxic cell survival through interactions with heme. J Biol Chem. 2004 Jun 4;279(23):24218-25.

Krivtsov AV, Twomey D, Feng Z, Stubbs MC, Wang Y, Faber J, Levine JE, Wang J, Hahn WC, Gilliland DG, Golub TR, Armstrong SA. Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9. Nature. 2006 Aug 17;442(7104):818-22.

Krummel MF, Allison JP. CD28 and CTLA-4 have opposing effects on the

response of T cells to stimulation. J Exp Med. 1995 Aug 1;182(2):459-65.

Kujoth GC, Hiona A, Pugh TD, Someya S, Panzer K, Wohlgemuth SE, Hofer T, Seo AY, Sullivan R, Jobling WA, Morrow JD, Van Remmen H, Sedivy JM, Yamasoba T, Tanokura M, Weindruch R, Leeuwenburgh C, Prolla TA. Mitochondrial DNA mutations, oxidative stress, and apoptosis in mammalian aging. Science. 2005 Jul 15;309(5733):481-4.

Kurachi M, Barnitz RA, Yosef N, Odorizzi PM, DiIorio MA, Lemieux ME, Yates K, Godec J, Klatt MG, Regev A, Wherry EJ, Haining WN. The transcription factor BATF operates as an essential differentiation checkpoint in early effector CD8+ T cells. Nat Immunol. 2014 Apr;15(4):373-83.

Lafferty KJ, Misko IS, Cooley MA. Allogeneic stimulation modulates the in vitro response of T cells to transplantation antigen. Nature. 1974 May 17;249(454):275-6.

Laidlaw BJ, Cui W, Amezquita RA, Gray SM, Guan T, Lu Y, Kobayashi Y, Flavell RA, Kleinstein SH, Craft J, Kaech SM. Production of IL-10 by CD4(+) regulatory T cells during the resolution of infection promotes the maturation of memory CD8(+) T cells. Nat Immunol. 2015 Aug;16(8):871-9.

Lander AD, Kimble J, Clevers H, Fuchs E, Montarras D, Buckingham M, Calof AL, Trumpp A, Oskarsson T. What does the concept of the stem cell niche really mean today? BMC Biol. 2012 Mar 9;10:19.

Lang A, Nikolich-Zugich J. Functional CD8 T cell memory responding to persistent latent infection is maintained for life. J Immunol. 2011 Oct 1;187(7):3759-68.

Lanzavecchia A, Sallusto F. Progressive differentiation and selection of the fittest in the immune response. Nat Rev Immunol. 2002 Dec;2(12):982-7.

Lassalle B, Bastos H, Louis JP, Riou L, Testart J, Dutrillaux B, Fouchet P, Allemand I. 'Side Population' cells in adult mouse testis express Bcrp1 gene and are enriched in spermatogonia and germinal stem cells. Development. 2004 Jan;131(2):479-87.

Lau LL, Jamieson BD, Somasundaram T, Ahmed R. Cytotoxic T-cell memory without antigen. Nature. 1994 Jun 23;369(6482):648-52.

Laurie GW. Charles Philippe Leblond, 1910–2007. Journal of Anatomy. 2007;211(1):I-III.

Law RH, Lukoyanova N, Voskoboinik I, Caradoc-Davies TT, Baran K, Dunstone MA, D'Angelo ME, Orlova EV, Coulibaly F, Verschoor S, Browne KA, Ciccone A, Kuiper MJ, Bird PI, Trapani JA, Saibil HR, Whisstock JC. The structural basis for membrane binding and pore formation by lymphocyte perforin. Nature. 2010 Nov 18;468(7322):447-51.

Leblond CP, Messier B, Kopriwa B. Thymidine-H3 as a tool for the investigation of the renewal of cell populations. Laboratory Investigation, 1959.

Leblond CP, Stevens CE, Bogoroch R. Histological Localization of Newly-formed Desoxyribonucleic Acid. Science. 1948 Nov 12;108(2811):531-3.

Leblond CP, Stevens CE. The constant renewal of the intestinal epithelium in the albino rat. Anat Rec. 1948 Mar;100(3):357-77.

Leblond CP. The time dimension in cell biology. FASEB J. 1995 Sep;9(12):1234-8.

Leblond, CP. Time dimension in cell biology. Protoplasma. 1991; 160, 5-38.

Lee E, Iskow R, Yang L, Gokcumen O, Haseley P, Luquette LJ 3rd, Lohr JG, Harris CC, Ding L, Wilson RK, Wheeler DA, Gibbs RA, Kucherlapati R, Lee C, Kharchenko PV, Park PJ; Cancer Genome Atlas Research Network. Landscape of somatic retrotransposition in human cancers. Science. 2012 Aug 24;337(6097):967-71.

Lefrançois L, Masopust D. The road not taken: memory T cell fate 'decisions'. Nat Immunol. 2009 Apr;10(4):369-70.

Leong YA, Chen Y, Ong HS, Wu D, Man K, Deleage C, Minnich M, Meckiff BJ, Wei Y, Hou Z, Zotos D, Fenix KA, Atnerkar A, Preston S, Chipman JG, Beilman GJ, Allison CC, Sun L, Wang P, Xu J, Toe JG, Lu HK, Tao Y, Palendira U, Dent AL, Landay AL, Pellegrini M, Comerford I, McColl SR, Schacker TW, Long HM, Estes JD, Busslinger M, Belz GT, Lewin SR, Kallies A, Yu D. CXCR5+ follicular cytotoxic T cells control viral infection in B cell follicles. Nat Immunol. 2016 Oct;17(10):1187-96.

Li W, Prazak L, Chatterjee N, Grüninger S, Krug L, Theodorou D, Dubnau J. Activation of transposable elements during aging and neuronal decline in Drosophila. Nat Neurosci. 2013 May;16(5):529-31.

Lin KK, Goodell MA. Purification of hematopoietic stem cells using the side population. Methods Enzymol. 2006;420:255-64.

Lin WW, Nish SA, Yen B, Chen YH, Adams WC, Kratchmarov R, Rothman NJ, Bhandoola A, Xue HH, Reiner SL. CD8+ T Lymphocyte Self-Renewal during Effector Cell Determination. Cell Rep. 2016 Nov 8;17(7):1773-1782.

Lindahl T. Instability and decay of the primary structure of DNA. Nature. 1993 Apr 22;362(6422):709-15.

Liu J, Yuan Y, Chen W, Putra J, Suriawinata AA, Schenk AD, Miller HE, Guleria I, Barth RJ, Huang YH, Wang L. Immune-checkpoint proteins VISTA and PD-1 nonredundantly regulate murine T-cell responses. Proc Natl Acad Sci U S A. 2015 May 26;112(21):6682-7.

Liu Z, Gerner MY, Van Panhuys N, Levine AG, Rudensky AY, Germain RN. Immune homeostasis enforced by co-localized effector and regulatory T cells. Nature. 2015 Dec 10;528(7581):225-30.

Loeb LA, Monnat RJ Jr. DNA polymerases and human disease. Nat Rev Genet.

2008 Aug;9(8):594-604.

Lombard DB, Chua KF, Mostoslavsky R, Franco S, Gostissa M, Alt FW. DNA repair, genome stability, and aging. Cell. 2005 Feb 25;120(4):497-512.

Lopez-Otin C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. Cell. 2013 Jun 6;153(6):1194-217.

Lorenzini A, Johnson FB, Oliver A, Tresini M, Smith JS, Hdeib M, Sell C, Cristofalo VJ, Stamato TD. Significant correlation of species longevity with DNA double strand break recognition but not with telomere length. Mech Ageing Dev. 2009 Nov-Dec;130(11-12):784-92.

Luckey CJ, Bhattacharya D, Goldrath AW, Weissman IL, Benoist C, Mathis D. Memory T and memory B cells share a transcriptional program of self-renewal with long-term hematopoietic stem cells. Proc Natl Acad Sci U S A. 2006 Feb 28;103(9):3304-9.

Ma S, Upneja A, Galecki A, Tsai YM, Burant CF, Raskind S, Zhang Q, Zhang ZD, Seluanov A, Gorbunova V, Clish CB, Miller RA, Gladyshev VN. Cell culture-based profiling across mammals reveals DNA repair and metabolism as determinants of species longevity. Elife. 2016 Nov 22;5. pii: e19130.

Mackay LK, Braun A, Macleod BL, Collins N, Tebartz C, Bedoui S, Carbone FR, Gebhardt T. CD69 interference with sphingosine-1-phosphate receptor function regulates peripheral T cell retention. J Immunol. 2015 Mar 1;194(5):2059-63.

Malaguarnera L, Cristaldi E, Malaguarnera M. The role of immunity in elderly cancer. Crit Rev Oncol Hematol. 2010 Apr;74(1):40-60.

Mandal PK, Ferreira LM, Collins R, Meissner TB, Boutwell CL, Friesen M, Vrbanac V, Garrison BS, Stortchevoi A, Bryder D, Musunuru K, Brand H, Tager AM, Allen TM, Talkowski ME, Rossi DJ, Cowan CA. Efficient ablation of genes in human hematopoietic stem and effector cells using CRISPR/Cas9. Cell Stem Cell. 2014 Nov 6;15(5):643-52.

Markowitz LE, Liu G, Hariri S, Steinau M, Dunne EF, Unger ER. Prevalence of HPV After Introduction of the Vaccination Program in the United States. Pediatrics. 2016 Mar;137(3):e20151968.

Marrero SL, Bloom DE, Adashi EY. Noncommunicable diseases: a global health crisis in a new world order. JAMA. 2012 May 16;307(19):2037-8.

Martin MD, Condotta SA, Harty JT, Badovinac VP. Population dynamics of naive and memory CD8 T cell responses after antigen stimulations in vivo. J Immunol. 2012 Feb 1;188(3):1255-65.

Martín Y, Domínguez-Kelly R, Freire R. Novel insights into maintaining genomic integrity: Wee1 regulating Mus81/Eme1. Cell Div. 2011 Dec 9;6:21.

Maruyama T, Miyazaki K, Masuda H, Ono M, Uchida H, Yoshimura Y. Human uterine stem/progenitor cells: Implications for uterine physiology and pathology.

Placenta. 2013 Mar;34 Suppl:S68-72.

Marzo AL, Klonowski KD, Le Bon A, Borrow P, Tough DF, Lefrançois L. Initial T cell frequency dictates memory CD8+ T cell lineage commitment. Nat Immunol. 2005 Aug;6(8):793-9.

Masopust D, Murali-Krishna K, Ahmed R. Quantitating the magnitude of the lymphocytic choriomeningitis virus-specific CD8 T-cell response: it is even bigger than we thought. J Virol. 2007 Feb;81(4):2002-11.

Masopust D, Vezys V, Wherry EJ, Ahmed R. A brief history of CD8 T cells. Eur J Immunol. 2007 Nov;37 Suppl 1:S103-10.

McCarron M, Osborne Y, Story CJ, Dempsey JL, Turner DR, Morley AA. Effect of age on lymphocyte proliferation. Mech Ageing Dev. 1987 Dec;41(3):211-8.

McClintock B. The origin and behavior of mutable loci in maize. Proc Natl Acad Sci U S A. 1950 Jun;36(6):344-55.

McCulloch EA & Till, JE. Perspectives on the properties of stem cells. Nat Med 2005.

McCulloch EA, Till JE. The radiation sensitivity of normal mouse bone marrow cells, determined by quantitative marrow transplantation into irradiated mice. Radiat Res. 1960 Jul;13:115-25.

Mendez-Ferrer S, Michurina TV, Ferraro F, Mazloom AR, Macarthur BD, Lira SA, Scadden DT, Ma'ayan A, Enikolopov GN, Frenette PS. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. Nature. 2010 Aug 12;466(7308):829-34.

Merrell AJ, Stanger BZ. Adult cell plasticity in vivo: de-differentiation and transdifferentiation are back in style. Nat Rev Mol Cell Biol. 2016 Jul;17(7):413-25.

Messier B, Leblond CP. Cell proliferation and migration as revealed by radioautography after injection of thymidine-H3 into male rats and mice. Am J Anat. 1960 May;106:247-85.

Metz PJ, Lopez J, Kim SH, Akimoto K, Ohno S, Chang JT. Regulation of Asymmetric Division by Atypical Protein Kinase C Influences Early Specification of CD8+ T Lymphocyte Fates. Sci Rep. 2016 Jan 14;6:19182.

Miller RA, Stutman O. T cell repopulation from functionally restricted splenic progenitors: 10,000-fold expansion documented by using limiting dilution analyses. J Immunol. 1984 Dec;133(6):2925-32.

Miller RA. The aging immune system: primer and prospectus. Science. 1996 Jul 5;273(5271):70-4.

Mills JC, Sansom OJ. Reserve stem cells: Differentiated cells reprogram to fuel repair, metaplasia, and neoplasia in the adult gastrointestinal tract. Sci Signal.

2015 Jul 14;8(385):re8.

Mohrin M, Bourke E, Alexander D, Warr MR, Barry-Holson K, Le Beau MM, Morrison CG, Passegué E. Hematopoietic stem cell quiescence promotes error-prone DNA repair and mutagenesis. Cell Stem Cell. 2010 Aug 6;7(2):174-85.

Moran AE, Holzapfel KL, Xing Y, Cunningham NR, Maltzman JS, Punt J, Hogquist KA. T cell receptor signal strength in Treg and iNKT cell development demonstrated by a novel fluorescent reporter mouse. J Exp Med. 2011 Jun 6;208(6):1279-89.

Morgan DA, Ruscetti FW, Gallo R. Selective in vitro growth of T lymphocytes from normal human bone marrows. Science. 1976 Sep 10;193(4257):1007-8.

Morrison SJ, Prowse KR, Ho P, Weissman IL. Telomerase activity in hematopoietic cells is associated with self-renewal potential. Immunity. 1996 Sep;5(3):207-16.

Moskophidis D, Cobbold SP, Waldmann H, Lehmann-Grube F. Mechanism of recovery from acute virus infection: treatment of lymphocytic choriomeningitis virus-infected mice with monoclonal antibodies reveals that Lyt-2+ T lymphocytes mediate clearance of virus and regulate the antiviral antibody response. J Virol. 1987 Jun;61(6):1867-74.

Mukherjee, S. The Emperor of All Maladies: A Biography of Cancer. Scribner, New York. 2010.

Muller M, Meijer C, Zaman GJ, Borst P, Scheper RJ, Mulder NH, de Vries EG, Jansen PL. Overexpression of the gene encoding the multidrug resistance-associated protein results in increased ATP-dependent glutathione S-conjugate transport. Proc Natl Acad Sci U S A. 1994 Dec 20;91(26):13033-7.

Murali-Krishna K, Altman JD, Suresh M, Sourdive DJ, Zajac AJ, Miller JD, Slansky J, Ahmed R. Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. Immunity. 1998 Feb;8(2):177-87.

Na Nakorn T, Traver D, Weissman IL, Akashi K. Myeloerythroid-restricted progenitors are sufficient to confer radioprotection and provide the majority of day 8 CFU-S. J Clin Invest. 2002 Jun;109(12):1579-85.

Nakagawa T, Sharma M, Nabeshima Y, Braun RE, Yoshida S. Functional hierarchy and reversibility within the murine spermatogenic stem cell compartment. Science. 2010 Apr 2;328(5974):62-7.

Nolan E, Savas P, Policheni AN, Darcy PK, Vaillant F, Mintoff CP, Dushyanthen S, Mansour M, Pang JB, Fox SB; Kathleen Cuningham Foundation Consortium for Research into Familial Breast Cancer (kConFab), Perou CM, Visvader JE, Gray DHD, Loi S, Lindeman GJ. Combined immune checkpoint blockade as a therapeutic strategy for BRCA1-mutated breast cancer. Sci Transl Med. 2017 Jun 7;9(393).

Nolz JC, Harty JT. Protective capacity of memory CD8+ T cells is dictated by

antigen exposure history and nature of the infection. Immunity. 2011 May 27;34(5):781-93.

Nowell PC, Cole LJ, Habermeyer JG, Roan PL. Growth and continued function of rat marrow cells in x-radiated mice. Cancer Res. 1956 Mar;16(3):258-61.

Okazaki T, Chikuma S, Iwai Y, Fagarasan S, Honjo T. A rheostat for immune responses: the unique properties of PD-1 and their advantages for clinical application. Nat Immunol. 2013 Dec;14(12):1212-8.

Olive PL and Banáth JP. The comet assay: a method to measure DNA damage in individual cells. Nat Protoc. 2006;1(1):23-9.

Olshansky SJ, Carnes BA, Cassel C. In search of Methuselah: estimating the upper limits to human longevity. Science. 1990 Nov 2;250(4981):634-40.

Olshansky SJ. Ageing: Measuring our narrow strip of life. Nature. 2016 Oct 13;538(7624):175-176.

Orenstein WA, Ahmed R. Simply put: Vaccination saves lives. Proc Natl Acad Sci U S A. 2017 Apr 18;114(16):4031-4033.

Paley MA, Gordon SM, Bikoff EK, Robertson EJ, Wherry EJ, Reiner SL. Technical Advance: Fluorescent reporter reveals insights into eomesodermin biology in cytotoxic lymphocytes. J Leukoc Biol. 2013 Feb;93(2):307-15.

Pallavicini MG, Summers LJ, Dean PN, Gray JW. Enrichment of murine hemopoietic clonogenic cells by multivariate analyses and sorting. Exp Hematol. 1985 Dec;13(11):1173-81.

Pancer Z, Cooper MD. The evolution of adaptive immunity. Annu Rev Immunol. 2006;24:497-518. Review.

Parashar, UD; Tate, JE, eds. (2016). "Health Benefits of Rotavirus Vaccination in Developing Countries". Clinical Infectious Diseases. 62 (Suppl 2): S91–S228.

Pardi N, Hogan MJ, Pelc RS, Muramatsu H, Andersen H, DeMaso CR, Dowd KA, Sutherland LL, Scearce RM, Parks R, Wagner W, Granados A, Greenhouse J, Walker M, Willis E, Yu JS, McGee CE, Sempowski GD, Mui BL, Tam YK, Huang YJ, Vanlandingham D, Holmes VM, Balachandran H, Sahu S, Lifton M, Higgs S, Hensley SE, Madden TD, Hope MJ, Karikó K, Santra S, Graham BS, Lewis MG, Pierson TC, Haynes BF, Weissman D. Zika virus protection by a single low-dose nucleoside-modified mRNA vaccination. Nature. 2017 Mar 9;543(7644):248-251.

Parry RV, Chemnitz JM, Frauwirth KA, Lanfranco AR, Braunstein I, Kobayashi SV, Linsley PS, Thompson CB, Riley JL. CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. Mol Cell Biol. 2005 Nov;25(21):9543-53.

Patt HM, Tyree EB, Straube RL, Smith DE. Cysteine Protection against X Irradiation. Science. 1949 Aug 26;110(2852):213-4.

Pauken KE, Sammons MA, Odorizzi PM, Manne S, Godec J, Khan O, Drake AM, Chen Z, Sen DR, Kurachi M, Barnitz RA, Bartman C, Bengsch B, Huang AC, Schenkel JM, Vahedi G, Haining WN, Berger SL, Wherry EJ. Epigenetic stability of exhausted T cells limits durability of reinvigoration by PD-1 blockade. Science. 2016 Dec 2;354(6316):1160-1165.

Pauken KE, Wherry EJ. SnapShot: T Cell Exhaustion. Cell. 2015 Nov 5;163(4):1038-1038.e1.

Pawelec G, Lustgarten J, Ruby C, Gravekamp C. Impact of aging on cancer immunity and immunotherapy. Cancer Immunol Immunother. 2009 Dec;58(12):1907-8.

Pircher H, Bürki K, Lang R, Hengartner H, Zinkernagel RM. Tolerance induction in double specific T-cell receptor transgenic mice varies with antigen. Nature. 1989 Nov 30;342(6249):559-61.

Posakony JW, England JM, Attardi G. Mitochondrial growth and division during the cell cycle in HeLa cells. J Cell Biol. 1977 Aug;74(2):468-91.

Rajagopal J, Stanger BZ. Plasticity in the Adult: How Should the Waddington Diagram Be Applied to Regenerating Tissues? Dev Cell. 2016 Jan 25;36(2):133-7.

Ramalho-Santos M, Willenbring H. On the origin of the term "stem cell". Cell Stem Cell. 2007 Jun 7;1(1):35-8.

Ramsey MJ, Moore DH, Briner JF, Lee DA, Olsen La, Senft JR, Tucker JD. The effects of age and lifestyle factors on the accumulation of cytogenetic damage as measured by chromosome painting. Mutat Res. 1995 Oct;338(1-6):95-106.

Rando TA. Stem cells, ageing and the quest for immortality. Nature. 2006 Jun 29;441(7097):1080-6.

Rao RR, Long JZ, White JP, Svensson KJ, Lou J, Lokurkar I, Jedrychowski MP, Ruas JL, Wrann CD, Lo JC, Camera DM, Lachey J, Gygi S, Seehra J, Hawley JA, Spiegelman BM. Meteorin-like is a hormone that regulates immune-adipose interactions to increase beige fat thermogenesis. Cell. 2014 Jun 5;157(6):1279-91

Restifo NP, Gattinoni L. Lineage relationship of effector and memory T cells. Curr Opin Immunol. 2013 Oct;25(5):556-63.

Reya T, Clevers H. Wnt signalling in stem cells and cancer. Nature. 2005 Apr 14;434(7035):843-50.

Reya T, Duncan AW, Ailles L, Domen J, Scherer DC, Willert K, Hintz L, Nusse R, Weissman IL. A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature. 2003 May 22;423(6938):409-14.

Rocha B, Dautigny N, Pereira P. Peripheral T lymphocytes: expansion potential and homeostatic regulation of pool sizes and CD4/CD8 ratios in vivo. Eur J

Immunol. 1989 May;19(5):905-11.

Rossi DJ, Bryder D, Seita J, Nussenzweig A, Hoeijmakers J, Weissman IL. Deficiencies in DNA damage repair limit the function of haematopoietic stem cells with age. Nature. 2007 Jun 7;447(7145):725-9.

Roychoudhuri R, Lefebvre F, Honda M, Pan L, Ji Y, Klebanoff CA, Nichols CN, Fourati S, Hegazy AN, Goulet JP, Gattinoni L, Nabel GJ, Gilliet M, Cameron M, Restifo NP, Sékaly RP, Flatz L. Transcriptional profiles reveal a stepwise developmental program of memory CD8(+) T cell differentiation. Vaccine. 2015 Feb 11;33(7):914-23.

Rutishauser RL, Martins GA, Kalachikov S, Chandele A, Parish IA, Meffre E, Jacob J, Calame K, Kaech SM. Transcriptional repressor Blimp-1 promotes CD8+ T cell terminal differentiation and represses the acquisition of central memory T cell properties. Immunity. 2009 Aug 21;31(2):296-308.

Ruzankina Y, Asare A, Brown EJ. Replicative stress, stem cells and aging. Mech Ageing Dev. 2008 Jul-Aug;129(7-8):460-6.

Sakaue-Sawano A, Kurokawa H, Morimura T, Hanyu A, Hama H, Osawa H, Kashiwagi S, Fukami K, Miyata T, Miyoshi H, Imamura T, Ogawa M, Masai H, Miyawaki A. Visualizing spatiotemporal dynamics of multicellular cell-cycle progression. Cell. 2008 Feb 8;132(3):487-98.

Sallusto F, Lanzavecchia A. Memory in disguise. Nat Med. 2011 Oct 11;17(10):1182-3.

Sallusto F, Lenig D, Förster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature. 1999 Oct 14;401(6754):708-12.

Sansoni P, Vescovini R, Fagnoni F, Biasini C, Zanni F, Zanlari L, Telera A, Lucchini G, Passeri G, Monti D, Franceschi C, Passeri M. The immune system in extreme longevity. Exp Gerontol. 2008 Feb;43(2):61-5.

Sansregret L, Patterson JO, Dewhurst S, López-García C, Koch A, McGranahan N, Chao WC, Barry DJ, Rowan A, Instrell R, Horswell S, Way M, Howell M, Singleton MR, Medema RH, Nurse P, Petronczki M, Swanton C. APC/C Dysfunction Limits Excessive Cancer Chromosomal Instability. Cancer Discov. 2017 Jan 9.

Sarkar S, Kalia V, Haining WN, Konieczny BT, Subramaniam S, Ahmed R. Functional and genomic profiling of effector CD8 T cell subsets with distinct memory fates. J Exp Med. 2008 Mar 17;205(3):625-40.

Sarkar S, Teichgräber V, Kalia V, Polley A, Masopust D, Harrington LE, Ahmed R, Wherry EJ. Strength of stimulus and clonal competition impact the rate of memory CD8 T cell differentiation. J Immunol. 2007 Nov 15;179(10):6704-14.

Schinkel AH, Jonker JW. Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. Adv Drug Deliv Rev. 2003 Jan 21;55(1):3-

29.

Schnyder S, Handschin C. Skeletal muscle as an endocrine organ: PGC-1α, myokines and exercise. Bone. 2015 Nov;80:115-25.

Schofield R. The relationship between the spleen colony-forming cell and the haemopoietic stem cell.

Blood Cells. 1978;4(1-2):7-25

Schoppy DW, Ragland RL, Gilad O, Shastri N, Peters AA, Murga M, Fernandez-Capetillo O, Diehl JA, Brown EJ. Oncogenic stress sensitizes murine cancers to hypomorphic suppression of ATR. J Clin Invest. 2012 Jan;122(1):241-52.

Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. Science. 2011 Mar 25;331(6024):1565-70.

Scott-Browne JP, López-Moyado IF, Trifari S, Wong V, Chavez L, Rao A, Pereira RM. Dynamic Changes in Chromatin Accessibility Occur in CD8+ T Cells Responding to Viral Infection. Immunity. 2016 Dec 20;45(6):1327-1340.

Seluanov A, Mittelman D, Pereira-Smith OM, Wilson JH, Gorbunova V. DNA end joining becomes less efficient and more error-prone during cellular senescence. Proc Natl Acad Sci U S A. 2004 May 18;101(20):7624-9.

Seo Y, Jothikumar P, Suthar M, Zhu C, Grakoui A. Local Cellular and Cytokine Cues in the Spleen Regulate In Situ T Cell Receptor Affinity, Function, and Fate of CD8+ T Cells. Immunity. 2016 Nov; Vol. 45, Issue 5, p988–998.

Shay JW, Wright WE. Hayflick, his limit, and cellular ageing. Nat Rev Mol Cell Biol. 2000 Oct;1(1):72-6.

Shaywitz DA, Melton DA. The molecular biography of the cell. Cell. 2005 Mar 25;120(6):729-31.

Shukla R, Upton KR, Muñoz-Lopez M, Gerhardt DJ, Fisher ME, Nguyen T, Brennan PM, Baillie JK, Collino A, Ghisletti S, Sinha S, Iannelli F, Radaelli E, Dos Santos A, Rapoud D, Guettier C, Samuel D, Natoli G, Carninci P, Ciccarelli FD, Garcia-Perez JL, Faivre J, Faulkner GJ. Endogenous retrotransposition activates oncogenic pathways in hepatocellular carcinoma. Cell. 2013 Mar 28;153(1):101-11.

Siminovitch L, McCulloch EA, Till JE. The distribution of colony-forming cells among spleen colonies. J Cell Comp Physiol. 1963 Dec;62:327-36.

Simon MC, Keith B. The role of oxygen availability in embryonic development and stem cell function. Nat Rev Mol Cell Biol. 2008 Apr;9(4):285-96.

Simons BD, Clevers H. Strategies for homeostatic stem cell self-renewal in adult tissues. Cell. 2011 Jun 10;145(6):851-62.

Smart N, Bollini S, Dubé KN, Vieira JM, Zhou B, Davidson S, Yellon D, Riegler J,

Price AN, Lythgoe MF, Pu WT, Riley PR. De novo cardiomyocytes from within the activated adult heart after injury. Nature. 2011 Jun 8;474(7353):640-4.

Smith-Garvin JE, Koretzky GA, Jordan MS. T cell activation. Annu Rev Immunol. 2009;27:591-619.

Spalding KL, Arner E, Westermark PO, Bernard S, Buchholz BA, Bergmann O, Blomqvist L, Hoffstedt J, Näslund E, Britton T, Concha H, Hassan M, Rydén M, Frisén J, Arner P. Dynamics of fat cell turnover in humans. Nature. 2008 Jun 5;453(7196):783-7.

Spalding KL, Bergmann O, Alkass K, Bernard S, Salehpour M, Huttner HB, Boström E, Westerlund I, Vial C, Buchholz BA, Possnert G, Mash DC, Druid H, Frisén J. Dynamics of hippocampal neurogenesis in adult humans. Cell. 2013 Jun 6;153(6):1219-27.

Spalding KL, Bhardwaj RD, Buchholz BA, Druid H, Frisén J. Retrospective birth dating of cells in humans. Cell. 2005 Jul 15;122(1):133-43.

Spangrude GJ, Heimfeld S, Weissman IL. Purification and characterization of mouse hematopoietic stem cells. Science. 1988 Jul 1;241(4861):58-62.

Spangrude GJ, Johnson GR. Resting and activated subsets of mouse multipotent hematopoietic stem cells.

Proc Natl Acad Sci U S A. 1990 Oct;87(19):7433-7.

Spradling A, Fan CM. Counterfeiting the family jewels. Cell Stem Cell. 2010 May 7;6(5):405-6.

Sprent J, Surh CD. Normal T cell homeostasis: the conversion of naive cells into memory-phenotype cells. Nat Immunol. 2011 Jun;12(6):478-84.

Stepp SE, Dufourcq-Lagelouse R, Le Deist F, Bhawan S, Certain S, Mathew PA, Henter JI, Bennett M, Fischer A, de Saint Basile G, Kumar V. Perforin gene defects in familial hemophagocytic lymphohistiocytosis. Science. 1999 Dec 3;286(5446):1957-9.

Stewart EJ, Madden R, Paul G, Taddei F. Aging and death in an organism that reproduces by morphologically symmetric division. PLoS Biol. 2005 Feb;3(2):e45.

Storey WF, Leblond CP. Measurement of the rate of proliferation of epidermis and associated structures. Ann N Y Acad Sci. 1951 Mar;53(3):537-45.

Strindhall J, Nilsson BO, Löfgren S, Ernerudh J, Pawelec G, Johansson B, Wikby A. No Immune Risk Profile among individuals who reach 100 years of age: findings from the Swedish NONA immune longitudinal study. Exp Gerontol. 2007 Aug;42(8):753-61.

Sun J, Ramos A, Chapman B, Johnnidis JB, Le L, Ho YJ, Klein A, Hofmann O, Camargo FD. Clonal dynamics of native haematopoiesis. Nature. 2014 Oct

16;514(7522):322-7.

Sun JC, Beilke JN, Lanier LL. Adaptive immune features of natural killer cells. Nature. 2009 Jan 29;457(7229):557-61.

Sun Y, Chen CS, Fu J. Forcing stem cells to behave: a biophysical perspective of the cellular microenvironment. Annu Rev Biophys. 2012;41:519-42.

Sung JH, Zhang H, Moseman EA, Alvarez D, Iannacone M, Henrickson SE, de la Torre JC, Groom JR, Luster AD, von Andrian UH. Chemokine guidance of central memory T cells is critical for antiviral recall responses in lymph nodes. Cell. 2012 Sep 14;150(6):1249-63.

Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006 Aug 25;126(4):663-76.

Takamura S, Roberts AD, Jelley-Gibbs DM, Wittmer ST, Kohlmeier JE, Woodland DL. The route of priming influences the ability of respiratory virus-specific memory CD8+ T cells to be activated by residual antigen. J Exp Med. 2010 Jun 7;207(6):1153-60.

Takeda N, Jain R, LeBoeuf MR, Wang Q, Lu MM, Epstein JA. Interconversion between intestinal stem cell populations in distinct niches. Science. 2011 Dec 9;334(6061):1420-4.

Tarlow BD, Pelz C, Naugler WE, Wakefield L, Wilson EM, Finegold MJ, Grompe M. Bipotential adult liver progenitors are derived from chronically injured mature hepatocytes. Cell Stem Cell. 2014 Nov 6;15(5):605-18.

Tata PR, Mou H, Pardo-Saganta A, Zhao R, Prabhu M, Law BM, Vinarsky V, Cho JL, Breton S, Sahay A, Medoff BD, Rajagopal J. Dedifferentiation of committed epithelial cells into stem cells in vivo. Nature. 2013 Nov 14;503(7475):218-23.

Tetteh PW, Basak O, Farin HF, Wiebrands K, Kretzschmar K, Begthel H, van den Born M, Korving J, de Sauvage F, van Es JH, van Oudenaarden A, Clevers H. Replacement of Lost Lgr5-Positive Stem Cells through Plasticity of Their Enterocyte-Lineage Daughters. Cell Stem Cell. 2016 Feb 4;18(2):203-13.

Thiery J, Keefe D, Boulant S, Boucrot E, Walch M, Martinvalet D, Goping IS, Bleackley RC, Kirchhausen T, Lieberman J. Perforin pores in the endosomal membrane trigger the release of endocytosed granzyme B into the cytosol of target cells. Nat Immunol. 2011 Jun 19;12(8):770-7.

Thiery J, Lieberman J. Perforin: a key pore-forming protein for immune control of viruses and cancer. Subcell Biochem. 2014;80:197-220.

Thomas L. Discussion of cellular and humoral aspects of the hypersensitivity states. In: Lawrence HS, ed. *Cellular and Humoral Aspects of Hypersensitivity*, 529–32. New York: Hoeber-Harper, 1959.

Thucydides. History of the Peloponnesian War; translated from the Greek by William Smith. New York: Derby & Jackson, 1858.

Tian H, Biehs B, Warming S, Leong KG, Rangell L, Klein OD, de Sauvage FJ. A reserve stem cell population in small intestine renders Lgr5-positive cells dispensable. Nature. 2011 Sep 18;478(7368):255-9.

Till JE, McCulloch EA. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat Res. 1961 Feb;14:213-22.

Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity. 1995 Nov;3(5):541-7.

Topalian SL, Drake CG, Pardoll DM. Immune checkpoint blockade: a common denominator approach to cancer therapy. Cancer Cell. 2015 Apr 13;27(4):450-61.

Tsukamoto H, Clise-Dwyer K, Huston GE, Duso DK, Buck AL, Johnson LL, Haynes L, Swain SL. Age-associated increase in lifespan of naive CD4 T cells contributes to T-cell homeostasis but facilitates development of functional defects. Proc Natl Acad Sci U S A. 2009 Oct 27;106(43):18333-8.

United Nations Department of Economic and Social Affairs, Population Division. (2012). Population Ageing and Development. Available at: http://www.un.org/en/development/desa/population/publications/pdf/ageing/2012PopAgeingandDev_WallChart.pdf

Van Braeckel-Budimir N, Martin MD, Hartwig SM, Legge KL, Badovinac VP, Harty JT. Antigen Exposure History Defines CD8 T Cell Dynamics and Protection during Localized Pulmonary Infections. Front Immunol. 2017 Jan 27;8:40.

van Es JH, Sato T, van de Wetering M, Lyubimova A, Gregorieff A, Zeinstra L, van den Born M, Korving J, Martens ACM, van den Oudenaarden A, Clevers H. Dll1+ secretory progenitor cells revert to stem cells upon crypt damage. Nat Cell Biol. 2012 Oct;14(10):1099-1104.

van Faassen H, Saldanha M, Gilbertson D, Dudani R, Krishnan L, Sad S. Reducing the stimulation of CD8+ T cells during infection with intracellular bacteria promotes differentiation primarily into a central (CD62Lhigh CD44high) subset. J Immunol. 2005 May 1;174(9):5341-50.

Van VQ, Raymond M, Baba N, Rubio M, Wakahara K, Susin SA, Sarfati M. CD47(high) expression on CD4 effectors identifies functional long-lived memory T cell progenitors. J Immunol. 2012 May 1;188(9):4249-55.

Vaupel JW. Biodemography of human ageing. Nature. 2010 Mar 25;464(7288):536-42.

Vaziri H, Dragowska W, Allsopp RC, Thomas TE, Harley CB, Lansdorp PM. Evidence for a mitotic clock in human hematopoietic stem cells: loss of telomeric

DNA with age. Proc Natl Acad Sci U S A. 1994 Oct 11;91(21):9857-60.

Vijg J. Somatic mutations, genome mosaicism, cancer and aging. Curr Opin Genet Dev. 2014 Jun;26:141-9.

Virchow, R. Editorial. Virchows Arch. Pathol. Anat. Physiol. Klin. Med. 3, 23 (1855).

von Furstenberg RJ, Buczacki SJ, Smith BJ, Seiler KM, Winton DJ, Henning SJ. Side population sorting separates subfractions of cycling and non-cycling intestinal stem cells. Stem Cell Res. 2014 Mar;12(2):364-75.

Waddington, C.H. The Strategy of the Genes: a Discussion of Some Aspects of Theoretical Biology. London: Allen & Unwin, 1957.

Walford RL. The Immunologic theory of aging. Copenhagen: Munksgaard, 1969.

Walunas TL, Lenschow DJ, Bakker CY, Linsley PS, Freeman GJ, Green JM, Thompson CB, Bluestone JA. CTLA-4 can function as a negative regulator of T cell activation. Immunity. 1994 Aug;1(5):405-13.

Weismann A. Essays upon heredity and kindred biological problems. Oxford: Clarendon Press, 1889.

Weissman IL. Clonal origins of the hematopoietic system: the single most elegant experiment. J Immunol. 2014 Jun 1;192(11):4943-4.

Weissman IL. Stem cells: units of development, units of regeneration, and units in evolution. Cell. 2000 Jan 7;100(1):157-68.

Wherry EJ, Barber DL, Kaech SM, Blattman JN, Ahmed R. Antigen-independent memory CD8 T cells do not develop during chronic viral infection. Proc Natl Acad Sci U S A. 2004 Nov 9;101(45):16004-9.

Wherry EJ, Blattman JN, Murali-Krishna K, van der Most R, Ahmed R. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. J Virol. 2003 Apr; 77(8):4911-27.

Wherry EJ, Kurachi M. Molecular and cellular insights into T cell exhaustion. Nat Rev Immunol. 2015 Aug;15(8):486-99.

Wherry EJ, Teichgräber V, Becker TC, Masopust D, Kaech SM, Antia R, von Andrian UH, Ahmed R. Lineage relationship and protective immunity of memory CD8 T cell subsets. Nat Immunol. 2003 Mar;4(3):225-34.

Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH. Viable offspring derived from fetal and adult mammalian cells. Nature. 1997 Feb 27;385(6619):810-3.

Wilson A, Laurenti E, Oser G, van der Wath RC, Blanco-Bose W, Jaworski M, Offner S, Dunant CF, Eshkind L, Bockamp E, Lió P, Macdonald HR, Trumpp A. Hematopoietic stem cells reversibly switch from dormancy to self-renewal during

homeostasis and repair. Cell. 2008 Dec 12;135(6):1118-29.

Wolchok JD, Kluger H, Callahan MK, Postow MA, Rizvi NA, Lesokhin AM, Segal NH, Ariyan CE, Gordon RA, Reed K, Burke MM, Caldwell A, Kronenberg SA, Agunwamba BU, Zhang X, Lowy I, Inzunza HD, Feely W, Horak CE, Hong Q, Korman AJ, Wigginton JM, Gupta A, Sznol M. Nivolumab plus ipilimumab in advanced melanoma. N Engl J Med. 2013 Jul 11;369(2):122-33.

Workman CJ, Cauley LS, Kim IJ, Blackman MA, Woodland DL, Vignali DA. Lymphocyte activation gene-3 (CD223) regulates the size of the expanding T cell population following antigen activation in vivo. J Immunol. 2004 May 1;172(9):5450-5.

Wu AM, Till JE, Siminovitch L, McCulloch EA. Cytological evidence for a relationship between normal hemotopoietic colony-forming cells and cells of the lymphoid system. J Exp Med. 1968 Mar 1;127(3):455-64.

Wu T, Shin HM, Moseman EA, Ji Y, Huang B, Harly C, Sen JM, Berg LJ, Gattinoni L, McGavern DB, Schwartzberg PL. TCF1 Is Required for the T Follicular Helper Cell Response to Viral Infection. Cell Rep. 2015 Sep 29;12(12):2099-110.

Wu X, Roth JA, Zhao H, Luo S, Zheng YL, Chiang S, Spitz MR. Cell cycle checkpoints, DNA damage/repair, and lung cancer risk. Cancer Res. 2005 Jan 1;65(1):349-57.

Xie T, Spradling AC. A niche maintaining germ line stem cells in the Drosophila ovary. Science. 2000 Oct 13;290(5490):328-30.

Xin A, Masson F, Liao Y, Preston S, Guan T, Gloury R, Olshansky M, Lin JX, Li P, Speed TP, Smyth GK, Ernst M, Leonard WJ, Pellegrini M, Kaech SM, Nutt SL, Shi W, Belz GT, Kallies A. A molecular threshold for effector CD8+ T cell differentiation controlled by transcription factors Blimp-1 and T-bet. Nat Immunol. 2016 Apr;17(4):422-32.

Yanger K, Knigin D, Zong Y, Maggs L, Gu G, Akiyama H, Pikarsky E, Stanger BZ. Adult hepatocytes are generated by self-duplication rather than stem cell differentiation. Cell Stem Cell. 2014 Sep 4;15(3):340-9.

Zammit DJ, Turner DL, Klonowski KD, Lefrançois L, Cauley LS. Residual antigen presentation after influenza virus infection affects CD8 T cell activation and migration. Immunity. 2006 Apr;24(4):439-49.

Zhang Y, Joe G, Hexner E, Zhu J, Emerson SG. Host-reactive CD8+ memory stem cells in graft-versus-host disease. Nat Med. 2005 Dec;11(12):1299-305.

Zhou S, Morris JJ, Barnes Y, Lan L, Schuetz JD, Sorrentino BP. Bcrp1 gene expression is required for normal numbers of side population stem cells in mice, and confers relative protection to mitoxantrone in hematopoietic cells in vivo. Proc Natl Acad Sci U S A. 2002 Sep 17;99(19):12339-44.

Zhou S, Schuetz JD, Bunting KD, Colapietro AM, Sampath J, Morris JJ, Lagutina

I, Grosveld GC, Osawa M, Nakauchi H, Sorrentino BP. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nat Med. 2001 Sep;7(9):1028-34.

Zhou X, Ramachandran S, Mann M, Popkin DL. Role of lymphocytic choriomeningitis virus (LCMV) in understanding viral immunology: past, present and future. Viruses. 2012 Oct 29;4(11):2650-69.

Zhou X, Yu S, Zhao DM, Harty JT, Badovinac VP, Xue HH. Differentiation and persistence of memory CD8+ T cells depend on T cell factor 1. Immunity. 2010 Aug 27;33(2):229-40.