Cytokine Signaling Control
of Naïve CD8\(^{+}\) T-Cell Homeostasis

by
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B.Sc.E. Engineering Chemistry
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Abstract

Mounting effective adaptive immune responses requires a large naïve T-cell population with a wide diversity of target specificity. Naïve CD8+ T-cells depend on T-Cell Receptor (TCR) and γ, cytokine signals for their homeostatic survival and proliferation, but differences in sensitivity to these homeostatic signals among T-cell clones have been generally attributed to differences in TCR specificity. This thesis describes the novel identification and characterization of intrinsic heterogeneity in the TCR-independent abilities of CD8+ T-cells to respond to homeostatic γ, cytokines, and survive in their absence. These differences were predictably marked by expression of CD5, a surrogate marker of TCR:spMHC binding avidity. In vitro, CD5hi T-cells proliferate more robustly to saturating levels of the γ, interleukin (IL) cytokines IL-7, IL-2 and IL-15, while CD5lo cells have prolonged survival in the absence of dedicated homeostatic cues. IL-7 is the most critical cytokine for naïve T-cell homeostasis, and a detailed analysis of IL-7 signaling revealed that IL-7 responsiveness is primarily determined by IL-7 receptor (IL-7R) expression, which is correlated with CD5 expression. While T-cells share common relationships between IL-7-induced signaling and responses, the signaling network encodes distinct signaling requirements for survival, proliferation and CD8α induction responses. As a result, all T-cells survive when treated with high doses of IL-7, but only cells with a critically high level of IL-7R expression can induce sufficient signaling to proliferate. IL-7 depletion also scales with IL-7R expression, and the ‘overconsumption’ of IL-7 by CD5hiIL-7Rh T-cells can compromise their prolonged survival. In vivo, lymphoreplete mice preserve the homeostatic diversity of CD5 expression by maintaining physiological IL-7 levels that promote neither preferential proliferation nor survival of CD5hiIL-7Rh and CD5loIL-7Rh T-cells. However, elevated IL-7 levels in lymphopenic mice or lymphoreplete mice administered with exogenous IL-7 yield preferential expansion of CD5hiIL-7Rh T-cell subsets, elevating the mean CD5 expression of the T-cell repertoire. This demonstration of functional intrinsic heterogeneities in IL-7R expression between CD8+ T-cells supports a previously under-appreciated role for IL-7 in maintaining not only the size but also the diversity of the T-cell repertoire. Furthermore, the exemplified potential for preferential expansion of more auto-reactive CD5lo T-cells subsets has important implications for the design of cytokine therapies.

Thesis Supervisor: Douglas A. Lauffenburger
Title: Professor
Dedicated to my loving family
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The pursuit of a PhD is by no means a solitary work.

My time as a graduate student has been outstanding both in the quality of the intellectual experience that the MIT community affords, and the amazing friendships that I have forged both in and out of lab. I cannot begin to thank all of the people who have contributed to my graduate school experience.

I do not have adequate words to express my gratitude to Douglas Lauffenburger for his mentorship during my time at MIT. Doug has an unparalleled commitment to educating young scientists, always holding them as his first priority. He truly believes that at the end of the day, science should be fun and exciting, and is always looking to make sure his students get the very most out of their graduate experience. Moreover, despite all his other commitments, his door is always open to talk not only about science, but also about other life issues that shape our experience during graduate school. I have the utmost respect for Doug’s mentorship style, always leading through encouragement instead of criticism. Even on the worst days, he finds the best in what I have done, and I walk out of our meetings feeling inspired, intelligent and ready to take on the next challenge. I also greatly admire that he trusts his students to take whatever avenue they think is most exciting and fruitful, and by only providing gentle but critical guidance, he lets students feel vital ownership of their work. This remarkable flexibility and positivity has made me feel confident and empowered to be as good of a scientist as I can possibly be. I can only hope that someday I can be as effective of an advisor to my students that I feel that Doug is to his.

Although Doug has been my official advisor, my PhD was really the product of a three-way advising situation in which my entire thesis committee was invested and engaged in steering the project. Darrell Irvine’s mentorship has been particularly important in my development as a scientist. Darrell was always prepared to get into the nitty, gritty details of experiments, and ask important questions about how we could improve upon, clarify or extend the results of our experiments. In particular, he taught me to prioritize my workflow, search for the best angle to tell a story, and find the cleanest way to demonstrate biological phenomena. He was always very prudent about keeping up with the literature, and pulling ideas from sometimes very distal areas of biology that shed important light upon our results.

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Chapter 1  Highlights of This Thesis

The human adaptive immune system has been evolved to enable specific responses to a wide array of foreign pathogens. A major component of our adaptive immune system are T-cells, each of which is capable of detecting a different set of foreign antigens comprised of peptides bound to major histocompatability complexes (pMHC) via their unique T-cell receptor (TCR). T-cells are continually produced in the thymus where they obtain their unique specificity and are selected for tolerance to binding to self-pMHC (spMHC). Functional mature T-cells are then exported into peripheral tissues where they survey for infection. In order to exploit our immune capacity, our bodies must therefore strive to maintain a peripheral T-cell repertoire of maximum size and functional diversity of TCR specificity within a defined physical space and with limited chemical resources. It is well established that the critical chemical factors regulating peripheral homeostasis are spMHC and interleukin-7 (IL-7) [1]. However, relatively little is known about the independent abilities of these homeostatic cues to stimulate survival and proliferation across the T-cell repertoire and how they coordinate to control homeostatic population size and diversity. This thesis undertakes a detailed analysis of CD8⁺ T-cell homeostasis, with a particular focus on the role of cytokine signaling. It characterizes novel functional differences among CD8⁺ T-cells in their intrinsic cytokine responsiveness and survival capacities in the absence of homeostatic stimuli. This work reveals an important role for IL-7R expression in regulating the ability of T-cells to respond to and manipulate their cytokine environment. These findings have critical implications for our understanding of the maintenance of T-cell homeostasis and how the T-cell repertoire might be altered by cytokine therapies.

1.1  Homeostasis of T-Cell Population Size and Diversity: Open Questions

The size of the T-cell pool remains stable throughout most of life [1]. This occurs despite changes in the rate of naïve T-cell production as we age, periodic bouts of infection invoking massive effector T-cell proliferation and subsequent population contraction, and accruement
of memory T-cells. Maintaining the T-cell population size therefore requires a careful
dynamic balance of generation, survival, proliferation and differentiation. In addition to
regulating population size, maintaining the homeostasis of functional diversity of the naïve
T-cell repertoire also requires balancing these effects across TCR specificities. The
maintenance of T-cell homeostasis is believed to occur via competition for homeostatic
survival factors in the form of IL-7 and spMHC. The presiding model is that competition
for a diverse pool of cognate spMHCs maintains the diversity in the T-cell repertoire [1-3],
while IL-7 availability regulates the overall size of the naïve T-cell population [4, 5].
However, exclusive attribution of the control of T-cell diversity to TCR interactions requires
two critical assumptions. The first maintains that T-cells with different TCR specificities
have equal responses to available cytokines. The second is that all T-cells have equivalent
requirement for survival signals, meaning they survive equally well in the absence of
homeostatic stimulation. However, neither of these assumptions has been explicitly tested.

Despite the stringency for thymic selection, the naïve T-cell repertoire comprises a
distribution of TCR:spMHC interaction strengths which are reflected in their level of TCR
signaling-induced CD5 expression [6-9]. Studies have suggested that CD5hi T-cells have a
competitive advantage as a result of their stronger avidity TCR:spMHC interactions [10, 11].
This poses a potential challenge for the homeostasis of T-cell diversity. As described by the
‘competition-diversity paradox’, this should result in a narrowing of diversity favoring
stronger avidity spMHC interactions [12, 13]. Interestingly, this narrowing is not observed in
normal T-cell replete mice, but enhanced proliferation of CD5hi T-cells can be observed in
lymphopenic environments with depleted T-cell pools [10, 11]. While differences had
heretofore been attributed solely to differences in spMHC availability, IL-7 levels also differ
between normal and lymphopenic environments. This offers prospect that IL-7 signaling
may contribute to the observed differences both between different environments and
amongst T-cells with varying avidity for spMHC. However, the ability for variations in IL-7
availability to influence the survival and proliferation of T-cells with different TCR avidities
has not been characterized.
This thesis aimed to address the aforementioned gaps in our understanding of the regulation of T-cell homeostasis by carefully examining the role of cytokine signaling in promoting survival and proliferation of CD8+ T-cells of varying TCR specificity.

1.2 Heterogeneity in T-Cell Cytokine Responsiveness and Turnover

Previous studies have attributed all variations in the responses of T-cells to homeostatic cues to differences in their TCR specificity. However, this thesis identifies novel heterogeneities among mature CD8+ T-cells in their functional responsiveness to IL-7 in the absence of TCR stimulation. We find that differences in IL-7 induced survival, proliferation at CD8α inductions between CD8+ T-cells are predictably marked by their relative CD5 expression, whose levels are independent of IL-7 stimulation and maintained in the absence of spMHC. Only T-cells with high levels of CD5 are able to proliferate under IL-7 stimulation. Conversely, while all T-cells can survive at high IL-7 doses, CD5hi T-cells have prolonged survival when deprived of IL-7R and TCR stimuli. The IL-7 environment can therefore be varied to favor CD5hi or CD5lo T-cells at high or low IL-7 doses respectively. Differences in IL-7 dependent responses and CD5 expression were present even within monoclonal TCR-transgenic populations, and were maintained following priming/differentiation, suggesting these properties are intrinsic to an individual T-cell. CD5hi T-cells also had enhanced responses to the related cytokines IL-2 and IL-15, indicating potential globally enhanced cytokine responsiveness.

Our data suggest the mature T-cell pool is comprised of populations that differ not only in their TCR sensitivity, but also in their intrinsic cytokine sensitivity. Furthermore, co-variation of responses with TCR:spMHC avidity suggests an unappreciated role for IL-7 signaling in previously observed heterogeneous T-cell responses. Whereas the enhanced LIP of CD5hi T-cell clones in IL-7 rich lymphopenic environments had been attributed to their greater avidity for spMHC [10, 11], our data suggests enhanced IL-7 signaling may also potentiate proliferation. IL-7 signaling may thereby play a role in shaping both the size and the diversity/composition of the T-cell pool.
1.3 IL-7R as a Critical Controller of IL-7 Responsiveness

While many of the critical signaling pathways induced by IL-7 have been identified, few studies have quantitatively characterized IL-7 signaling across T-cell specificities in the absence of spMHC stimulation. To determine the signaling network basis of heterogeneous IL-7-induced responses among T-cells, we therefore undertook a detailed analysis of IL-7 signaling in CD5⁺ and CD5⁻ T-cells. Intriguingly, our data suggests that high IL-7R expression on CD5⁺ T-cells is the primary determinant of their enhanced IL-7 responsiveness. While proximal signaling capacity scaled directly with IL-7R expression, T-cells shared common relationships between their cytokine-induced signaling and responses, including survival, proliferation and CD8α induction. However, the signaling network encodes distinct signaling requirements for each response, such that all cells induced sufficient signaling to survive, but only cells with critically high IL-7R expression could initiate proliferation. This sharp signaling threshold is thereby responsible for generating large differences in functional cytokine responses from a comparably narrow distribution of IL-7R expression across the T-cell repertoire. Thus, while different IL-7 dose requirements for survival versus proliferation had been suggested [14], our data suggest both the IL-7 environment and IL-7R expression determine an individual cell's response.

1.4 IL-7R as Regulator of the IL-7 Environment

Tight control over IL-7R expression is thought to be critical for regulating competition for limiting pools of IL-7 amongst T-cells [5]. In the altruistic model of IL-7 regulation, negative feedback to IL-7R expression maximizes the naïve T-cell population size via scaling receptor expression with the need for IL-7 signaling amongst naïve T-cells cycling in and out of IL-7 rich lymphoid organs [5, 15]. However, this model makes assumptions of equal basal IL-7R expression and requirements for survival signaling that our data shows to be invalid. We therefore sought to characterize the ability of cells with heterogeneous IL-7R expression to deplete their IL-7 environment and survive in limiting IL-7 conditions. We demonstrate that the cellular consumption of IL-7 scales directly with IL-7R expression. However, depletion
of IL-7 doesn’t necessarily scale with persistence, as IL-7R\textsuperscript{hi} cells can ‘overconsume’ IL-7 early in culture, compromising their later survival. This suggests that the population size may not necessarily be maximized with available IL-7. Furthermore, while IL-7 levels \textit{in vivo} were thought to scale inversely with total cell numbers [4], our data suggests possible cytokine scaling inversely with total IL-7R expressed amongst those T-cells. Interestingly, we found that T-cells require periodic contact with IL-7 on timescales that match their proposed cycling times through IL-7 rich lymphoid organs [16, 17]. As IL-7 signaling is known to induce expression of key trafficking molecules [2, 18], heterogeneous IL-7R expression may therefore be converted to heterogeneous trafficking patterns.

1.5 IL-7 As Controller of the Homeostasis of CD5 Expression \textit{In Vivo}

While our data revealed TCR-independent heterogeneities in IL-7 signaling between T-cells, \textit{in vivo} encounters with cytokine occur in the presence of spMHC stimulation. To determine the physiological consequences of cytokine-dependent heterogeneities amongst T-cells, we therefore examined the relative capacity of CD5\textsuperscript{hi} and CD5\textsuperscript{lo} T-cells to survive and proliferate in both normal mice and when IL-7 is elevated during lymphopenia or IL-7 therapy. Intriguingly, normal lymphoreplete mice were found to maintain a homeostatic balanced between CD5\textsuperscript{hi} and CD5\textsuperscript{lo} subsets. At first this is somewhat perplexing, as co-variation of cytokine responsiveness with TCR:spMHC avidity could be expected to accentuate the problem posed by competition/diversity paradox, and favor population dominance by CD5\textsuperscript{hi}IL-7R\textsuperscript{hi} T-cells. However, an \textit{in vivo} bioassay indicated that lymphoreplete mice maintain IL-7 concentrations that favor neither selective persistence nor survival CD5\textsuperscript{lo} or CD5\textsuperscript{hi} T-cells. However, when IL-7 levels become increased during lymphopenic IL-7 treatment, CD5\textsuperscript{lo} T-cells have a proliferation advantage, and the resulting T-cell repertoire shifts towards higher CD5 expression.

Our data indicates that IL-7 is not only a regulator of the size of the T-cell population, but also significantly impacts the diversity of TCR avidities \textit{in vivo}, reflected in the distribution of CD5 expression across the repertoire. Expansion of more self-reactive clones has been
linked to autoimmune progression [19, 20], and enhanced IL-7 signaling has been proposed as a contributing factor in leukemia [21]. Therefore while the therapeutic use of IL-7 may expand overall T-cell numbers, it may have the unintended consequence of preferentially expanding undesirable T-cell populations. Given the increasing interest in IL-7 based therapies, further investigation of the intrinsic differences in the signaling networks across T-cell populations will help us understand the clinical impact of skewing the T-cell repertoire toward a CD5^hi^IL-7R^hi^ or CD5^lo^IL-7R^lo^ phenotype.

1.6  Integrated Quantitative Systems Analysis of T-Cell Homeostasis

While many of the critical mediators of spMHC -and cytokine- induced signaling have been identified, there is relatively little know about how these signals integrate collectively control T-cell responses [1]. While IL-7 induced signals are the most critical for naïve T-cell homeostasis, related cytokines, such as IL-2 and IL-15, have partially overlapping functions mediated by shared receptor components and downstream signaling pathways [22]. TCR-mediated signaling cross-talk with cytokine-induced signaling has also been proposed to yield both synergistic [23] and inhibitory [24] responses. Moreover, there exist multiple modes of feedback regulating cell responses, encoded both within cell-intrinsic signaling networks, and between networks of cells in their coordinated regulation of production, consumption and access to homeostatic spMHC and cytokine resources. Understanding the often non-intuitive behavior resulting from these complex interactions begs for an integrated quantitative systems approach. Using mathematical modeling to interpret and inform the design of experiments measuring quantitative and dynamic signaling and responses has proven to be a powerful method for deconvoluting key regulatory relationships from complex network behavior [25]. This thesis begins by undertaking a theoretical examination of the properties of the IL-7R signaling network architecture that may contribute to regulatory control under stimulation with IL-7 alone or in the context of other TCR and cytokine cues. Although the remainder of this thesis is devoted to the experimental characterization of T-cell behavior, its design is motivated by this quantitative approach, and opportunities to use data to inform quantitative models that may extend our understanding.
of the system behavior is highlighted throughout. In particular, we describe how the data presented here, combined with mathematical modeling, can be potentially used to inform the improved design of cytokine therapies.

1.7 Outline of Chapters

Chapter 2 reviews IL-7R and TCR signaling and current models for their regulation of the homeostasis of T-cell population size and diversity.

Chapter 3 undertakes a theoretical examination of the properties of the IL-7 signaling network architecture contributing to signaling control under stimulation with IL-7 alone or in the context of other TCR and cytokine cues.

Chapter 4 presents novel experimental findings supporting heterogeneous intrinsic cytokine responsiveness among T-cells in the absence of TCR stimulation that correlates with CD5 expression. Also demonstrated are previously unappreciated differences in the survival of T-cells in the absence of homeostatic stimulation.

Chapter 5 delves into a quantitative signaling analysis of IL-7-induced responses that reveals IL-7R expression as the dominant controller of cytokine responsiveness. While T-cells are shown to share common signal-response relationships, the IL-7 signaling network is revealed to encode distinct IL-7R signaling requirements for survival versus proliferation.

Chapter 6 determines how observed in vitro variations in IL-7 sensitivity and turnover translate to the in vivo environment, specifically examining how IL-7 levels in vivo maintain homeostasis and the effects of IL-7 therapy on the composition of the T-cell pool. Observed variations in IL-7 levels are used to re-interpret previous data in support of TCR-mediated variations between T-cells from different TCR-transgenic mice.
Chapter 7 ascertains the relative ability of T-cells with heterogeneous IL-7R expression to deplete their IL-7 environment, and examined the consequences of consumption on cell persistence and maintenance of population size.

Chapter 8 discusses in detail the implications of heterogeneity in IL-7R expression, cytokine responsiveness and turnover amongst T-cells, and proposes revised models for the regulation of population size and diversity with particular regard to the application of IL-7 therapies.

Chapter 9 proposes future studies in which experimental and computational approaches are used to clarify how signaling originating from TCR and other cytokine receptors interacts with IL-7, and design optimal therapeutic IL-7 dosing strategies.
Chapter 2  Signaling Control of T-Cell Homeostasis

2.1  Homeostasis of T-Cell Population Size & Diversity

2.1.1  T-Cell Development and Function

T-cells function to specifically recognize foreign pathogens during infection. Critical to this function is the expression of a unique T-cell receptor (TCR) by individual T-cells. Through genetic recombination of TCR genes during early development, upwards of $10^{15}$ TCR variants can be expressed [3]. In the thymus, a small fraction of T-cells are selected for the ability for their TCR to weakly recognize endogenous peptide fragments bound to major histocompatibility complex (MHC) molecules on the surface of antigen presenting cells (APCs). In this process – termed positive selection – those T-cells that don’t recognize self-peptide-MHC (spMHC) die by neglect. Potentially autoreactive TCRs that bind spMHC ligands strongly are eliminated by a negative selection process. During thymic maturation, T-cells also acquire one of two TCR co-receptors, CD4 or CD8, the latter of which is of interest to the studies discussed in this thesis. These fully mature ‘naïve’ T-cells leave the thymus and enter the peripheral lymphoid organs, tissues and circulation.

In the periphery, naïve T-cells must remain tolerant of weak interactions with self-peptides. While selection eliminates T-cells bearing TCRs that interact with spMHC too strongly or too weakly, there remains a distribution of different TCR:spMHC avidities over which tolerance is maintained. During infection, T-cells encounter foreign or ‘agonist’ peptide-MHC (pMHC) ligands not present during thymic development, and those T-cells capable of binding strongly to agonist MHC are activated by strong signaling through their TCR. These cells undergo clonal expansion and differentiation to produce a large number of ‘effector’ T-cells which perform functions specific to pathogen clearance. After clearance, the majority of effector cells die in a contraction phase. However, a small population of ‘memory’ T-cells remain which are able to mount a quicker immune response upon reencounter with the same foreign peptide than their naïve counterparts.
2.1.2 T-Cell Homeostasis

Maintaining an extensive diversity of TCR expression is critical to ensuring the presence of T-cells in the periphery that are strongly reactive to any particular foreign peptide, but not to self peptides. This diversity is strongly connected to overall size of the T-cell repertoire [3]. The objective of immune system homeostasis at its simplest can therefore be summarized as how to maintain the maximum number of tolerant T-cells within a defined physical space with limited chemical resources. T-cells are maintained in the periphery at very stable numbers over most of an animal’s lifetime [26, 27]. A careful balance of T-cell generation, survival, death, proliferation and differentiation shapes the naïve T-cell repertoire (Fig 2.1). Export of newly developed naïve T-cells from the thymus continually replenishes the T-cell pool, while naïve T-cell loss occurs via activation and differentiation into memory cells, as well as death during normal T-cell turnover.

Naïve T-cells in ‘lymphoreplete’ hosts - those with a full T-cell compartment - are normally quiescent and only proliferate only when encountering a foreign antigen [1, 2]. However, T-cell proliferation can occur in T-cell-deficient – or ‘lymphopenic’ - hosts, termed ‘homeostatic’ proliferation or ‘lymphopenia-induced’ proliferation (LIP), which results in partial restoration of T-cell population sizes [2, 28]. Regulation of T-cell numbers is therefore frequently studied by adoptively transferring T-cells into lymphopenic recipients. There is considerable debate over the relevance of LIP to T-cell maintenance in normal T-cell replete hosts [2, 29, 30], but there are arguably common mechanisms controlling cell viability and turnover [1, 2, 31]. Furthermore, LIP likely also contributes significantly to recovery of T-cell counts where the hematopoietic system is severely compromised, such as arising from whole body irradiation, chemotherapy or viral immunodeficiency [32, 33]. Lymphopenia also occurs in both early and late stages of life where thymic output is insufficient to fill the T-cell pool, and its study is therefore relevant to understanding how T-cell numbers and diversity are initially established and are impacted by ageing.
Figure 2.1. Dynamic Regulation of T-Cell Homeostasis

A schematic diagram of the dynamic balance of inputs and outputs required to maintain homeostasis of the naïve T-cell repertoire. New naïve T-cell cells are continually exported from the periphery. Mature cells then rely on signaling from limited amounts of survival ‘resources’ in the form of cytokines and peptides released by and presented on stromal cells. While naïve T-cells are normally quiescent, under conditions where resources are in excess, cells can undergo limited division, termed lymphopenia-induced proliferation (LIP). Cells exit the naïve T-cell pool when they become activated via contact with foreign antigens. These cells clonally expand and differentiate into effector cells. Most cells are clonally deleted after the infection is cleared, but a fraction persists as memory cells. Direct exit of T-cells from the naïve T-cell repertoire also occurs via normal T-cell turnover when faced with resource limitations. Lastly, this system must also respond when faced with acute system perturbations such as cell or cytokine therapies, or severe T-cell depletion after chemotherapy or viral attack.

One interesting feature of LIP is that proliferating cells acquire phenotypic and functional similarity to memory T-cells [2, 28, 33, 34]. Naïve T-cells undergoing division in lymphopenic hosts upregulate the memory cell markers CD44, CD122 (IL-2Rβ) and lymphocyte 6 complex (Ly6C) [35]. When activated with an agonist, they also acquire the ability to make IFN-γ, upregulate CD69 and downregulate TCR quicker than their naïve predecessors [35]. The lack of both effector cell formation and CD28 and IL-2 signaling requirements during memory T-cell generation by LIP distinguishes it from antigen-induced memory cell production [28]. However, both processes share common features including
TCR ligation and cell division [28, 36], and the resulting expression profiles are closely matched [31].

### 2.1.3 Homeostatic Signals Regulating T-Cell Population Size and Diversity

Once naïve T-cells have exited the thymus, they persist for at least several weeks in the periphery [2]. Their turnover is not regulated by an intrinsic survival clock, but rather by competition for limiting amounts of ‘resources’ required for their continued persistence. The two most critical homeostatic signals regulating both the survival and proliferation of T-cells have been identified as T-cell receptor (TCR) binding to self-peptide MHC (spMHC) and interleukin 7 binding to the IL-7 receptor (IL-7R) [1, 2] (Fig. 2.2). Competition for these limiting resources – also referred to as ‘space’ – determines both the size and the diversity (or composition) of the T-cell repertoire. The presiding model is that the diversity of the T-cell repertoire is determined primarily via competition for a diverse pool of cognate spMHCs. In contrast, IL-7 availability is thought to regulate the overall size of the naïve T-cell population, with each T-cell responding equally to IL-7. However, there is increasing evidence that a model supposing distinct roles for IL-7R and TCR signaling may be too simplified. Recent studies have suggested that IL-7R signaling in T-cells may vary with the strength of their spMHC signaling as a result of cross-talk between the TCR and IL-7R signaling pathways [24]. Yet these studies still imply that differences in TCR specificity underlie heterogeneities in ability of T-cells to respond to homeostatic cues. One particularly important point of contention with this model is that assumptions of equal reliance on and responses to cytokines amongst T-cells of different TCR specificities have not been rigorously tested. Potential heterogeneities in IL-7-induced proliferation and survival responses amongst naïve T-cells with different specificities would call for a re-examination of the classical roles of TCR and IL-7R signaling in the homeostasis of T-cell population size and diversity.
Figure 2.2. T-Cell Homeostatic Cues

Naïve T-cell survival and proliferation are dependent upon TCR binding to self peptides (sp) found to major histocompatibility complex (MHC) presented on antigen presenting cells (APCs) as well as soluble interleukin-7 (IL-7) secreted by stromal epithelia, which binds to the heterodimeric IL-7 receptor (IL-7R) composed of IL-7Ra (CD127) and the common gamma chain (γc/CD132).

In the following sections we discuss the evidence supporting the importance of TCR and IL-7R signaling in the regulation of homeostatic naïve T-cell survival and proliferation. We discuss both initial experiments supporting their distinct roles in the control of T-cell population size and diversity and new data that challenges this simplified model. We highlight unaddressed questions regarding the homeostasis of the T-cell repertoire, with a particular focus on those relating to the role of cytokine signaling that this thesis aims to address.

2.2 Role of TCR in T-Cell Homeostasis

2.2.1 Requirement for spMHC:TCR Engagement for Survival/Proliferation

Although strongly self-reactive cells are eliminated during selection, positive selection requires some reactivity with self [37]. Several experiments have demonstrated the continued
requirement for weak spMHC-induced TCR signaling for mature T-cell survival, proliferation and maintenance of function in the periphery [2]. Transfer of T-cells into hosts lacking relevant MHC ligands leads to their death [38-40], as does the inducible loss of TCR expression [41] or the Src kinases Lck and Fyn required for TCR signaling [42]. While these studies all involved lymphopenic hosts, a recent study has demonstrated a requirement for TCR:spMHC engagement within normal replete hosts [43]. Microscopy reveals that in the absence of infection, naïve T-cells are continually establishing transient contacts with spMHC within lymph nodes [44]. These low-affinity interactions upregulate CD3 phosphorylation that is abrogated upon loss of contact [42, 45]. However, it is controversial whether this signaling is functional or whether TCR:spMHC engagement elicits other signals or guides cell localization to receive additional stimulation, such as IL-7. Each T-cell receptor is able to bind to a variety of different peptides, albeit with differing affinity [3, 46, 47]. It is generally thought that the same self-peptides mediating positive selection in the thymus are responsible for maintaining survival and inducing proliferation in the periphery [34, 38, 40, 48, 49].

2.2.2 Mechanisms for TCR-Mediated Control of Diversity

Although it is the general consensus that diversity in the T-cell repertoire is maintained via a requirement for TCR:spMHC engagement, there is some controversy over the mechanism [1, 2]. Some studies suggest that there is intraclonal competition between cells of the same specificity, but not between cells of different specificity [50-52] (Figure 2.3a). As such, the expression and access to cognate spMHC expression limits the diversity of the T-cell repertoire. This model for competition is supported by recent work looking at clonal abundance (~20-200 for each epitope) [53], and the selective survival/proliferation of T-cell clones when their numbers are shifted above or below this apparent steady state [54]. Other work suggests there is a hierarchy between T-cells of different specificity in their capacity to compete for spMHC [10, 11, 55, 56]. There are two proposed models for a hierarchy in competitiveness [2]. The first suggests that TCR promiscuity, or the diversity of cognate spMHC that a T-cell can engage, determines competitiveness [56] (Figure 2.3b). The second suggests that not all interactions with spMHC are equal, and that stronger and/or more frequent interactions directly provide stronger survival signals [10, 11] or allow cells enhanced ability to compete for other survival factors [55] (Figure 2.3c).
Intracional Competition

Interclonal Dominance Through Promiscuity

Interclonal Dominance Through Fitness

Figure 2.3. Models of T-Cell Competition

Schematic of proposed models for the mechanisms of homeostatic competition regulation the size and diversity of the T-cell repertoire. (a) In the ‘interclonal competition’ model, T-cell diversity is maintained strictly via competition between T-cells of a given specificity for the available number of spMHC. T-cells of one specificity die or proliferate independently of another specificity to use all available resources. (b) In the ‘interclonal dominance through promiscuity’ model T-cells with the broadest array of spMHC specificity have enhanced capacity to compete for limited spMHC. (c) In the ‘interclonal dominance through fitness’ model T-cells with a greater ability to compete for homeostatic factors, either directly via higher avidity specific TCR:spMHC interactions, or resulting increased access to cytokine resources, are able to dominate the population. Adapted from Takada K and SC Jameson, 2009 [2].

2.2.3 Role of TCR Affinity in Regulating Homeostasis

Several studies examining competition between T-cells have suggested an important role for the affinity of TCR-spMHC interactions in regulating T-cell homeostasis [10, 11, 57]. When TCR-transgenic (TCRtg) T-cells of different TCR specificity were adoptively transferred into lymphopenic hosts, their relative ability to survive and proliferate strongly correlates with CD5 expression [10, 11, 57]. In particular, donor T-cells were only able to expand upon adoptive transfer into TCRtg hosts when the recipient CD5 expressing was less than that of the donor [10]. CD5 is a putative negative regulator of TCR signaling [58] that is up-regulated upon spMHC engagement [7]. Thus, CD5 expression levels are thought to reflect the strength of TCR-spMHC interactions [6-9] (Figure 2.4). As such, CD5high T-cells are thought to have increased homeostatic survival and proliferation properties as a result of greater ability to compete for spMHC. One proposed implication of selective expansion of higher avidity T-cells is that LIP may skew the T-cell repertoire toward greater self-reactivity [59]. Although, as noted by Takeda & Jameson [2], even cells with the greatest ability to compete will still reach some maximum number limited by cognate spMHC expression and other survival resources.
Figure 2.4. CD5 as an Indicator of TCR:spMHC Signal Strength
CD5 expression is increased as a result of spMHC interactions, and CD5 is therefore often used as a proxy for TCR avidity for spMHC. Here, we show a panel of TCR-transgenic T-cells which vary in their CD5 expression in the order F5<2C<OT1, and this hierarchy is reflected in their capacity to proliferate under lymphopenia (Ge, Bai et al. 2004).

2.2.4 TCR Signaling in Naïve T-Cell Survival and Proliferation
Aside from knowledge of some downstream TCR signaling activity following spMHC binding (evidenced by zeta chain phosphorylation), the exact signaling pathways critical for spMHC dependent survival and proliferation are not well characterized. For cells undergoing slow proliferation in lymphopenic hosts, microarray analysis suggests that signaling activity resembles that typical of IL-7 and TCR under activating stimuli, though at much lower levels [31]. Potentially critical to the regulation of spMHC signaling is feedback control to TCR signaling. As highlighted above, TCR signaling is believed to induce negative feedback to its own signaling via upregulating expression of CD5, a scavenger receptor whose intracellular tail binds phosphotases that inhibit TCR signaling [6]. TCR:spMHC signaling is also proposed to modulate expression of the CD8 coreceptor, which promotes binding to MHC [24]. TCR signaling is therefore believed to tune its relative activation thresholds via balancing feedback to CD5 and CD8α expression, though the mechanisms regulating this feedback, and whether they equalize signaling activity across different affinity TCRs, remain unknown.
2.2.5 Summary: TCR Signaling Control of T-Cell Homeostasis

In summary, it is clear that contact with spMHC is essential for regulating T-cell survival and function in vivo. Current studies suggest that the diversity of spMHC presentation determines the diversity of TCR expression of the T-cell repertoire, though there is some debate over whether there is a hierarchy in competitiveness between T-cells for overlapping spMHC specificity or access to spMHC and/or other resources.

2.3 Role of Cytokine Signaling in T-Cell Homeostasis

2.3.1 Homeostatic Requirement for IL-7 & Regulation of Population Size

Many experiments have demonstrated the requirement of IL-7 in both naïve and memory T-cell survival and proliferation [60-62]. IL-7Ra−/− and IL-7−/− mice are lymphopenic because IL-7 is critical to normal thymic development [63, 64]. The first demonstrations that IL-7 was involved in T-cell maintenance were therefore performed by treating thymectomized mice with anti-IL-7 antibodies, which resulted in severe T-cell depletion [65]. Similar results were seen upon transfer of naïve T-cells into IL-7−/− hosts [64]. Conversely, transgenic expression of IL-7 or exogenous IL-7 treatment results in increased T-cell expansion [66-68]. This lead to the suggestion that IL-7 might act as a limiting factor for the size of the T-cell pool [4, 5].

Other interleukin cytokines may also play a role in naïve T-cell survival. For instance, IL-15 can often substitute for IL-7, though it appears to more critical for memory cell maintenance [69, 70]. Similarly, IL-2 can also provide some survival signaling to naïve T-cells, though its presence in vivo is usually restricted to periods of production by effector T-cells following their activation, and these cells are much more sensitive to and reliant on IL-2 than naïve T-cells. There is some evidence that naïve T-cells in vitro are also maintained by IL-4, IL-6 and TSLP [71-73]. However, in vivo IL-7 appears to be the most important and other interleukin family cytokines are either not required or delivering redundant signals [62, 64, 74].

IL-7 is not produced by T-cells themselves, but is rather produced primarily by fibroblastic reticular cells in the T-cell zones of secondary lymphoid organs (SLOs) [75]. IL-7
concentrations in the serum have been estimated to be at or below the current limit of detection of approximate ~1 pg/mL [76]. However, IL-7 is believed to remain highly localized at its sites of production by virtue of binding to the extracellular matrix following secretion, as has been shown for other related cytokines [77]. While recent studies suggest some potential mechanisms for feedback to IL-7 production by dendritic cells following binding of displayed spMHC by CD4+ T-cells [76], for the most part IL-7 is thought to be produced at relatively constitutive rates [1]. The balance of constitutive production and cell-mediated consumption and degradation is therefore thought to regulate IL-7 concentrations in vivo, such that there is a linear inverse scaling of population size with in vivo IL-7 levels [4].

In accordance with its purported role as a limiting factor for population size, cell-mediated consumption of IL-7 levels is thought to drive cytokine to very low levels barely sufficient to support T-cell survival [2]. However, in lymphopenic hosts, the rise in IL-7 levels resulting from reduced cellular consumption are believed to play an important role alongside spMHC in potentiating LIP [2]. Proliferation would thereby continue until IL-7 and/or spMHC resources are depleted. In addition to regulating survival and proliferation responses, IL-7 signaling is thought to be critical for maintaining cell function [1]. In particular, IL-7 signaling may act to prime cells, lowering their TCR signaling threshold for activation [78].

2.3.2 IL-7R Signaling Control of T-Cell Survival and Proliferation

IL-7 binds to a dimeric receptor composed of the IL-7Rα chain (CD127) which is specific to IL-7 binding, and the common gamma chain γc (CD132) which is a shared component of the multimeric receptors of several related cytokines including IL-2, IL-4, IL-9, IL-15 and IL-21 [22, 70]. These cytokines are often referred to as IL-2 family or γc cytokines. As γc is expressed ubiquitously on the cell surface, responsiveness to IL-7 at the receptor level is controlled primarily by IL-7Rα abundance. IL-7 binding to IL-7R is thought to regulate T-cell responses primarily via activation of two signaling pathways which are important across multiple γc cytokines: Jak-Stat and PI3K/Akt [1, 2, 22] (Figure 2.5).
Interleukin-7 is thought to regulate T-cell responses primarily via activation of the Jak/Stat and PI3K/Akt pathways. Shown are the major pathways controlling cell survival, proliferation, migration/trafficking and metabolism. While Jak/Stat and PI3K/Akt have been shown to be independently important in each of these responses, it is clear that there is some degree of signaling pathway crosstalk. Adapted from Takada and Jameson (2009) [2].

Jak-Stat Signaling

The primary signaling pathway initiated by IL-7 is Jak-Stat activation. IL-7 binds to IL-7Rα with high affinity, mediating recruitment of the γ chain. This leads to reciprocal tyrosine phosphorylation of Janus kinases (Jaks) which are constitutively associated with the cytoplasmic tails of the receptor chains: Jak1 and Jak3 with the α and γ chains respectively. Activated Jaks phosphorylate tyrosine residues on the cytoplasmic tails of the opposing receptor chain. These serve as docking sites for the SH2 domain of the Signal Transducer and Activator of Transcription (Stat) proteins 1, 2, 3 and 5. Stat5 appears to be the most critical for IL-7 signaling, though it is activated by many other γ cytokines, notably IL-15 and IL-2 [79]. Jak-mediated Stat5 phosphorylation leads to Stat5 dimerization. Dimerized Stats then translocate to the nucleus where they regulate transcription of many genes, including γ cytokines [80]. Stat5−/− mice have decreased T-cell numbers [81-83], while overexpression enhances peripheral expansion of T-cells [84], suggesting a critical role for
Stat5 in peripheral T-cell homeostasis. Jak-Stat signaling also induces negative feedback through transcriptional upregulation of CIS and Soes1 [85]. Soes1 binds to Jak kinases, inhibiting their activity, whereas CIS inhibits Stat recruitment to the cytokine receptors [85].

**PI3K/Akt signaling**

IL-7 binding also induces the activation of PI3K and one of its key downstream targets, Akt. PI3K and Akt are critically implicated in many cytokine survival signals and can also induce cell cycling and gene transcription [22, 86, 87]. However, what little is known about their mechanisms of action is largely derived from studies of other IL-2 family cytokines and the relevance to IL-7 signaling is often uncertain. The activation of PI3K is linked to receptor signaling by the activity of the Jak kinases, which phosphorylate PI3K upon recruitment via its SH2 binding domain [88].

**Survival Signaling**

The major mechanism by which IL-7 is thought promote cell survival is by altering the balance between pro- and anti-apoptotic factors of the Bcl-2 family, which regulate the intrinsic mitochondrial cell death pathway [2, 89]. IL-7 induces the anti-apoptotic proteins Bcl-2 and Mcl-1 [90], and inhibits pro-apoptotic proteins Bax [91], Bad [92], and Bim [93]. Both Jak-Stat and PI3K-Akt signaling are thought to contribute to this mechanism of pro-survival signaling [2]. However, overexpression of Bcl-2 or Bim gene knockouts only partially rescues T-cell survival in IL-7R deficient mice [94, 95], suggesting other IL-7-induced signaling pathways contribute to survival. PI3K-mediated activation of mTOR [72] and upregulation of the glucose transporter Glut1 [96] are thought to be critical for preventing cell atrophy upon cytokine removal [97]. IL-7-induced PI3K-Akt signaling can also affect rates of T-cell trafficking to sites of IL-7 production at SLO via Foxo1 inhibition [98], as Foxo1 upregulates important homing molecules such as CCR7 and CD62L (L-selectin) [18].
Proliferation Signaling

Comparably less is known about signaling implicated in IL-7-mediated proliferation responses than its regulation of survival. Studies in IL-7-dependent T-cell lines have demonstrated mechanisms by which IL-7 may directly control proliferation. Li et al. show that IL-7 withdrawal leads to post-transcriptional upregulation of the Cdk2 inhibitor p27\(^{kip1}\) leading to G1 cell arrest, while cytokine re-addition lead to PKC\(\theta\)-mediated p27\(^{kip1}\) degradation and S phase entry [99]. The same group also found that IL-7 stimulates the phosphatase Cdc25a to remove an inhibitory phosphate on Cdk2 [100]. Akt modulates PKC\(\theta\) activity, suggesting a potential role for the PI3K-Akt pathway in mediating proliferation responses. Akt-mediated Foxo1 inhibition may also be involved in IL-7-mediated proliferation, as Foxo1 also inhibits P27\(^{kip1}\) [97, 99]. Thus while mechanisms for IL-7 induced proliferation have for the most part not been shown directly in primary cells, these studies suggest a role for IL-7 signaling beyond simply mediating T-cell survival.

2.3.3 Regulation of IL-7R Expression

As \(\gamma_c\) is expressed ubiquitously on the surface of almost all hematopoietic cells and is shared amongst multiple cytokine receptors, responsiveness to IL-7 at the cell surface is controlled primarily via IL-7Ra expression [5]. Multiple signals regulate IL-7Ra expression, including TCR signaling, other cytokines, and IL-7 itself [5, 15, 22, 101]. In addition to surface receptor loss due to internalization following IL-7 binding [102], IL-7R signaling leads to transcriptional downregulation of IL-7Ra expression [15]. Receptor downregulation occurs via induction of the transcriptional repressor GFI1, a mechanism common to multiple cytokines regulating IL-7R expression [15].

Negative feedback to IL-7R expression is thought to be a critical mechanism for rationing limited IL-7 resources amongst naïve T-cells to allow support for the maximum T-cell population size [5, 15]. The notion that IL-7R levels are optimally tuned via feedback control to maximize the population size under limited IL-7 conditions originated in studies of enforced IL-7R expression on mature T-cells [15]. In contrast to the expected increase in total population size from increased survival signaling, there was a paradoxical decrease in the peripheral T-cell population. This unexpected decrease was attributed to increased
consumption of limiting IL-7 beyond an individual cell’s survival requirements. The ‘altruistic’ model of IL-7R regulation therefore proposes that cells that have recently received survival signals downregulate IL-7R in order to not compete with cells that have sustained longer cytokine deprivation (Figure 2.6). This is thought to be particularly important in the context of cells cycling in and out of IL-7-rich SLOs, a notion further supported by the modulation of homing molecules by IL-7 signaling (Figure 2.6a). The broad distribution of IL-7R expression observed among naïve T-cells in vivo is therefore attributed largely to spatiotemporal variations in their contact with IL-7 as they move throughout the body [5, 15].

Figure 2.6. ‘Altruistic’ Model for Interleukin-7 Regulation
Schematic representation of the ‘altruistic’ model of IL-7 regulation, both within naïve T-cell populations, and between naïve and rapidly expanding effector T-cell populations. (a) In the absence of antigen stimulation, the periphery is occupied by the maximum number of T-cells that can be supported by the limiting amount of IL-7 in vivo. As T-cells enter IL-7-rich lymphoid organs, they bind IL-7, receive survival signals and downregulate their receptor so as not to compete with other cells entering these regions which still require survival signaling. When cells leave the periphery, survival signals dissipate and receptor is re-expressed. This allows for T-cells to maintain clonal diversity and optimally allocate cytokine survival resources. (b) When T-cells encounter a specific activating antigen, they begin to secrete their own survival cytokines, in this case, IL-2. The rapidly expanding population becomes dependent on these self-produced cytokines, and downregulates IL-7R so as not to compete for survival resources with the naïve T-cell population. In this way, antigen-induced proliferation can occur independent of the naïve T-cell pool and not inflict clonal loss. Adapted from Park et al (2004) [15].
Strict regulation of IL-7R expression is also thought to be critical for mediating competition between different T-cell populations. In both immature and mature cells, IL-7R expression is downregulated on rapidly expanding populations (Figure 2.6b). Downregulation of IL-7R by DN cells as they proliferate and differentiate into DP cells during thymic selection is thought to prevent them from competing with less abundant DN and SP cells [5]. Consistent with this idea, enforced expression of IL-7R throughout development results in an increase in DN cell death [103]. A similar mechanism is employed by activated cells, which lose IL-7R expression and become dependent on IL-2, thereby preventing their rapid expansion from affecting the naïve T-cell pool [15].

**2.3.4 Summary: IL-7R Signaling Control of T-Cell Homeostasis**

In summary, IL-7 induces Jak-Stat and PI3K-Akt mediated signaling critical to T-cell survival, proliferation and function. Strict control of IL-7Ra expression is critical for regulating sensitivity to cytokines, and mediating competition both within and between T-cell populations to maximize population size. IL-7 production is largely constitutive, and its availability is believed to limit the total T-cell population with the assumption that T-cells have equal cytokine requirements and sensitivity. While IL-7 levels in replete hosts are barely sufficient for survival, elevated IL-7 levels during lymphopenia are thought to be important for potentiating proliferation. IL-7 is therefore believed to control naïve T-cell population size, but not its composition.

**2.4 Interaction Between TCR and IL-7R**

While the studies discussed in the previous sections strongly indicate that TCR and IL-7R signals each influence both T-cell survival and proliferation, the mechanisms by which they interact in vivo to regulate overall T-cell homeostasis remain unclear. Several studies have suggested positive interactions between TCR and IL-7R signaling. In one model, stronger TCR:spMHC interactions promote more frequent and/or sustained access to IL-7 produced by APCs and/or bound to their surrounding ECM [55]. Similarly, higher TCR interactions have been suggested to sensitize cells to IL-7 signaling [11, 78], and vice versa [104].
Interestingly, many IL-7 target genes are overlapping with genes induced by TCR activation [22, 105], leading to suggestions that these two signaling pathways may cooperate to enhance each others signal strength or duration at the transcriptional level [22]. Experiments using T-cells lacking Lck, a kinase required for homeostasis signaling through the TCR, suggested that both TCR and IL-7R signals operate independently to promote lymphopenia-induced expansion, however in combination they synergize to give overall greater proliferative capacity [23]. One interesting potential result of proposed synergies in TCR and IL-7R signals is further reinforcement of what has been referred to as the ‘competition-diversity’ paradox [12, 13]. According to this model, the T-cell population should be dominated by those cells with the greatest ability to compete for survival/proliferation stimuli. Cells with both increased TCR and IL-7R signaling could therefore theoretically overrun the T-cell population to the extent that spMHC/IL-7 becomes limiting.

![Image](image.png)

**Figure 2.7. Co-Receptor Tuning Model**

(a) Schematic illustration of the co-receptor tuning model for balancing IL-7R and TCR mediated homeostatic signaling among T-cells of different TCR:spMHC avidity [24]. In this model, mutual feedback between IL-7R and TCR results in ‘tuning’ of CD8α co-receptor expression inversely with the TCR avidity of the T-cell clone. IL-7 signaling upregulates CD8α expression, but TCR signaling then feeds back to inhibit IL-7R signaling, which is reflected by less efficient IL-7R suppression. (b) This model is supported by an inverse correlation of CD8α expression with both CD5 and IL-7R expression for a panel of freshly isolated TCR-tg CD8+ T-cell clones.
In contrast to suggested synergistic interactions of TCR and IL-7R signals, Park et al (2007) [24] have proposed a model whereby signaling crosstalk between the two receptors acts to balance net homeostatic signaling. In what they have termed the 'co-receptor tuning' model, CD8α expression is regulated by mutual feedback between the IL-7Rα and TCR signaling pathways: IL-7Rα signaling induces the transcription of CD8α to increase TCR signaling, which negatively feeds back to reduce IL-7 signaling (Figure 2.7a). Reduced CD8α co-receptor expression on CD5hi T-cells is then proposed to 'tune down' excessive spMHC-induced TCR signaling. In support of this model, freshly isolated CD5hi male HY TCR-tg CD8+ T-cells lack the ability to phosphorylate Stat5 in response to IL-7. Also observed in support of their model is a positive correlation of IL-7Rα, and inverse correlation of CD8α, with CD5 expression for a panel of freshly isolated TCR-tg cells (Figure 2.7b). However, the observation that LIP of TCRtg T-cells varies with their TCR avidity for spMHC as reflected by their CD5 expression [10, 11] suggests that TCR and IL-7R signals are not fully balanced in some environments.

Thus, while it is increasingly evident that TCR and IL-7R signals interact to control T-cell responses, the specific mechanisms of interaction, and whether they reinforce or counteract the effects of varying TCR:spMHC affinity, are largely unknown.

2.5 IL-7 & IL-7 Therapies in Health & Disease

Because of its diverse roles in promoting T-cell development and function, there is much interest in the therapeutic manipulation of IL-7 levels in the treatment of multiple pathologies. IL-7 therapy has been shown to expand T-cell populations in normal mice by both thymic-dependent and independent mechanisms [106-108]. Under conditions of lymphopenia, such as following chemotherapy of whole-body irradiation, IL-7 addition also accelerates lymphoid regeneration [109-111]. IL-7 also has prospective uses as a vaccine adjuvant. When administered in combination with immunization, IL-7 enhanced T-cell responses to subdominant antigens and promoted the formation of long-lived memory cells [112, 113]. This has made IL-7 therapy attractive for use in cancer immunotherapies, as
tumor antigens are typically weak self-antigens with low immunogenicity. A recent study demonstrated that IL-7 enhances tumor specific immunity by multiple mechanisms, though it is inefficient in the absence of vaccination [113]. IL-7 may also find use in enhancing the ex-vivo expansion of patient cells for adoptive immunotherapy [114] and prolonging the survival of those cells in vivo after transfer.

IL-7 is also being explored in the treatment of HIV. Naïve T-cell proliferation under IL-7 therapy may be able to counteract the reduction in regeneration of lost CD4+ T-cells during HIV infection [115-117]. IL-7 may also help restore HIV-specific T-cells that are normally lost early in infection [114, 118, 119]. Despite its potential therapeutic uses, deregulation of IL-7 signaling has been implicated in HIV progression. IL-7 levels rise as T-cell numbers decrease, and increased IL-7 has been shown to increase HIV replication [120] and cell entry [121] and may predispose T-cells to Fas-mediated death [122].

Deregulation of IL-7 signaling has also been implicated in other disease pathologies. Patients with single nucleotide polymorphisms (SNPs) in IL-7, IL-7Ra and SOCS1 genes also have much higher risk for multiple sclerosis (MS), suggesting deregulated IL-7 signaling may be connected to the disease pathology [123-125]. Whilst IL-7 is being investigated for use in cancer immunotherapy, enhanced IL-7R signaling has been proposed to contribute to T-cell leukemogenesis [21]. Selective expansion of more self-reactive clones in response to increased IL-7 has also been proposed to contribute to development and progression of autoimmune disorders [19, 20]. Thus while IL-7 therapies may expand overall T-cell numbers, they may have the unintended consequence of preferentially expanding undesirable T-cell population subsets.

Two rhIL-7 phase I clinical trials have recently been completed in adult patients with refractory cancer [126, 127]. In both trials, CD8+ and CD4+ T-cells showed an IL-7 dose-dependent increase in population size, but no accompanying anti-tumor activity. Interestingly, authors saw a concomitant increase in the diversity of TCR VB usage amongst T-cells, which they attributed to predominant expansion of the more ‘diverse’ recent thymic emigrant (RTE) population [127]. The resulting TCR repertoire usage resembled that of healthy young adults, suggesting therapeutic use of IL-7 in rejuvenating the T-cell repertoire
of elderly or lymphopenic patients whose restricted repertoire may limit their immune competence [128]. However, significant questions remain as to how this increase in diversity of TCR Vβ segment usage translates to functional diversity, as contrary to increased thymic export, division of mature cells does not expand the number of unique TCRs in the periphery and may not translate to increase ability to respond to new antigenic challenges.

While the therapeutic potential of cytokines has been recognized for decades, there has been mixed success in their application. Early clinical trials of cytokine therapies were conducted prior to a good understanding of cytokine biology, particularly regarding the diversity of different responsive cell types and the subtleties involved in predicting their responses and half-lives upon systemic delivery [129]. Administration of high doses cytokines was found in some cases to invoke severe immunotoxicity [129-131]. These early failed attempts stalled the rapid development of related therapies, but nevertheless many successful applications for multiple cytokines, including IL-2, have been approved and/or are in clinical trials [129]. Given the diversity of different disease contexts for which IL-7 is being considered therapeutically relevant, and the number of IL-7 clinical trials already underway, a careful study into the consequences of IL-7 therapies and how they might be optimized is timely and critical to their successful development.

2.6 Unaddressed Aspects of Homeostasis Signaling Control & Aims of This Thesis

There is ample evidence supporting critical roles for both IL-7R and TCR signaling in regulating survival and proliferation responses. However, the mechanisms by which these signals integrate to regulate the homeostasis of the T-cell repertoire remain unclear. The presiding model is that competition for TCR engagement with spMHC maintains diversity in the T-cell pool [1-3] while IL-7 availability regulates the overall size of the naïve T-cell population but not its composition [4, 5]. This simplified model assumes that all variations in the homeostatic capacity of T-cells arise from the strength and/or frequency of engagement of their specific TCR with spMHC. It also implies that IL-7R signaling is uniform amongst
all T-cell populations, and that T-cells survive equally well in the absence of homeostatic stimuli. However, few studies have rigorously tested these assumptions. Recent studies suggest IL-7R signaling in T-cells may vary with the strength of their spMHC signaling as a result of TCR and IL-7R crosstalk [24], but these again imply that differences in TCR signaling underlie heterogeneities in the response to homeostatic cues. One potential problem with studies in support of distinct role for spMHC and IL-7 signaling is that in most cases responses induced by either TCR or IL-7R stimulation have not been examined independently of potentially heterogeneous signaling via the other receptor. This greatly confuses the ability to attribute variations in individual responses across T-cells to differences in either TCR or IL-7R signaling alone.

There are many instances in which the source of variations in T-cell responses to homeostatic signals may be arguable. For example, variation in the rates of LIP of naïve T-cell clones has been attributed solely to their strength of TCR signaling, indicated by their CD5 expression level [10, 11]. However, whether IL-7R signaling also varies with TCR specificity and potentiates proliferation responses and has not been examined. Expression levels of key homeostatic signaling components, including IL-7R [5] and CD5 [8], are known to vary among naïve CD8+ T-cell clones. While these differences in expression have been attributed to the interaction of T-cells with heterogeneous IL-7 and spMHC environments [7, 15, 24], they could also reflect stable cell-intrinsic differences in signaling pathways among mature T-cells. The potential misattribution of heterogeneous T-cell responses to variations in TCR signaling and/or the local environment calls for a carefully controlled re-examination of T-cell responses to individual homeostatic stimuli.

The general aim of this thesis is therefore to specifically examine the role of cytokine signaling in the regulation of CD8+ T-cell homeostasis. We first undertake a theoretical examination of the properties of the IL-7 signaling network that contribute to signaling control in the context of TCR and other cytokines. Prior assumptions regarding equal cytokine responsiveness and turnover across T-cells of different specificities are then experimentally tested in the absence of TCR stimulation. Taking a rigorous quantitative signaling analysis approach, we determine relationships between receptor expression and signaling activity to cytokine-induced survival and proliferation responses. In *vivo*
examinations of T-cell survival and proliferation in vivo are then used to determine how IL-7 signaling contributes to homeostatic control of population size and diversity, and examine the potential of cytokine therapies to alter the T-cell repertoire. Turning the tables, we then determine the capacity for the T-cell repertoire to alter their cytokine environment. This detailed analysis of cytokine signaling control is used to re-interpret previous models for the control of T-cell population size and diversity in both normal and disease contexts. By these means, we aim to clarify the role of cytokine signaling in the control of T-cell homeostasis.
Chapter 3 Perspectives on the Integrated Systems Analysis of IL-7 Receptor Signaling

In recent years, the analysis of cell signaling networks using quantitative systems biology approaches has revealed emergent phenomena that are not obvious based on the study of the individual network components [132]. Further, it has yielded a molecular understanding of previously observed phenomena at the level of the cell, such as cellular responses occurring only over a narrow range of ligand stimulation, and at the level of the entire organism, such as tissue homeostasis. Of particular relevance to the biology of IL-7 are lymphocyte differentiation checkpoints at various stages of development and the regulation of lymphocyte population size at the level of the organism. It is becoming increasingly clear that the IL-7R signaling network is regulated at multiple hierarchical levels in the signaling pathways. What is unclear is the relative contribution of the various arms of the IL-7R regulatory network in controlling IL-7R signaling efficacy and the IL-7R signaling network behavior as a whole in stimulating survival, proliferation and differentiation throughout lymphocyte development.

In this chapter, we present an analysis of the IL-7R signaling network and network behavior from an integrated systems perspective. A scheme of the IL-7R signaling network and its connectivity with other signaling networks in T-cells is depicted in Figure 3.1. We have highlighted the occurrence of the following mechanisms of signaling control: shared receptor components, signaling pathway crosstalk, common interaction domains, feedback regulation, integrated gene regulatory control, multimerization and ligand competition. In subsequent figures (Figures 3.2-3.8), we have illustrated the potential of each ‘network motif’ to affect the properties of the IL-7R signaling network including the strength and duration of IL-7R signaling. Because the quantitative properties of many of the system interactions are poorly characterized at present, we employ idealized models with assumed parameters in presenting these illustrations. We find that the interacting network components have the potential to affect the biology of the IL-7R signaling network in important but non-intuitive ways. We discuss the implications of these IL-7R signaling ‘network motifs’ on lymphocyte biology in health and disease. Since we have used idealized
models, the predictions presented in this review are best viewed as hypothetical system properties that will require confirmation using quantitative experimental and analytical approaches. Model details associated with each figure are outlined in the Appendix.

Figure 3.1. Control Mechanisms in the IL-7R Signaling Network
A schematic diagram of the IL-7R signaling network and its connectivity with interacting signaling networks, including TSLP, IL-15 and the TCR, is depicted. Important 'network motifs' of signal control are labeled with circled numbers.

3.1 Shared Receptor Components

Several cytokine receptors are multimeric complexes made up of two or more different component proteins, which are often shared between multiple cytokine receptors. This can
impact the relative availability of each receptor component, thereby limiting the extent of cytokine receptor signaling. Common examples of shared cytokine receptor components include sharing of the common gamma chain (\(\gamma_c\)) between the receptors for IL-2, -4, -7, -9, -15 and -21, the sharing of a common \(\beta\) chain between the receptors for IL-3, IL-5 and GM-CSF [133], and the sharing of the \(\beta\) chain between IL-2 and IL-15 receptor complexes [134]. In the case of the IL-7R complex, both receptor subunits are components of other receptors. As discussed in chapter 2, the \(\gamma_c\) chain is shared with five other cytokine receptors, all of which regulate T-cell growth and differentiation. As signaling specificity through the \(\gamma_c\) cytokine receptors is largely controlled by expression of the cytokine-specific \(\alpha\) chains (as well as the \(\beta\) chain for IL-2 and IL-15), these subunits are kept under tight transcriptional control throughout development. The IL-7R\(\alpha\) chain can also form a heterodimeric receptor complex on binding to the TSLPR, which is homologous to the \(\gamma_c\) chain [135]. IL-7R\(\alpha\) and TSLPR are co-expressed on T-cells, pre-B cells and dendritic cells (DCs) [136]. The ligand for the IL-7R\(\alpha\):TSLPR complex is TSLP, which is a cytokine that is homologous to IL-7, and is produced by cells of epithelial origin in the thymus, lung, gut and skin [137]. The presence of shared receptor components among cytokines can result in competition for the shared components among their respective ligands.

The association between the \(\gamma_c\) chain and IL-7R\(\alpha\) is required for the generation of a functional receptor complex and is mediated either by the binding of IL-7 or the activation status of T-cells [138]. The presence of competing cytokines can sequester IL-7 receptor components and adversely impact the ability to form functional IL-7R signaling complexes. For instance, IL-7 and TSLP can compete for IL-7R\(\alpha\). Similarly, IL-7 and IL-15 compete for the \(\gamma_c\) chain (Fig 3.2a). One potential repercussion of shared receptor components among cytokines is an upper limit on the total amount of cytokine-specific signaling that a cell can receive in response to multiple cytokines. For instance, IL-15 can compete for the shared \(\gamma_c\) component when its expression is limiting [139]. An example of the quantitative effects of this sharing is shown in (Fig 3.2b), which illustrates how the number of IL-7:IL-7R complexes may decrease on exposure to increasing levels of IL-15 in the presence of a constant level of IL-7. However, the net signal strength down any given pathway may
depend on the extent to which the γc chain is complexed with IL-7Rα or IL-15Rα. The use of shared receptor components also partly helps explain the overlap in many cytokine receptor signaling pathways. As discussed in subsequent sections, this pleiotropy can also lead to competition for intracellular binding partners and downstream signaling effectors that can affect the propagation of signals from a particular receptor when multiple cytokines are present.

Aberrant receptor competition may be related to pathological outcomes. For instance, atopic dermatitis and asthma involves pathological Th2 differentiation induced by dendritic cells primed with TSLP [140]. The effects of TSLP on T-cells are largely mediated by modulating the function of DCs [141]. IL-7 can also modulate DC function. For example, IL-7 is produced by inflamed synoviocytes in rheumatoid arthritis and it induces cell contact-dependent Th1 cytokine production in cocultures of synovial T-cells and monocytes [142]. Interestingly, skin keratinocytes have been shown to produce both IL-7 and TSLP, especially on exposure to certain pathogens [143]. The competing effects of TSLP and IL-7 on dendritic cells may therefore influence their ability to bias Th2 cell differentiation of CD4+ T-cells that they activate. Further, we speculate that the possible ability of IL-7 to modulate the effects of TSLP by ligand competition may open new therapeutic avenues for asthma or atopic dermatitis.
3.2 Shared Downstream Signaling Components

Many of the main signaling components of the IL-7R signaling pathway, including both positive and negative regulators of cell proliferation and survival, are shared with other cytokine receptors. During such an interconnected signaling network response, multiple input cues work together through a small set of signaling network effectors that propagate down and spread to a number of downstream targets. Competition for shared signaling mediators can result in a hierarchy of responses controlled at the level of abundance and relative binding affinities to upstream regulators of the response.

The cross-specificity in Jak and Stat activation is one of the major nodes of cytokine signaling crosstalk. The Jak kinase family is comprised of four members: Jak1, Jak2, Jak3 and Tyk2, each of which is found to be associated with multiple cytokine receptors [144]. For instance, Jak1 is associated with the α subunits of γ, cytokines such as IL-7Rα and IL-4Rα. Jak3 is associated with the γ chain [145, 146]. Cytokine binding stimulates the trans-phosphorylation of receptor associated Jak kinases, which in turn phosphorylate tyrosine residues on the receptors themselves. The receptor phosphotyrosines serve as docking sites for SH2 domain proteins including the Stat family of transcription factors which are activated by Jak-mediated phosphorylation. Signaling crosstalk due to shared Jak kinases likely underlies many of the redundant signaling activities observed among interleukin family pro-survival cytokines. Cytokines, as well as many other growth factors, activate overlapping subsets of the seven Stat family members (Stat1, 2, 3, 4, 5a, 5b, and 6) [144]. Thus, phosphorylation of multiple Stats by the Jak kinases also results in considerable crosstalk [83]. Yet another point of crosstalk exists in the induction of suppressor of cytokine signaling (SOCS) family members. The SOCS proteins include eight family members (SOCS 1-7 and CIS), each of which can inhibit signaling induced by multiple cytokines and growth factors by several mechanisms, including binding the Jak catalytic site, occupying the receptor Stat docking site, and targeting signaling proteins for degradation [147]. SOCS1 is the major SOCS family protein involved in IL-7R signaling regulation and is induced by other cytokines, especially IFNγ. Another major point of signaling crosstalk with the IL-7R signaling network is the PI3K-Akt pathway which is involved in a number of signal transduction networks that regulate cell survival [148]. Each level of cross-specificity in these
pathways, at the level of Jaks, Stats, SOCSs and PI3K-Akt targets, makes it harder to assign protein-specific effects and to deconvolve multi-cytokine responses.

![Diagram](image)

**Figure 3.3. Influence of Pathway Crosstalk on Signaling**

(a) Signaling through the common Jak-Stat pathway downstream of IL-7Rα and IL-15R. (b) Example of the anticipated kinetics of Stat5 phosphorylation after stimulation with either IL-7 or IL-15. Here we assume equal IL-7 and IL-15 receptor numbers and ligand concentration but that IL-7 binds to its receptor with 10-fold greater affinity than IL-15, as well as first order Stat dephosphorylation. In this case, the combined IL-7 and IL-15 signal give more rapid Stat5 phosphorylation than each of the cytokines alone, but a less than additive level of phosphorylation due to limiting amounts of Stat5. The combined signaling would approach the predicted additive signal upon increasing downstream Stat5 target expression.

Currently little is known about how pathways signaled by IL-7 are quantitatively regulated, especially in the context of concurrent signals derived from other cytokines or the T-cell receptor (TCR). When signaling originates from multiple cytokines, it is likely that limiting amounts of common downstream targets can result in less than additive activation profiles. This phenomenon is illustrated in Figure 3.3, which examines the combined activation of the Jak1-Stat5 pathway by IL-15 and IL-7 in the presence of limiting Stat5. When both cytokine receptors are simultaneously ligated, the combined cytokine signals results in an increase in the rate of the response but little increase in the magnitude of the response over individual cytokine treatments. For simplicity, we have treated IL-15 as a soluble ligand like IL-7 in this example. However, it is now established that IL-15 is primarily bound to IL-15Rα by IL-15 producing cells in vivo [149]. This is reminiscent of the biological activities of surface-tethered growth factors such as EGF, which induce sustained signals as they prevent the internalization of the receptor complex [150]. The effects of possible differences in
receptor internalization rates of cytokines like IL-7, which is soluble, and IL-15, which is presented by IL-15Rα, have not been investigated. These differences could impact the dynamics of downstream signal competition. Well-designed quantitative experiments at the network level will be required to develop strategies for therapeutic manipulation of the network due to complex interconnected effects of multiple pathway activation.

3.3 Common Interaction Domains

The vast inter-connectivity of signaling networks is largely a result of overlapping binding specificities of multiple proteins for the same target motif, which often occurs in several distinct signaling proteins. Thus, many kinases have numerous substrates, and signaling scaffold proteins can recruit several different signaling mediators to the same domain. Yet, cytokines often predominantly activate only a small subset of these signaling mediators. Simultaneous stimulation with multiple cytokines which share overlapping downstream partners can also influence the strength and dynamics of the signal. Multiple cytokines can compete for the same binding site on a limiting number of receptors. Alternatively, the presence of the same signaling domain on multiple receptors can lead to competition for limiting downstream signaling mediators. Response specificity, timing and prioritization for pathway activation is thus dictated by the relative abundance and binding strength of interacting signaling proteins.

Several conserved interaction domains are found in the intracellular domain of the IL-7Rα chain. The cytoplasmic tail of IL-7Rα has two regions, Box1 and Y449, which are thought to be of particular importance for signal propagation regulating survival, proliferation and thymocyte development. Box1 is an eight amino acid membrane proximal motif that binds Jak1 and is found in all type I cytokine receptors. Y449 is one of three tyrosines in IL-7Rα, which is conserved between humans and mice, and recruits SH2 domain-containing Stat family members when it is phosphorylated by receptor-associated Jak1. Although Stat5 is the major Stat recruited to the Y449 site on IL-7R signaling, SH2 domain homology with other Stat family members could lead to competition among the Stats for binding to the Y449 site (Figure 3.4a). In particular, Stat1, 3 and 5 have been shown to be activated by IL-7R
signaling [83, 102]. However, a mutation at the Y449 site does not completely abrogate Stat1 and Stat3 signaling [102], suggesting additional routes for their activation by IL-7. Additional phosphotyrosine binding proteins like the Shc adaptor protein and insulin receptor substrate proteins may also compete with Stat5 for binding to the Y449 site [22]. Competition at the Y449 site affecting Stat5 access could alter the timing and magnitude of Stat signaling. A hypothetical example of such competition between Stat5 and Stat3 is shown in Figure 3.4b. We have assumed that the Stat5:pY449 interaction is 10-fold stronger than the Stat3:pY449 binding and that the rates of Jak-mediated phosphorylation are equal for both Stats. The resulting delay in the kinetics of Stat3 phosphorylation, given limiting amounts of pY449 binding sites, is illustrated. Conversely, the presence of phosphotyrosines on other proteins that can also bind the SH2 domain of Stat5 could sequester Stat5 and hinder its binding to IL-7Rα. It has also been proposed that a second survival signal originating from the Y449 site arises from the recruitment of PI3K [88]. The difference in binding kinetics of PI3K and Stat5 to the Y449 site could regulate the extent and timing of the signal through these two pathways, which may influence the downstream integration of survival signals. Quantitative experiments of signal dynamics under varying IL-7Rα, PI3K or Stat5 levels will be needed to determine how competition for binding sites impacts propagation of survival signals.

![Figure 3.4 Influence of Conserved Binding Domain on Signaling](image)

(a) Competition between the SH2 domains of Stat3 and Stat5 for binding the phosphorylated Y449 residue in the IL-7Rα chain. (b) Hypothetical differences in the kinetics of activation of Stat5 and Stat3, which bind the same site (pY449) on the IL-7Rα receptor, with different affinities. Here we assume that the affinity of the pY449 site on IL-7Rα for Stat5 is 10-fold higher than that for Stat3, and that Stat3 and Stat5 phosphorylation rates by Jak1 are equal.
3.4 Signaling Feedback Control

Feedback loops comprise key mechanisms by which signal inhibition and propagation is controlled within cells. Signaling through a receptor may lead to signal inhibition via receptor internalization, induction of inhibitory phosphatases or transcriptional changes in receptor or regulator expression. Likewise, positive signaling feedback can be generated by inducing transcription of the receptor or its positive regulators or by autocrine secretion of a stimulatory ligand. Signaling feedback control is an important regulatory process in IL-7R signaling that allows avoidance of pathway saturation, establishment of signaling thresholds and fine-tuning of the signal at an optimal level for cell survival. Understanding the balance of positive and negative feedback loops will be essential for a complete understanding of cytokine responses.

Negative feedback loops are particularly important in the regulation of IL-7Rα expression. Receptor ligation leads to endocytic loss of the receptor from the surface, contributing to signal attenuation. In addition to receptor loss from internalization, several γc cytokines, including IL-7, activate both negative and positive feedback loops to modulate receptor mRNA expression. In CD8+ T-cells, downregulation is mediated by the transcriptional repressor Gfi1, which is upregulated upon IL-7R signaling, as well as signaling by other interleukin family members [15]. A second transcriptionally mediated negative feedback loop involves upregulation of Socs1 expression upon cytokine signaling (Figure 3.5). Socs1 can directly inhibit Jaks by acting as a pseudosubstrate through its kinase inhibitory region, as well as by ubiquitin-mediated degradation of the signaling complex itself [147]. In addition, cytokine-independent regulation of Socs1 also plays critical roles in regulating signaling by IL-7 and other γc cytokines throughout development. For example, Socs1 is expressed at high levels in DP cells during thymic development to prevent IL-7R signaling and possible aberrant positive selection [151]. Socs1 knockout mice show spontaneous activation of lymphocytes even in a pathogen free environment [152]. A number of negative regulators of the Stats have also been identified such as the PIAS family of proteins [153]. However, the relative contribution of these mechanisms of signal feedback inhibition to the overall control of IL-7R signal attenuation is yet to be elucidated.
IL-7R signaling also elicits positive feedback loops which contribute to signal amplification and sharp response thresholds. In developing B cells, IL-7R signaling causes upregulation of the transcription factors EBF and E2A, which in turn upregulate IL-7Ra, leading to a self-sustaining positive feedback loop [154]. Feedback loops in IL-7R signaling play a critical role in B cell development by maintaining B cell lineage commitment among differentiating common lymphoid progenitors. In addition, sustained IL-7R signaling is necessary for survival of pro-B cells [155]. EBF and E2A coordinately regulate the initiation of the B cell gene expression program as well as rearrangement of the immunoglobulin heavy chain loci [153]. The signal feedback loops between these proteins ensure normal development of B cell precursors through various checkpoints in B cell development.

It was recently shown that in macrophages, Tat protein produced by the human immunodeficiency virus (HIV) can cause upregulation of IL-7Ra and increase IL-7R signaling. Increased signaling in turn promotes early infection events including viral entry, and ultimately efficient viral production [156]. Interestingly, the effect of HIV Tat protein on CD8 T-cells was the opposite of that seen in macrophages, where it instead decreased IL-7Ra expression, inhibiting cell survival signaling [157]. A detailed analysis of the complex, cell-specific feedback mechanisms will help better understand how the IL-7R signaling network is exploited by pathogens.
3.5 Integrated Gene Regulatory Control

Interaction between signaling pathways at the gene regulatory level gives rise to a coordinated response and synergy in outcomes. Survival, activation and proliferation programs that are driven by antigen-receptor signaling and various γ cytokines are characteristic of lymphocytes. Microarray profiling has allowed for the high-throughput querying of gene expression programs regulated by cytokine and TCR signaling and their relationships with a variety of biological processes. High levels of overlap have been observed amongst the several hundred genes regulated by IL-2, IL-7 and IL-15. However, approximately 73% of these genes are also regulated by TCR signaling, and less than 20% of genes are unique to cytokine stimulation [105, 158]. Socs1 and Gfi1 are genes that are known to be transcriptionally induced during T-cell activation as well as by pro-survival cytokine signaling. Socs1 and Gfi1 inhibit the IL-7R signaling pathway at the post-translational and transcriptional levels respectively. IL-7Ra itself is transcriptionally downregulated by antigen-receptor signaling. In fact, it has been suggested that there is a greater overlap among TCR and interleukin-induced genes than amongst the genes induced by interleukin family members themselves [105]. This strongly suggests that gaining a complete understanding of IL-7 induced signaling will require consideration of how related cytokines and TCR signaling influence the transcriptional network response.

As a specific example of the impact of interacting gene regulatory control, we have illustrated the phenomenon of coreceptor tuning which is proposed to allow CD8+ T-cells to maintain their antigen-receptor signaling at levels just below the threshold of autoimmunity (Figure 3.6a) [24]. The levels of CD8 are a critical determinant of the responsiveness of a T-cell to self-peptide MHC (spMHC) as CD8 promotes the kinetics of binding of TCR to a spMHC complex [159]. Interestingly, CD8α is transcriptionally induced by IL-7R signaling while at the same time, IL-7R signals are inhibited by spMHC-induced TCR signal transduction. This leads to a mutual feedback loop at the level of gene regulation which results in co-regulation of T-cell survival and antigen responsiveness [24]. This allows CD8+ T-cells to adapt to the self-specificity of their unique TCRs so that they receive sufficient survival signals without losing self-tolerance. Figure 3.6b illustrates how the combined TCR and IL-7 survival signal would be expected to change as a function of the strength of
interaction between TCR and spMHC in the presence and absence of the coreceptor tuning effect. In the absence of coreceptor tuning, cells with higher self-reactivity would receive greater survival stimuli, making them potentially autoreactive. Coreceptor tuning may also allow T-cells with a weak responsiveness to spMHC to better receive other pro-survival signals from IL-7.

**Figure 3.6. Influence of Integrated Gene Regulatory Control on Signaling**

(a) Co-regulation of TCR and IL-7R induced signaling according to the CD8 coreceptor tuning model. IL-7 transcriptionally increases CD8α expression to promote TCR- self peptide-MHC engagement, but TCR signaling impairs IL-7R signaling. (b) The predicted effects of coreceptor tuning are shown by plotting how TCR and IL-7R signals would be expected to vary as a function of TCR input signal strength ('affinity' for spMHC) with constant IL-7 input signal, either with or without TCR mediated IL-7R signal inhibition as described by the coreceptor tuning model. In the absence of tuning, TCR signaling is enhanced by IL-7 signaling and increases with the TCR input strength, whereas negative feedback to IL-7 dampens the TCR signaling induced by high 'affinity' ligands.

Studying the circuitry of gene regulatory programs may help bring important new insights into the contribution of IL-7R signaling to autoimmunity and cancer. Exposure of CD8⁺ T-cells to low levels of IL-7 in vitro for a few hours can pre-dispose them to an enhanced syngeneic mixed lymphocyte reaction with syngeneic dendritic cells [24]. This phenomenon suggests that autoimmune phenotypes may involve deregulation of coreceptor tuning machinery. It has also been suggested that elevated responses to pro-survival cytokines may contribute to the ability to avoid death by lymphoblastic leukemia cells [21]. The altered responses of these leukemic cells likely reflect critical changes in gene expression.
potential therapeutic targets in the IL-7R signaling pathway will therefore require a comprehensive understanding of how normal control is achieved, and the transcriptional processes contributing to its pathological deregulation.

### 3.6 Multimeric Signaling Complexes and Combinatorial Complexity

The Stat proteins are transcription factors involved in γ cytokine signaling that are expressed constitutively at high levels in the cytosol of resting cells which facilitates their ability to induce a rapid response on phosphorylation by Jak kinases [144]. Tyrosine phosphorylation of Stats leads to their dimerization through their SH2 domain interactions. Both homodimers (all Stats except Stat2) and heterodimers (Stat1 and Stat2, Stat1 and Stat3, and Stat5a and Stat5b) are formed [22, 144]. Furthermore, tetramerization of Stats has been shown to occur in the case of Stat3, Stat4 and Stat5. Such higher order complex formation is mediated by the N-terminal domains of Stat proteins. The Stat dimers and corresponding tetramers could have differences in their respective DNA binding specificities [160].

The presence of several Stat protein multimerization states (monomer, dimer and tetramer) simultaneously present in the cell, each with different cellular function, complicates the ability to directly relate Stat phosphorylation state to activity and phenotypic outputs. Multimerization kinetics can also cause higher order oligomerization states to be turned on over narrower ranges of input signal (Figure 3.7). IL-7 has been shown to promote survival versus proliferation at different concentrations (0.1 and 1ng/mL respectively) in recent thymic emigrants, but the effect is not correlated with total Stat5 phosphorylation [161]. One potential explanation could be that Stat dimers and tetramers could preferentially induce cell survival or cell cycle entry respectively. As shown in Figure 3.7b, one possibility is that Stat5 dimers may be more abundant than tetramers upon weak IL-7 signals, with the opposite effect being seen for high IL-7 signals.
Figure 3.7. Influence of Component Multimerization on Signaling

(a) IL-7 induced formation of phosphorylated Stat5 dimers and tetramers, each with unique influences on gene expression programs. (b) Potential Stat5 phosphorylation response showing relative steady state levels of pStat5 monomers, dimers and tetramers with varying IL-7 stimulation dose assuming equal rates of dimerization and tetramerization and first order Stat dephosphorylation. There exist IL-7 concentrations at which dimer abundance is either greater or lesser than tetramer abundance due to a sharper dose response with higher multimerization states.

Due to cooperative effects, Stat tetramers may selectively increase the activity of certain promoters that have lower affinity binding sites for Stats. Indeed, mutational analysis of Stat5a-binding DNA oligonucleotides has demonstrated that it is possible to introduce specific mutations that virtually abolish Stat5a dimer binding without affecting binding as tetramers. Increased promoter occupancy may change the threshold for transcriptional activity while widening the gene transcription spectrum [160]. Some responses to cytokine signaling may be explained by the presence of conditions that favor the generation of dimers over tetramers or vice versa. In fact, the presence of high levels of Stat5 tetramers has been consistently seen in some leukemias [161]. Furthermore, combinatorial complexity in the dimerization of Stats can lead to additional diversity in the nature of signals transduced by IL-7. Stat5a and Stat5b are two closely related Stats with subtle differences in their DNA binding specificity. These differences are likely to be reflected in the DNA-binding specificities of Stat5a/b homodimers and heterodimers and also in their higher order complexes. Combinatorial complexity in these interactions can also arise from the heterodimerization of other Stats (Stat1/Stat3 or Stat1/Stat2).
3.7 Multicellular Ligand Competition

Lymphocyte homeostasis refers to the maintenance of the numbers and diversity of lymphocytes through an organism’s lifetime. The homeostasis of naïve T-cells is maintained by the competition for limiting amounts of pro-survival cytokines such as IL-7 and regular contact with spMHC [155]. Despite stiff competition for pro-survival ligands among T-cells, the homeostasis of T-cell numbers as well as diversity is maintained [3]. This can be better explained by studying how features of the IL-7R signaling network affect the behavior of the entire cell population.

T-cells have evolved strategies to maximize the efficiency of utilization of limiting amounts of IL-7 to maintain homeostasis. IL-7Rx downregulation, which is induced by IL-7R signaling, has been proposed to serve as a mechanism to maximize population size among cells competing for limiting IL-7 by allowing the maximum possible number of cells to receive pro-survival signals [15]. In this ‘altruistic’ model of IL-7R signaling, cells signaled by IL-7 transiently downregulate surface expression of IL-7Rx and thus become unresponsive to the continued presence of IL-7. The remaining IL-7 in the extracellular milieu can then be used to signal as of yet unsignaled cells. Cells are hypothesized to indefinitely cycle between signaled and unsignaled states with low and high receptor expression respectively [5]. It is not known if such cycling occurs in vivo and if it does occur, what the time scales of the cycling in receptor levels are. A further point of contention that remains unexplored is whether ‘altruistic’ behavior must be realized by two populations of ‘signaled’ and ‘unsignaled’ cells in vivo, driven for example by their respective arrival at the IL-7 production sites in the lymph node, or whether the entire population is maintained at constant receptor levels in equilibrium with extracellular IL-7. Furthermore, it is possible that the comparatively faster processes of IL-7Rx internalization are sufficient to regulate signal strength and depletion. This ‘altruistic’ behavior has been extended to explain the need for IL-7Rx downregulation upon T-cell activation (Figure 3.8) [15]. Activated T-cells undergo rapid expansion and their numbers can occasionally reach almost half of the total number of T-cells during some acute infections [162]. On activation, T-cells gain responsiveness to other cytokines such as IL-2 and downregulate IL-7Rx and IL-7 consumption, presumably
in order to preserve the naïve repertoire that is critically dependent on IL-7. Figure 6.8b shows the possible decrease in total naïve T-cell population numbers that might be expected in the absence of IL-7Rα downregulation in the activated pool as compared to what would be seen for an ‘altruistic’ activated pool. A similar interaction is believed to exist between double negative (DN) and double positive (DP) thymocytes. The DN population of lymphocyte precursors in the thymus is critically dependent on IL-7 and gives rise to DP cells that constitute a large fraction of the cells in the thymus. As forced expression of IL-7Rα on DP thymocytes results in reduced numbers in the thymus, it has been suggested that the physiological downregulation of IL-7Rα on DP cells results in sparing of the limiting IL-7 resource for the DN population [103].

IL-7 has been tested as a therapy for lymphopenic disorders [126]. A population level model of the IL-7R signaling network can be used to estimate optimal IL-7 therapeutic dosing regimens that minimize simultaneous activation of negative feedback loops. This will require careful determination of the effects of both receptor downregulation at increasing doses in combination with consideration of the effects of multi-cellular competition.
Figure 3.8. Influence of Multicellular Ligand Competition Effects on Signaling

(a) Competition between naïve and activated cells in the presence or absence of 'altruistic' IL-7Rα downregulation by activated cells. According to this model, downregulation of IL-7Rα prevents depletion of available IL-7 by the expanding population of activated cells, which is required for naïve T-cell survival. (b) An example of how the abundance of naïve and activated T-cells might be expected to change once activated T-cells undergo rapid IL-7 independent expansion (days 2-7) and either (i) downregulate their receptors ('altruism') or (ii) do not ('no altruism') according to the model in (a). The naïve T-cell population sizes decrease in the absence of altruism due to IL-7 ligand consumption.
3.8 Conclusions: An Integrated Systems Analysis of IL-7R Signaling

We have discussed several examples of pathological perturbations in the IL-7R signaling network. However, the involvement of IL-7R signaling in many diseases is not completely understood. For instance, a likely causal SNP (rs6897932) in the IL-7Rα gene, was recently identified in a population of multiple sclerosis (MS) patients [124]. This SNP results in aberrant expression of a soluble isoform of the IL-7Rα by putatively disrupting an exonic splicing silencer. Simultaneously, the level of the normal membrane-bound IL-7Rα is reduced. This may have an impact on the pathogenesis of MS by altering the dynamics of the IL-7 or TSLP signaling network in multiple cell types. Notably, TSLP-activated DCs are involved in CD4+ T-cell homeostasis [163] and T regulatory cell development in the thymus [164]. In another recent development, a low level of IL-7Rα was shown to be an excellent marker for human T regulatory cells and Foxp3 expression [165]. This further suggests that there may be hitherto unrecognized roles for the IL-7R signaling network in human diseases.

The IL-7R signaling network provides a good example of the utility of a systems perspective in examining how multiple levels and mechanisms of regulation interact to produce a dynamic cellular response. A view of the pathway that is focused on single components provides an incomplete picture of the regulatory network because IL-7R simultaneously signals through multiple shared pathways and IL-7R signaling is modulated through multiple concurrent mechanisms. We can reliably model the complex biology of IL-7R signaling only by integrating what is known about all the factors involved in signal propagation, repression and modulation. A comprehensive quantitative evaluation of the IL-7R signal network will extend our understanding of complex relationships involved in lymphocyte development and homeostasis. In addition, a detailed network analysis of IL-7R signaling may shed light on other cytokine signaling networks as well because it shares many components with other cytokine signaling networks. New technologies that allow quantitative network level analysis of signaling will improve our understanding of the immunobiology of IL-7R signaling.
Chapter 4  Intrinsic Heterogeneity in Cytokine-Dependent Naïve T-Cell Survival and Proliferation

The rates of LIP among different naïve T-cell clones vary depending upon the specificity of their TCRs, and correlate with their CD5 expression levels [10]. CD5 is a putative negative regulator of TCR signaling [58] that is up-regulated upon spMHC engagement [7]. Thus, CD5 expression levels are thought to reflect the strength of TCR-spMHC interactions [6-9]. Based on these data, variations in LIP have been attributed to differences in the avidity of T-cells for cognate spMHC [10, 11], with the implicit assumption that naïve T-cells bearing different TCRs have equal cytokine signaling capacity. Examinations of the lifespan of naïve T-cells have also inherently assumed that T-cells survive equally well in the absence of dedicated homeostatic cues [166-169]. Nonetheless, expression levels of key homeostatic signaling components, including IL-7R [5] and CD5 [8], are known to vary among naïve CD8’ T-cell clones. These differences in expression have been attributed to the interaction of T-cells with heterogeneous IL-7 and spMHC environments [7, 15, 24], but could also theoretically reflect stable cell-intrinsic differences in signaling pathways among mature T-cells. To explore this possibility, we tested the assumption that cytokine responsiveness is intrinsically uniform among distinct mature naïve CD8’ T-cell clones in the absence of spMHC signals. Our focus was primarily on responses to IL-7, as it is considered to be the most critical for naïve T-cell responses [1, 2]. However, we also briefly examine responses to the related gamma-chain cytokines IL-2 and IL-15, which have partially redundant roles in supporting naïve T-cell survival [1].

4.1  Heterogeneity in Cytokine-Induced Proliferation of CD8’ T-Cells

4.1.1  Heterogeneity in IL-7-Induced Proliferation of CD8’ TCR-tg T-cells

We sought to determine whether T-cells with a common genetic background but different antigen specificities respond similarly to IL-7. We began by studying responses between
 naïve T-cells isolated from TCR-tg mice as a model system for identifying potential variations across polyclonal T-cell repertoires. Naïve CD8$^+$ T-cells isolated from OT1 and F5 TCR-transgenic (TCR-tg) B6.Rag$^\text{2/2}$ mice (hereafter referred to as OT1 or F5 cells) were chosen for comparison as both cells are selected in the H-2b MHC background, but OT1 cells exhibit markedly greater LIP than F5 cells [10]. In support of studies linking LIP to TCR sp-MHC avidity, OT1 cells also have higher CD5 expression than F5 T-cells. Comparison of freshly isolated unpurified OT1 and F5 CD8$^+$ T-cells revealed a CD44$^+$ population in OT1 cells that was not present in F5 populations (Figure 4.1a). To ensure we were directly comparing naïve T-cell populations, we therefore sorted OT1 and F5 cells on their CD44$^+$ 'naïve' fraction for all experiments. Freshly isolated OT1 cells had slightly higher CD44, CD62L and TCR expression, and approximately equal CD8α expression, compared to F5 cells (Figure 4.1b).

![Figure 4.1](image_url)

**Figure 4.1. Surface Expression Profiles of OT1 and F5 TCR-tg CD8$^+$ T-Cells**

(a) CD44 expression on freshly isolated unsorted OT1 and F5 TCR-tg naïve CD8$^+$ T-cells. (b) CD44, CD62L, CD5, TCR and CD8α expression profiles of freshly isolated OT1 and F5 TCR-tg naïve (CD44$^+$) CD8$^+$ T-cells.

To compare the IL-7 proliferation responses of OT1 and F5 cells, we cultured CFSE-labeled cells at low density in media containing 10 ng/mL IL-7 for seven days. We were surprised to find that in vitro IL-7 stimulation alone had disparate effects on naïve OT1 and F5 cells: a fraction of the OT1 cells proliferated with 1-3 rounds of division while essentially none of the F5 cells divided (Figure 4.2a).
Figure 4.2. OT1 and F5 TCR-tg CD8+ T-Cells Differ in their Proliferation Responses to IL-7

(a) CD5 surface expression of freshly isolated OT1 and F5 TCR-tg naïve (CD44lo) CD8+ T-cells (top panel) and their proliferation when cultured in vitro at low density (~2x10^5 cells/mL) with 10 ng/mL of IL-7, as assayed by CFSE dilution after 7 days (middle panels), and quantified as the fraction of cells divided (bottom panel). (b) As described in (a), except for OT1 and F5 TCR-tg CD8+ in vitro memory-like cells generated via activation of naïve (CD44lo) OT1 and F5 TCR-tg CD8+ T-cells with plate-coated αCD3 and 20 ng/mL IL-2 for 3 days, followed by incubation with 40 ng/mL IL-15 for 3 days and overnight rest in cytokine-free media prior to IL-7 stimulation.

We next sought to determine whether this difference in IL-7 sensitivity was maintained between OT1 and F5 cells after activation and differentiation to memory-like cells. OT1 and F5 memory-like cells were generated in vitro using minor modifications to a procedure previously described [35]. Briefly, cells were activated on plate-coated anti-CD3 in the presence of 20 ng/mL IL-2 for 3 days, differentiated in 40 ng/mL IL-15 for 3 days, and then cultured overnight in the absence of cytokine. OT1 and F5 memory-like cells showed similar differences in proliferation in response to IL-7 compared to the naïve T-cells from which they were derived: OT1 cells underwent several rounds of division, while F5 cells were unable to proliferate (Figure 4.2b). These results suggested that T-cells of different TCR specificities have intrinsically different capacities to proliferate in response to IL-7 that are robustly heritable through several rounds of division.
4.1.2 Stability in the Relative CD5 Expression Between T-Cell Clones

In addition to maintaining their relative sensitivity to IL-7, OT1 and F5 cells maintained their relative CD5 expression following priming/differentiation (Figure 4.3c-d). CD5 is often used as a surrogate measure of the strength of spMHC-mediated signaling based on the requirement for continuous TCR engagement with spMHC to maintain CD5 levels [7]. We also found that CD5 levels on OT1 and F5 cells decayed over time during *in vitro* culture, independent of IL-7 stimulation (Figure 4.3a). However, naïve OT1 cells maintained their approximately 3-fold higher level of CD5 at all time-points (Figure 4.3b). Relative CD5 expression between OT1 and F5 cells was also maintained during IL-7 culture for memory-like cells (Figure 4.3c). This suggests that the basal CD5 expression is intrinsic to a particular T-cell clone, independent of IL-7 levels and TCR engagement.
Figure 4.3. Intrinsic Heterogeneity in IL-7-Independent Basal CD5 Expression
(a-b) Decay of CD5 surface expression of freshly isolated OT1 and F5 TCR-tg naïve (CD44lo) CD8+ T-cells cultured with 0, 0.1, 1 or 10 ng/mL IL-7, normalized to (a) initial expression for each cell type or to (b) initial CD5 expression on OT1 cells. (c-d) CD5 expression profiles before and after low density (~2x10^5 cells/mL) *in vitro* culture with 10 ng/mL for 7 days as flow cytometry histograms (left two panels) and quantified relative to the CD5^{hi} population for (c) OT1 and F5 TCR-tg naïve (CD44lo) CD8+ T-cells (d) OT1 and F5 TCR-tg CD8+ *in vitro* memory-like cells.
4.1.3 CD5 Marks Heterogeneity in the IL-7-Induced Proliferation Capacity of Polyclonal CD8+ T-cells

Maintenance of relative CD5 expression levels led us to hypothesize that CD5 expression might generally stratify T-cell populations with varying IL-7 sensitivity. To test this hypothesis in polyclonal T-cell populations, we sorted polyclonal naïve (CD44^lo) CD8^+ T-cells from B6 mice into fractions expressing the top and bottom ~20% CD5^hi and CD5^lo fractions. Similar to OT1 and F5 TCR-tg cells, polyclonal CD5^hi CD8^+ T-cells had slightly higher CD44 expression than CD5^lo cells (Figure 4.4). IL-7 sensitivity also showed similar trends to TCR-tg cells: Culturing CFSE-labeled cells at low density with 10 ng/mL IL-7 for 7 days yielded greater proliferation for polyclonal CD5^hi T-cells compared to CD5^lo cells (Figure 4.5a). CD5^hi and CD5^lo cells also maintained their relative CD5 expression levels following IL-7 treatment (Figure 4.5b). Similar differences in IL-7 responsiveness were observed when TAP1^+/ CD5^hi and CD5^lo T-cells were compared (Figure 4.5c,d), suggesting that the differential response to IL-7 is due to intrinsic differences in the ability of cells to respond to IL-7 and not a result of differential interaction with spMHC complexes presented on T-cells themselves. These data show that CD5 expression levels stratify the polyclonal naïve CD8^+ T-cell repertoire in terms of proliferation responses to the homeostatic cytokine IL-7, with higher CD5 levels marking cells with more robust proliferation.

Figure 4.4. Surface Expression Profiles of Polyclonal B6 CD5^hi and CD5^lo CD8^+ Naïve T-cells

CD44 and CD5 expression profiles of C57BL/6 naïve (CD44^lo) CD8^+ T-cells sorted into CD5^hi and CD5^lo expressing fractions compared to unsorted cells.
Figure 4.5. CD5 Expression Levels Stratify a Hierarchy in the IL-7-Induced Proliferation Capacities of Polyclonal CD8+ T-cells
(a) CD5 surface expression of freshly isolated C57BL/6 naïve (CD44o) CD8+ T-cells sorted into CD5hi and CD5lo expressing fractions (top panel) and their proliferation when cultured in vitro at low density (~2x10^5 cells/mL) with 10 ng/mL of IL-7, as assayed by CFSE dilution after 7 days (middle panels), and quantified as the fraction of cells divided (bottom panel). (b) As described in (a), except for freshly isolated TAP-/ C57BL/6 naïve (CD44o) CD8+ T-cells sorted into CD5hi and CD5lo expressing fractions. (c-d) CD5 expression profiles before and in vitro culture with 10 ng/mL for 7 days quantified relative to the CD5hi population for C57BL/6 naïve (CD44o) CD8+ T-cells from (c) wild type, and (d) TAP-/ mice.
4.1.4 Heterogeneity in IL-7-Induced Proliferation Marked by CD5 Expression

Even within a population of TCR-tg T-cells, CD5 expression levels are distributed with some variance. We hypothesized that CD5 levels might also stratify monoclonal T-cell populations in their responsiveness to IL-7. To test this idea, we sorted OT1 cells into CD5<sup>hi</sup> and CD5<sup>lo</sup> fractions (Figure 4.6a). Importantly, these cells express identical levels of TCR (Figure 4.6b). Following 6 days in low-density culture with 10 ng/mL IL-7, OT1 CD5<sup>hi</sup> cells showed a modest but statistically significant increase in proliferation over CD5<sup>lo</sup> cells (Figure 4.6a). This indicates that CD5 levels are intrinsic to an individual T-cell, even intraclonally and independent of TCR expression level, and can be used as a marker for the proliferation capacity of naive CD8<sup>+</sup> T-cells.

![Graph showing CD5 expression and proliferation](image)

Figure 4.6. CD5 Expression Marks Intraclonal Differences in IL-7-Induced Proliferation capacity of Naïve CD8<sup>+</sup> T-cells

(a) CD5 surface expression of OT1 TCR-tg naïve (CD44<sup>−</sup>) CD8<sup>+</sup> T-cells sorted into CD5<sup>hi</sup> and CD5<sup>lo</sup> expressing fractions (top panel) and their proliferation when cultured <i>in vitro</i> at low density (~2x10<sup>5</sup> cells/mL) with 10 ng/mL of IL-7, as assayed by CFSE dilution after 7 days (middle panels), and quantified as the fraction of cells divided (bottom panel).
4.1.5 CD5 Expression as a Global Marker of T-Cell Proliferation Capacity Across Multiple γ, Cytokines

We hypothesized that CD5 expression levels might also indicate responsiveness to other related pro-survival cytokines. IL-2 and IL-15 are considered to be more critical for supporting survival of effector and memory T-cells respectively compared to naïve T-cells, responses thought to be regulated via expression of the cognate receptor components and local cytokine availability [1]. However, IL-2 and IL-15 have overlapping signaling pathways with IL-7 (Chapter 3), and have been shown to partially compensate for IL-7 in supporting peripheral naïve T-cell survival in IL-7 depleted environments [69, 70]. Culturing CFSE-labeled OT1 and F5 cells in saturating levels of IL-7, IL-2 and IL-15 for 7 days revealed even more robust proliferation responses for OT1 cells in response to IL-2 and IL-15 compared to IL-7 (Figure 4.7a,b). In contrast, F5 cells did not divide in any of the cytokine cultures. Similar trends were seen between CD5hi and CD5lo sorted polyclonal CD8+ T-cells (Figure 4.7c,d).

Examination of the surface expression profiles of proliferated OT1 cells indicated an activated phenotype in IL-2 stimulated cells characterized by increases in CD44, CD25 and CD69 expression and a decrease in CD62L expression (Figure 4.8). In contrast, IL-15 and IL-7 induced a milder increase in CD44 expression, and both showed little or no change in any other indicator of activation. Again, similar trends were observed for polyclonal CD5hi cells that had undergone cytokine-induced proliferation (Figure 4.9).

These data suggest that CD5hi T-cells may have globally enhanced responsiveness to related homeostatic pro-survival gamma chain cytokines. However, high doses of these cytokines may be sufficient to induce activated T-cell phenotypes. Despite IL-7 yielding weak proliferation compared to IL-2 or IL-15, we focused for the remainder of this thesis on characterizing IL-7 responses, as IL-7 is considered essential for naïve T-cell survival, while IL-2 and IL-15 provide only overlapping or partially redundant signals which may be additionally restricted via production/localization [1].
Figure 4.7. CD5 Expression Marks Differences in Proliferation Responses to Multiple Pro-Survival Cytokines

(a-b) Proliferation of OT1 and F5 TCR-tg naïve (CD44lo) CD8+ T-cells when cultured in vitro at low density (~2x10^5 cells/mL) with either 10 ng/mL of IL-7 (top panels), 20 ng/mL IL-2 (middle panels) or 40 ng/mL IL-15 (bottom panels), as assayed by (a) CFSE dilution after 7 days, and (b) quantified as the fraction of cells divided (bottom panel). (c-d) As described in (a-b), except for C57BL/6 naïve (CD44lo) CD8+ T-cells sorted into CD5hi and CD5lo expressing fractions.
Figure 4.8. Surface Expression Profiles of OT1 TCR-tg T-cells Proliferating in Response to Pro-Survival Cytokines

CD44, CD25, CD5, CD62L, TCR, CD69 and CD122 surface expression versus CFSE dilution for OT1 TCR-tg naïve (CD44+) CD8+ T-cells following 7 day in vitro culture with either 10 ng/mL IL-7 (left column), 20 ng/mL IL-2 (middle column) or 40 ng/mL IL-15 (right column).
Figure 4.9. Surface Expression Profiles of B6 CD5<sup>hi</sup> CD8<sup>+</sup> Naïve T-cells Proliferating in Response to Pro-Survival Cytokines
CD44, CD25, CD5, CD69 and CD62L surface expression versus CFSE dilution for CD5<sup>hi</sup> sorted C57BL/6 naïve (CD44<sup>lo</sup>) CD8<sup>+</sup> T-cells following 7 day <i>in vitro</i> culture with either 10 ng/mL IL-7 (left column), 20 ng/mL IL-2 (middle column) or 40 ng/mL IL-15 (right column).

4.1.6 Enhanced IL-7 Responsiveness of CD44<sup>hi</sup> T-Cells
OT1 mice have a CD44<sup>hi</sup> peripheral T-cell population <i>in vivo</i> (Figure 4.10a), and can exhibit a mild increase in CD44 expression upon IL-7 treatment <i>in vitro</i> (Figure 4.8). We therefore hypothesized that high CD44 expression might mark cells with greater cytokine sensitivity. We therefore sorted OT1 cells into CD44<sup>hi</sup> and CD44<sup>lo</sup> fractions and examined their proliferation in response to 10ng/mL IL-7 for 7 days. While both CD44<sup>lo</sup> and CD44<sup>hi</sup> populations underwent division, CD44<sup>hi</sup> T-cells had more robust proliferation (Figure 4.10b,c). CD44<sup>hi</sup> cells also showed an increase in CD44 expression and a decrease in CD62L expression indicative of conversion to a memory-like phenotype that was not observed in the CD44<sup>lo</sup> population (Figure 4.10b). These data suggest that the CD44<sup>hi</sup> population observed in OT1 mice may represent cells that have already undergone LIP and conversion to a memory-like phenotype, and these cells are imparted with an enhanced sensitivity to IL-
7 compared to their CD44<sub>i</sub> counterparts. To facilitate direct comparison of the same T-cell population across different TCR specificities, we focused on characterizing responses of the CD44<sub>i</sub> 'naïve' T-cell population for the remainder of this thesis.

Figure 4.10. OT1 CD44<sub>hi</sub> CD8<sup>+</sup> T-cells Have Enhanced IL-7-Induced Proliferation
(a) CD44, CD5 and CD8α expression profiles of OT1 TCR-tg CD8<sup>+</sup> T-cells either freshly isolated or sorted into CD44<sub>lo</sub> or CD44<sub>hi</sub> expressing populations. (b) Proliferation as assayed by CFSE dilution for OT1 TCR-tg CD8<sup>+</sup> T-cells sorted into CD44<sub>lo</sub> or CD44<sub>hi</sub> expressing fractions when cultured in vitro at low density (~2x10<sup>5</sup> cells/mL) for 7 days with 10 ng/mL of IL-7. CFSE dilution profiles are shown as histograms or against the expression of CD44, CD5 or CD62L. (c) Quantification of proliferation as the fraction of cells divided for the conditions described in (b).
4.2 Heterogeneity in the Cytokine-Deprived Survival Capacities of CD8⁺ Naïve T-Cells

4.2.1 Prolonged Survival of CD5⁺ T-Cells in the Absence of Homeostatic Signaling

Having observed differences in the IL-7-induced proliferation capacities of naïve T-cells, we next asked whether there were comparable differences in the ability of IL-7 to support survival in CD5⁺ and CD5⁻ cells. We first examined survival responses of OT1 and F5 cells. However, freshly isolated cells undergo an acute, IL-7 independent crash in survival in the first 8-12 hours in culture (Figure 4.11) that is unpredictably variable depending on the age and sex of the mouse and the isolation procedures used. Furthermore, residual signaling from heterogeneous IL-7 and spMHC environments between TCR-tg mice could convolute examination of responses directly following isolation. Therefore, for all experiments, we cultured cells in cytokine-free media overnight (~16 hours) before stimulating with IL-7.

![Figure 4.11. Initial IL-7-Independent Acute Death Following Isolation of TCR-tg CD8⁺ T-Cells](image)

Viability of freshly isolated OT1 and F5 TCR-tg naïve (CD44⁺) CD8⁺ T-cells cultured at low density (~2x10⁵ cells/mL) in vitro in media containing IL-7 at concentrations ranging from 0.001-10ng/mL at varying time points over 24 hours showing an early IL-7 independent acute death immediately following isolation.

To examine survival responses, overnight rested OT1 and F5 cells were cultured in IL-7-supplemented or cytokine-free cultures. Both OT1 and F5 naïve T-cells exhibited ~100% survival over three days in the presence of saturating doses of IL-7 (Figure 4.12a). However, F5 cells survived better in vitro than OT1 cells in the absence of IL-7. Polyclonal CD5⁺ and CD5⁻ naïve CD8⁺ T-cells behaved similarly to their TCR-tg counterparts, with CD5⁻ cells
surviving better in cytokine-deprived culture though differences were milder (Figure 4.12b). Thus, while CD5lo and CD5hi CD8+ T-cells have different abilities to proliferate in response to IL-7, they also have different abilities to survive in the absence of homeostatic stimuli.

4.2.2 Increased Glucose Uptake by CD5hi T-Cells
IL-7 supports survival in part by increasing glucose uptake via up-regulation of the Glut1 glucose transporter [96]. Upon cytokine withdrawal, Glut1 is internalized and degraded, and cells lose glucose uptake and atrophy. To determine whether OT1 and F5 cells differed in glucose uptake, we measured their radioactive glucose incorporation following overnight culture in the presence or absence of IL-7. While both cells increased glucose uptake with IL-7 treatment, F5 cells had greater uptake than OT1 in both the presence and absence of IL-7 (Figure 4.13). Greater glucose uptake in F5 cells may therefore support prolonged cell
survival in cytokine-depleted conditions, whereas in the presence of cytokine, both cells may have sufficient glucose uptake.

![Image](image.png)

**Figure 4.13. F5 T-cells Have Increased Glucose Uptake**
Radioactive glucose uptake for OT1 and F5 TCR-tg naïve (CD44°) CD8+ T-cells cultured 16 hours +/- 10 ng/mL IL-7, rested 30 minutes in serum- and glucose-free media, and then incubated for 45 minutes with 0.1mM 2-deoxy-D[1-3H] glucose (4 μCi/mL).

4.2.3 Increased Sensitivity to PI3K Inhibition of CD5hi T-Cells

PI3K is a critical mediator of IL-7-mediated glucose uptake [96]. We therefore hypothesized that F5 cells might have increased PI3K signaling activity compared to OT1 cells. Low Akt phosphorylation levels in the absence of cytokine signaling prevented accurate quantification of phospho-Akt as a proxy for signaling activity (data not shown). We therefore took an alternative approach and determined the sensitivity of OT1 and F5 viability to PI3K inhibition. Inhibition of PI3K by LY294002 did not significantly decrease OT1 or F5 survival over 24 hours in the presence 0.1 ng/mL IL-7 (Figure 4.14a). However, F5 cells had lower sensitivity to PI3K inhibition in the absence of cytokine, with an IC50 of 25uM LY294002 compared to 10uM for OT1 cells (Figure 4.14b). Similar differences in sensitivity were observed with a second PI3K inhibitor, PI-103 (Figure 4.14c). This suggests that greater baseline PI3K activity in CD5hi T-cells may support their prolonged survival in cytokine-deprived conditions via PI3K-dependent mechanisms, including glucose uptake.
Figure 4.14. F5 T-Cells Have Decreased Sensitivity to PI3K inhibition in the Absence of IL-7
(a-b) Sensitivity of viability to PI3K inhibition for OT1 and F5 TCR-tg naïve (CD44<sup>+</sup>) CD8<sup>+</sup> T-cells rested overnight (~16 hours) in cytokine-free media and then treated 24 hours with varying doses of the PI3K inhibitor LY294002 in (a) cultures containing 10ng/mL IL-7, and in (b) cytokine-deprived cultures, normalized to uninhibited viabilities. The LY294002 IC<sub>50</sub> for the viability of OT1 and F5 T-cells in the absence of cytokine is shown to be 10 μM and 25 μM respectively. (c) Same as (b), but for PI3K inhibition with 6.25μM PI-103.

4.2.4 Cell Size Variation with CD5 Expression

IL-7 is known to be an important regulator of cell size that is often linked to its ability to support metabolism and cell survival via PI3K-dependent pathways [72]. Despite the fact that F5 cells appeared to have enhanced metabolic capacity in the absence of cytokine as measured by prolonged survival and PI3K activity, F5 cells have smaller cell size (as measured by Forward Scatter in Flow Cytometry Measurements) than OT1 cells (Figure 4.15a,c). This same trend is seen between CD5<sup>+</sup> and CD5<sup>-</sup> polyclonal CD8<sup>+</sup> T-cells (Figure 4.15b,c). This suggests that cell size may be more closely linked to IL-7 responsiveness than cell survival capacity in the absence of cytokine.
4.3 Different IL-7 Dose Requirements for Survival and Proliferation

Our examinations of cytokine responses thus far had focused on saturating doses of IL-7 >1ng/mL. However, IL-7 levels in vivo are thought to be much lower [2, 76], and still support cell survival. We therefore titrated the level of IL-7 in vitro and examined the 24 hour survival and 5 day proliferation of OT1 and F5 cells (Figure 4.16). The fraction of cells expressing the proliferation antigen Ki67 was used as a more sensitive readout of proliferation. IL-7 concentrations >= 0.1ng/mL were able to support complete survival of both OT1 and F5 cells (Figure 4.16a), while concentrations >=1ng/mL were required to induce proliferation in OT1 cells (Figure 4.16b). Different IL-7 dose requirements for survival and proliferation have previously been identified for polyclonal recent thymic emigrant T-cell populations [14]. However, our data suggests that the IL-7 requirements for survival are similar among T-cell clones, and while greater IL-7 doses can support proliferation for some cells, the extent of the response depends on the particular T-cell examined.
Figure 4.16. Different IL-7 Dose Requirements for T-Cell Survival versus Proliferation
(a) 24 hours viability and (b) 5 day proliferation (as assessed by Ki67+ fraction) of OT1 and F5 TCR-tg naïve (CD44+CD8+) T-cells rested overnight (~16 hours) in cytokine-free media and then cultured in vitro at low density with IL-7 concentrations ranging from 0.0001-100ng/mL.

4.4 Summary: Heterogeneity in IL-7 Responsiveness and Turnover Among the Naïve T-Cell Repertoire

We have identified differential IL-7 responsiveness amongst mature TCR-tg and polyclonal CD8+ T-cell populations in the absence of TCR signaling that is correlated with CD5 expression. CD5 levels are IL-7-independent, and relative differences in basal CD5 expression are maintained in the absence of spMHC. At saturating doses of IL-7, all T-cells survived efficiently, but only cells with high levels of CD5 were capable of proliferating. Conversely, at low doses of IL-7, CD5- cells had prolonged survival, that was also reflected in their PI3K-dependent glucose uptake. Differences in IL-7 responses are presents even intra-clonally within populations bearing the same TCR specificity, and persist through several rounds of activation-induced proliferation. CD5hi populations also have increased responsiveness to the related γc cytokines IL-2 and IL-15. This suggests that the T-cell repertoire is comprised of populations with both intrinsic differences in TCR specificity and cytokine responsiveness. Thus, while the enhanced LIP of CD5hi T-cell clones has been previously attributed to their greater avidity for spMHC [11], our data suggests differential cytokine signaling may also potentiate proliferation.
Chapter 5  Quantitative Signaling Analysis of IL-7 Responsiveness

In Chapter 4, we characterized heterogeneous abilities among naïve T-cells to proliferate in response to IL-7 stimulation that was predictably marked by their CD5 expression. We hypothesized that differences in cellular responses to IL-7 would be encoded by IL-7-dependent signaling pathway activation. Previous studies have identified differences in IL-7-induced signaling and responses between T-cells, but these were attributed to different strengths of TCR-mediated negative feedback to IL-7 signaling [24]. We therefore sought to characterize potential differences in IL-7 induced signaling in CD5hi and CD5lo cells in the absence of spMHC signaling, and determine how these give rise to heterogeneous IL-7 responses. While many of the signaling components critical for mediating IL-7-induced responses in T-cells have been identified (Section 2.3.2, Figure 2.5), few studies have quantitatively analyzed IL-7 signaling, particularly across T-cell populations of different TCR specificity. In this chapter we undertake a detailed analysis of IL-7 signaling in CD5hi and CD5lo T-cells that suggests IL-7R expression is a critical mediator of downstream responses. Receptor expression limits the ability of cells to achieve distinct signaling requirement for different downstream responses, including survival, proliferation and induction of the TCR co-receptor CD8α.

5.1  Heterogeneity in IL-7R Expression Among CD8+ T-Cells

5.1.1  Heterogeneous CD8+ T-Cell IL-7R Expression Correlated With CD5 Levels

We first characterized IL-7Rα surface expression of OT1 and F5 cells. As IL-7 signaling suppresses transcription of IL-7Rα [15] and in vivo IL-7 levels may differ between OT1 and F5 mice, we compared IL-7Rα expression in both freshly isolated T-cells and cells placed in cytokine-free culture for 16 hours (Figure 5.1a). Freshly isolated OT1 cells had approximately 2-fold higher IL-7Rα expression than F5 cells, and this relative difference was maintained following cytokine-free culture, exposing a higher ‘basal’ (i.e. uninhibited) receptor expression in OT1 cells (Figure 5.1a). In contrast, culture of either OT1 or F5
cells for 16 hours with 10 ng/mL IL-7 completely suppressed surface IL-7Rα expression. Polyclonal naïve CD8⁺ CD5⁺ and CD5⁻ T-cells also showed more modest but statistically significant differences in IL-7Rα levels (Figure 5.1b), suggesting that CD5 levels mark intrinsic differences in IL-7Rα expression. IL-7Rα surface expression differences between OT1 and F5 cells were reflected in the IL-7Rα mRNA expression profiles (Figure 5.2), though IL-7Rα was not completely suppressed in IL-7 culture, supporting the notion that IL-7Rα expression is primarily, but not fully, controlled at the level of gene expression. Naïve CD8⁺ T-cell populations are therefore heterogeneous in their IL-7R expression, which is correlated with CD5 expression, and regulated at the level of gene expression.

![Figure 5.1](image1.png)

**Figure 5.1. CD5⁺ Naïve CD8⁺ T-cells have higher IL-7R expression**
(a-b) Surface IL-7Rα expression of (a) OT1 and F5 TCR-tg naïve (CD44⁺) CD8⁺ T-cells and (b) C57BL/6 naïve (CD44⁺) CD8⁺ T-cells sorted on CD5⁺ or CD5⁻ expressing fractions, for cells freshly isolated from lymph nodes or cells cultured overnight (16 hours) +/- 10 ng/mL IL-7.

![Figure 5.2](image2.png)

**Figure 5.2. IL-7Rα Surface Expression Reflected by mRNA Expression**
IL-7Rα mRNA expression of (a) OT1 and F5 TCR-tg naïve (CD44⁺) CD8⁺ T-cells for cells freshly isolated from lymph nodes or cells cultured overnight (16 hours) +/- 10 ng/mL IL-7.
5.1.2 IL-7 Dose Requirements for IL-7R Suppression

Negative feedback to IL-7Rα expression after IL-7 binding occurs via at least two mechanisms: transcriptional suppression of IL-7Rα expression [15], and endocytic internalization of ligand-bound receptors [170]. To determine whether differences in IL-7 responses may be reflected in negative feedback to IL-7Rα expression, we characterized the IL-7 dose requirements for suppression of surface receptor expression in OT1 and F5 cells (Figure 5.3). IL-7Rα expression 24 hours after treatment with varying concentrations of IL-7 showed similar dose responses between OT1 and F5 cells, with complete receptor suppression for IL-7 >0.1ng/mL, no suppression at IL-7 <0.01ng/mL and partial suppression at 0.01ng/mL. Notably, only at IL-7 doses leading to surface receptor suppression do we observe support of cell survival and proliferation (Figure 4.16b), supporting a strong relation between receptor levels and responses.

![Figure 5.3. IL-7 Dose Requirements for Receptor Downregulation](image)

(a-b) Surface IL-7Rα expression for OT1 and F5 TCR-tg naïve (CD44lo) CD8+ T-cells rested overnight (~16 hours) in cytokine-free media and then cultured in vitro at low density with IL-7 concentrations ranging from 0.0001-10 ng/mL, normalized to (a) OT1 expression at 0 ng/mL IL-7 or (b) individual cell expression at 0 ng/mL IL-7.

Signaling arising from TCR binding to spMHC is also proposed to inhibit IL-7 signaling mediated suppression of IL-7R expression [24, 171]. As an additional control to determine whether T-cells were interacting with spMHC on their own surface and influencing responses, OT1 cells were cultured for 24 hours with varying doses of IL-7 in the presence of known positively selecting self-peptides of varying affinity for the OT1 TCR, or foreign
antigenic peptides (Figure 5.4). IL-7Rα expression, survival and CD8α expression were unchanged in cultures with self-peptides compared to the no peptide control. However, addition of antigenic peptide to cultures suppressed IL-7Rα expression and supported partial viability, independent of IL-7 dose. Conversely, IL-7 dose-dependent increases in CD8α expression were conserved in cultures with antigen, but there was a large increase in absolute CD8 levels. This provided further supporting evidence that the signaling and response we were characterizing were specific to IL-7R and not being influenced by TCR engagement with self-peptides under the examined culture conditions.

Figure 5.4. IL-7R, Viability and CD8α Responses to IL-7 are Not Influenced by Self-Peptides

(a-c) (a) Surface IL-7Rα expression, (b) viability and (c) CD8α of OT1 and F5 TCR-tg naïve (CD44lo) CD8+ T-cells rested overnight (~16 hours) in cytokine-free media and then cultured in vitro at low density with IL-7 concentrations 0.001-10ng/mL and either no peptides, known positively selecting self peptides (1uM ISFKFDHL or RTYTYEKL) or known antigenic peptides (1uM SIINFEKL).

5.2 Enhanced IL-7 Signaling of CD5hi T-Cells Mediated by High IL-7R Expression

5.2.1 Differential Dependence of Signaling and Responses on Jak and PI3K Activation

IL-7 induces activation of the Jak/Stat and PI3K/Akt pathways [1, 2], however there is some uncertainly as to the relative importance of these pathways in the control of downstream responses and their potential points of crosstalk. We therefore examined signaling and responses in OT1 cells in the presence and absence of IL-7 in the presence of
either the Jak family inhibitor Jak Inhibitor I, or the PI3K inhibitors LY294002 or PI-103 (Figure 5.5). We also wished to identify a downstream proxy for PI3K signaling activity, and therefore examined phosphorylation of two kinases purportedly regulated by IL-7 dependent PI3K activation: GSK3 and Akt. Interestingly, we did not see any IL-7 dependent increases in Akt phosphorylation, but total phosphorylation was decreased in both cytokine-supplemented and cytokine-free cultures under either Jak or PI3K inhibition (Figure 5.5a). These same trends were observed when Akt phosphorylation was detected by SDS-PAGE, and notably, pAkt was only expressed at very low levels near the limit of accurate quantization (data not shown). Conversely, GSK3 phosphorylation exhibited a strong Jak and PI3K dependent increase with IL-7 treatment (Figure 5.5b). We therefore chose to use GSK3 as a proxy for PI3K and/or Jak activity in future assays. This data also suggests that PI3K dependent responses to IL-7 may not be primarily transduced via Akt, and rather, through other downstream targets of PI3K.

Our examination of cellular responses to IL-7 surprisingly revealed that IL-7-dependent increases in viability were Jak- but not PI3K-dependent, while only PI3K inhibition significantly decreased viability in cytokine-free cultures (Figure 5.5c). Decreases in viability in the presence of IL-7 under Jak inhibition were reflected in the Bcl2 profiles, but not under cytokine-free PI3K inhibition (Figure 5.5d). This supports the notion that while Bcl2 induction is a critical mediator of the anti-apoptotic effects of Bcl2 inhibition, PI3K activity is essential for other mechanisms supporting cell survival. Suppression of IL-7R by IL-7 also occurs via a Jak-dependent but PI3K-independent pathway (Figure 5.5e). Conversely, basal levels of CD8a are not affected by inhibition of either pathway, and IL-7-induced increases in CD8a expression are both Jak- and PI3K-dependent (Figure 5.5f). These data support a network structure whereby all IL-7 dependent responses are Jak-dependent, and that Jak-dependent PI3K pathway activation is critical to some responses (CD8a induction) but not others (Viability, Bcl2 induction, IL-7R suppression). Furthermore, PI3K is essential for basal cytokine-deprived survival.
Figure 5.5. Different Dependence of IL-7 Responses on PI3K- and Jak-Mediated Signaling
(a-f) Influence of PI3K and Jak inhibition on cellular responses to IL-7. OT1 TCR-tg naïve (CD44
CD8+ T-cells were rested overnight (~16 hours) in cytokine-free media and then cultured in vitro at for 24 hours at low density in media +/- 1 ng/mL IL-7 alone or in the presence of the Jak family inhibitor Jak Inhibitor I (1 uM), the PI3K Inhibitors LY294003 (10 uM) or PI-103 (10 uM), or a DMSO vehicle control. After 24 hours in culture, (a) pAkt (S473), (b) pGSK3β, (c) Viability, (d) Bcl2, (e) IL-7Ra surface expression, and (f) CD8α surface expression were assayed by flow cytometry. (g) Putative signaling network connectivity map derived from signaling inhibition studies shown in (a-f).
5.2.2 Enhanced IL-7-Dependent Signaling Pathway Activation for CD5hi T-Cells

We sought to determine whether the modest differences in IL-7R expression between CD5hi and CD5lo T-cells elicits differential signaling pathway activation. To characterize both Jak/Stat and PI3K dependent signaling we measured levels of phosphorylated Stat5 and GSK3 (pStat5 and pGSK3) and increases in Bcl2 and CD8α expression following IL-7 stimulation. T-cells were rested overnight before measuring signaling to eliminate the potential effects of heterogeneous signaling received in vivo. OT1 cells treated with IL-7 showed higher pStat5 (at 20 minutes) and pGSK3 (at 24 hours) than F5 cells (Figure 5.6a), which was independent of total Stat5 or GSK3 expression (Figure 5.6b). IL-7 also induced a greater increase in CD8α expression at 24 hours in OT1 cells. While the fold induction of Bcl2 at 24 hours was similar between the two cells, OT1 cells had higher basal and IL-7-induced Bcl2 levels. Polyclonal naïve CD8+ CD5hi and CD5lo cells exhibited the same trends as TCR-tg cells, although the differences were less pronounced, reflecting the smaller differences in their initial IL-7Rα expression (Figure 5.6c). Differences in IL-7-induced pStat5 between OT1 and F5 cells could also be seen immediately following isolation, when IL-7Rα is partially suppressed (Figure 5.6d). These results demonstrate that even mild differences in baseline IL-7R levels between CD5hi and CD5lo cells are associated with significant differences in IL-7-induced signaling pathway activation.
Figure 5.6. CD5hi Naïve CD8+ T-cells Have Higher IL-7-Induced Signaling Capacity
(a) IL-7-induced signaling in OT1 and F5 TCR-tg naïve (CD44lo) CD8+ T-cells as assessed by Stat5 phosphorylation at 20 minutes, and GSK3α/β phosphorylation, Bcl2 and surface CD8α expression at 24 hours, in cells rested overnight and then treated with +/- 10 ng/mL IL-7. All measurements made by intracellular flow cytometry. (b) Total expression of Stat5, GSK3 and β-actin (loading control) as assessed by SDS-PAGE (left panel) and quantified by densitometry (right panel) in OT1 and F5 TCR-tg naïve (CD44lo) CD8+ T-cells rested overnight in cytokine-free media. (c) As described in (a), except for C57BL/6 naïve (CD44lo) CD8+ T-cells sorted on CD5hi or CD5lo expressing fractions. (d) Stat5 phosphorylation at 20 minutes for freshly isolated OT1 and F5 TCR-tg naïve (CD44lo) CD8+ T-cells treated with +/- 10ng/mL IL-7.

5.2.3 Receptor-Proximal Signaling Capacity Directly Related to IL-7R Expression
It was not clear that higher IL-7-induced signaling in CD5hi T-cells was due to greater IL-7R expression alone. To elucidate the relationship between IL-7R expression and signaling, OT1 and F5 cells were treated with varying IL-7 doses and their pStat5 and IL-7Rα surface expression dynamics measured over 6 hours (Figure 5.7a,b). Both OT1 and F5 cells showed the same linear relationship between loss of surface IL-7Rα at 6 hours and the level of Stat5 signaling induced (quantified either as the pStat5 level at 10 minutes or integrated over 6 hours) (Figure 5.7c,d). This suggests equivalent proximal signaling per receptor bound and internalized for OT1 and F5 cells, and that the enhanced signaling capacity of OT1 cells is directly related to their higher IL-7R expression.
5.3 CD8⁺ T-Cells Share Common Signal-Response Relationships

5.3.1 Methodology for Signal-Response Analysis

We next asked whether OT1 and F5 cells translated a given amount of signaling into equivalent functional responses. We compared two methods for quantitatively titrating the level of receptor-proximal signaling: (1) varying the IL-7 concentration, or (2) treating with a saturating dose of IL-7 and varying amounts of a Jak family inhibitor (Figure 5.8a,b). There is a strong linear correspondence between early (20 minute) pStat5 and pGSK3 signaling and agreement between varying IL-7 and varying inhibitor conditions for both OT1 and F5 cells (Figure 5.8c). There also exists a strong linear relationship between (20 minute) and late (24
hour) pGSK3 levels for cells with a varying inhibitor treatment, indicative of sustained signaling. However, this relationship is lost for OT1 cells in the varying IL-7 treatment, with low IL-7 doses showing decreased late signaling. Less sustained signaling resulted from ligand depletion from the media (explored further in Chapter 7), whose effects are more apparent for OT1 than F5 cells due to their higher IL-7R expression. The effects of ligand depletion severely impact the interpretation of signal response relationships across different timescales. For instance, in examining the relationship between early 20 minute pStat5 signaling and 24 hour viability, titrating signaling via different IL-7 concentration yields a linear relationship between signaling and responses (Figure 5.8d). Only in eliminating the effects of ligand depletion by titrating the inhibitor dose do we expose that viability actually quickly saturates as a function of pStat5 signaling.
Figure 5.8. Determination of Signal-Response Relationships Requires Signal Inhibition Approach
(a) Comparison of strategies for examining the relationship between IL-7-induced signaling and responses. IL-7 signaling can be modulated by either increasing IL-7 dose, or using a constant saturating dose of IL-7 and varying the levels of a receptor proximal signaling inhibitor such as Jak Inhibitor I. (b) 20 minute Stat5 phosphorylation for OT1 and F5 TCR-tg naïve (CD44o) CD8+ T-cells rested overnight (~16 hours) without cytokine and then subject to either the varying IL-7 dose (left panel) or varying Jak inhibitor I with constant 1 ng/mL IL-7 dose (right panel) treatments for titrating IL-7 signaling as described in (a). (c) Correspondence between 20 minute Stat5 phosphorylation and 20 minute GSK3 phosphorylation (left panel) and between 20 minutes and 24 hour GSK3 phosphorylation (right panel) for OT1 and F5 TCR-tg naïve (CD44o) CD8+ T-cells when IL-7-induced signaling is titrated by varying the IL-7 dose versus using a constant saturating dose of IL-7 and varying inhibitor treatments as described in (a-b). (d) Relationship between 20 minute Stat5 phosphorylation and 24 hour viability for OT1 TCR-tg naïve (CD44o) CD8+ T-cells under varying inhibitor of varying IL-7 dose treatments as described in (a). An arrow indicates the effect of ligand depletion on the signal-response curve at low IL-7 doses.
5.3.2 Common Relationships Between Signaling and Viability, CD8α Induction

Given a common relationship between receptor expression and proximal signaling, we asked whether OT1 and F5 similarly translated a given amount of proximal signaling into downstream proliferation responses or CD8α induction. To eliminate the effects of ligand depletion, cells were treated with a high dose of IL-7 (1 ng/mL) and signaling was titrated by varying the amount a Jak family inhibitor, Jak Inhibitor 1. OT1 and F5 cells exhibited common relationships between IL-7 signaling (20 minute pStat5 or 24 hour pGSK3) and viability or CD8α expression at either 24 or 48 hours, but had different dynamic ranges (Figure 5.9). Viability responses were nonlinear with IL-7 signaling and were readily saturated, with ~100% cell survival induced by very low levels of IL-7 signaling (Figure 5.9a,b,e,g). A consequence of this sensitive viability response is that the uninhibited signaling capacities of OT1 and F5 cells, though reaching different maxima, were each sufficient to yield complete survival of either T-cell at high IL-7 doses. In contrast, while OT1 and F5 cells also shared a common relationship between signaling and induction of CD8α expression, this relationship was linear (Figure 5.9c,d,g,h). Thus the maximum signaling capacity of F5 cells was insufficient to yield the maximum CD8α induction achievable by OT1 cells. A naïve T-cell’s capacity to survive and induce CD8α in response to IL-7 is therefore directly related to its IL-7R receptor expression, and in abundant cytokine conditions, a mild decrease in receptor expression limits CD8α induction, but not viability.
Figure 5.9. IL-7R Expression Thresholds Naive CD8+ T-Cell Responses to IL-7
(a-d) Relationship of IL-7-induced signaling to (a-b) viability and (c-d) CD8α expression at (a,c) 24 hours and (b,d) 48 hours for ‘early’ signaling quantified as 20 minute Stat5 phosphorylation for OT1 and F5 TCR-tg naive (CD44+) CD8+ T-cells rested overnight (~16 hours) in cytokine-free media and then treated with 1 ng/mL of IL-7 and varying concentrations of the Jak family inhibitor Jak Inhibitor I. (e-f) As described in (a-d) except for ‘late’ signaling quantified as GSK3 phosphorylation at 24 hours.
5.4 IL-7R Expression Limits Naïve CD8⁺ T-Cell Proliferation Capacity

5.4.1 Attempts to Modulate IL-7R Expression of CD8⁺ T-Cells

We hypothesized that similar to CD8α induction and viability, there may exist a threshold level of IL-7 signaling required to promote T-cell proliferation. We initially sought to test this hypothesis by determining whether increasing IL-7R expression on F5 cells would allow for their proliferation in response to IL-7.

5.4.1.1 Functional Expression of a Retroviral IL-7R Expression Vector

Full-length murine IL-7Rα cloned into the pMig retroviral expression plasmid (MSCV-Ires-GFP) was either obtained from Scott Durum (Mig7sd) or prepared independently by Vinay Mahajan (Mig7vsm). To test expression and function of the IL-7Rα vector, we first examined retroviral infection of the murine cell line 58⁺⁻, a variant of the DO-11.10.7 CD4 CD8 mouse hybridoma that lacks TCR α and β chains [172] (Figure 5.10). 24 hours after infection, infected cells cultured in cytokine-free media showed a strong increased in IL-7Rα expression that was highly correlated with GFP expression (Figure 5.10a,b). Culture of infected cells 24 hours with 10 ng/mL IL-7 led to only partial receptor downregulation (Figure 5.10b). As the introduced vector is not driven by the endogenous IL-7Rα promoter, and thus not subject to the same transcriptional suppression, surface receptor loss likely results from endocytic internalization. Stimulation of cytokine-deprived Mig7 infected cells lead to an increase in Stat5 phosphorylation over uninfected cells that was also correlated with GFP expression (Figure 5.10c,d), indicating the Mig7 retroviral vector could be functionally expressed in murine cells.
Figure 5.10. IL-7Rα Retroviral Expression Vector is Expressed, Functional in a Murine Lymphocyte Cell Line

(a-b) IL-7Rα surface expression for uninfected (left panel) or Mig7sd infected (right panel, MOI=10) 58/- cells after overnight (~16 hour) culture +/− 10 ng/mL IL-7, shown as histograms (a) or as a function of GFP expression (b). (c-d) As described in (a-b), but staining for intracellular pStat5.

5.4.1.2 Lack of IL-7R Overexpression Suggests Tight Regulation of Surface IL-7Rα

Having tested the functionality of the IL-7Rα expression vector, we next sought to express the vector in F5 cells. There is a lack of efficient and reliable methods for the introduction of foreign genetic material into naïve T-cells, and we therefore attempted three different approaches. We first tried to directly transfect naïve F5 cells using the Amaxa electroporation system (see Methods). Unfortunately, infection rates for the IL-7Rα vector were <5%, resulted in a severe loss in cell viability, and electroporation even decreased IL-7Rα expression in control samples that did not include DNA (Figure 5.11).

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As we also observed differences in IL-7 responsiveness between OT1 and F5 in vitro derived memory-like cells, we next tried to overexpress IL-7Rα in F5 memory cells via retroviral spin infection of blasting cells during activation. Briefly, cells were activated via either plate coated αCD3 or PMA/Ionomycin stimulation in the presence of 20 ng/mL IL-2 with two round of spin infections with either Mig7sd or Mig7vsm virus (MOI=10) at 24 hours and 48 hours after activation, washing cells 24 hours after each round of infection. Cells were then differentiated in 10ng/mL IL-7 or 40ng/mL IL-15 for three days. Unfortunately, infected F5 in vitro memory cells showed no GFP+ population, or increase in IL-7Rα surface expression over uninfected controls (Figure 5.12), and did not proliferate in response to IL-7 (not shown).
Figure 5.12. No Increase in IL-7Ra for F5 in vitro Memory Cells after Mig7 Spin Infection

IL-7Ra surface expression for F5 TCR-tg CD8+ in vitro memory-like T-cells uninfected (left panels), Mig7vsm infected (middle panels) or Mig7sd infected (right panels). In vitro memory-like cells were generated via activation of naive (CD44O) F5 TCR-tg CD8+ T-cells with either (i) plate-coated αCD3 or (ii) PMA/Ionomycin, in the presence of 20 ng/mL IL-2 for 3 days, followed by incubation with either (i) 40 ng/mL IL-15 or (ii) 10 ng/mL IL-7 for 3 days and overnight rest in cytokine-free media prior to assessment of IL-7Ra levels. Two rounds of spin infection with retrovirus (MOI=10) were performed 24 hours and 48 hours after activation, with media exchanges 24 hours after each infection.

Failing to directly transfect mature T-cells, we subsequently attempted to make bone marrow chimeras in which F5 bone marrow-derived stem cells retrovirally transduced with IL-7Ra were adoptively transferred into Rag-/- hosts. While modulation of IL-7Ra expression and signaling is critical to thymic development, IL-7R overexpression in thymic T-cell precursors has been shown to produce mature peripheral T-cells with normal phenotypes (Trop S Blood 2001). To produce chimeras, the stem cell fraction of F5 bone marrow was enriched, infected and expanded using minor modification of a protocol previously described (Zhang CC et al 2006). In brief, purified F5 bone marrow stem cells were infected with Mig7sd or Mig7vsm at 24 hours and 48 hours (MOI~10) and expanded for 7 days. Expanded cells were then retro-orbitally injected into sublethally irradiated (600 Rad) Rag-/- hosts (~10⁵ cells/mouse). Bone marrow chimeras were produced for each of the two Mig7sd and Mig7vsm virus preparations, for a total of ~30 mice. Tail bleeds of mice 6 weeks later
showed ~18 mice with significant CD8’ peripheral reconstitution, and GFP+ fractions ranged from 30-70%. Isolation of the lymph node and spleen of one mouse produced from each of the four virus infection batches confirmed a GFP’ mature CD8’ T-cell population in the lymphoid organs (Figure 5.13a). However, examination of IL-7Rα surface expression in freshly isolated cells or cells rested overnight in the presence of 10 ng/mL of IL-7 or in cytokine-free media revealed identical surface receptor expression in infected F5 cells compared to cells from uninfected F5 control mice (Figure 5.13b,c). Complete loss IL-7Rα surface expression after cytokine treatment also suggested that all receptors expressed were subject to transcriptional repression, indicating the presence of protein from only the endogenous gene, and not a combination of endogenous and exogenous expression. As the Mig7 vector produces a single GFP-fused mRNA transcript, GFP expression without surface expression suggests additional regulatory mechanisms, such as translational suppression or other post-transcriptional modifications regulating protein localization. Despite no apparent increases in total receptor surface expression, we wanted to confirm whether exogenous IL-7R expression altered F5 cell responses to IL-7. After 5 days of treatment with 10ng/mL of IL-7, pStat5 and pGSK3 levels and CD8 induction in Mig7 infected F5 cells were actually slightly below levels observed in uninfected F5 cells (Figure 5.13d-f). Infected F5 cells also had identical CD5 expression levels to their uninfected counterparts (Figure 5.13g). Lastly, there was no change in viability with infection (Figure 5.13h), and cells were not able to proliferate (Figure 5.13i).
Figure 5.13. Transgenic Expression of IL-7Rα Does Not Increase IL-7Rα Surface Expression or IL-7 Induced Proliferation in F5 Bone Marrow Stem Cell Chimeras

(a) GFP expression showing infection efficiency for CD8+ T-cells isolated from the lymph node and spleen of Mig7sΔ-infected F5 bone marrow chimeras 6 weeks after adoptive transfer compared to those from F5 or OT1 mice. (b-c) IL-7Rα surface expression of naïve CD8+ T-cells isolated from the lymph node and spleen OT1, F5 and Mig7sΔ-infected F5 bone marrow chimeras sorted on either the whole population (F57) or the GFP+ fraction (F57 GFP+) for freshly isolated cells or cells cultured overnight (16 hours) +/- 10g/mL IL-7. Histograms are shown in (b) are quantified in (c). (d-i) IL-7-induced signaling and responses for naïve CD8+ T-cells isolated from the lymph node and spleen of OT1, F5 and Mig7sΔ-infected F5 bone marrow chimeras (F57) showing (d) Stat5 phosphorylation (e) GSK3 phosphorylation (f) CD8α surface expression (g) CD5 surface expression (h) viability, and (i) proliferation as assessed by the KI67+ fraction, all at 5 days.
Altogether these data suggested that the level of IL-7Rα expression on mature T-cells of a given TCR specificity might be tightly developmentally regulated. Previous studies using similar approaches to study overexpression of IL-7Rα via bone marrow chimeras only examined thymic populations, without commenting on IL-7Rα expression in the mature population [103, 173, 174]. While IL-7R overexpression on mature peripheral cells has been achieved, these studies have used mice with an embryonic knock-in of transgenic IL-7Rα driven off of the CD2 or CD4 promoter/enhancer, allowing it to bypass earlier thymic developmental checkpoints [15, 173, 175].

5.4.2 Inhibiting OT1 Signaling to F5 Levels Stops Proliferation, Not Viability

Given the difficulties encountered in undertaking gain-of-function studies by overexpressing IL-7Rα in F5 cells, we adopted an alternate approach, and asked whether lowering IL-7-induced signaling in OT1 cells to levels achievable by F5 cells abolished proliferation. OT1 cells were treated with 10 ng/mL IL-7 and a dose of Jak or PI3K inhibitor sufficient to bring pGSK3 signaling at 24 hours to levels in untreated F5 cells (Figure 5.14). Reduced signaling capacity was also reflected by lowering of CD8α induction at 5 days in Jak- or PI3K-inhibited OT1 cells to approximately F5 levels. In uninhibited OT1 cells, ~26% of cells expressed the nuclear proliferation antigen, Ki67, after 5 days, whereas F5 cells had no Ki67+ fraction. The OT1 proliferating fraction disappeared upon Jak or PI3K inhibition of signaling to F5 levels but had little or no effect on survival. This suggests that even in the presence of abundant cytokine, F5 IL-7R expression limits the maximum IL-7 signaling capacity to levels capable to promoting survival, but not proliferation.
Figure 5.14. F5 CD8+ T-Cell IL-7-Induced Signaling Capacity is Insufficient to Promote Proliferation

(a-b) Comparison of IL-7 induced responses in OT1 cells with signaling reduced to levels achievable by F5 cells. (a) Schematic of the signaling inhibition approach to compare OT1 and F5 responses at the same level of signaling, using inhibition of either Jak by Jak Inhibitor I or PI3K by PI-103. (b) 24 hour GSK3 phosphorylation and 5 day CD8α surface expression, viability and Ki67+ proliferating fraction for untreated OT1 and F5 TCRtg naïve (CD440) CD8+ T-cells compared to OT1 cells treated with 0.0625 μM of Jak Inhibitor I or 1 μM of the PI3K inhibitor PI-103.

5.4.3 Distinct Thresholds for Varying Downstream Responses Encoded by the IL-7 Signaling Network

While OT1 signaling at levels achievable by F5 cells was insufficient to promote proliferation, we wondered precisely what level of IL-7 induced signaling was required for eliciting proliferation. Varying the dose of Jak Inhibitor I for OT1 cells treated with 10 ng/mL IL-7 revealed a sharp increase in proliferation at 5 days over a very narrow range of pGSK3 signaling (Figure 5.15). Furthermore, this sharp increase in proliferation occurred at ~75% of the maximum OT1 signaling, which is well above the maximum signaling obtainable by F5 cells. This was in distinct contrast to the linear increase in CD8α induction, and quickly saturating viability responses, as a function of signaling. These data suggest that
the IL-7 signaling network encodes distinct signaling thresholds for different downstream responses, with the capacity to achieve different responses determined by surface IL-7R expression.

![Figure 5.15. Distinct Signaling Requirements for IL-7-Induced Viability, Proliferation, CD8α Expression](image)

Relationship between 24 hour signaling (pGSK3 phosphorylation) and 5 day responses (viability, CD8α surface expression, and proliferation (as measured by % Ki67+ cells) for OT1 and F5 TCR-tg naïve (CD44o) CD8+ T-cells treated with 10 ng/mL IL-7. Signaling in OT1 cells was titrated by varying the dose of Jak Inhibitor I. Responses are normalized to OT1 uninhibited controls.

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**5.5 Foxo1 as a Putative Regulator of Basal IL-7R expression**

In a recent study, Kerdiles et al (2009) [18] found that Foxo1 is transcription factor for IL-7R, and consequently knocking out Foxo1 expression lead to decreased IL-7R expression in CD8+ T-cells. Foxo1 knockout mice also had decreased lymph node population sizes, and CD8+ T-cells from these mice had decreased expression of Bcl2 and CD62L. As we observed these same trends in comparing F5 cells to OT1 cells (Figure 4.1b, Figure 5.6a), we wondered whether there might be also be differences in Foxo1 expression between these cells. Indeed, SDS-PAGE of freshly isolate OT1 and F5 cells revealed a ~2 fold decrease in Foxo1 expression in F5 cells compared to OT1 cells (Figure 5.16). Foxo1 is a downstream target of the PI3K/Akt pathway, and Kerdiles et al (2009) [18] also show that inhibition of
the PI3K phosphatase PTEN results in decreased IL-7Ra expression. Intriguingly, our data examining the sensitivity of OT1 and F5 cells to PI3K inhibition suggests higher PI3K activity in F5 cells (Figure 4.12b), which would be consistent with lower PTEN levels in these cells. These data could suggest an interesting model whereby PI3K activity regulates basal IL-7Ra levels and survival in the absence of cytokine, which in turn regulates the ability to activate Jak-dependent signaling pathways, including PI3K, in the presence of cytokine.

![Figure 5.16. F5 T-Cells Have Decreased Foxo1 Expression](image)

Total Foxo1 expression as measured by SDS-PAGE (left panel) and quantified by densitometry (right panel) for viable OT1 and F5 TCR-tg naïve (CD44o) CD8+ T-cells after rested overnight (~16 hours) in cytokine-free media. Viable cells were isolated by Ficoll-Paque gradient separation after culture.

5.6 Summary: IL-7R Expression as the Primary Determinant of CD8+ T-Cell IL-7 Responsiveness

Our data indicate that while T-cells share common relationships between IL-7-induced signaling and responses, their maximum signaling capacity is limited by IL-7R expression. Furthermore, there exist distinct signaling requirements for inducing survival, proliferation and CD8α expression. While survival in abundant cytokine conditions only requires low levels of IL-7R expression and IL-7 induced signaling, proliferation is only observed at much levels of signaling. This suggests that increased IL-7Ra expression on CD5 hi T-cells is the major determinant of their ability to selectively proliferate in response to saturating IL-7, while all T-cells are able to survive. Different IL-7 dose requirements for T-cell proliferation
versus survival have been identified for polyclonal T-cell populations [14]. In contrast, our study extends this work by demonstrating that responses to IL-7 are encoded at the level of signaling induced, which is determined both by the IL-7 environment and IL-7R expression.
Chapter 6  Physiological Implications of Heterogeneous IL-7 Responsiveness

IL-7 provides critical survival signals to T-cells in vivo, but IL-7 signaling is received in the context of TCR engagement with spMHC as well as additional cytokine and growth factor signaling. While T-cells in lymphoreplete hosts are normally quiescent, T-cells are able to proliferate in response to lymphopenia or exogenous IL-7 therapy [2]. In vitro, we observed that CD5hi CD8+ T-cells have more robust proliferation responses to IL-7, while CD5hi cells have prolonged survival in cytokine-deprived cultures (Chapter 4). We hypothesized that variations in the concentration of available IL-7 in vivo may also cause the selective proliferation or persistence of CD5hi or CD5lo T-cell populations. We therefore examined in this chapter the relative ability of CD5hi and CD5lo T-cells to survive and proliferate in both normal lymphoreplete mice, and when IL-7 is elevated during lymphopenia or exogenous IL-7 treatment. We find that IL-7 levels in lymphoreplete mice promote a homeostatic balance between CD5hi and CD5lo populations, but elevated IL-7 levels during lymphopenia or IL-7 treatment yield selective proliferation of CD5lo T-cells. These findings suggest that IL-7 is a significant regulator of both T-cell population size and diversity.

6.1 Homeostasis of CD5 Expression in Lymphoreplete Hosts

The amount of IL-7 in vivo is thought to be limiting, thereby preventing the spontaneous proliferation of naïve T-cells in lymphoreplete animals [2]. If the in vivo level of IL-7 is indeed highly limiting, our in vitro results suggest that it may favor the relative survival of CD5hi cells. To test this, we transferred naïve B6.Thyl.2* CD8+ T-cells into age- and sex-matched B6.Thyl.1* mice and followed their CD5 profile over three weeks (Figure 6.1). The number of transferred cells declined steadily with a half-life of ~8.4 days (Figure 6.1b). However, there was no change in their CD5 profiles compared to the naïve CD8+ T-cells of the recipient (Figure 6.1a). This suggests that at physiological levels of IL-7, CD5hi and CD5lo cells have equivalent population kinetics in vivo. There is little to no proliferation of naïve T-cells in untreated lymphoreplete hosts. Consequently, maintenance of the CD5 profile would require equivalent turnover between CD5hi and CD5lo clones. Additionally, this
suggests that CD5\(^{hi}\) and CD5\(^{lo}\) T-cells are replenished by thymic export of newly developed cells at equivalent rates. Thus, IL-7 levels in lymphoreplete hosts favor neither CD5\(^{hi}\) nor CD5\(^{lo}\) expressing T-cells and can be said to maximize the diversity of CD5 expression.

**Figure 6.1. Homeostasis of CD5 Expression in Lymphoreplete Hosts**

(a) Comparison of CD5 expression profiles for donor versus recipient naïve (CD44\(^{lo}\)) CD8\(^{+}\) T-cells over 3 weeks for donor C57BL/6.Thyl.2\(^{+}\) CD44\(^{lo}\) CD8\(^{+}\) T-cells adoptively transferred into age and sex-matched C57BL/6.Thyl.1\(^{+}\) recipients. (b) Percent of donor cells amongst naïve (CD44\(^{lo}\)) CD8\(^{+}\) T-cells recovered from recipient spleens at varying times after adoptive transfer as described in (a).

### 6.2 Selective CD5\(^{hi}\) T-Cell Proliferation At Elevated IL-7 Levels in vivo

#### 6.2.1 Enhanced Proliferation of CD5\(^{hi}\) T-Cells During Lymphopenia

IL-7-induced proliferation of T-cells occurs *in vivo* when there is an excess of IL-7 even in the absence of foreign antigen, for instance during lymphopenia or exogenous IL-7 therapy [2]. In the setting of excess IL-7 availability caused by lymphopenia, CD5\(^{hi}\) cells have a greater rate of proliferation than CD5\(^{lo}\) cells [10, 11], which has so far been attributed to the greater responsiveness of CD5\(^{lo}\) cells to cognate spMHC. While our *in vitro* results suggest that naïve T-cells may also differ in their ability to respond to IL-7, this is difficult to demonstrate *in vivo*, as these cells may simultaneously receive different levels of spMHC signaling through their TCRs. Thus, to determine whether CD5\(^{hi}\) T-cells have an intrinsically
enhanced responsiveness to IL-7 that can impact proliferation in vivo, we exploited the fact that even within a population of TCR-tg T-cells, there is some variance in CD5 expression. We sorted naïve OT1 cells into CD5$^{hi}$ and CD5$^{lo}$ populations with a ~2.5-fold difference in mean CD5 levels (Figure 6.2a) but equivalent TCR expression (Figure 6.2b), and transferred the cells into syngeneic Rag$^{-/-}$ hosts. Five days post transfer, on average 85% of CD5$^{hi}$ OT1 cells had undergone division compared to 64% of CD5$^{lo}$ OT1 cells (Figure 6.2a). Thus, even in the presence of spMHC, T-cells expressing the same TCR at equivalent levels can have different rates of IL-7-driven proliferation in vivo. These data suggest that naïve T-cell populations may be comprised of cells with intrinsically different sensitivity to IL-7 overlaid on their varying spMHC and foreign antigen responsiveness.

![Figure 6.2. Enhanced Proliferation of Intraclonal CD5$^{hi}$ OT1 T-Cells in Lymphopenic Hosts](image)

(a) CD5 expression of OT1 TCR-tg naïve (CD44$^{lo}$) CD8$^{+}$ T-cells sorted into CD5$^{hi}$ and CD5$^{lo}$ expressing fractions (top panels), and their proliferation 5 days after adoptive transfer into syngeneic Rag$^{-/-}$ hosts, as assessed by CFSE dilution of donor cells recovered from recipient spleens and quantified as the percent of cells divided (bottom panels). (b) CD5 and TCR surface expression for OT1 TCR-tg naïve (CD44$^{lo}$) CD8$^{+}$ T-cells gated on CD5$^{hi}$ and CD5$^{lo}$ expressing fractions.

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6.2.2 Selective Proliferation of CD5^hi T-Cells After Exogenous IL-7 Treatment

Having observed differential proliferation of OT1 CD5^hi and CD5^lo cells in response to elevated IL-7 levels during lymphopenia, we next asked whether exogenous IL-7 treatment in polyclonal lymphoreplete mice had similar effects. We first confirmed that our IL-7 treatment protocol was capable of inducing detectable proliferation. CFSE-labeled naïve Thy1.2^+ CD8^+ were adoptively transferred into Thy1.1^+ congenic mice bearing mini-osmotic pumps that released PBS or 5 ug IL-7 over 7 days. A fraction of labeled cells recovered from mice receiving IL-7 underwent 1-2 divisions, while little to no proliferation was observed in PBS control mice (Figure 6.3). To then identify whether there is heterogeneous proliferation across the transferred population, sorted naïve Thy1.2^+ CD8^+ T-cells into CD5^hi and CD5^lo fractions were individually transferred into congenic mice bearing IL-7-releasing pumps (Figure 6.4). After 7 days, a greater fraction (35%) of CD5^hi T-cells underwent division than CD5^lo T-cells (7.9%). Again, no proliferation was seen in control mice that received PBS only (not shown). IL-7 therapy is therefore capable of inducing selective proliferation of CD5^hi T-cells.

Figure 6.3. Proliferation of CD8^+ T-Cells in Lymphoreplete Mice With IL-7 Treatment

Proliferation of donor C57BL/6.Thy1.2^+ CD44^lo CD8^+ T-cells adoptively transferred into age and sex-matched C57BL/6.Thy1.1^+ recipients given 5 µg IL-7 by mini-osmotic pump over seven days, as assessed by CFSE dilution of donor cells recovered from recipient spleens and quantified as the percent of cells divided.
6.2.3 Upwards Shift in the Distribution of CD5 Expression Upon IL-7 Treatment

While IL-7 treatment induces proliferation of mature peripheral cells, it may also alter the rates of T-cell turnover or thymic export of newly developed cells [106]. To determine how these combined effects affect the overall resulting peripheral T-cell population, we examined the surface expression profiles of CD8⁺ T-cells in mice receiving IL-7 treatment. We observed significant shifts towards higher CD5 expression in the total CD8⁺ population of young mice receiving IL-7 versus PBS over 7 days (Figure 6.5a). Greater IL-7 signaling in T-cells upon exogenous IL-7 addition was also reflected by an increase in surface CD8α expression (Figure 6.5b), and there was a significant increase in cell numbers in the lymph node and spleen over PBS treated mice (Figure 6.5c). Interestingly, we also observed an increase in the CD44⁺ expressing population in IL-7 treated mice (Figure 6.5d). However, it was unclear whether this resulted from selective proliferation of the CD44⁺ population or an increase in CD44 expression by proliferating CD44⁻ cells. We observed both of these effects in vitro (Figure 4.8-9), and conversion to a CD44⁺ memory-like phenotype has been reported for cells undergoing LIP [34]. Nevertheless, CD8⁺ cells gated on the CD44⁻ fraction still show an increase in CD5 and CD8 with IL-7 treatment (Figure 6.5e,f), though
the shift in CD5 is less significant. These results therefore demonstrate that exogenous IL-7 therapy has the potential to skew the naïve T-cell repertoire towards higher CD5 expression, in part by inducing selective proliferation of CD5hi cells.

6.2.4 No Shift in CD5 Expression Distribution in IL-7 Depleted Mice

At low concentrations of cytokine in vitro, CD5hi cells have a reduced ability to survive compared to CD5lo T-cells. We hypothesized that enhanced survival of CD5hi T-cells might also occur in vivo under conditions of IL-7 deficiency and shift the CD8+ T-cell repertoire to lower CD5 expression levels. To test this idea, lymphoreplete B6 mice were injected i.p. with 1mg of the IL-7 depleting antibody M25 or an isotype control antibody every alternate day for 14 days. In contrast to IL-7 treatment, mice receiving IL-7-depleting antibodies showed no difference in their distribution of CD5, CD44 or CD8α expression compared to isotype-treated controls (Figure 6.6a-b,d). However, M25-treated mice did show a slight decrease in overall CD8+ lymph nodes and spleen cell numbers (Figure 6.6c). Extrapolating from our in vitro signaling studies (Figure 5.9), the lack of a decrease in CD8α expression implies that IL-7 signaling is already at extremely low levels in lymphoreplete animals. Antibody deletion of IL-7 may therefore be insufficient to inhibit IL-7 signaling to levels where mild differences in survival between CD5hi and CD5lo clones can be observed on these time scales. In this case, differences might only be observed on longer timescales with greater doses of IL-7 blocking antibodies or in hosts deficient in IL-7 production. Alternatively, the presence of self-peptide stimulation in vivo, which was excluded in our in vitro survival studies, may equalize CD5hi and CD5lo T-cell turnover in vivo in cytokine-starved conditions.
Figure 6.5. IL-7 Treatment Shifts the CD5 Expression Profile of the Naïve T-Cell Repertoire

(a-d) Comparison of the (a) CD5 and (b) CD8α surface expression, (c) total cell numbers and (d) CD44 surface expression for CD8+ T-cells recovered from the lymph nodes or spleen of lymphoreplete C57BL/6 mice given PBS vs. 5 μg IL-7 by mini-osmotic pump over 7 days. (a-b) As described for (a-b) except for cells gated on the CD44lo expressing fraction.
Figure 6.6. IL-7 Depletion May be Insufficient for Observing Shifts in CD8+ T-Cell CD5 Expression
(a-d) Comparison of the (a) CD5 and (b) CD8α surface expression, (c) total cell numbers and (d) CD44 surface expression for CD8+ T-cells recovered from the lymph nodes or spleen of lymphoreplete C57BL/6 mice given injections of 1mg of the IL-7-binding antibody M25 vs. an isotype control antibody every alternate day for 14 days.

6.3 In vivo IL-7 Levels Suggest Mechanism for Maintaining Homeostasis

6.3.1 IL-7R Expression in vivo Suggest Physiological Range of Cytokine Levels
We sought to determine if the physiological range of IL-7 concentrations at steady state in vivo is tuned to maintain the homeostatic diversity of CD5 expression in CD8+ T-cells. Measurement of IL-7 levels in serum isolated from the blood of OT1, F5 and B6 mice by ELISA confirmed previous reports [76] that IL-7 serum levels are close to or below the detection limit of 1 pg/mL (Figure 6.7). However, serum levels of IL-7 may not reflect the
local availability of IL-7 in lymphoid organs [5, 176, 177], in part because IL-7 is sequestered by binding to the extracellular matrix [77]. We therefore took an alternate approach, and used IL-7Rα and CD8α expression on freshly isolated CD8\(^+\) cells as an indicator of the amount of effective cytokine signaling received in lymphoid organs \textit{in vivo}. We first examined IL-7Rα expression of freshly isolated OT1 and F5 cells and compared them to their IL-7Rα levels 24 hours after treatment with varying concentrations of IL-7 \textit{in vitro} (Figure 6.8a). This suggested that equivalent \textit{in vivo} cytokine concentrations in these mice correspond to \textit{in vitro} IL-7 concentrations of approximately 0.01-0.1 ng/mL. Interestingly, at this range of IL-7 concentrations \textit{in vitro}, both cells had equal viability (Figure 6.8b), but neither received sufficient IL-7 signaling to proliferate (Figure 6.8c). This suggests that the homeostatic balance between CD5\(^{hi}\) and CD5\(^{lo}\) T-cells \textit{in vivo} may be achieved by maintaining IL-7 at a level sufficient to promote equal survival among T-cells without inducing selective proliferation (Figure 6.9).

![Figure 6.7. Serum IL-7 Concentrations At or Below the Limit of Detection](image-url)

\textbf{Figure 6.7. Serum IL-7 Concentrations At or Below the Limit of Detection}

IL-7 concentration in the serum fraction of blood isolated from C57BL/6, OT1 TCR-tg or F5 TCR-tg mice as measured by ELISA, in comparison to the reported limit of detection for this measurement technique.
Figure 6.8. IL-7Rα Expression Indicate in vivo Cytokine Levels Promote Neither Selective Survival Nor Proliferation

(a) IL-7Rα expression of OT1 and F5 TCR-tg naïve (CD44<sup>+</sup>) CD8<sup>+</sup> T-cells rested overnight in cytokine-free media and treated 24 hours with varying IL-7 concentrations ranging from 0.0001-100 ng/mL, compared to the IL-7Rα expression of freshly isolated cells, indicating effective in vitro cytokine concentrations reflect in vivo IL-7 concentrations of 0.01-0.1 ng/mL. (b-c) 24 hour viability (b) and 5 day proliferation (%Ki67<sup>+</sup> fraction) (c) for OT1 and F5 TCR-tg naïve (CD44<sup>+</sup>) CD8<sup>+</sup> T-cells treated with varying IL-7 concentrations in vitro as described in (a), showing that at the proposed effective in vivo IL-7 concentration range, both cells survive, but do not proliferate.
Figure 6.9: Model for IL-7-Mediated Regulation of the Diversity of CD5 Expression in vivo
Model of skewing of ratio of relative CD5lo versus CD5hi CD8+ T-cell abundance with varying IL-7 concentrations in vivo. At IL-7 concentrations in normal lymphoreplete hosts, the diversity of CD5 expression is maximized, while selective proliferation of CD5hi subsets at high [IL-7] shifts towards high CD5 expression and selective survival of CD5lo subsets at low [IL-7] shifts towards low CD5 expression.

6.3.2 In vivo Bioassay Revealing Variations in IL-7 Levels Across Hosts
While we were able to estimate the in vitro correlate of in vivo IL-7 concentrations for OT1 and F5 mice, it was not clear how this related to the IL-7 concentration in normal B6 lymphoreplete mice. To compare the relative IL-7 levels between different mice, we adoptively transferred 2C TCR-tg Rag1Δ/Thy1.1+ T-cells into B6, Rag1Δ, OT1, 2C and F5 hosts (Figure 6.10a) and examined the relative IL-7Rα and CD8α expression on the transferred cells in the recipient spleens after 18 hours as a surrogate measure of IL-7 levels in vivo (Figure 6.10b-c). While this assay avoids problems inherent to comparing resident populations across TCR-tg mice which differ in their TCR specificity and basal IL-7R expression, it implicitly assumes that access to spMHC signaling is equivalent for donor cells bearing the same TCR across hosts.

Low IL-7Rα and high CD8α expression of donor cells in Rag1Δ mice indicated relatively high IL-7 levels in these mice compared to OT1, 2C, F5 and B6 recipients that bear full CD8+ T-cell compartments (Figure 6.10b). Our assay indicates that IL-7 levels are similar among OT1, 2C, F5 and B6 mice, albeit with subtle differences in the order: F5 > 2C > B6 > OT1. These data additionally suggest that the effective in vivo cytokine concentration in B6 mice also corresponds to in vitro IL-7 concentrations of 0.01-0.1 ng/mL observed to support
survival but not proliferation. However, Rag-/- mice have relatively elevated IL-7 levels, presumably sufficient to promote selective proliferation of CD5hi clones. Donor cell IL-7Rα expression showed a strong correlation with the number of CD8+ T-cells in the spleens of the recipient mice (Figure 6.10c), supporting the notion that in vivo IL-7 levels scale approximately with the number of T-cells consuming IL-7 [4, 5]. Altogether, these data support a model whereby physiological IL-7 levels are optimally regulated to maintain the diversity of CD5 expression in the naïve CD8+ T-cell population by supporting T-cell survival but not selective proliferation (Figure 6.9). Increasing IL-7 levels have the capacity to cause selective proliferation of CD5hi T-cells; conversely, depleting IL-7 may favor selective persistence of CD5hi T-cells.

Figure 6.10. Bioassay Indicating Variations in IL-7 Levels Between Mice Scaling with Population Size (a-c) Bioassay for in vivo IL-7 levels in which 10^6 Thy1.1+ 2C TCR-tg naïve (CD44o) CD8+ T-cells were transferred into Thy1.2+ B6, OT1 TCR-tg Rag/-, 2C TCR-tg Rag/-, F5 TCR-tg Rag/- or Rag/- hosts (a), and the relative IL-7 levels between hosts were inferred from the IL-7Rα and CD8α expression of Thy1.1+ donor cells (b). IL-7Rα expression of donor cells showed a strong linear correspondence with number of CD8+ T-cells recovered from the recipient spleens (c).
6.3.3 Alternate Interpretation of Varied Rates of LIP in Different TCR-tg Hosts

Our IL-7 bioassay suggests that there are variations in effective cytokine levels across different TCR-tg mice. Interestingly, CD5hi CD8+ TCR-tg T-cells are found to proliferate when adoptively transferred into certain syngeneic TCR-tg Rag-/- hosts only when the recipient CD8+ T-cells have lower CD5 expression [10] (Figure 6.11). While this was interpreted as being due to the enhanced ability of donor CD5hi T-cells to compete for spMHC, our results suggest that these differences could also be impacted by variations in the in vivo IL-7 levels between different TCR-tg hosts. For instance, OT1 cell proliferation after adoptive transfer scales in the order Rag-/- > F5 > 2C > OT1, and our assay indicates in vivo IL-7 availability also increases in the same order (Figure 6.10b). 2C cell proliferation across hosts also scales in the same order, but these cells have lower proliferation than OT1 cells, potentially because their reduced receptor expression compared to OT1 cells (data not shown) leads to reduced signaling in the same environment. This suggests that both the cell-intrinsic ability to respond to IL-7 and extrinsic variations in IL-7 availability can shape the naïve T-cell pool.

![Donor Cell Expansion when CD8 # Divisions in 30d]

<table>
<thead>
<tr>
<th># Divisions in 30d</th>
<th>0-1</th>
<th>2-4</th>
<th>6-7</th>
<th>8+</th>
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Donor Cell IL-7 Induced Proliferation Capacity

Host [IL-7]

Figure 6.11: In vivo IL-7 Availability May Potentiate Variations in LIP Across TCR-tg Hosts

Schematic of results from Ge et al (2004) [10], demonstrating the average number of divisions of CFSE-labeled donor naïve (CD44hi) CD8+ T-cells 30 days after adoptive transfer into recipient mice for donor cells isolated from OT1, F5 or 2C TCR-tg mice and transferred into OT1, F5 or 2C TCR-tg and Rag-/- recipients. Also shown is the relative donor cell proliferation capacity in response to IL-7 stimulation alone, and the relative estimated IL-7 levels, demonstrating how variations in IL-7 levels of the host and intrinsic IL-7 responsiveness of the donor may contribute to observed differences in proliferation, as opposed to TCR avidity differences between host & donor alone.
6.3.4 Implications of in vivo IL-7 Heterogeneity For the Co-receptor Tuning Model

Diversity in the levels of CD5, CD8α and IL-7Rα expressed by naïve T-cells in vivo has been largely explained in the context of heterogeneous extrinsic signals from both spMHC and IL-7 [2, 5, 7, 15, 24]. In what they have termed the ‘co-receptor tuning’ model, CD8α expression is regulated by mutual feedback between the IL-7Rα and TCR signaling pathways: IL-7Rα signaling induces the transcription of CD8α to increase TCR signaling, which negatively feeds back to reduce IL-7 signaling [24]. Reduced CD8α co-receptor expression on CD5hi T-cells is then proposed to ‘tune down’ excessive spMHC-induced TCR signaling (Figure 6.12a). The ‘co-receptor tuning’ model is supported in part by a positive correlation of IL-7Rα, and inverse correlation of CD8α, with CD5 expression for a panel of freshly isolated TCR-tg cells (2C, F5 and female HY Rag-/- mice). Indeed, we also observe these trends for OT1, 2C and F5 mice, however at more modest levels (data not shown). While feedback between IL-7R and TCR signals may dampen TCR signals in CD5hi cells (Figure 6.12b), observed trends in CD5, CD8α and IL-7Rα expression may also be explained by differences in IL-7 availability in these mice (Figure 6.12c). Indeed, our data suggests that OT1, 2C and F5 mice have decreasing numbers of CD8’ cells and conversely increasing levels of available IL-7 (Figure 6.10b), which could partly explain the progressively higher levels of CD8α in these mice.
Figure 6.12: Variations in IL-7 Levels Across TCR-tg Hosts May Partially Account for Correlations in CD8, IL-7R, CD5 Attributed to Co-Receptor Tuning

(a) Schematic of co-regulation of TCR and IL-7R induced signaling according to the CD8 coreceptor tuning model [24]. IL-7 transcriptionally increases CD8α expression to promote TCR-self peptide-MHC engagement, but TCR signaling then feeds back to impair IL-7R signaling. Low CD8 expression on CD5hi IL-7Rlo cells resulting from this mutual feedback is proposed to 'tune down' excessive TCR-spMHC signaling. (b-c) Relationship between CD5, CD8α and IL-7Rα for a panel of TCR-tg mice observed in vivo versus at varying levels of IL-7 in vitro. The observed in vivo trends could result from spMHC signaling leading to non-linear feedback to CD8 expression assuming the same in vivo IL-7 levels across hosts (b). Alternatively, trends may be accounted for solely by differences in the in vivo IL-7 levels between hosts from which cells are isolated (c).
6.4 Summary: IL-7 as a Critical Controller of the Homeostasis of CD5 Expression

Altogether, these data suggest that variations in IL-7 responsiveness across the T-cell repertoire are present and functional in vivo even in presence of spMHC stimulation. IL-7 levels in lymphoreplete hosts are such that the homeostatic diversity of CD5 expression is maintained by promoting neither selective survival nor proliferation of CD5 <sup>a</sup> and CD5 <sup>b</sup> T-cell subsets. Conversely, the relative in vivo abundance of T-cell clones is shifted towards favoring high CD5 expressing clones when IL-7 is elevated during lymphopenia or IL-7 treatment. This suggests an important role for IL-7 in controlling both the size and the composition of the T-cell pool, and adds the selection of certain T-cell subsets as an additional consideration for the design of IL-7 therapies. Our studies also suggest a putative reinterpretation of previous studies presented in support of TCR-mediated differences in LIP and surface expression profiles across TCR-tg mice in which extrinsic variations in the IL-7 environment potentiate responses. This reinforces the notion that both cell-intrinsic variations in IL-7 responsiveness, and cell-extrinsic variations in the cytokine environment, play critical roles in shaping T-cell responses.
Chapter 7  Quantitative Analysis of Heterogeneous IL-7 Depletion Amongst CD8^{+} T-Cells

Increased IL-7R expression on CD5^{+} CD8^{+} T-cells imparts a signaling advantage that allows for enhanced proliferation in response to elevated IL-7 levels \textit{in vitro} (Chapter 4) and \textit{in vivo} (Chapter 6). However, one putative disadvantage of higher receptor expression is higher depletion of IL-7 from the environment, which may limit long-term survival when faced with limiting cytokine reserves. While previous chapters have focused on characterizing the effect of varying IL-7 environments on IL-7 responses, this chapter focuses on characterizing the ability of cells to modulate the IL-7 environment. We find that T-cells have heterogeneous capacities to deplete IL-7 that scales with their receptor expression. Furthermore, T-cells require sustained signaling to support viability, IL-7R\alpha suppression and CD8\alpha induction. Fast IL-7 depletion for high IL-7R expressing populations therefore poses a potential survival disadvantage in IL-7 limited conditions. This reinforces the concept that the naïve T-cell pool is shaped by a complex interplay between heterogeneities in both the ability to consume, and respond to, available IL-7.

7.1 Sustained IL-7 Signaling Required for Complete Survival, Receptor Suppression, CD8 induction

IL-7 is highly localized to its sites of production, which include the bone marrow, thymus, lymph nodes, spleen and liver [2]. T-cells cycle between these IL-7 rich regions and the relatively IL-7 depleted circulatory system. The transient receipt of cytokine signals during cycling is believed to play a major role in directing T-cell trafficking to and/from lymphoid organs via the modulation of the expression of the CCR7 chemokine receptor [18, 178]. To gain a better understanding of the consequences of this cycling behavior, we sought to characterize the differences in T-cells responses to the transiently pulsed versus sustained presence of IL-7.

A range of T-cell residence times in and out of the secondary lymphoid organs have been reported. During normal homeostasis, T-cells can spend up to 24-48 hours in lymphoid
organs, while transitions times between blood and lymph node vary highly, peaking at ~24 hours [16, 17, 179]. As a model of the behavior of T-cells which have just entered lymph nodes versus those that have recently exited into circulation, we compared OT1 and F5 T-cells treated continuously with varying levels of IL-7 with those receiving a 1 hour ‘pulse’ of cytokine followed by washing and culture in cytokine-free media for the remaining culture time (Figure 7.1). Cells under pulsed cytokine treatment had reduced viability (Figure 7.1a) and CD8α induction (Figure 7.1b) and incomplete surface IL-7Rα suppression (Figure 7.1c) at 24 hours compared to cells left in the presence of cytokine. This effect was more apparent after 48 hours in culture. Increasing the pulse time from 1 to 4 hours only slightly decreased the effect of cytokine removal compared to sustained IL-7 presence (not shown). Lack of differences between pulsed and sustained IL-7 treatments at high doses (>10 ng/mL) likely reflects difficulty in completely washing off large amounts of cytokine bound to surface IL-7R, as well potential recycling to the media of cytokine already internalized. Interestingly, this net difference in responses between pulsed and sustained dosing conditions is slightly greater for OT1 cells than F5 cells. For instance, at 0.1 ng/mL IL-7, CD8α induction is greater for OT1 than F5 cells under sustained signaling, but both cells have approximately equivalent CD8α levels in the pulsed condition (Figure 7.1c).

T-cells therefore require re-encounter with cytokine on time scales <24 hours in order to support complete viability and CD8α induction, and the net effect of cytokine deprivation may be heterogeneous across clones. These time scales are in accordance with the proposed T-cell cycling times outside the lymph nodes peaking at ~24 hours. Furthermore, re-upregulation of receptor surface expression over these time scales is in line with the proposed ‘altruistic’ model of IL-7 sharing whereby cells that have just entered the lymph node from the circulation have increased receptor expression (Figure 2.6). Cells most needing survival signals therefore have improved capacity to compete for, and signal in response to, limiting cytokine reserves.
7.2 Heterogeneous IL-7 Depletion Scales with IL-7R Expression

In the proposed ‘altruistic’ model, IL-7 is optimally utilized to support the maximum T-cell population by scaling receptor expression with the relative need for IL-7 signaling [5, 24] (Figure 2.6). However, these studies discuss heterogeneity in IL-7R expression amongst naïve T-cells originating only from extrinsic differences in the spatiotemporal presentation of
IL-7. Furthermore, they do not directly quantify ligand depletion to determine whether it is truly optimally utilized amongst all T-cells. We therefore sought to directly measure IL-7 depletion, and characterize the consequences of the observed heterogeneities in basal IL-7R expression amongst naïve T-cells on their relative rates of IL-7 depletion.

To measure rates of IL-7 depletion, OT1 and F5 cells were cultured at varying densities (1x, 2x, or 3x 3.3x10^5 cells/mL) in the presence of 0.1 ng/mL IL-7. IL-7 remaining in the supernatant of cell-containing and cell-free cultures after 24 or 48 hours was quantified by ELISA, and cell viability and IL-7Ra expression was determined by flow cytometry (Figure 7.2). IL-7 depletion from the media was both time- and cell density-dependent, but OT1 cells gave rise to significantly greater IL-7 depletion from the media (Figure 7.2a). Furthermore, while F5 cells at all densities maintained complete receptor downregulation throughout the full 48 hours in culture, OT1 cells already showed re-upregulated receptor expression that varied with cell density by 24 hours (Figure 7.2b). Despite differences in the rates of ligand depletion from the media, cells across all conditions maintained ~100% viability over the two days examined (Figure 7.2c). Quantifying the ligand depletion over 24 hours in 1x cell density conditions reveals ~2 fold increase in ligand depletion in OT1 cells over F5 cells (Figure 7.2d). However, depletion is approximately equivalent on a per-receptor basis (Figure 7.2e). Nevertheless, both cells have equal viabilities on these timescales (Figure 7.2c), and in previous experiments we have shown that the IL-7 dose of 0.1 ng/mL used is insufficient for signaling a proliferation response for OT1 cells (Figure 4.16). T-cells therefore have heterogeneous capacities to deplete IL-7 from their environment that scales with receptor expression, and greater consumption does not necessarily lead to an enhanced phenotypic response.
Figure 7.2: IL-7 Consumption Scales with IL-7Ra Expression

(a-c) OT1 and F5 TCR-tg naive (CD446) CD8+ T-cells rested overnight (~16 hours) in cytokine-free media, and then viable cells were plated at either 1x, 2x or 3x (3.3x10^5) cells/mL density and then treated with 0.1 ng/mL of IL-7. After 24 or 48 hours in culture, (a) IL-7 remaining in the media was measured by ELISA and (b) IL-7Ra surface expression and (c) viability were measured by flow cytometry. (d-e) IL-7 molecules consumed after 24h for OT1 and F5 T-cells cultured at 1x density, either (d) per cell and (e) per receptor.
7.3 Early IL-7 Depletion Compromises Late Survival

While OT1 cells depleted more IL-7 than F5 cells when treated with 0.1 ng/mL IL-7, it did not increase their relative viability over the 2-day time scales examined, nor induce them to proliferate. As we have observed that complete viability responses require the sustained presence of cytokine (Section 7.1), we hypothesized that this early ‘over-consumption’ of cytokine might compromise viability over longer time scales when cells are faced with limited IL-7. To test this hypothesis, we extended culture times to 9 days, and compared daily behavior in intermediate IL-7 doses (0.1 ng/mL) to those in abundant cytokine conditions (10 ng/mL IL-7) and in cytokine-free cultures (Figure 7.3).

Figure 7.3: High IL-7R Expression and IL-7 Consumption Poses Survival Disadvantage When IL-7 is Limited
Viability (top row), IL-7Rα surface expression (middle row) and CD8α surface expression (bottom row of panels) over 9 days for OT1 and F5 naïve (CD44lo) CD8+ T-cells rested overnight in cytokine-free media and treated with 0 ng/mL IL-7 (left column), 0.1 ng/mL IL-7 (middle column) or 10 ng/mL IL-7 (right column).
At high concentrations of cytokine, there appears to be no effect of differences in IL-7 uptake, as both OT1 and F5 cells maintain ~100% survival over the full 9 days at high cytokine conditions. However, at an intermediate cytokine dose, we reveal the consequences of greater early IL-7 consumption by OT1 cells: while both cell types survive equivalently for the first ~5 days, OT1 cells rapidly die off in the days thereafter. Early IL-7 depletion from OT1 cultures in reflected in the upregulation of receptor after 2 days in culture, whereas F5 cells only start to re-express receptor after 7 days. Receptor is not fully re-expressed, though comparison with cytokine-free cultures reveals this is not due to presence of residual cytokine. Rather, this may reflect overall decreases in protein expression and/or metabolism after removal of cells from their in vivo environment, including stimulation by spMHC. Differences in the duration of signaling between OT1 and F5 cells at 0.1ng/mL are also apparent in their CD8α profiles: while OT1 has greater initial CD8α expression, it is surpassed by F5 later in culture. CD8α expression for OT1 cells also begins to decrease later in culture at 10 ng/mL IL-7, which may result from differences in cytokine levels which only becomes functionally apparent at later time points when starting within initially high cytokine doses. Overall, these data suggest that increased IL-7R expression leads to greater ligand consumption than is required to support early survival, which poses a survival disadvantage in limiting cytokine conditions.

7.4 Summary: IL-7R Expression Drives Heterogeneous IL-7 Consumption

Our data reveals that while heterogeneous IL-7R expression among T-cells drives their differential responses to the IL-7 environment, it also determines their capacity to alter that environment via cytokine consumption. IL-7 consumption scales with IL-7R expression, thus CD5hiIL-7Rh T-cells more quickly deplete their IL-7 environment. However, T-cells require periodic stimulation with IL-7 to support prolonged survival. Thus, under IL-7 limited conditions, early ‘overconsumption’ of cytokine by IL-7Rh T-cells poses a survival disadvantage for T-cells later in culture. Differential use and demand for cytokine resources across the T-cell repertoire calls for re-examination of the notions that IL-7 levels in vivo...
scale with the number of cells consuming IL-7, and that IL-7 levels are optimally regulated to support the maximum population size.
Chapter 8  Conclusions and Discussion

8.1 Intrinsic Heterogeneity in Cytokine Responsiveness Across the CD8+ T-Cell Repertoire

T-cells have been increasingly divided into numerous functionally distinct sub-populations such as naïve, effector, memory and regulatory cells based on defined sets of surface marker expression. Functional differences between T-cells within sub-populations have been thought to arise from genetically distinct TCRs with varying avidity for self or foreign antigens. Stochastic heterogeneity in protein levels has also been reported to result in significant diversification of activation responses even among individual naïve T-cell clones [180]. However, variability in these responses is limited by expression of opposing signaling co-regulators. While stochastic fluctuations in protein state can be transmitted from mother to daughter, they result in transient heritability in cell fate [181]. In contrast, here we have described an additional novel layer of functional heterogeneity in T-cells that appears distinct from non-genetic cell-to-cell variability within cell populations [182, 183]. Our data suggests that in the context of homeostatic survival and proliferation, even modest differences in surface IL-7Rα expression levels among naïve T-cells have predictable functional consequences that are robustly heritable.

While IL-7 has a well-documented role in promoting T-cell survival and proliferation, responses have been generally been in examined in the context spMHC signaling. Here we have shown that even in the absence of TCR-spMHC engagement, IL-7 can promote both CD8+ T-cell survival and proliferation (Chapter 4). Furthermore, while all T-cells examined could survive in response to IL-7, their IL-7 induced proliferation capacities were correlated with their CD5 and IL-7R expression levels. As CD5 expression is thought to reflect the avidity of TCR-spMHC interactions [6-9], the enhanced LIF of CD5hi T-cell clones has been previously attributed to their enhanced spMHC signaling [10, 11]. However, our data suggests differential IL-7R expression and resulting responsiveness to IL-7 may also potentiate proliferation.
The TCR-independent IL-7-induced proliferation characterized in this thesis is reduced in magnitude compared to the combined stimulation with spMHC-expression dendritic cells \textit{in vitro} [184] or during LIP in lymphopenic mice (see, for example, [10]). TCR stimulation with spMHC is not sufficient for proliferation, as cells die the absence of cytokine stimulation [64]. Similarly, while IL-7 can support complete T-cell survival over short time scales \textit{in vitro}, spMHC signaling is required for long-term survival \textit{in vivo} [1, 2]. This suggests that functional differences in survival and proliferation \textit{in vivo} result from a complex interplay between TCR and IL-7R signals. The idea that IL-7 requirements and/or responsiveness may vary with TCR affinity, and that IL-7 may potentiate spMHC signaling is almost a decade old [185], though only recently have potential mechanisms been proposed [78]. One putative mechanism of synergy supported by recent work suggests that TCR signaling may maintain IL-7R surface expression for more sustained IL-7 signaling (Park J.H. et al, unpublished results). However, these studies still generally assume equal TCR-independent IL-7 signaling capacities. Deconvolution of the contribution of heterogeneous IL-7R versus TCR signaling to responses will require systems for the combined quantitative presentation of both spMHC and IL-7 and precise measurements of their downstream signaling and responses. Subsequent determination of the key modes of response regulation encoded within the TCR- and IL-7R- signaling network will greatly benefit from the development of mathematical models to interpret and guide experimental designs (Discussed further in Chapter 9).

In addition to their enhanced responsiveness to IL-7, CD5\textsuperscript{hi} T-cells also selectively proliferated in response to IL-2 and IL-15 stimulation (Figure 4.7). CD5 may therefore serve as a marker for globally enhanced responsiveness of T-cells to homeostatic cytokines. IL-2 and IL-15 are classically thought to be more specific to effector and memory T-cell populations [1]. However, recent proposals suggest that naïve T-cells can also proliferate robustly to these cytokines, but their \textit{in vivo} cell responses are limited via cytokine production and localization [1]. One putative underlying mechanism for heightened cytokine responsiveness may reside in CD5\textsuperscript{hi} T-cells having already undergone division, or be primed to divide, upon their isolation. LIP is known to induce naïve T-cell conversion to a 'memory-like' phenotype [34]. Indeed, we observed that OT1 mice have a ‘memory-like’ CD44\textsuperscript{hi} subpopulation of CD8\textsuperscript{T} cells that presumably arises from LIP (Figure 4.1). CD44
expression is increased even among the CD44\(^+\) subpopulation compared to CD5\(^-\) F5 mice. This CD44\(^+\) population has more robust cytokine-induced proliferation, although we still observe preferential cytokine-induced proliferation for CD5\(^+\) cells under stringent sorting for CD44\(^+\) T-cells (Figure 4.10b). CD5\(^+\) T-cells also have increased cell size compared to CD5\(^-\) cells (Figure 4.15), and there is thought to be a critical cell size requirement to initiate division [186]. This increase in cell size may also be supported by 'priming' by homeostatic signals \textit{in vivo} and may potentiate the observed cytokine responses. Altogether, these data suggest that prior \textit{in vivo} cytokine and spMHC interactions may have complex effects on global cytokine responsiveness that we observe both \textit{in vitro} and \textit{in vivo}. It will also be interesting for future studies to look across a panel of IL-2 family cytokine receptor to see whether their increased expression is also the mechanism for enhanced responsiveness in CD5\(^+\) T-cells, as we have shown for IL-7. As IL-2 and IL-15 have multiple shared receptor components under feedback control, mathematical modeling of receptor dynamics will also undoubtedly be useful for understanding their signaling control of heterogeneous T-cell responses (Discussed further in Chapter 9).

In addition to differences in cytokine responsiveness, our studies reveal an intrinsic difference in the rate of cell death amongst T-cell clones that also correlates with their CD5 expression (Section 4.2). While T-cells survive equally in the presence of saturating levels of cytokine, CD5\(^+\) T-cells having prolonged survival when withdrawn from both TCR and IL-7R stimuli. CD5\(^-\) clones have a survival advantage despite their lower Bcl2 expression (Figure 5.6) and a smaller cell size (Figure 4.15) that would indicate reduced anti-apoptotic survival signaling and basal metabolism respectively. It therefore remains uncertain whether prolonged survival results from differential utilization of death pathways, compensatory differences in other Bcl-2 family members regulating apoptosis [1], or the relative ability of these cells support metabolism under conditions of starvation through mechanisms such as autophagy [187, 188]. Interestingly, \textit{in vivo} adoptive transfer experiments suggest that IL-7 levels in replete mice are sufficient to maintain equal turnover between clones with different CD5 expression (Figure 6.1). Furthermore, even in the presence of IL-7 depleting antibodies, we were not able to observe \textit{in vivo} differences in survival on short time scales, nor decreases in IL-7 induced signaling (Figure 6.6), suggesting IL-7 is already highly limiting. In lymphoreplete mice, equal turnover may therefore be maintained by balancing
the requirement for homeostatic signals with the ability to compete for these signals. In other words, CD5<sup>+</sup> T-cells have a shorter half-life in the absence of homeostatic signals compared to CD5<sup>-</sup> T-cells, but their higher IL-7R expression and TCR avidity may allow them greater access to limiting IL-7 and/or spMHC to make up for their basal survival deficiency. Discerning whether homeostasis is maintained primarily by IL-7 levels, or a balance of IL-7 and access to spMHC, will require better tools to measure local IL-7 abundance and TCR and IL-7R signaling. Mathematical models which are able to capture and dissect the subtle quantitative effects of variations in homeostatic availability and cell responsiveness may be useful in discriminating the primary mechanisms controlling the homeostatic diversity of CD5 expression.

The presiding model for regulation of the naïve T-cell pool posits that T-cell diversity results from competition for a correspondingly diverse pool of cognate spMHCs [1-3], while IL-7 availability limits the overall size of the naïve T-cell population [5, 185]. This model has recently been supported by several computational models of the regulation of T-cell population and size in both normal hosts, and during peripheral reconstitution following thymus transplantation in DiGeorge syndrome [166, 168, 169]. However, these studies implicitly assume equal turnover in the absence of signaling, and ability to respond to non-TCR-dependent stimuli. Our demonstration of heterogeneous abilities amongst naïve T-cells to respond to IL-7 and survive in the absence of TCR-and IL-7R signaling therefore suggests a new model whereby IL-7 plays a critical role in regulating both the size and diversity of the T-cell repertoire. Revisions of these previous mathematical models to include heterogeneities in cytokine-dependent responses across T-cell of differing specificity may help reconcile differences in current conceptual models for how T-cell repertoires are established and maintained (discussed further in Chapter 9).

8.2 IL-7R as the Primary Regulator of IL-7 Responses

We found that CD5 expression is a stable marker of predictable correlated differences in TCR-independent T-cell survival and proliferation capacities in response to IL-7 stimulation (Chapter 4). However mechanistically, increased IL-7Rα expression on CD5<sup>+</sup> T-cells
appears to be the major determinant of their increased responsiveness to IL-7 (Chapter 5). Receptor proximal signaling scaled linearly with loss of IL-7R from the cell surface following IL-7 binding and internalization (Figure 5.7), indicating receptor expression limits the maximum achievable upstream signaling capacity. However, T-cells with different IL-7R expression had conserved relationships between their receptor-proximal signaling and downstream responses, including survival, proliferation and CD8α induction (Figure 5.9).

There are several different mechanisms by which IL-7 sensitivity could vary across individual T-cells (discussed in detail in Chapter 3), including differential expression of proteins such as the Jak-Stat inhibitor SOCS1, or altered receptor trafficking dynamics. However, downstream changes in the signaling network impacting feedback to signal durations would be reflected in differences in the relationship between early signals and late responses in T-cells, which we fail to observe (Figure 5.9). Thus, while there may be additional subtle network-wide changes affecting IL-7 responses, our data suggest that heterogeneous IL-7R expression is sufficient to give rise to the observed variation in IL-7 sensitivity across T-cell clones.

Our data suggest that even the modest differences in IL-7Rα expression observed across the naïve T-cell pool can give rise to large differences in the functional responses to IL-7 (Chapter 4). Our detailed analysis of signal-response relations in the IL-7R signaling network revealed that this broadening of responses results from distinct signaling relationships for varying downstream responses (Figure 5.15). Survival rapidly saturates as a function of signaling, with signaling even by low IL-7R expressing cells supporting complete survival. In contrast, CD8α induction scales linearly with receptor expression and increased continuously with IL-7 treatment. Variations in proliferation were the most dramatic differences in IL-7 induced response between T-cells, with only cells expressing a critical level of IL-7R capable of proliferating in the presence of abundant cytokine. The underlying signaling relationships suggest this is due to a sharp increase in proliferation over a narrow range in signaling, with a critical signaling threshold lying well above that required for survival. Different IL-7 dose requirements for T-cell proliferation vs. survival have been identified [14]. In contrast, our study indicates that responses to IL-7 are encoded at the level of signaling induced, which is determined both by the IL-7 environment and IL-7R
expression. Determination of the molecular mechanisms underlying varying responses will require both continued identification of the critical downstream effectors of IL-7 induced signaling and further analysis of how the network topology can give rise to distinct signal-response relationships (discussed in detail in Chapter 3).

Identifying conserved dynamic signal-response relationships between T-cells required an alternative approach for titrating signaling which could remove the effects of varying receptor-mediated ligand depletion between cells (Section 5.3.1). If instead of modulating signaling by inhibiting the initial receptor-proximal signaling events we had taken the more traditional approach of varying the cytokine dose, very different signaling relationships would have been identified, with significant interclonal divergence at low ligand doses (Figure 5.8). Previous investigations into the Stat signaling requirements for survival and proliferation in polyclonal T-cell populations have not taken these effects into consideration [14], and future studies into the signaling differences between T-cell clones will need to take precautions to discriminate whether less sustained signaling arises from altered intracellular signaling, or extracellular resources. This can be achieved both by careful experimental design, and use of mechanistic models of receptor trafficking that can capture these dynamics effects [189].

While IL-7 can induce proliferation in CD5<sup>hi</sup> T-cells, not all cells proliferate (e.g. Figure 4.2). Similar to our observations for CD5 (Figure 4.6), there are variations in IL-7R expression even within TCR-tg T-cell populations. Furthermore, IL-7R is expressed at relatively low levels on T-cells – estimates range from ~1000-3000 receptors/cell – and signaling induces strong negative feedback to IL-7R expression [1, 184]. Stochastic fluctuations in receptor expression, or among its downstream regulators – combined with local heterogeneities in IL-7 concentration around an individual T-cell - may therefore result in only a subset of cells sustaining sufficient signaling strengths and/or durations in order to initiate proliferation response. Similar effects have been attributed to heterogeneities amongst cells undergoing apoptosis [181, 190]. Recent work indicates that receptor suppression plays a strong role in restricting proliferation, as expression of a non-repressible form of IL-7Rα in OT1 T-cells at the same levels as uninhibited endogenous IL-7Rα induced robust proliferation among the entire T-cell population [191]. This reinforces the need to examine cell responses on an
individual cell basis, and indicates that functionally discrete T-cell subpopulations are not required to give rise to the distinct behavioral responses observed within naïve T-cell populations.

Our data indicates that heterogeneous surface IL-7R receptor levels are controlled at the level of gene expression (Figure 5.2), and are intrinsic to an individual T-cell. IL-7R levels may therefore be imprinted during development, and these set expression levels then determine mature peripheral T-cell cytokine responses. Initial attempts to identify methylation or other epigenetic modifications of the IL-7Rα promoter revealed no significant differences between T-cells [184]. IL-7R may therefore not be the direct target of imprinting, and rather its expression regulated by the net effect of network-wide changes affecting one or more regulators of IL-7R expression. One potential candidate regulator of IL-7R expression levels is the transcription factor Foxo1, whose deletion in lymphocytes results in significant loss of IL-7R expression [18]. Indeed, we observe significantly lower expression of Foxo1 in IL-7R<sup>hi</sup> F5 cells compared to IL-7R<sup>hi</sup> OT1 cells (Figure 5.16). F5 cells also show the same traits of decreased Bcl2 (Figure 5.6) and CD62L (Figure 4.1) expression, and reduced thymocyte numbers [10] observed for mice with Foxo1 KO T-cells [18]. Our data also indicated that F5 cells have increased PI3K signaling activity (Figure 4.14), and PI3K is a known negative regulator of Foxo1 activity [192, 193]. Regulation of Foxo1 therefore offers a possible connection between decreased IL-7R expression and increased PI3K dependent survival in the absence of cytokine.

IL-7R expression is tightly controlled in a cell-type and stage-specific manner during thymic development. For both DP and mature activated T-cells, downregulation of IL-7R is proposed to remove these rapidly expanding populations from competition for IL-7 with less abundant DN T-cell and relatively quiescent naïve T-cell fractions [5]. IL-7 signaling is also thought to be critical for CD8<sup>+</sup> vs. CD4<sup>+</sup> lineage selection [194] and IL-7R expression is proposed to identify effector subsets destined for memory cell formation [195-197]. While negative feedback to IL-7R expression is thought to be critically important for maximizing naïve T-cell populations [15], all variations in IL-7R expression were attributed to extrinsic spatiotemporal variations in access to IL-7 [5]. Thus while the importance of modulating IL-7R expression throughout the developmental lifespan of T-cell is well recognized, our work
reveals an underappreciated role for IL-7R in controlling interclonal differences in the ability of naïve T-cells to respond and compete for cytokine signals.

8.3 Intrinsic Versus Extrinsic Regulation of IL-7R, CD5, CD8

Diversity in CD5, CD8α and IL-7Rα expression amongst naïve T-cells in vivo has been largely attributed to extrinsic heterogeneities in the local spMHC and IL-7 environments [7, 15, 24]. However, our data suggest that basal CD5 and IL-7R levels are maintained by intrinsic mechanisms, and that their expression patterns can then be further altered via extrinsic heterogeneities in the signaling environment. Conversely, basal CD8α levels appear to be relatively equal amongst T-cells, though heterogeneous signaling down intrinsically different IL-7R- and TCR-mediated pathways results in more widely varied expression when subject to homeostatic signaling in vivo.

Based on the requirement of naïve T-cells to engage spMHC for the maintenance of their CD5 levels in vivo [7], CD5 expression has been interpreted as a surrogate measure for the strength of spMHC-induced signaling in the periphery [7, 8, 10, 11]. In this thesis we have present several lines of evidence that differences in basal CD5 levels among naïve T-cell clones are stably maintained as T-cells undergo activation/differentiation or after withdrawal from spMHC signals (Figure 4.3). These findings suggest that measured differences in CD5 levels are maintained by both extrinsic and intrinsic mechanisms. The degree of induction of CD5 levels above baseline may reflect spMHC signals received in the periphery [7], with the baseline CD5 level indicating the strength of thymic selection [8]. Similarly, while the broad distribution of IL-7R expression among naïve T-cells in vivo was attributed solely to the heterogeneity in the spatiotemporal presentation of IL-7 between T-cells cycling in and out of IL-7 rich lymphoid organs [5], our data suggest intrinsic differences in basal IL-7R expression also underlie these differences.

While we find that IL-7R expression is sufficient to explain the heterogeneous IL-7 responsiveness of mature T-cells (Chapter 4), the correlation between CD5 and IL-7R levels
may have developmental connections. One hypothesis is that imprinting of CD5 and IL-7R arises from a one-time epigenetic remodeling event during thymic selection. Spatiotemporal variability in the spMHC repertoire encountered by T-cells undergoing selection can give rise to thymic selection of the same TCR into different lineages even within the same TCR transgenic mouse [9]. It is therefore conceivable that stochastic variations in TCR:spMHC interactions variation also effect epigenetic remodeling events involved in any particular lineage commitment. CD5 expression is thought to reflect the strength of cognate spMHC interactions during thymic selection [9], and underlying remodeling events controlling CD5 expression may also predispose IL-7R expression levels. As discussed above (Section 8.2), these may not directly target IL-7R or CD5, but rather be the result of many subtle network-wide changes across indirect regulators of IL-7R and CD5. The unique developmental history of spMHC interactions of an individual T-cell clones might therefore determine its homeostatic capacity as a mature T-cell.

A recent study has suggested that mutual feedback between TCR and IL-7R signaling pathways underlies correlations between IL-7R, CD5 and CD8α expression among mature naïve T-cells [24]. In what is termed the ‘co-receptor tuning’ model, IL-7Rα signaling induces the transcription of CD8α to increase TCR signaling, which negatively feeds back to reduce IL-7R signaling (Figure 2.7). Reduced CD8α co-receptor expression on CD5hi T-cells is then proposed to ‘tune down’ excessive spMHC-induced TCR signaling. Lack of IL-7-induced Stat5 signaling in freshly isolated CD5hi male HY TCR-tg CD8+ T-cells has been used to support this model. However, we did not find comparable signaling defects in naïve OT1 or polyclonal CD5hi CD8+ T-cells (Figure 5.6d), which may reflect differences in their thymic development compared to male HY T-cells, which are selected on agonist ligands. The ‘co-receptor tuning’ model is also supported by a positive correlation of IL-7Rα, and inverse correlation of CD8α, with CD5 expression for a panel of freshly isolated TCR-tg cells. Indeed, we also observe these trends for OT1, 2C and F5 mice, however at more modest levels (data not shown). While feedback between IL-7R and TCR signals may dampen TCR signals in CD5hi cells, observed trends in CD5, CD8α and IL-7Rα expression may also be explained by differences in IL-7 availability in these mice. Our data suggests that OT1, 2C and F5 mice have decreasing numbers of CD8+ cells and conversely increasing
levels of available IL-7, which could partly explain the progressively higher levels of CD8α in these mice (Section 6.3.4).

Altogether, these data suggest that observed variations in IL-7R, CD5 and CD8 expression amongst naïve T-cells are a complex function of both intrinsic heterogeneities in expression with possible developmental connections, and extrinsic heterogeneities in the homeostatic signaling environment that vary both locally within hosts and between different animals. Determining the underlying relationships giving rise to these correlated differences and functional responses will require accurate quantification of the local IL-7 and spMHC environment the resulting TCR and IL-7R signaling across TCR specificities and host environments for individual T-cell clones. Analysis of these subtle variations will undoubtedly benefit from coupling of these data with mathematical systems modeling (discussed in Chapter 9).

8.4 IL-7R as an Important Regulator of the IL-7 Environment

Our data suggests that while high IL-7R expression imparts enhanced IL-7 induced signaling capacity (Chapter 5), it also leads to more rapid depletion of IL-7 from the environment (Figure 7.2). This is potentially disadvantageous under limiting cytokine environments, as cells require periodic contact with cytokine to survive (Figure 7.1), and rapid early depletion compromises late survival responses (Figure 7.3). The observation of heterogeneous manipulation of the IL-7 environment by T-cells has several important consequences for previous models for how IL-7 resources are distributed amongst the naïve T-cell population in vivo.

It has been hypothesized that signaling feedback to IL-7R expression ensures optimal sharing of limited cytokine resources between T-cells by scaling receptor expression with the need for cytokine signals [5, 15] (Fig 2.6). That is, cells deprived of cytokine for the longest periods of time (i.e. those re-entering the lymph nodes from circulation) re-upregulate their receptor and bind ligand preferentially over those cells that recently received cytokine signals and downregulated receptor expression (i.e. cells already residing in the lymph nodes). The
broad distribution of IL-7R expression seen in vivo is also attributed to this cycling behavior. While our studies confirm that the dynamics of receptor expression are in line with this type of homeostatic cycling behavior (Section 7.1), it suggest that there also exist heterogeneous abilities to compete for cytokine even amongst cells simultaneously encountering cytokine-rich regions (Section 7.2). Furthermore, cells with higher receptor expression are capable of ‘over-consuming’ cytokine beyond what is required to support certain responses, such as viability. This potentially non-optimal use of cytokine would deplete the cytokine environment for all cells, limiting overall population size below that which is theoretically achievable (Section 7.3). Thus, while IL-7 levels have been generally believed to scale inversely with the number of cell consuming IL-7 [4, 5], our studies suggest it scales more closely with the total receptor expression amongst the IL-7 consuming pool.

While consequences of unequal competition between T-cells have not been previously characterized within the naïve T-cell population, there have been studied in the context of competition between different T-cell subpopulations. Overexpression of transgenic IL-7Rα throughout thymic development was found to decrease overall thymic cellularity [103]. As DP cells normally silence IL-7R expression, reduced cell numbers were thought to result from depletion of limiting IL-7 by the relatively abundant DP cells, which then deprive DN and SP cells of IL-7. Similarly, activated cells are proposed to downregulate IL-7R expression and become reliant on other cytokines such as IL-2 for pro-survival signaling, so that the rapidly expanding population does not compete for IL-7 with the naïve T-cell population [15]. While immunohistochemistry is not sensitive enough to directly confirm whether these populations do in fact compete in vivo [2], our results suggests that heterogeneous expression can result in different capacities to deplete the IL-7 environment. Furthermore, it demonstrates that this competition may be important not only between functionally distinct T-cell populations, but also within T-cell subpopulations.

Our in vitro studies of depletion differ from in vivo environments in several potentially important ways. First, at least for CD4+ cells, strong TCR-spMHC binding affinity may enhance the ability of cells to compete for IL-7: TCR engagement signals to IL-7 production in dendritic cells and higher TCR binding keeps cells in proximately to this localized source of IL-7 [76]. However, our in vitro depletion measurements do not take into account the
effects of TCR engagement. Our experimental model also looks at a potentially extreme example of differences in consumption by beginning with cells with complete receptor upregulation. However, we still observe heterogeneities in IL-7R expression in freshly isolated cells (Figure 5.1), so differences in consumption rates may be comparatively smaller, but still present. Lastly, we look explicitly at cell responses when faced with a single limiting dose of IL-7, but in vivo, IL-7 is continually replenished. The relative homeostatic rates of IL-7 uptake at the pseudo-steady-state concentrations of IL-7 in lymph nodes will therefore depend highly on the rate of receptor and ligand recycling to the surface when receptor is partially suppressed. As IL-7 signaling influences migration [18], measurements of total consumption also needs to take into consideration the distribution of T-cells in the periphery. Accounting for all these effects will require advancements in the ability to experimentally monitor IL-7 levels and cycling times in vivo.

Whilst IL-7 conditions in normal replete mice do not appear to favor CD5^hi or CD5^hi populations, there must remain a balance between total IL-7 production and consumptions by the naïve T-cell population. Despite ‘overconsumption’ of IL-7 by OT1 cells, there is a paradoxical greater naïve T-cell population size in OT1 TCR-tg mice than in F5 TCRtg mice. One possibility is that although OT1 T-cells consume more IL-7, IL-7 levels in these relatively lymphopenic mice (which lack other IL-7 consuming populations such as B-cells) may still allow OT1 T-cells to proliferate and expand their population whereas F5 cells cannot. Expanded cell populations may also gain memory-like phenotypes with more reliance upon other cytokines, such as IL-15, whose expression may not be as limiting. As most TCR-tg mice fail to attain the steady state population sizes of replete mice [10], it is also possible that their IL-7 production rates differ (due to TCR stimulated feedback, as discussed above) and/or that the cytokine pool does not become limiting. In the latter case, the naïve T-cell population size achievable by F5 cells may represent the maximum steady state population capacity in the absence of peripheral expansion. Lastly, as IL-7R signaling is also a key regulator of migration in and out of the lymphoid organs [18], altered rates of cycling due to different IL-7R expression may skew the fraction of the population occupying these sites and bias estimates of total population size.
Altogether, these data highlight the importance of IL-7R expression in the regulation of both the ability of T-cells to respond to IL-7 and to impact their IL-7 environment. Deconvolution of the relative effects of receptor expression, feedback to receptor expression and rates of IL-7 production, and lymphoid trafficking in controlling the homeostatic levels of IL-7, and cell numbers and diversity, would likely highly benefit from rigorous quantitative experiments in combination with mathematical systems modeling (discussed in Chapter 9).

8.5 Physiological and Therapeutic Implications

Competition between T-cells for limited extraneous resources in the form of spMHC presented on dendritic cells and cytokines produced by stromal cells is believed to set the limits on the numbers and diversity of a T-cell population. The pro-survival cytokines are currently believed to regulate total cell numbers while spMHC regulates the structural (TCR) diversity of the T-cell repertoire [2]. This general model has been supported by several mathematical models [166, 168, 169], however, these studies typically implicitly assume equal reliance on non-TCR specific resource, and equal turnover in the absence of TCR signals. Rather, our demonstration of heterogeneities IL-7R signaling and basal survival among naïve T-cells suggest that IL-7 does not strictly control population size alone, and suggests that the diversity of naïve T-cell repertoire is shaped by a complex interplay between TCR and IL-7R signaling.

The ‘competition-diversity paradox’ describes how competition for survival factors would ultimately result in the domination of the repertoire by T-cell clones with the best ability to compete for (or respond to) homeostatic signals [12, 13]. Yet despite CD5^h T-cells having both increased TCR-spMHC avidity and increased ability to respond to IL-7, our in vivo studies indicate that normal replete mice maintain a homeostatic balance between CD5^h and CD5^n T-cells (Figure 6.1). Avoidance of dominance of the T-cell repertoire by CD5^h clones according to the competition-diversity paradox may rely on the basal differences in survival when deprived of homeostatic signals. One putative model is that a T-cell’s greater ability to
compete for resources, via higher spMHC:TCR affinity and IL-7Rα expression in CD5hi cells, is balanced by a greater demand for available spMHC and IL-7 resources. Our findings suggest that T-cell survival and proliferation is balanced at a state of stable equilibrium associated with an IL-7 level that does not promote selective proliferation or persistence of CD5hi or CD5lo cells [4, 5]. This steady state may arise by balancing the overall size of the T-cell population consuming IL-7 with its production. Maintaining this limiting optimal IL-7 level may allow for avoidance of the competition-diversity paradox. However, exogenous IL-7 therapy temporarily disrupts homeostasis, shifting the environment to one in which CD5hi T-cells have a selective advantage. However, it is plausible that once IL-7 therapy is stopped, the expanded T-cell population would then deplete remaining IL-7 at an increased rate due to higher basal IL-7R expansion and consumption of the expanded CD5hi T-cells. This may eventually lead to IL-7 depleted conditions where CD5lo T-cells have a survival advantage, returning the overall population to homeostasis. Confirming if, and how, populations return their basal homeostatic diversity of CD5 expression after IL-7 treatment has stopped will require longer in vivo experiments examining the dynamics of both population size and CD5 distributions. Moreover, determining precisely how subtle heterogeneities in IL-7R expression and TCR avidity integrate to influence the size and diversity of the T-cell repertoire during normal homeostasis and under cytokine therapies will likely require rigorous quantitative models describing the balance of production, consumption and access to homeostatic resources among cells with varying survival and proliferation capacities (discussed in Chapter 9).

A number of studies have shown that the T-cell population is expanded by thymic-dependent and independent mechanisms in both normal and lymphopenic mice following infusion of IL-7 [106, 185]. IL-7 administered in combination with immunization also enhances T-cell responses and promotes memory cell generation [112, 113]. This work has sparked much interest in the therapeutic use of IL-7 to help restore compromised immune systems following chemotherapy or viral attack, and as an adjuvant for vaccines and cancer immunotherapies [113, 114, 198]. Two rhIL-7 Phase I clinical trials have recently been completed in adult patients with refractory cancer [126, 127]. In both trials, CD8+ and CD4+ T-cells showed an IL-7 dose-dependent increase in population size and a concomitant
increase in the diversity of TCR Vβ usage which they attributed to predominant expansion of the more ‘diverse’ recent thymic emigrant (RTE) population [127]. However, it is unclear whether this increase in TCR Vβ diversity translates to functional ability to respond to new antigenic challenges. Our study demonstrates an additional mechanism by which IL-7 affects the diversity of the T-cell repertoire: regulating the diversity of TCR-spMHC avidities, indicated by CD5 expression, via correlated differences in their IL-7R expression and IL-7 responsiveness. While TCR Vβ diversity increases with IL-7 therapy, our model suggests that the homeostatic diversity of CD5 expression amongst T-cell clones is maximized at physiological IL-7 levels (Figure 6.9).

Our studies also add an important consideration to the design of potential IL-7 therapies. Although in normal replete mice we saw no survival advantage between CD5hi and CD5ki cells, IL-7 treatment can result in the preferential expansion of CD5hi T-cells (Figure 6.4). Preferential expansion of more autoreactive CD5ki T-cells in response to increased IL-7, either systemically or at local sites of inflammation, has been proposed to contribute to development and progression of autoimmune disorders [19, 20]. Yet selective expansion of self-reactive T-cells may prove to be desirable for the use of IL-7 as an adjuvant in cancer immunotherapies [113, 199]. Enhanced IL-7R signaling has also been proposed to contribute to T-cell leukemogenesis [21]. Thus while IL-7 therapies may expand overall T-cell numbers, they may have the unintended consequence of preferentially expanding undesirable T-cell population subsets. Given the increasing interest in IL-7 based therapies, further investigation of the intrinsic differences in the signaling networks across T-cell populations will help us understand the clinical impact of skewing the T-cell repertoire toward a CD5hiIL-7Rhi or CD5kiIL-7Rki phenotype.

The potential therapeutic promise of IL-7 therapies for expanding or enhancing the function of T-cell populations combined with the potential detrimental effects of skewing of the T-cell repertoire calls for careful design of therapeutic dosing strategies. While the aforementioned rhIL-7 clinical trials observed an IL-7 dose-dependent increase in the population size of the T-cell repertoire, at high cytokine doses, receptor suppression appeared to limit overall responses [127]. Determining the optimal IL-7 dose size and timing
to maximize beneficial therapeutic effects can be guided by mathematical models that capture the dynamic feedback between receptor suppression, population size and composition and the cytokine environment. Furthermore, characterizing the key physicochemical parameters regulating these processes may help guide the design of IL-7 mutants with improved therapeutic efficiency (discussed in Chapter 9).
Chapter 9 Perspectives on Future Work

Chapter 8 highlighted many outstanding questions surrounding how heterogeneities in IL-7R signaling across naïve T-cells arise developmentally and are manifested \textit{in vivo} in the context of TCR and other cytokine signaling. Of particular importance are questions relating to how IL-7 therapies currently in clinical trials might impact the T-cell repertoire, and whether they can be further optimized. In the following sections we discuss in detail particular areas that are ripe for future exploration and potentially addressable by a combination of quantitative experimental and mathematical modeling approaches.

9.1 IL-7 Signaling Cross-Talk with TCR & Other Related Cytokines

It is becoming increasingly evident that there are multiple mechanisms of both cell-intrinsic and cell-extrinsic crosstalk between TCR and IL-7R signaling. Moreover, multiple cytokines present \textit{in vivo} within the T-cell niche have partially overlapping roles with IL-7 and affect IL-7R expression [1]. Chapter 3 discusses many of the interactions that may give rise to non-intuitive cellular responses under stimulation with multiple cytokine and/or TCR cues. Here we offer a few specific questions that directly follow from the conclusions of this thesis that might be of particular interest for future studies, and some initial perspectives on how they might be addressed.

9.1.1 Relationships Between IL-2, IL-15 & IL-7 Signaling

Is the heterogeneous responsiveness of CD5$^{hi}$ and CD5$^{lo}$ T-cells to IL-2 and IL-15 controlled at the level of receptor expression? Do these related cytokines share common signal-response relationships with IL-7?

\textit{In vivo}, the pro-survival cytokines IL-2 and IL-15 are thought to have partially overlapping roles with IL-7 [1], and are proposed to partially account for maintenance of the small T-cell
population still present in IL-7-deficient mice [64]. While the focus of this thesis was to characterize responses to IL-7, many of the same mechanisms regulating cell responses to IL-7 may also apply generally across IL-2 family cytokines [200]. Indeed, we observed that in addition to their increased responsiveness to IL-7, CD5b T-cells also had more robust proliferation responses to IL-2 and IL-15 (Figure 4.7). IL-2-based therapies have found use in treating a diversity of disease conditions [201], and manipulation of IL-15 levels has been proposed for vaccine adjuvants [202] or autoimmune therapies [203]. It will be critical to study whether these and other related cytokines have similar abilities to shift the diversity of TCR affinities across the T-cell repertoire as we have observed for IL-7.

This thesis offers a potential ‘roadmap’ to characterizing signal-response relationships of T-cells to other cytokines. First, while we have established differential responsiveness to IL-2 and IL-15 across CD8+ T-cells of different CD5 expression, it is unclear whether this enhanced sensitivity is determined primarily by their receptor expression, as we have observed for IL-7. Future work should therefore begin by characterizing the receptor expression and proximal signaling dynamics as we’ve undertaken for IL-7 (Chapter 4). In contrast to negative signaling feedback to receptor expression for IL-7, IL-2 signaling causes upregulation of the IL-2-specific IL-2Rα/CD25 receptor chain [22]. IL-15 poses further complexities, as its unique α chain is thought to transpresented, though signaling can still originate from IL-15Rα on the cell surface [204, 205]. An Fc/IL-15Rα fusion can be pre-complexed with IL-15 to mimic transpresentation for signaling studies (R&D systems) [206]. Furthermore, both IL-2 and IL-15 have an additional shared receptor component, IL-2Rβ/CD122. These complexities highlight the need for precise measurements of multiple receptor components over time to fully characterize whether differences in signaling and responses are determined by receptor expression dynamics. Mechanistic ODE-based models of receptor-ligand dynamics will likely be useful in quantitatively relating signaling and receptor dynamics [189].

Our initial signaling profiles for IL-7 indicated that Stat5 and PI3K were the dominant upstream signaling pathways and their individual measurements were sufficient to predict responses (Section 5.3.2). However, in many systems, predicting responses often requires
network-wide activity measurements across multiple signaling pathways (see for example [207, 208]). In the particular case of IL-2 and IL-15 stimulation, MAPK pathway signaling is likely to play a significant regulatory role in addition to Jak/Stat and PI3K/Akt signaling [22]. Nevertheless, it will be interesting to see to what extent viability, proliferation and CD8α induction responses to IL-2 and IL-15 can be individually predicted from their Stat5 or pGSK3 phosphorylation levels as we’ve observed for IL-7, and whether this signal-response relationship is common among all three cytokines. Where discrepancies exist, additional dynamic measurements of MAPK pathways components may improve response predictions. These studies will likely benefit from statistical modeling techniques which determine a hierarchy of predictive power amongst multiple signals, and can be used to identify critical underlying regulatory relationships [25]. As all of these cytokines can be simultaneously present in vivo, and share both downstream signaling pathways and receptor components, models built on dynamic data under an array of combined cytokine stimulations will be useful for deconvoluting overall systems behavior in complex cytokine environments present in vivo.

9.1.2 IL-7R Control in the Context of Other Cytokines

Do alterations in IL-7R expression induced by other cytokines translate directly to IL-7 signaling capacity and responses?

We found that IL-7R expression was the primary determinant of responsiveness to IL-7 among T-cells. However we, and others [15], have observed that multiple cytokines influence IL-7R expression. IL-4, IL-6, IL-2, IL-15 and IFNγ all decrease IL-7R surface protein expression, while TNFα increases expression (Figure 9.1). While each of these cytokines have unique roles in T-cell development and function [200], they may also alter responses to IL-7 either directly via receptor expression or via downstream signaling crosstalk (Chapter 3). Furthermore, manipulation of receptor expression with other cytokines might be an alternative approach to studying variations in IL-7R expression.
Figure 9.1: IL-7R Expression is Influenced by Multiple Cytokines

IL-7Rα surface expression of F5 TCR-tg naïve (CD44<sup>lo</sup>) CD8<sup>+</sup> T-cells rested overnight (~16 hours) in cytokine-free media and then treated with 10ng/mL of IL-2, IL-4, IL-6, IL-7, IL-15, IFNγ, TNFα or medium alone.

We were unable to increase IL-7R expression on F5 cells via infection of bone marrow stem cells with retroviral IL-7Rα, presumably because of tight development controls on expression (Section 5.4.1). However, it might be possible to pre-treat F5 cells with TNFα to increase IL-7R expression, followed by treatment with IL-7, to see whether this leads to sufficient signaling for proliferation. As TNFα induces cell death pathways [209] whereas IL-7 induces pro-survival signaling, these studies will also be informative as to how T-cells balance pro- and anti-apoptotic signaling via their receptor dynamics. Pre-treatment or simultaneous stimulation with a variety of cytokines types and doses and IL-7 could therefore be used in a similar way to varying inhibitor treatments to titrate IL-7 signaling responses. As highlighted in the previous section, a variety of mechanistic and statistical modeling approaches might be useful to deconvoluting effects that result from either manipulation of receptor levels or influencing downstream pathway activation. These studies would help inform a more complete understanding of how IL-7 responses are regulated via other cytokines present in vivo.
9.1.3 Interactions Between TCR and IL-7R Signaling

What is the effect of spMHC stimulation on IL-7-induced downstream signaling activity and responses and vice versa?

Work in this thesis has focused on IL-7 responses in the absence of TCR stimulation, yet it is increasingly clear that T-cell survival and proliferation is controlled by integration of both TCR and IL-7R signals. Park et al. (2007) have proposed a model whereby mutual feedback between these pathways balance overall homeostatic signaling [24]. In contrast, others suggest that cytokine signaling primes T-cells for more robust TCR-pMHC interactions [78]. The ability to carefully study the mechanisms of integrated signaling control of cellular responses to TCR and IL-7R stimulation has been hindered in several ways. Of foremost difficulty is directly quantifying and comparing low-affinity spMHC:TCR interactions, especially across TCR specificities [210]. While an increasing number of estimates of binding affinities have been made, it is not always clear these directly relate to the multivalent interactions of T-cells with spMHC on antigen presenting cell surfaces [210, 211]. There are also likely dynamic changes in the local spMHC and cytokine environment during these cell-cell interactions whose effects are not accounted for in artificial spMHC presentation systems. Lastly, there is in many cases a lack of reliable probes to monitor signaling activity on a single-cell basis by flow cytometry, immunohistochemistry, or live cell imaging. Despite these limitations, discussed here are a few simple studies that might provide some initial insight into TCR-IL-7R interactions.

There exist several TCR-tg T-cell systems for which a library of peptides of varying TCR affinity has been identified. For instance, the 2C TCR system has been highly characterized [46]. Naturally occurring self-peptides that mediate both positive selection and peripheral homeostasis have also been identified for the 2C and OT1 TCR systems [47, 212]. While the exact affinity of these peptide-TCR interactions have not all been measured, in many cases, their affinities can be rank ordered. New peptide-exchange systems [213] allow for simple and quick presentation of a diversity of pMHC complexes either on artificial surfaces, on antigen presenting cells, or as MHC tetramers [214]. Another method for quantitatively varying TCR signaling is to take a similar approach to our cytokine signaling titration
As a first step, future studies could profile T-cell survival, proliferation and CD8α induction responses \textit{in vitro} using one of the aforementioned spMHC presentation systems for varying TCR signaling in combination with varying cytokine signaling. This data should reveal in what treatment regimes there is divergent behavior from spMHC or IL-7 signaling alone, and the minimal individual and combined requirements for homeostasis. Monitoring critical IL-7R and TCR signaling pathway components such as Stat5 and Erk respectively and downstream effectors of survival such as Bcl2 will provide an initial indication of IL-7R/TCR signaling pathway crosstalk. These pathways offer the additional advantage of having good flow cytometry-validated antibodies for their detection (BD Bioscience PhosFlow System), allowing for single-cell analysis of signaling even in the context of co-culture systems for spMHC presentation. Simply assaying IL-7 signaling in the presence and absence of stimulation by positively selecting spMHC will help resolve discrepancies between the TCR signaling mediated inhibition of IL-7 signaling reported by Park et al (2007) [24] that was not observed in our studies.

9.1.4 Predictive Models of Signaling Control of T-Cell Responses

Can we predict survival and proliferation responses as a function of combined spMHC and cytokine signaling?

We may not be able to fully mimic \textit{in vitro} all the components present within \textit{in vivo} T-cell environments. However, work by our lab and other suggest that signaling assays of network activity may be more robustly predictable of cellular responses than the particular cell treatment (combination of cytokines and spMHC), which will undoubtedly present other contextual stimulatory cues (see for example [207]). We can gain significant understanding of cellular control by undertaking a ‘cue-signal-response-analysis’: examining how a constrained set of stimulatory cues (or treatments) give rise to signaling pathway activation and then are translated into responses. However, in translating these relationships to complex \textit{in vivo}
environments containing other potentially unknown cues, signal-response relationships are more likely to be preserved than cue-response relationships. This suggests that even with our limited ability to directly mimic in vivo environments, systematic profiling of T-cell signaling and responses under a wide range of cytokine and TCR stimulations might inform a useful model for predicting in vivo cellular outcomes based on their signaling state when isolated from their in vivo environments.

We have the experimental methodology to extend the study of control of T-cell homeostasis from one cue (IL-7) to many cues (multiple cytokines and/or spMHC complexes). However, discerning critical regulatory relationships from this expanded data set comprising multiple stimulations, downstream signals and response requires more sophisticated data analysis and visualization tools that the one component analysis framework we have largely employed in this thesis. Many of these tools have been developed in our lab and successfully applied to a wide array of cell systems, including a T-cell hybridoma system under varying levels of activating stimuli [208]. We might similarly be able to use a comprehensive and quantitative data set for primary T-cells under combined stimulation to help understand & predict how T-cells resolve their complex cytokine and TCR environment into functional responses.

Using these models to infer mechanisms for integrated signaling control in vivo will require murine models from which we can either quantitatively measure the homeostatic environment or independently control access to either spMHC or IL-7. We’ve observed potential variations in IL-7 levels across TCR-tg hosts (Section 6.3.2), enforcing the need to compare survival and proliferation responses of T-cells with specificities within identical hosts. In vivo levels of IL-7 in a single host can then be manipulated via infusion of cytokine or cytokine-neutralizing antibodies as we’ve described in Chapter 7. In may be possible to compare relative homeostatic capacities of a given T-cell specificity across varying spMHC stimulations in vivo by genetically varying TCR expression or using ‘single’ spMHC mice [48, 49, 217]. Cells can then be assayed for their resulting signaling network following adoptive transfer into each host environment. Thus, whereas models derived from in vitro stimulation data can help derive relationships between signals and responses, these in vivo studies can clarify the physiological relationships between stimulatory cues and signaling. These coupled
approaches may help define the roles of spMHC versus IL-7 signals in regulating homeostatic T-cell survival and proliferation responses.

9.2 Optimization of Therapeutic IL-7 Dosing Strategies

IL-7 is currently being administered in multiple clinical trials aimed at treating a wide variety of pathological diseases including cancer, HIV, and a variety of autoimmune diseases, and has shown early promise in restoring compromised immune systems and as an adjuvant in vaccines [218]. However, significant questions remain as to how to optimize therapeutic dosing strategies for IL-7. Early cytokine immunotherapy trials evoked adverse immunological reactions that were partially attributed overdosing, and these studies have hindered the rapid development and approval of related therapies [129-131]. This highlights the need for careful design of cytokine therapies during initial stages of development and early clinical trials. Human clinical have shown dose-dependent expansion of the T-cell pool, but total expansion achievable was limited in both size and duration [126, 127]. However, the therapeutic capacity of cytokines may be altered by genetic mutations affecting receptor/ligand binding and resulting trafficking properties [219], or using cytokines pre-complexed or directly fused to antibodies [220-223]. Here we highlight several questions relating to IL-7 dosing strategies and potential ways to begin addressing them with quantitative modeling, protein engineering and in vivo studies.

9.2.1 Examination of Receptor Feedback-Limited Dosing

Does receptor suppression limit the net population expansion during IL-7 therapy?

In one of the first human clinical trial of IL-7 therapy, a cohort of adults with refractory cancer was subjected to varying doses of IL-7 injected every other day for two weeks [127]. Clinicians observed a dose-dependent increase in the CD4⁺ and CD8⁺ lymphocyte populations which peaked at ~300% and ~400% of initial population size respectively at 21...
days after the initiation of treatment. However, the percentage of cycling cells, as well as intracellular Becl2 levels, peaked after only 7 days into the treatment, and coincided with loss of IL-7R expression at both the surface protein and mRNA levels. Investigators interpreted loss in IL-7R expression as a self-limiting mechanism preventing further cell expansion with continued IL-7 treatment. However, at lower doses of IL-7, despite reduced total expansion than with high doses, cells did not completely lose receptor expression. Furthermore, IL-7R expression increased after cessation of IL-7 treatment in all cases. This implies that either using lower IL-7 doses over longer durations, or higher doses at less frequent intervals may allow for continued and/or periodic re-expression of receptor, allowing cells to continue to be receptive to IL-7 stimulation. Authors also observed that several weeks after treatment was halted, growth in the lymphocyte population ceased, and in some cases population sizes began to fall. This might indicate the initial stage of a return to a lower homeostatic population size. However, patients were not monitored sufficiently long to confirm whether they re-establish their baseline population sizes prior to treatment or maintain a net increase in lymphocyte numbers.

Determining IL-7 dosing parameters for promoting an optimal sustained increase in population size begs for a rigorous quantitative analysis of different therapeutic strategies - both dose size and duration/frequency - and how they are impacted by negative feedback to receptor expression. In particular, a simple mathematical differential equation model relating receptor trafficking dynamics and signaling to survival, proliferation and receptor suppression [189] would greatly benefit the interpretation of both in vitro and in vivo studies. Such a model would need to be informed by measured kinetic rates of receptor dynamics and empirical signal-response relationships similar to those derived in this thesis. However, it could then be used to evaluate in silico a much wider array of IL-7 treatment regimes than is feasible with in vivo studies to determine critical parameters controlling population dynamics as a function of treatment dose and time. The capacity of the model to predict population size outcomes could then be evaluated for a more restricted set of treatment regimes in mouse models. These in vivo studies could also monitor CD5 expression profiles throughout and after treatment, in particular to determine whether CD5 and population size returns to initial homeostatic states after exogenous IL-7 is depleted. If true, this would suggest that
while IL-7 therapy may accelerate population recovery in the short term, populations will eventually return to the initial size governed by rates of IL-7 production.

9.2.2. Improving the Therapeutic Design of IL-7

Can IL-7 be rendered more efficacious by altering its receptor binding properties and/or pre-complexing with antibody?

Work in our lab has shown that the receptor binding properties of cytokines can be altered via genetic mutation, which impacts cell signaling and responses as well as rates of cytokine recycling and degradation [219, 224-228]. The overall impact of changing binding properties on cell behavior is often non-intuitive, but can be their design can be guided, and effects interpreted, by the use of mathematical models [219]. Development of these models require quantitative dynamic data to determine kinetic rate parameters, and our group has developed experimental methodology to independently determine rates of ligand binding, internalization, uptake, degradation and recycling. Models of behavior with wild type IL-7 can therefore be constructed using data in this thesis and supplemented with additional measurements using either radioactive isotope or fluorescent labeling of IL-7. Sensitivity analysis of ligand binding parameters would then inform hypotheses as to whether cell behavior and/or ligand depletion may be significantly altered via mutation. IL-7 with mutations that meet desired biophysical parameters can be isolated using yeast-two-hybrid screening approaches [229]. It may therefore be possible to develop recombinant mutant IL-7 that is more therapeutically efficacious than wild type IL-7.

Another approach to altering the in vivo activity and persistence of cytokines is to pre-complex them or fuse them directly to antibodies and/or soluble receptor components [221-223]. IL-7:αIL-7mAb complexes have been shown to elicit more robust B- and T-cell expansion and more potent CD8+ T-cell antigen-specific activation responses [230]. IL-2:IL-15 antibody complexes and IL-15 pre-associated with IL-15Ra have similarly increased biological activity [206, 220, 231]. There is some debate over the mechanisms of increased activity for cytokine-antibody complexes [220, 222]. Antibody complexation may increase in
half-lives of cytokines by reducing cell-mediated-degradation and clearance [232]. They may also directly affect receptor-binding properties [232]. Soluble isoforms of IL-7R first identified in humans [233, 234], and just recently observed in mice [235] have also been proposed to have similar action to cytokine mAbs. However, presence of the Fc portion of antibodies is required for the full activity of some IL-7/mAb complexes, suggesting additional cellular interactions unique to antibody complexation [230]. Mathematical modeling might provide a useful tool for critically evaluating these possible mechanisms in a quantitative framework. Extending the model to include extracellular soluble antibodies and/or receptors would be a simple extension of the prospective model described in the previous section. Similar to the studies described above, we may also be able to use this model to inform whether changes in the antibody-ligand binding properties might further enhance the efficacy of antibody-cytokine complexes and how their dosing might be optimized.

9.2.3 Modeling Homeostasis with IL-7R Heterogeneity

What are the implications of heterogeneous IL-7R signaling capacities for current models for the development and reconstitution of the T-cell pool?

Several mathematics models have recently been published which describe how the size and diversity of the T-cell pool is initially established, maintained during normal homeostasis, and how it recovers following periods of lymphopenia [166, 168, 169]. These models reach similar conclusions: T-cell population diversity is regulated by TCR specificity, while IL-7 places limitations on the total population size achievable. However, these models made the implicit assumptions that T-cell turnover and ‘demand’ for non-TCR-dependent recourses such as IL-7, and sensitivity to these cytokines, is equal across TCR specificities. As we’ve demonstrated that heterogeneities in both cytokine responses and cytokine- and spMHC-deprived survival vary across the T-cell repertoire, these models need to be revised. Future work could involve the simple extension of these models to include additional variation in reliance on cytokine signaling, and basal turnover rates, that co-vary with TCR sensitivity. Determining whether these models allow for establishment of steady state diversity in
TCRspMHC avidity (CD5 expression) and population size would help determine whether feedback between TCR and IL-7R pathways is in fact required for homeostasis. It would also allow for more detailed evaluation of how IL-7 resources are shared among the T-cell pool and how population sizes scale with the availability of IL-7. These revised models would be very useful in furthering our understanding of which factors influence the development and maintenance of immune function and advise the further development of cytokine therapies.
Methods

M.1 Mice
C57BL/6J (B6) mice, B6 TAP−/− mice and B6.CD90.1/Thy1.1 congenic mice (B6.PL.Thy1a/Cy) were purchased from the Jackson Laboratory. OT1, 2C and F5 TCR-tg mice were backcrossed onto the B6.Rag−/− background for >20 generations. Age- and sex-matched mice, between 6 and 16 weeks of age, were used for experiments. Mice were maintained in the MIT animal facilities and used according to the guidelines of the Institutional Committee on Animal Care.

M.2 T-Cell Purification and in vitro Culture
CD8⁺ T-cells were purified from single cell suspensions of lymph nodes or spleen using an EasySep mouse CD8⁺ T-cell enrichment kit (Stem Cell Technologies). For enrichment of the naïve (CD44⁺) lymphocyte fraction, T-cells were either (1) stained with phycoerythrin-conjugated anti-CD44 and the stained fraction removed using anti-phycoerythrin microbeads (Miltenyi Biotech) or (2) stained with anti-CD44 and removed using fluorescent cell sorting. For all experiments involving sorting on CD5 expression, viable CD8⁺CD44⁺ T-cells expressing the maximum 20% and the minimum 20% levels of CD5 were sorted into CD5⁺ and CD5⁻ fractions. Purified T-cells were cultured in complete RPMI-1640 medium containing 10% FCS (see Culture Media) at 37°C and 5% CO₂. Cells were assayed either immediately following isolation, or where indicated, cells were rested overnight (~16 hours) in medium without cytokine to eliminate any TCR and cytokine signaling prior to treatment. Unless otherwise indicated, cells were cultured at densities of 2-3.5x10⁵ cells/mL.

M.3 Culture Media
Unless otherwise indicated, cells were grown in RPMI-1640 with glutamine (VWR), 10% charcoal/dextran stripped FBS (HyClone), penicillin (100 U/ml), streptomycin (100ug/ml), nonessential amino acids (1x), HEPES (10 mM), sodium pyruvate (1 mM), and 50 uM β-mercaptopetoethanol (all VWR).
M.4 Peptides, Cytokines & Inhibitors

Recombinant murine IL-7, IL-2, IL-15, IL-4, IL-6, IL-9, TGFα, and IFNγ (all Peprotech, H₂O with 5% BSA stock) were added to culture media at concentrations ranging from 0.0001 to 100 ng/mL. The PI3K inhibitors LY2904002 (0.1-200 μM), PI-103 (0.01-10 μM) and the Jak family inhibitor, Jak Inhibitor I (0.004-2 μM) (all Calbiochem, DMSO stock) were added to cultures for 30 minutes prior to cytokine addition at the indicated concentrations. RTY (RTYTYEKL), ISF (ISFKFDHL) and SII (SIINKEKL) peptides (MIT Biopolymers facility, H₂O stock) were added to cultures at the concentrations indicated simultaneously with cytokines.

M.5 In vitro Memory Cell Generation

OT1 and F5 memory-like T-cells were generated via slight modification of protocols previously described [35]. In brief, OT1 and F5 cells were activation by one of two methods, as indicated: (1) cells were cultured in on plate-coated anti-CD3 antibody (BD Biosciences) for 3 days in the presence of 20 ng/mL mIL-2 (Peprotech), or (2) cells were cultured in media containing 25 ng/mL PMA + 500 ng/mL ionomycin for 24 hours, washed 3x with media, and cultured in media containing 20 ng/mL IL-2 for 2 days. Following activation and blasting, cells were then washed 3x in cytokine-free media, and culture in either 5 ng/mL IL-7 or 40 ng/mL IL-15. Cell responses to cytokines were then measured following cell culture overnight in media without cytokines after washing 3x with cytokine-free media.

M.6 Antibodies

The following antibodies were used (clone names in parentheses): Bcl-2 (3F11), Stat5 (89), pStat5 Y694 (47), Ki67 (B56), Mouse IgG1,κ isotype (MOPC-21) (all BD Biosciences), pGSK3 (37F11), GSK3 (9315), β-Actin (8H10D10) (all Cell Signaling Technologies), IL-7Rα/CD127 (SB/199), CD5 (53-7.3), CD44 (IM7), CD69 (H1.2F3), CD62L (MEL-14), TCR, CD8α (53-6.7), CD25 (PC61), CD122 (TM-b1), Thy1.1/CD90.1 (OX-7), Thy1.2/CD90.2 (30-H12), mouse IgG2α,κ isotype (MOPC-173), Rat IgG2α,κ isotype (RTK2758) and Rat IgG2β,κ isotype (RTK4530) (all Biolegend). Optimal antibody dilutions were determined by titration.
M.7 CFSE staining

T-cells were suspended at 1x10^7 cells/ml in 2mL serum-free RPMI media containing 5mM CFSE (carboxyfluorescein diacetate succinimidyl diester; Invitrogen) and incubated at 37°C for 5 minutes. Staining was stopped by addition of 10mL of serum-containing media and placing cells on ice for 5 minutes. Cells were washed twice prior to in vitro culture or adoptive transfer.

M.8 Flow Cytometry: Live Cell Detection of Surface Epitopes

Cells were washed in PBS containing 0.5% BSA and resuspended in PBS containing 0.5% BSA, 0.1% NaN₃ and 2.5 μg/mL Fc Block™ for 10 minutes at 4°C. Fluorescently-tagged antibodies were added to a final concentration of 1.25μg/mL for 40 minutes at 4°C. Nonspecific background fluorescence was determined by staining with isotype-matched antibodies. Cells were washed twice with PBS containing 0.5% BSA and 0.1% NaN₃ and kept on ice prior to multi-parameter flow cytometric detection on FACS LSRII (Becton Dickinson). Cell viabilities were determined by adding 1.5 mM DAPI (Invitrogen) to the final resuspension.

M.9 Flow Cytometry: Detection of Cell Surface & Intracellular Antigens

For detection of intracellular signaling, Live/Dead Fixable Blue Dead Cell Stain (Invitrogen) was added to cells for 10 minutes before the end of culture. Cells were placed on ice and washed 1x with ice-cold PBS. Cells were fixed by resuspending in with 4% formaldehyde for 10 minutes. Cells were then permeabilized by resuspending in ice-cold 90% MeOH for >2 hours at -20°C. Cells were washed twice and then resuspended in PBS with 0.5% BSA and 2.5 μg/mL Fc Block™ (2.4G2) (BD Biosciences) for 10 minutes at 4°C. One of two protocols was then followed depending on the nature of the antibodies used for staining:

**Primary conjugated antibodies only (pStat5, pErk, Ki67):**

Fluorescently-tagged antibodies against intracellular and cell surface antigens were added for 40 minutes at room temperature. Final concentrations for antibodies against cell surface antigens were 1.25μg/mL (typically 1:200 dilution for Biolegend antibodies) unless otherwise
indicated. For intracellular antigens, final concentrations of 1 µg/mL (typically 1:20 dilution for BD Phosflow antibodies) were used unless otherwise indicated. Cells were then washed twice and resuspended in PBS containing 0.5% BSA and kept on ice prior to multi-parameter flow cytometric detection on FACS LSRII (Becton Dickinson).

**Primary conjugated and unconjugated antibodies (pGSK3, Bcl2, Akt):**
Unconjugated primary antibodies against intracellular antigens were added for 40 minutes at room temperature. Unless otherwise indicated, primary antibodies were added at 5µg/mL (typically 1:20 dilution Cell Signaling Technologies). Cells were washed twice with PBS containing 0.5% BSA, and resuspended in secondary antibodies and antibodies against cell surface antigens in PBS with 0.5% BSA and 2.5 µg/mL Fc Block™ for 40 minutes at room temperature. Final concentrations for antibodies against cell surface antigens were 1.25µg/mL (typically 1:200 dilution for Biolegend antibodies) unless otherwise indicated. Secondary antibodies were added at 2µg/mL (typically 1:500 dilution for Invitrogen antibodies) unless otherwise noted. Nonspecific background fluorescence was determined by staining with isotype-matched antibodies and/or secondary antibody staining only as indicated. Cells were washed twice with PBS containing 0.5% BSA and kept on ice prior to multi-parameter flow cytometric detection on FACS LSRII (Becton Dickinson).

**M.10 Flow Cytometry Analysis**
All flow cytometry analysis was performed using FlowJo software (Treestar). In nearly all cases, cell gatings were performed in the following order: (1) FSC/SSC for non-debris populations (2) FCS-A vs. FSC-H for non-doublet population (3) CD8⁺ fraction (4) PI- or DAPI- viable cell fraction (5) additional markers.

**M.11 Western Blot Analysis of Total Protein Expression**
T-cells were cultured in cytokine-free media for 16 hours, and viable T-cells were isolated by Ficoll-Paque Plus gradient separation (GE Healthcare). T-cells were lysed in RIPA lysis buffer (Thermo Scientific) containing PhosStop Phosphotase Inhibitor, Complete Mini Protease Inhibitor (Roche) and 0.1 M PMSF. Lysates equivalent to 6x10⁵ cells were subjected to SDS-PAGE (Invitrogen). Western blots transferred onto nitrocellulose were probed with
primary antibodies against Stat5 (1:1000, Becton Dickinson), β-actin (1:10000) and GSK3 (1:1000) (both Cell Signaling Technologies) in Licor blocking buffer (Licor Biosciences) overnight at 4°C, washed 3x with PBS with 0.1% Tris (PBS-T) for 10 minutes, probed with Infrared secondary antibodies (1:10000 Licor Biosciences), washed 3x with PBS with 0.1% Tris (PBS-T) for 10 minutes and imaged on the LICOR Odyssey Infrared Imaging system (Licor Biosciences).

M.12 Glucose Uptake
Glucose uptake was measured as previously described [14] with minor modifications. T-cells were cultured for 24 hours in presence or absence of 10 ng/mL IL-7. Viable cells were isolated by Ficoll-Paque Plus gradient separation. T-cells (8.5x10⁵) were incubated for 30 minutes in serum- and glucose-free RPMI 1640 media. Glucose uptake was initiated by adding labeled 2-deoxy-D[1-³H] glucose (Amersham Pharmacia Biosciences) to a final concentration of 0.1mM (4 µCi/mL). Cells were incubated for 45 minutes at 37°C, washed 3x in cold glucose- and serum-free RPMI 1640 media and solubilized in 525 µL of 1% SDS. Radioactivity in 470 µL of the sample was measured by liquid scintillation.

M.13 Adoptive Transfer Assays
For all experiments involving adoptive transfer of T-cells, purified naïve CD8⁺ T-cells were resuspended in Hank’s buffered salt solution and injected retro-orbitally into age- and sex-matched recipients. Unless otherwise indicated, ~1x10⁶ cells were injected in ~100µL.

M.14 In vivo IL-7 Addition
In vivo addition of IL-7 or PBS was done by subcutaneous implantation of mini-osmotic pumps which release 0.5 µL/hr over 7 days (#1007D, Alzet). Prior to implantation, pumps were filled with 100 µL of PBS or PBS containing 5 µg of IL-7 (Peprotech). Pumps were implanted into a 2 cm long subcutaneous pocket inside a 0.5 mm mid-scapular incision over the right flank and closed using a wound clip with application of a Betadine® antiseptic on the incisions site. Lymph nodes and spleen were harvested after 7 days.
M.15 **In vivo IL-7 Depletion**

*In vivo* IL-7 depletion was achieved by i.p. injection of 1mg anti-IL-7 antibody M25 or the mouse IgG2b isotype MPC-11 (BioXCell) every other day for two weeks (days 0, 2, 4, 6, 8, 10, 12). Lymph nodes and spleen were harvested after 14 days.

M.16 **Bioassay for Comparison of in vivo IL-7 Levels Across Mice**

Purified Thy1.1* 2C TCR-tg naïve CD8* T-cells (~10⁶) were adoptively transferred into syngeneic Thy1.2* mice and the relative IL-7 levels *in vivo* in different recipients were determined by comparing IL-7Rα and CD8α expression on the Thy1.1* donor cells recovered from the recipient spleens and lymph nodes after 18 hours. A minimum of three mice of each genotype (B6, OT1 Rag−, 2C Rag−, F5 Rag−, Rag−) were examined for each experiment.

M.17 **Plasmids & Virus Production**

Coding sequence for the full-length mIL-7R was cloned into the pMIG vector (MSCV-IRES-GFP) by Vinay Mahajan, designated Mig7vsm. A second supposedly identical construct was also obtained from the Scott Durum’s lab, designated Mig7sd. For production of virus, 293FT/− cells were transfected with 2ug transfer vector, 1ug MLV gag-pol and 1ug VSVg expression vectors using Fugene 6 (Roche) per the manufacturer’s protocols. Medium was changed 24 h after transfection. After 48 h, media was collected, passed through a 0.45uM filter and ultracentrifuged to concentrate virus. To titer the virus and demonstrate all constructs were expressed on surface of murine cells, 58* cells [172] were transfected with retrovirus supernatants and 4ug/mL polybrene for 24 hours and surface IL-7Rα and GFP expression subsequently measured by flow cytometry. Virus titers were determined to be approximately ~7x10⁴/uL (Mig7sd) and 5x10⁴/uL (Mig7vsm).

M.18 **Amaxa Transfection of Naïve T-Cells**

10⁶ F5 T-cells freshly isolated from lymph nodes were transfected with pMaxGFP (2.5ug), Mig7sd (4ug) or Mig7vsm (4ug) vectors by electroporation using the Amaxa T-Cell Nucleofactor Kit (VPA-1006, Lonza) as per the manufacturer’s directions. Transfected cells
were rested for 24 hours in media with or without 10 ng/mL for 24 hours prior to cell surface staining and detection by flow cytometry.

M.19 Spin Infection of in vitro Memory Cells

Memory cells were generated as described in Section M.5 with the following changes. At 24h and 48h after activation (plating on anti-CD3 or addition of PMA/Ionomycin), Mig7sd or Mig7vsm was added to cells at an MOI of 10. The culture plate was sealed with saran wrap and spun at 1000g for 90 minutes. Cells were placed back in culture at 37°C and 5% CO₂, and cells were washed 3x with media and resuspended in fresh media (with IL-2 as indicated) 24 hours after each infection.

M.20 F5 Retroviral IL-7Rα Overexpressing Bone Marrow Chimeras

Purified F5 bone marrow stem cells were isolated according to procedures previously described (Zhang CC et al Nature med 2006). In brief, bone marrow was isolated from the femur and tibia of F5 mice and made into single cell suspensions. The stem cell fraction was enriched by depleting cells with the following surface markers by magnetic separation (MACS): Ter119 (erythroid), CD11b (macrophage), Gr1 (myeloid), CD8 (T lymphoid), CD19 (B lymphoid), DX5 (NK cells), using ~1uL antibody per 4x10⁶ total cells. Purified cells were plated at low density (10000 cells/150uL) in a U-bottom 96-well plate in stemspan serum-free medium (StemCell Technologies) supplemented with 10μg/mL heparin (Sigma), 10ng/mL mouse SCF, 20ngmL mouse TPO, 20ng/mL mouse IGF-2 (all from R&D systems) and 10ng/mL human FGF-1 (Invitrogen), (STIF medium) with 100ng/mL Flag Angptl2. Cells were infected with Mig7sd, Mig7vsm or an empty control vector (MOI=10) with 4ug/mL polybrene at 2 and 3 days after cell isolation. After 7 days in culture, cells were washed and resuspended in HBSS, and checked for the presence of GFP’ expressing fractions. Expanded cells were then retroorbitally injected into sublethally irradiated (600 Rad) Rag’ hosts (~10⁶ cells/mouse). Bone marrow chimeras were produced for each of the two Migs7d and Mig7vsm virus preparations, for a total of ~30 mice. Tail bleeds of mice 6 weeks later showed ~18 mice with significant CD8’ peripheral reconstitution, and GFP’ fractions ranged from 30-70%. Mature cells were then isolated from the lymph nodes and spleen 8 weeks after adoptive transfer for analysis.
M.21 Pulsed versus Sustained Signaling Treatments

Purified naïve (CD44+) OT1 and F5 T-Cells were rested overnight in cytokine-free media. Cells were resuspended in media with varying concentrations of IL-7 (0, 0.0001-100ng/mL) at ~5x10⁴ cells/150uL in a 96-well plate. After 1 or 4 hours in culture, ‘pulsed’ treatment samples were washed 3x with 200uL of cytokine-free media and resuspended in 200uL of cytokine-free media for the remainder of culture (24 or 48 hours). ‘Sustained’ samples did not receive media changes, but were centrifuged and resuspended alongside pulsed samples during washings.

M.22 In vitro IL-7 Depletion Measurements

T-cells were rested overnight in cytokine free media and then suspended at 0x, 1x, 2x, or 3x 3.3x10⁵ cells/mL and cultured with 0.1ng/mL of IL-7 for 24h. After 24 hours, supernatant media was collected and IL-7 remaining in cell containing versus cell free cultures measured by ELISA (R&D Systems). IL-7Rα surface expression and viability of cells in cultures were assayed by flow cytometry.

M.23 Statistical Analysis

Prism software was used for statistical analyses. P-values were calculated with two-tailed Student’s t-test. P-values of less than 0.05 were considered significant.

M.24 Mathematical Modeling Analysis

Ordinary differential equation models were executed using the Matlab software package (The Mathworks).
Appendix

A.1 Model Reaction Schemes for Chapter 3

The model reaction schemes and parameter values used to generate the plots for the figures presented in Chapter 3 are outlined below. These schemes are manifestly idealized in a highly simplified manner with many major approximations and assumptions. Some aspects of the network topology are not fully characterized and many of the system parameters are undetermined or poorly defined, so model results represent merely a reasonably conceptualized set of outcomes. These models should therefore not be considered simulations of true system function, but rather theoretical explorations of the modulation of system behavior that could arise from effects of various network interaction processes. In each case, simplifying assumptions and parameter values have been selected to highlight the key effects explored in that particular figure. We believe that these explorations raise interesting and potentially significant hypotheses that could motivate more in-depth and detailed modeling and quantitative experimental efforts in this system.
Figure 3.2

Reactions

\[ IL-7R\alpha + \gamma_\epsilon \xrightleftharpoons{k_f^1}{k_r^1} IL-7R\alpha\gamma_\epsilon \]
\[ IL-15R\beta + \gamma_\epsilon \xrightleftharpoons{k_f^2}{k_r^2} IL-15R\beta\gamma_\epsilon \]
\[ IL-7R\alpha\gamma_\epsilon + IL-7 \xrightleftharpoons{k_f^3}{k_r^3} IL-7R\alpha\gamma_\epsilon\gamma \]
\[ IL-15R\beta\gamma_\epsilon + IL-15 \xrightleftharpoons{k_f^4}{k_r^4} IL-15R\beta\gamma_\epsilon\gamma \]

Rate constants

\[ k_f^1 = k_f^2 = k_f^3 = 1 \text{ nM}^{-1} \text{ min}^{-1} \]
\[ k_f^4 = 0.1 \text{ nM}^{-1} \text{ min}^{-1} \]
\[ k_r^1 = k_r^2 = k_r^3 = k_r^4 = 0.1 \text{ min}^{-1} \]

Initial Conditions

\[ IL-7R\alpha_{t=0} = IL-15R\beta_{t=0} = \gamma_{t=0} = 1000/\text{cell} \]
\[ IL-7=1\text{nM} \quad IL-15=0.1-1000\text{nM} \]
\[ Y=100000 \text{ cell/L} \]
Figure 3.3

Reactions

\[ IL-7 + IL-7R \cdot Jak1 \xrightleftharpoons{kr1}{kf1} IL-7 \cdot IL-7R \cdot Jak1 \]
\[ IL-15 + IL-15R \cdot Jak1 \xrightleftharpoons{kr2}{kf2} IL-15 \cdot IL-15R \cdot Jak1 \]
\[ IL-7 \cdot IL-7R \cdot Jak1 \xrightarrow{k3} IL-7 \cdot IL-7R \cdot pJak1 \]
\[ IL-15 \cdot IL-15R \cdot Jak1 \xrightarrow{k4} IL-15 \cdot IL-15R \cdot pJak1 \]
\[ IL-7 \cdot IL-7R \cdot pJak1 + Stat5 \xrightarrow{kr5}{kf5} IL-7 \cdot IL-7R \cdot pJak1 \cdot Stat5 \xrightarrow{k5} IL-7 \cdot IL-7R \cdot pJak1 + pStat5 \]
\[ IL-15 \cdot IL-15R \cdot pJak1 + Stat5 \xrightarrow{kr6}{kf6} IL-15 \cdot IL-15R \cdot pJak1 \cdot Stat5 \xrightarrow{k6} IL-15 \cdot IL-15R \cdot pJak1 + pStat5 \]
\[ IL-7 \cdot IL-7R \cdot pJak1 \xrightarrow{k7} IL-7 \cdot IL-7R \cdot Jak1 \]
\[ IL-15 \cdot IL-15R \cdot pJak1 \xrightarrow{k8} IL-15 \cdot IL-15R \cdot Jak1 \]
\[ pStat5 \xrightarrow{k9} Stat5 \]

Rate constants

\[ kf1 = kf3 = kf4 = kf5 = 1 \text{ nM}^{-1} \text{ min}^{-1} \]
\[ kf2 = 0.1 \text{ nM}^{-1} \text{ min}^{-1} \]
\[ kr1 = kr2 = kr3 = kr4 = kr5 = 0.1 \text{ min}^{-1} \]
\[ k3 = k4 = k5 = k6 = k7 = k8 = k9 = 0.1 \text{ min}^{-1} \]

Initial Conditions

\[ IL-7R_{i=0} = IL-15_{i=0} = 1000/\text{cell} \]
\[ IL-7 = IL-15 = 1 \text{nM} \]
\[ Stat5 = 1500/\text{cell} \]
\[ Y = 100000 \text{ cell/L} \quad V = 10^{-12} \text{ L/cell} \]
Figure 3.4

Reactions

\[
\begin{align*}
\text{IL-7} + \text{IL-7R} \cdot \text{Jak} & \rightarrow \text{IL-7} \cdot \text{IL-7R} \cdot \text{Jak} \\
\text{IL-7} \cdot \text{IL-7R} \cdot \text{Jak} & \rightarrow \text{IL-7} \cdot \text{IL-7R} \cdot \text{pJak} \\
\text{IL-7} \cdot \text{IL-7R} \cdot \text{pJak} + \text{Stat5} & \rightarrow \text{IL-7} \cdot \text{IL-7R} \cdot \text{pJak} \cdot \text{Stat5} \\
\text{IL-7} \cdot \text{IL-7R} \cdot \text{pJak} \cdot \text{Stat5} & \rightarrow \text{IL-7} \cdot \text{IL-7R} \cdot \text{pJak} + \text{pStat5} \\
\text{IL-7} \cdot \text{IL-7R} \cdot \text{pJak} \cdot \text{Stat3} & \rightarrow \text{IL-7} \cdot \text{IL-7R} \cdot \text{pJak} \cdot \text{Stat3} \\
\text{IL-7} \cdot \text{IL-7R} \cdot \text{pJak} & \rightarrow \text{IL-7} \cdot \text{IL-7R} \cdot \text{Jak} \\
p\text{Stat5} & \rightarrow \text{Stat5} \\
p\text{Stat3} & \rightarrow \text{Stat3}
\end{align*}
\]

Rate constants

\[
\begin{align*}
k_f1 = k_f3 &= 1 \text{ nM}^{-1}\text{min}^{-1} \\
k_f4 &= 0.1 \text{ nM}^{-1}\text{min}^{-1} \\
k_r1 = k_r3 = k_r4 &= 0.1 \text{ min}^{-1} \\
k_2 = k_3 = k_4 &= k_5 = k_6 = k_7 = 0.1 \text{ min}^{-1}
\end{align*}
\]

Initial Conditions

\[
\begin{align*}
\text{IL-7R}_{t_0} &= 1000/\text{cell} \\
\text{IL-7R} \cdot \text{pJak}_{t_0} &= 1000/\text{cell} \\
\text{Stat3}_{t_0} = \text{Stat5}_{t_0} &= 1500/\text{cell} \\
Y &= 100000 \text{ cell/L} \\
V &= 10^{-12} \text{ L/cell}
\end{align*}
\]
Figure 3.5

Reactions

\[ \text{IL-7} + \text{IL-7R} \rightarrow \text{IL-7} \times \text{IL-7R} \rightarrow \text{IL-7} \times \text{IL-7R} + \text{pJak} \]

\[ \text{IL-7} \times \text{IL-7R} \rightarrow \text{IL-7} \times \text{IL-7R} + \text{pJak} \]

\[ \text{IL-7} \times \text{IL-7R} + \text{Stat5} \rightarrow \text{IL-7} \times \text{IL-7R} + \text{pJak} \times \text{Stat5} \rightarrow \text{IL-7} \times \text{IL-7R} + \text{pJak} \times \text{pStat5} \]

\[ \varnothing \rightarrow \text{Stat5} \]

\[ \text{IL-7} \times \text{IL-7R} + \text{Stat5} \rightarrow \text{IL-7} \times \text{IL-7R} \times \text{pJak} \times \text{Stat5} \]

\[ \text{IL-7} \times \text{IL-7R} \times \text{pJak} \rightarrow \text{IL-7} \times \text{IL-7R} + \text{pJak} \]

\[ \text{pStat5} \rightarrow \text{Stat5} \]

Rate constants

\[ kf1 = kf2 = kf3 = 1 \text{ nM}^{-1} \text{min}^{-1} \]

\[ kr1 = kr3 = 0.1 \text{ min}^{-1} \]

\[ k5 = 0 \text{ min}^{-1} \text{ or } 1 \text{ min}^{-1} \]

\[ k2 = k4 = k6 = k7 = 0.1 \text{ min}^{-1} \]

\[ k3 = 1 \text{ min}^{-1} \]

Initial Conditions

\[ \text{IL-7}_{t=0} = 1 \text{nM} \]

\[ \text{IL-7R} \times \text{pJak}_{t=0} = 1000 / \text{cell} \]

\[ \text{Stat5}_{t=0} = 1500 / \text{cell} \]

\[ Y = 100000 / \text{cell} \]

\[ V = 10^{12} \text{ L/cell} \]
Figure 3.6

Reactions

\[
\text{[IL-7 signal]} = \frac{\text{[IL-7 input]}}{1 + \alpha \text{[TCR signal]}}
\]

\[
\text{[TCR signal]} = \text{[TCR input]}(1 + \text{[IL-7 signal]})
\]

Rate constants

\[\alpha_{\text{tuning}} = 1 \quad \alpha_{\text{no tuning}} = 0\]

Initial Conditions

\[\text{[IL-7 input]} = 3\]

\[\text{[TCR input]} = 1 - 6\]
Figure 3.7

Reactions

IL-7+IL-7R•Jak1 $\overset{k_f 1}{\underset{k_r 1}{\rightleftharpoons}}$ IL-7•IL-7R•Jak1

IL-7•IL-7R•Jak1 $\overset{k_2}{\rightarrow}$ IL-7•IL-7R•pJak1

IL-7•IL-7R•pJak1+Stat5 $\overset{k_f 3}{\underset{k_r 3}{\rightleftharpoons}}$ IL-7•IL-7R•pJak1•Stat5 $\overset{k_3}{\rightarrow}$ IL-7•IL-7R•pJak1 + pStat5

pStat5+pStat5 $\overset{k_f 4}{\underset{k_r 4}{\rightleftharpoons}}$ pStat5$_{\text{Dimer}}$

pStat5$_{\text{Dimer}}$ + pStat5$_{\text{Dimer}}$ $\overset{k_f 5}{\underset{k_r 5}{\rightleftharpoons}}$ pStat5$_{\text{Tetramer}}$

IL-7•IL-7R•pJak1 $\overset{k_6}{\rightarrow}$ IL-7•IL-7R

pStat5 $\overset{k_f 7}{\rightarrow}$ Stat5

Rate constants

$k_f 1 = k_f 3 = k_f 4 = k_f 5 = 1 \text{nM}^{-1}\text{min}^{-1}$

$k_r 1 = k_r 2 = k_r 4 = k_r 5 = 0.1 \text{min}^{-1}$

$k_2 = k_3 = k_4 = k_6 = k_7 = 1 \text{min}^{-1}$

Initial Conditions

IL-7$_{\text{eq}}$ = 1nM

IL-7R•pJak1$_{\text{eq}}$ = 1000/cell

Stat5$_{\text{eq}}$ = 10000/cell

Y = 100000 cell/L \quad V = 10^{-12} \text{L/cell}
Figure 3.8

Reactions

\[
\begin{align*}
\text{IL-7+IL-7R} & \xrightarrow{k_f} \text{IL-7+IL-7R} \\
\text{IL-7+IL-7R} & \xrightarrow{k_c\text{endo}} \emptyset \\
\text{IL-7R} & \xrightarrow{k_r\text{endo}} \emptyset \\
\text{IL-7+IL-7R} & \xrightarrow{k_{\text{sig}}} \text{Signal} \\
\emptyset & \xrightarrow{k_{\text{synth}}} \text{IL-7R} \\
\emptyset & \xrightarrow{k_{\text{IL-7\_prod}}} \text{IL-7} \\
\emptyset & \xrightarrow{k_{\text{Cell\_prod}}} \text{Cells\_naive} \\
\emptyset & \xrightarrow{k_{\text{Cell\_prod}}} \text{Cells\_activated} \\
\text{Signal} & \xrightarrow{k_{\text{sig\_deg}}} \emptyset \\
\text{Cells\_naive} & \xrightarrow{k_{\text{cell\_death}}} \emptyset \\
\text{Cells\_activated} & \xrightarrow{k_{\text{cell\_death\_activated}}} \emptyset
\end{align*}
\]

Rate constants

\[
\begin{align*}
k_f &= 1.3 \text{ nM}^{-1} \text{ min}^{-1} \\
k_r &= 0.1 \text{ min}^{-1} \\
k_c\text{endo} &= 0.1 \text{ min}^{-1} \\
k_r\text{endo} &= 0.01 \text{ min}^{-1} \\
k_{\text{sig}} &= 1 \\
k_{\text{synth\_naive, non-altruistic activated}} &= 10 \text{ min}^{-1} \\
k_{\text{synth\_altruistic activated}} &= 1 \text{ min}^{-1} \\
k_{\text{IL-7\_prod}} &= 0.005 \text{ nM min}^{-1} \\
k_{\text{Cell\_prod}} &= 10^8 \text{ L}^{-1} \text{ min}^{-1} \\
k_{\text{expand}} &= 0.0015 \text{ min}^{-1} \\
k_{\text{sig\_deg}} &= 0.003 \text{ min}^{-1} \\
k_{\text{death}} &= 0.002 \text{ min}^{-1} \\
k_{\text{death\_activated}} &= 0.000004 \text{ in}^{-1} \\
k_{\text{in\_death}} &= 0.001
\end{align*}
\]
References


