microRNA Regulation of Cellular Immunity

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of Philosophy
in the Department of Immunology in the Graduate School of Duke University

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ABSTRACT

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Abstract

Immunity is broadly defined as a mechanism of protection against non-self entities, a process which must be sufficiently robust to both eliminate the initial foreign body and then be maintained over the life of the host. Life-long immunity is impossible without the development of immunological memory, of which a central component is the cellular immune system, or T cells. Cellular immunity hinges upon a naïve T cell pool of sufficient size and breadth to enable Darwinian selection of clones responsive to foreign antigens during an initial encounter. Further, the generation and maintenance of memory T cells is required for rapid clearance responses against repeated insult, and so this small memory pool must be actively maintained by pro-survival cytokine signals over the life of the host.

T cell development, function, and maintenance are regulated on a number of molecular levels through complex regulatory networks. Recently, small non-coding RNAs, miRNAs, have been observed to have profound impacts on diverse aspects of T cell biology by impeding the translation of RNA transcripts to protein. While many miRNAs have been described that alter T cell development or functional differentiation, little is known regarding the role that miRNAs have in T cell maintenance in the periphery at homeostasis.

In Chapter 3 of this dissertation, tools to study miRNA biology and function were developed. First, to understand the effect that miRNA overexpression had on T cell

responses, a novel overexpression system was developed to enhance the processing efficiency and ultimate expression of a given miRNA by placing it within an alternative miRNA backbone. Next, a conditional knockout mouse system was devised to specifically delete miR-191 in a cell population expressing recombinase. This strategy was expanded to permit the selective deletion of single miRNAs from within a cluster to discern the effects of specific miRNAs that were previously inaccessible in isolation. Last, to enable the identification of potentially therapeutically viable miRNA function and/or expression modulators, a high-throughput flow cytometry-based screening system utilizing miRNA activity reporters was tested and validated. Thus, several novel and useful tools were developed to assist in the studies described in Chapter 4 and in future miRNA studies.

In Chapter 4 of this dissertation, the role of miR-191 in T cell biology was evaluated. Using tools developed in Chapter 3, miR-191 was observed to be critical for T cell survival following activation-induced cell death, while proliferation was unaffected by alterations in miR-191 expression. Loss of miR-191 led to significant decreases in the numbers of $CD4^+$ and $CD8^+$ T cells in the periphery lymph nodes, but this loss had no impact on the homeostatic activation of either $CD4^+$ or $CD8^+$ cells. These peripheral changes were not caused by gross defects in thymic development, but rather impaired STAT5 phosphorylation downstream of pro-survival cytokine signals. miR-191 does not specifically inhibit STAT5, but rather directly targets the scaffolding protein, IRS1, which

in turn alters cytokine-dependent signaling. The defect in peripheral T cell maintenance was exacerbated by the presence of a Bcl-2^{YFP} transgene, which led to even greater peripheral T cell losses in addition to developmental defects. These studies collectively demonstrate that miR-191 controls peripheral T cell maintenance by modulating homeostatic cytokine signaling through the regulation of IRS1 expression and downstream STAT5 phosphorylation.

The studies described in this dissertation collectively demonstrate that miR-191 has a profound role in the maintenance of T cells at homeostasis in the periphery. Importantly, the manipulation of miR-191 altered immune homeostasis without leading to severe immunodeficiency or autoimmunity. As much data exists on the causative agents disrupting active immune responses and the formation of immunological memory, the basic processes underlying the continued maintenance of a functioning immune system must be fully characterized to facilitate the development of methods for promoting healthy immune function throughout the life of the individual. These findings also have powerful implications for the ability of patients with modest perturbations in T cell homeostasis to effectively fight disease and respond to vaccination and may provide valuable targets for therapeutic intervention.

Dedication

I dedicate my dissertation to my family. To my grandparents, whose passion for discussion, hard-work, and integrity have been an inspiration throughout my life. To my parents, who afforded me the opportunity to see the world and pursue my intellectual curiosity and have been unwavering sources of support and wisdom. To my siblings, whose own great accomplishments and respect have spurred me ever onward and upward. And to my wife, Jacquie, whose intelligence and focus humble me and whose optimism and support give me the strength and desire to be my very best. Without you, this work would not have been possible. The fact that I have all of you in my life makes Lykken, which derives from the Norwegian word for good fortune, a truly appropriate surname.

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1. Introduction

1.1 The Immune System

Immunity, broadly defined as a mechanism of protection against a non-self entity, is so critical to the survival of an organism that it can be found in essentially every living organism, from archaea, bacteria, and plants through higher order species. In mammals, the immune system comprises a smorgasbord of cell types and specialized tissue niches that chiefly serve to protect the host from pathogens and malignancies. Immune cells are able to traverse and take up residence throughout the tissues of the host, actively surveying for foreign interlopers and cellular anomalies. Upon recognition of a non-self entity, immune cells are tasked with its elimination, but the clearance mechanisms require tight regulation to prevent aberrant tissue destruction.

The power of the immune system's recognition of non-self and subsequent elimination is apparent in the rejection of transplanted tissues, which is why many of the earliest defining discoveries in immunology derive from studies of tissue transplant. Vertebrates express a series of histocompatibility markers, which can be proteins, carbohydrates, or lipids, on the surface of their cells. With the exception of blood type antigens, the most important of these markers in driving transplant rejection is the expression of major histocompatibility complex I (MHC-I). While many minor histocompatibility markers that drive transplant rejection also exist, MHC-I protein is expressed on the surface of every nucleated cell and is constantly surveyed by immune

cells. MHC-I binds and displays peptide fragments generated during specialized protein degradation within the cell and therefore serves as a window for intracellular processes. If the immune system recognizes an unusual peptide displayed on MHC-I, it indicates that something is wrong within the cell, and the immune system will move to eliminate the threat.

To prevent immune cells from attacking healthy host cells, a process referred to as autoimmunity, adaptive immune cells that recognize and strongly react to normal self-derived peptides in the context of MHC-I ideally are eliminated during development. However, such selection is only able to occur against the version of MHC-I expressed by the host, and MHC-I is a codominantly expressed polygenic protein deriving from three main genes with a variety of polymorphisms. The codominant expression of these polymorphisms results in an exponential diversity for the potential MHC-I molecules expressed in the human population, with each human expressing MHC-I molecules distinct from the vast majority of the human population. Thus, without considering the similarity in MHC-I molecules expressed between donor and recipient, a recipient's immune system sees self-peptides displayed in the context of donor MHC-I as foreign and acts to destroy the transplant. Such responses form the basis of histocompatibility.

While desirable in modern medicine, transplants are not a naturally occurring phenomena and highlight the need to fully understand how the immune system is

regulated so that we might manipulate it to achieve more desirable outcomes. One major facet of immune regulation is that the different types of immune cells work in concert to orchestrate a protective, but controlled response. Broadly, one can classify immune cells as belonging to one of two distinct, yet interactive arms: cells of the innate immune system, which provide a rapid, general response, and cells of the adaptive immune system, which provide a specific response and are capable of immunologic memory. Innate immune cells predominantly operate at the onset of an immune response as a first line of defense. They rapidly respond by recognizing conserved molecular patterns within various components of a pathogen and proceed to secrete soluble signaling proteins known as cytokines to promote an appropriately inflammatory environment for the immune response. Once the threat is eliminated, innate immune cells will produce anti-inflammatory cytokines to temper the response and prevent unnecessary collateral damage. Meanwhile, adaptive immune cells, though slower in initial responses, can recognize a much wider diversity of molecules than innate immune cells and are able to develop "memory" for those molecules to which there has been a previous exposure, allowing for more rapid subsequent responses. Adaptive immune cells can also produce cytokines to promote or temper the response as needed and can kill infected cells directly by secreting either cytotoxic effector molecules or antibodies, which are proteins specific for a wide diversity of protein epitopes. Antibodies coat pathogens and thereby both prevent infection and also signal innate immune cells to phagocytose (i.e. engulf)

the pathogen. Thus, the innate and adaptive arms of the immune system interact and support each other to promote rapid, regulated immunity.

1.1.1 The Innate Immune System

Recent work examining the innate immune system suggests a division of innate immune cells into two functional classes: effectors and "scouts". Innate effectors include innate lymphoid cells (ILCs), mast cells, basophils, eosinophils, and neutrophils. These cells primarily function to promote inflammatory responses, either by creating a cytokine milieu tailored to eliminate a particular class of pathogen or, in the case of natural killer cells and neutrophils, directly killing infected cells or pathogens, respectively. The rapid effector response is critical to the health of the host, as it provides protection even in the absence of adaptive immunity. However, a lack of adaptive immune responses leaves an individual vulnerable to recurrent infection and rarely permits long-term viability. Innate scouts, however, function more to discern the nature of the pathogen and raise the appropriate alarm to attract adaptive and other innate immune cells ideally suited to handle the pathogen. Monocytes, macrophages, and dendritic cells (DCs) are members of the scout class. While these cell types share some functions, they also have particular strengths: macrophages are exceptional consumers and are able to directly eliminate pathogens or dying cells through phagocytosis, whereas dendritic cells, while also able to consume microorganisms, are essential for proper T cell activation. The different members of the scout class can be further divided

into many subpopulations with varying specialties; ultimately, the function of the scouts is to survey throughout the body, usually through phagocytosis or pinocytosis, for pathogens or cellular abnormalities and then instigate inflammatory responses as necessary. As part of initiating an immune response, scout innate immune cells will produce pro-inflammatory cytokines, but perhaps more critically, will also directly interact with adaptive immune cells to communicate the nature of a threat and drive the appropriate effector response. Crucial to the function of scout innate immune cells, then, is to discern the nature of a pathogen. This process relies on the recognition of conserved molecular patterns within various components of the pathogen and is heavily conserved; indeed, this process was first described in flies. A variety of surface and intracellular receptors, broadly known as pattern recognition receptors (PRRs), such as toll-like receptors (TLRs), mediate the recognition event, with each receptor specialized towards a particular pattern (e.g. lipopolysaccharide (LPS), double-stranded RNA, and unmethylated CpG oligodeoxynucleotide (CpG)). The nature of the conserved molecular patterns and the subcellular localization of the associated PRR helps the scout innate immune cell interpret the infection. For example, double-stranded RNA is found in RNA viruses and associates with an intracellular TLR, thereby prompting the release of anti-viral cytokines and driving an adaptive immune response focused on the killing of infected cells and neutralization of free virus. Meanwhile, LPS is an outer-membrane component of gram-negative bacteria and associates with an extracellular TLR,

prompting a more innate-like immune response focused on engulfment and neutralization of the bacteria. There are also instances where scouts are activated in response to sterile injury or the developmental need for tissue remodeling; these responses are then designed to minimize the recruitment of other, more pathogen-focused immune cells. Pattern recognition is therefore a powerful tool that enables scout innate immune cells to tailor immune responses and communicate the broad nature of a threat, a process akin to communicating various types of hazard with different colored flares. However, scout innate immune cells have another, more specific threat communication mechanism: MHC-I and MHC-II.

While all nucleated cells are able to present peptides on MHC-I, only scout innate immune cells are able to license adaptive immune cells to respond to a particular peptide:MHC-I complex wherever they may next encounter it, making the proper activation and function of scouts critically important to the regulation of adaptive immunity. Further, scouts are able to present peptides via MHC-II, which is only expressed by a small subset of immune cell types. Unlike MHC-I, which presents peptides predominantly derived from intracellular processes and infections, MHC-II presents peptides predominantly derived from extracellular sources (i.e. objects picked up through phagocytosis or pinocytosis), thus enabling a scout innate immune cell to also prepare adaptive immunity to fight extracellular pathogens. When coupled with threat recognition through a PRR, presentation of peptides on MHC-I and MHC-II by

scout innate immune cells communicates the presence and broad nature of a threat, but also provides adaptive immune cells with a highly specific "threat signature" so that a productive initial response can lead to specialized, protective memory responses should the host encounter the pathogen again.

1.1.2 The Adaptive Immune System

Immunity is impossible without the adaptive immune response and its provision of immunological memory. Adaptive immune responses are executed by lymphocytes and are classically divided into two distinct but highly interactive arms: humoral, or antibody-dependent, responses driven by B cells, and cellular responses driven by T cells. T cells are further divided into $CD4^+$ T-helper (T_H) cells, which recognize peptide:MHC-II complexes and largely function to secrete cytokines to tailor the immune response to a specific pathogen, and $CD8^+$ cytotoxic T cells, which recognize peptide:MHC-I complexes and act to find and kill infected cells. Both B and T cells express eponymous cell surface receptors: the B cell receptor (BCR) and T cell receptor (TCR), respectively. Each cell expresses around 100,000 copies(*1*) of a single receptor, which are selected during development and derive from semi-random rearrangement of the respective receptor loci in a process requiring recombination activating gene (RAG) expression, followed by site-specific mutation, specific chain pairing, and functional selection. Lymphocytes with receptors that recognize self are eliminated through negative selection or are otherwise suppressed once past development through one of

several mechanisms that constitute peripheral tolerance. This process then yields a pool of naïve lymphocytes, each expressing a single receptor, capable of recognizing an exponential diversity of non-self entities. Post-development, further modification of BCRs can occur through both receptor editing, which involves further genetic editing, and affinity maturation, which involves hypermutation and T cell-driven selection in the context of an ongoing immune response.

B cells are best known as the origin of the humoral response, which refers to their ability to produce antibody, the secreted form of the antigen recognition subunit of the BCR. Antibodies play a number of critical roles in the adaptive immune response. Antibodies can function as a natural barrier to infection through direct neutralization of a pathogen (i.e. bind to pathogens to prevent pathogen attachment and/or entry into a cell), and can bind to and "highlight" a pathogen as a phagocytic target for innate immune cells; both processes generally permit the clearance of unwanted organisms without eliciting significant inflammation. Because antibodies often elicit minimal inflammation, weakly self-reactive antibodies help to clear cellular debris resulting from normal cell turnover at homeostasis (i.e. in the absence of an ongoing immune response). Antibodies are also able to elicit strong immune responses. For example, mast cells expressing surface receptors for circulating antibody can be activated following antibody recognition of a specific antigen, which in turn causes the mast cell to release granules filled with inflammatory mediators; this response forms the basis of many

allergic responses. Similarly, antibodies drive complement-mediated lysis of bacteria or cells. Thus, antibody production provides another example highlighting how multiple immune cells work together to generate an appropriate and well-regulated response.

Following development in the bone marrow, immature B cells migrate to peripheral lymphoid tissues (e.g. the spleen and lymph nodes). These naïve immature B cells are capable of activating and producing antibodies in response to BCR recognition of its cognate antigen. However, to ensure that B cells do not respond inappropriately, there is a signaling threshold to permit activation. Generally this threshold can only be crossed when the B cell receives "help" from T_H cells: B cells present peptide:MHC-II complexes derived from the antigen captured on the BCR, and those T_H cells with a TCR recognizing the same cognate antigen provide cytokine and other co-stimulatory signals to the B cells. While there are certainly cases where B cells do not require T cell help, such as when there is co-ligation of other receptors, including TLRs, or the antigen induces multivalent BCR cross-linking, many aspects of adaptive immunity are predicated on the productive interactions between T and B cells. Indeed, this interaction does not end after the initial activation stage. Activated B cells will either differentiate into short-lived plasma cells, which secrete a significant amount of antibody into the periphery for the duration of an ongoing immune response, or will proliferate in lymphoid follicles and then migrate into the germinal center (GC) with follicular dendritic cells and $CD4^+$ T follicular helper cells (T_{FH}, a differentiated subset of T_H cells).

Within the GC, B cells cycle between stages of proliferation and antigen engagement. During antigen engagement, B cells tear antigen off the surface of follicular dendritic cells and present peptide:MHC-II complexes to the T_{FH} cells. T_{FH} cells then provide B cells with co-stimulatory signals and cytokines that induce antibody isotype switching and affinity maturation. Isotype switching is a genetic rearrangement of the BCR that leads to antibodies with specialized properties for different types of infections. Affinity maturation is also a genetic process in which the binding region of the BCR is mutated to yield B cells with BCRs expressing a range of affinities for the same cognate antigen. This process, which is a beautiful example of Darwinian evolution observable over days, leads to the selection of higher affinity B cell clones that then form the B cell memory compartment. Memory B cells may go on to differentiate into long-lived plasma cells, which secrete isotype-switched antibody, and also respond more readily upon subsequent exposure to the same pathogen.

While T cells critically facilitate aspects of B cell immunity, B cells broadly facilitate cellular immune responses as key regulators of lymphoid tissue organogenesis and architecture. Indeed, mice congenitally deficient in B cells have impaired follicular dendritic cells and secondary lymphoid tissue development(*2*, *3*) as well as reduced T cell numbers in the spleen(*4*). B cells also provide critical immunomodulatory cytokines that promote T cell activation and polarization(*5*). A recently defined subset of B cells that secrete IL-10 (B10 cells) to restrain immune responses, playing key roles in

regulating antibody-dependent tumor depletion(*6*), bacterial clearance(*7*), and autoimmune diseases(*8-10*). Importantly, B cells are also one of the critical antigen-presenting cell populations for T_H cells. Recent studies using B cell depletion in wild-type hosts, which avoids the complications that arise due to altered lymphoid tissue structure, have shown B cells are critical for T cell homeostasis(*11*) and activation, particularly T_H activation, and B cell depletion consequently leads to impaired anti-bacterial(*12*), anti-viral(*11*), anti-tumor(*13*), and memory responses(*12, 14*). Thus, in addition to their important role as antibody producers, B cells reciprocate the help received from T cells, weaving yet another thread through the complex tapestry of immune responses and regulation.

1.1.2.1 T Cell Development

Most cells in the hematopoietic lineage, including B lymphocytes, develop from hematopoietic stem cells (HSCs) in the bone marrow and further mature once in the periphery. T cells, however, develop in an entirely separate immunological tissue: the thymus. T cell development begins with the differentiation of an HSC into one of several distinct lymphoid progenitors(*15*), including lymphoid primed multipotent progenitors (LMPPs), though other progenitors under specific conditions have been reported to give rise to T cells(*16*). After migrating to the thymus, the progenitors immediately differentiate into early T cell progenitors (ETPs). ETPs are T cell progenitors that are in the double-negative one (DN1; named double-negative because the cells express neither

CD4 nor CD8 coreceptors) phase of T cell development. There are four stages within the DN developmental phase, a process that leads to the expression of a rearranged surface TCR, and each DN stage is associated with distinct cytokine environments and cellular interaction partners within the thymus. As the T cell progenitors progress through the DN1 and DN2 stages, they are progressively altering their transcriptional programs in response to signals from thymic epithelial cells (TECs). In addition to providing differentiation signals, the TECs are also providing critical pro-survival signals, namely Notch signaling and interleukin-7 (IL-7), both of which remain important pro-survival factors throughout the life of a T cell. At the end of DN2, the progenitors earnestly begin the process of becoming a functional T cell and transition into DN3, where they must successfully re-arrange and express a TCR.

From a genetic perspective, there two classes of TCR: the $\alpha\beta$ TCR, expressed by $CD4^+$ and $CD8^+$ T cells, and the $\gamma\delta$ TCR, expressed by $\gamma\delta$ T cells, which function mostly in the epithelium and mucosa-associated lymphoid tissues. β genes can rearrange simultaneously with γ and δ gene sets, though once a productive re-arrangement has occurred, which is determined by expression of and subsequent signaling feedback through the TCR, only an $\alpha\beta$ or $\gamma\delta$ TCR will be expressed. In preparation for signaling through either the $\gamma\delta$ TCR or the pre-TCR (re-arranged β chain with an invariant α chain), cells will activate the proximal promoter for lymphocyte-specific protein tyrosine kinase (Lck; a critical molecule for the intracellular events following extracellular TCR

engagement) at the later stages of DN2(*17*). Whether a developing T cell retains an αβ or γδ TCR is determined by TCR expression and the strength of signaling through the TCR(*18*). Indeed, transgenic γδ TCRs receiving strong or weak signals can move down the γδ or αβ developmental path, respectively(*19, 20*). Notably, γδ T cell development also requires IL-7 signaling to promote accessibility at the γ locus(*21-23*). Should the cell express a pre-TCR following the productive re-arrangement of the β chain, a process known as β selection, a developing αβ T cell will progress through DN4 into the intermediate single positive stage (ISP; $CD8^+$ in mice, $CD4^+$ in humans), then will rearrange the α chain and transition to the double-positive (DP; $CD4^+CD8^+$) stage, where positive selection occurs. DP cells are notable because they only rely on TCR signaling to provide survival cues, a fact that ensures that only T cells capable of recognizing antigens as displayed by host-MHC will ever make it to the periphery. A T cell that receives a positive selection signal must then make another fate decision: whether to exist as a $CD4^+$ or $CD8^+$ T cell. There has been much debate on how this decision is made, but recent work has brought much clarity through the development of the kinetic signaling model of CD4/CD8 lineage choice(*24*). The kinetic signaling model posits that, after receiving a positive selection signal, all T cells down-regulate expression of CD8, resulting in $CD4^+CD8^{low}$ cells(*25, 26*). If the positively selecting TCR signal persists, IL-7 signaling is blocked, and $CD4^+$ cells are born. If the TCR signal ceases, IL-7 signaling drives expression of the CD8 transcriptional program, and $CD8^+$ cells emerge. These

newly-minted single positive (SP) cells rely on the expression of key transcription factors to silence the molecular program of the alternative lineage for the lifespan of the T cell(*27-29*). Before entering the periphery, SP cells must undergo negative selection to reduce the potential for autoimmunity. This type of developmental regulation of autoimmunity is known as central tolerance. For T cells, central tolerance results from the expression of self-proteins on MHC, a process made more effective by the autoimmune regulator (AIRE) protein(*30*), which is a transcription factor that enables the broad expression of otherwise tissue-specific genes(*31*). Expression of AIRE in the thymus enables the negative selection of T cells that might recognize antigens present only in the heart or other peripheral tissues and is therefore indispensable for central tolerance. The mediators of peripheral tolerance, regulatory $CD4^+CD25^+$ T cells (T_{Regs}), also develop during negative selection in the thymus by reacting to self-peptide:MHC complexes. However, the master transcriptional regulator forkhead-box P3 (FOXP3), which drives T_{Reg} development, can be expressed as early as the $CD4^+CD8^{low}$ stage(*32*). T_H cells can also be induced to express FOXP3 in the periphery, generating so-called inducible-T_{Regs} (iT_{Regs}), in contrast to naturally-occurring T_{Regs} (nT_{Regs}), which develop in the thymus. Critically, all T_{Regs} rely on IL-2 signaling for their development and survival, and are therefore reliant on the expression of CD25 (IL-2Rα). The development and survival of T_{Regs} is critical for the prevention of autoimmunity as evidenced by the fact that mice with a mutated *Foxp3* locus rapidly develop lethal autoimmunity(*33*). Once

through the negative selection gauntlet, naïve $CD4^+$, $CD8^+$, and $CD4^+CD25^+$ are ready to leave the thymus and enter the peripheral tissues, where they can play a multitude of roles in the adaptive immune response.

1.1.2.2 The Cellular Immune Response

Once in the periphery, naïve T cells migrate into the peripheral lymphoid tissues and wait for the initiation of an immune response. Classically, a primary adaptive immune response is initiated by the innate immune system. Scout innate immune cells will collect non-self-antigens and, with the recognition of a threat through PRRs, will migrate to the peripheral lymphoid tissues and provide those antigens to follicular dendritic cells, which in turn activate B cells. The scout innate immune cells also secrete inflammatory cytokines and present peptide:MHC-II complexes and co-stimulatory signals to T_H cells, which then become activated once TCR and co-stimulatory signals (e.g. CD28 and ICOS) surpass the activation threshold. Given the cytokine milieu generated by the innate immune response, naïve T_H cells that recognize peptide:MHC-II will become activated and then proliferate and differentiate into effector cells capable of producing a cytokine cocktail best suited for the threat at hand. For example, a viral infection can drive innate immune cells to create a cytokine milieu rich in IL-12, a cytokine that encourages naïve T_H cells to differentiate to T_H-type 1 (T_H1) cells, which then produce interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α). As activated T_H1 cells are interacting with DCs through the TCR, they can also license the

DCs to activate a $CD8^+$ T cell response, provided the $CD8^+$ T cells are simultaneously interacting with the DC(*34*). These activated $CD8^+$ T cells can then go on to directly kill virally-infected cells, which are identified by the expression of viral antigen on MHC-I, through the directed secretion of large amounts of cytotoxic molecules (e.g. perforin and granzyme B) as well as cytokines (e.g. IFN- γ). Some of the activated T_H cells will interact with B cells and become T_{FH} cells, which go on to promote an anti-viral antibody response.

The destruction wrought by the inflammatory processes of the immune response makes it inevitable that self-peptides derived from damaged tissues will be presented with MHC molecules by activated antigen-presenting cells (APCs) , creating a permissive environment for the development of autoimmunity. Fortunately, T_{Regs} recognize the presentation of self-antigens and work to dampen local immune activation, often through the secretion of IL-10 and transforming growth factor-beta (TGF-β). Notably, anti-tumor responses directed against a tumor expressing a non-self-antigen feature many of the same components as an anti-viral response. However, the balance of pro- and anti-inflammatory mediators is often quite different owing to differences in immune accessibility, antigen persistence, and the largely self-nature of tumors. Thus, anti-tumor immunity is a response in which regulatory processes must be overcome in order to reach the desired therapeutic outcome.

Responses to extracellular pathogens (e.g. extracellular bacteria, helminths, or fungi) will be quite different, often yielding little productive immunity from $CD8^+$ T cells. Helminth infection typically induces a response in which T_H2 cells secrete the anti-helminth cytokine IL-13, which in turn induces innate cells like eosinophils to release inflammatory granules that promote helminth expulsion. Meanwhile, T_{FH} cells will produce IL-4 and IL-21(*35*) to generate an IgE antibody response, which in turn primes mast cells, eosinophils, and basophils for future helminth encounters. Fungal infections will induce T_H17 responses, resulting in the secretion of IL-17, which dramatically exacerbates specific inflammatory processes.

It is important to note that the TCR is also a critical determinant in effector T cell differentiation, as TCR signaling is not a binary system. Indeed, TCR dwell time, which is the aggregate half-life of TCR-peptide:MHC interaction(*36*, *37*), correlates with both proliferation and effector T cell differentiation(*37*, *38*). While it is tempting to interpret duration of TCR engagement and the number of TCR molecules engaged in a given signaling event as the only determinants of TCR signaling strength, and thus the determinants of the outcomes of TCR signaling (e.g. proliferation, survival, or differentiation), there is ample evidence for the tuning of TCR signaling strength(*39*), including developmentally determined regulation based on the avidity of the TCR for the selecting antigen(*40-42*). These observation highlight the importance of a diverse

naïve T cell repertoire where the integration of TCR and cytokine signals across multiple T cell clones is most likely to elicit an effective primary response.

Should the effector response be enough to eliminate the threat and permit survival of the host, a small minority of responding T cells will enter the memory T cell pool and lay in wait for future encounter with the pathogen. Upon subsequent exposure to the same pathogen, memory T cells are readily re-activated, as they no longer require significant assistance from other cells. These memory cells then rapidly clear the infection, often with significantly reduced inflammation. The signals that determine whether responding T cells enter the memory pool or undergo controlled cell death (apoptosis) is still an area of intense investigation. It is known, however, that both cytokine and TCR signaling play important roles in determining the selection of either fate. Even after clearance of the pathogen, antigen will persist for a finite period, and responding T cells exposed to high levels of IL-2 are primed for cell death should they have a secondary encounter with a peptide:MHC complex(*43*). The strength of TCR signaling downstream of the TCR will also play a role, with the available data suggesting a distinction between the best TCR for primary and memory responses(*42*). Alternatively, even in the absence of secondary antigen-encounter, T cells that do not receive sufficient pro-survival cytokine signals, including IL-2(*44*), will undergo apoptosis. Thus, T cell effector responses are diverse, and, following pathogen clearance,

yield a small memory pool that must be actively maintained by pro-survival cytokine signals over the life of the host.

1.1.2.3 T Cell Homeostasis

Cellular immunity hinges upon a naïve T cell pool of sufficient size and breadth to enable Darwinian selection of clones responsive to foreign antigens. Additionally, the generation and maintenance of memory T cells is required for rapid clearance responses against repeated insult. Age-accelerated loss of thymic output post-puberty reduces the T cell input necessary for peripheral protection and thus highlights the importance of maintaining pools of both naïve and memory T lymphocytes over the life of the host.

Much work has addressed the longevity of naïve and memory cell pools(*45, 46*) as well as the pro-survival signals that maintain resting T cells in the periphery. Naïve and memory T cells have different strategies and requirements for long-term survival. Naïve T cells, especially naïve $CD8^+$ T cells, are maintained *in vivo* with minimal proliferation and require tonic signaling through the TCR(*47*) as well as the pro-survival cytokine IL-7(*48*). T_{Regs} also require tonic TCR signaling, but shift in cytokine dependence toward IL-2(*49, 50*), although recent data suggests that resting iT_{Regs} require IL-7(*51*). Of special note, two recent lines of evidence suggest that pro-survival tonic TCR signals result from engagement with their specific cognate peptide:MHC: first, transgenic T cells with different TCR specificities do not compete with each other during homeostatic survival(*52, 53*); second, in transfers of transgenic T cells with a single TCR specificity,

transfer of large numbers of the transgenic T cells yielded short T cell lifespans, while the transfer of a smaller number yielded a relatively prolonged T cell lifespan(*54*). Together, these data also suggest that there is limited availability of a given peptide:MHC, and so only small naïve clonal frequencies are able to exist. Indeed, studies in mice suggest an average naïve clonal frequency of thirty T cells at homeostasis(*54-56*). Intriguingly, the requirement for tonic TCR signaling may thus ensure the sustained diversity of the naïve T cell pool.

In contrast to naïve cells, memory cells are more frequently found in cell cycle, and their maintenance depends much less on TCR signaling(*57*). Instead, memory cells rely primarily on cytokine signals; memory $CD4^+$ cells require IL-7(*58-60*), and memory $CD8^+$ cells require IL-7 and IL-15(*48, 61, 62*), although IL-15 is thought to be more important for proliferation than survival(*61, 63*). Given limited sources of pro-survival cytokines, naïve, memory, and regulatory T lymphocytes are constantly competing for survival. Thus, the efficiency of responses to these specific and limited pro-survival cytokines is essential for maintaining T lymphocytes at homeostasis.

On the surface of T lymphocytes, the pro-survival cytokines IL-7, IL-2, and IL-15 engage with receptors sharing a common subunit: the common gamma chain (γc, CD132). These three cytokines also share at least one common signaling pathway downstream of the receptor: activation via phosphorylation of janus kinases 1 and 3 (JAK1 and JAK3) and subsequent activation of signal transducer and activator of

transcription 5 (STAT5)(*64*). STAT5 promotes the expression of anti-apoptotic molecules from the B-cell lymphoma 2 (BCL-2) family(*65*). This leads to pro-survival signaling convergence on a shared mechanism, creating a recipe for systemic collapse of adaptive immunity following major perturbation of this pathway. Such systemic collapse is seen in severe combined immunodeficiency (SCID), which arises from the loss of key components required to develop and maintain lymphocytes (e.g. CD127, CD132, and JAK3)(*66*). However, small perturbations seem unlikely to collapse the system, instead merely forcing it towards a new equilibrium. Such perturbations are unlikely to present as striking clinical immunodeficiency, but may still have serious implications for immunity throughout life of the host and influence the outcome of immunotherapies. As such, investigation into the mechanisms by which the immune system fine-tunes the maintenance of resting T cells may provide new insights to diagnose minor immunodeficiencies and offer new approaches for therapies to promote immunocompetency throughout the life of the host.

1.2 microRNAs

1.2.1 Biogenesis and Function of microRNAs

microRNAs (miRNAs) are approximately 22-nucleotide small RNAs that function to suppress the translation of larger target messenger RNAs (mRNAs) by forming a stem loop structure with the mRNAs, similar to short-hairpin RNAs

(shRNAs). Although tempting to believe that mammalian miRNAs are transcribed by RNA polymerase III (Pol III) like several other classes of small RNA (e.g. the U6 spliceosomal RNA) and some viral miRNAs(*67*), miRNAs are actually predominantly transcribed by RNA polymerase II (Pol II)(*68*), a protein that is also responsible for the vast majority of mRNA transcription. It may come as little surprise then to know that miRNAs are often found within the introns of mRNA transcripts, although they do not necessarily share a promoter with the host gene(*69*), while exonic miRNAs also exist. miRNAs may also exist within polycistronic clusters where the miRNAs are all transcribed as a single unit, but are not necessarily subject to equivalent processing efficiency(*70*). Immediately post-transcription, miRNAs exist as stem-loop structures embedded in a much larger pri-miRNA, which rapidly undergoes several processing steps to yield mature miRNAs. First, while still within the nucleus, the pri-miRNA is cleaved to a pre-miRNA by the Microprocessor complex(*71-73*), which consists principally of Drosha and DGCR8. While the exact biochemical structure of the Microprocessor complex have proven elusive, it is clear that cleavage of the pri-miRNA requires recognition of the stem-loop structure and is aided by several sequence motifs (e.g. a UG 14 bases before the 5′ Drosha cleavage site and CNNC 17 bases after the 3′ Drosha cleavage site)(*74*). One special feature of Drosha cleavage is that it generates a 2 base overhang at the 3′ end of the pre-miRNA. The pre-miRNA is exported to the cytoplasm by exportin 5(*75-77*), and Dicer then binds the pre-miRNA using the 2 base

overhang at the 3′ end. In cases where the 5′ end of the pre-miRNA is thermodynamically unstable, Dicer will also bind the 5′ phosphorylated end. Acting largely as a molecular ruler, Dicer then cuts the pre-miRNA approximately 22 bases from either end(*78-80*). The accuracy of this cutting event is at least partially determined by the location of loops and bulges within the pre-miRNA(*81*). The miRNA now exists as two strands (the 5′ or 5p strand and the 3′ or 3p strand, with the 5′ and 3′ designations referring to the orientation in the pre-miRNA) in a small RNA duplex with overhangs on either end. At this stage, the miRNA is ready for loading into the Argonaute (AGO) protein-containing RNA-Induced Silencing Complex (RISC). Although the duplex is loaded into the pre-RISC, the mature RISC only permits one strand from the duplex to carry out the function of the miRNA, so one strand must be discarded. The functional strand of the miRNA is known as the guide strand, while the other strand is known as the passenger strand. For a given microRNA, with a few notable exceptions, there is near complete bias for the 5p or 3p strand to function as the guide. This bias can be explained biochemically due to the need to unwind the duplex. The strand with the weakest 5′ pairing is the easiest to begin unwinding from and is selected as the guide(*82*). In cases where no obvious bias exists in the strength of 5′ pairing, selection of the guide is essentially stochastic, although AGO proteins favor guides with a U as the first nucleotide(*83*).

With the guide stranded loaded onto AGO, the RISC can use the seed sequence (nucleotides 2-8) to find target mRNAs, typically through matches in the 3' untranslated region (UTR) of the mRNAs. The RISC will then suppress the translation of the mRNAs either through physical blockade of the translation machinery or through degradation of the mRNA(*84*). mRNA degradation is a multi-step process in which the mRNA is deadenylated(*85-87*), then decapped and degraded 5'-3'(*85, 88, 89*). Whether translational repression or mRNA degradation are the dominant mechanism through which a miRNA exerts its regulatory affect has been a subject of much interest as it determines whether high-throughput assays assessing mRNA levels are an appropriate readout of miRNA targeting; translational repression would leave the mRNA intact for detection but prevent protein formation, while mRNA degradation will eliminate the mRNA, thereby preventing its translation. Recent work suggests that mRNA levels are indeed a reasonable readout for miRNA effects, because mRNA degradation following deadenylation is the major mechanism through which mammalian microRNAs function(*90*). It should be noted that physical blockade of the translation machinery does occur, though it functions mostly as an early suppression mechanism. In other species, notably flies, mRNA degradation occurs through AGO-mediated slicing of the guide:target duplex. However, of the four AGO proteins in mice and humans, only AGO2 is capable of slicing(*91, 92*). While this slicing function is known to occur *in vivo*, as it is essential for development(*93*) and for siRNA function, there is some debate as to

the extent to which slicing occurs for mouse and human miRNA:mRNA duplexes(*94*). Furthermore, in humans, AGO1-4 show no obvious preferences for different miRNAs(*95-97*). Together, these data suggest that slicing may not be a dominant mechanism of miRNA-mediated mRNA degradation in mice and humans. If slicing is not a dominant mechanism for mammalian miRNAs, then the expression level of a miRNA relative to other miRNAs and to its target mRNA(s) is critical; mRNA deadenylation and degradation occur much more slowly than slicing, thus dramatically reducing the number of target mRNA copies a single copy of miRNA can degrade over its lifetime. Further complicating the issue is that RISC expression is likely a limiting factor, especially given that miRNAs are estimated to regulate the majority of protein coding genes. Taken together, it seems likely that a miRNA's relative abundance is a critical factor in whether or not it plays a meaningful regulatory role in the biology of a cell. Also important is that a single miRNA can target many mRNAs, and a single mRNA is likely targeted by multiple miRNAs, setting the stage for a complex regulatory network where the relative expression levels of mRNA targets and other miRNAs with mutual or opposing influences on a molecular pathway further refine the outcome of miRNA expression alterations(*98-101*). Thus, depending on the network's structure and the relative expression of factors within the network, miRNAs function on a spectrum from fine-tuning mRNA abundance to acting as a genetic switch(*102, 103*).

1.2.2 microRNAs and T Cell Function

miRNAs are expressed in every nucleated mammalian cell and are critical to cellular development and function. Indeed, germline deletion of Drosha or Dicer results in embryonic lethality in mice(*104, 105*). Cells of the immune system and T cells in particular are favored for miRNA studies due to their ease of access and culture, their importance for medical therapy, and the vast array of complicated differentiation and effector responses where miRNA regulation offers the promise of both basic discovery and therapeutic manipulation.

Recent studies have highlighted that miRNAs regulate diverse aspects of T cell biology. miR-181a modulates TCR signal strength, thereby influencing both positive and negative selection and controlling the peripheral activation threshold(*39*). miR-146a and miR-23a regulate the survival of effector $CD4^+$ T cells(*106, 107*). The miR-17-92 cluster as well as miR-155 and miR-146a regulate $CD4^+$ T cell differentiation to T_H1, T_H2, T_H17, T_{FH}, and T_{Reg} subsets(*108-112*). miR-146a and miR-23a directly impact function of $CD4^+$ and $CD8^+$ T cells, respectively(*113, 114*). However, only a handful of studies have investigated how miRNAs might regulate immune homeostasis. These studies have reported either dramatic alterations in lymphocyte development that then lead to peripheral defects(*115*) or involve the use of global knockouts and see multiple confounding disruptions(*116-118*). Thus, these studies highlight a need for further

investigation into what miRNAs influence immune homeostasis and the mechanisms of such control.

2. Materials and Methods

2.1 Study Design

These studies are comprised of controlled laboratory experiments using mice and publicly available mouse and human data. All data, including outliers, were included in the presented experiments. Unless otherwise noted, all experiments were performed at least twice with multiple samples to ensure reproducibility.

2.2 RNA secondary structure prediction and design

All RNA secondary structure prediction was performed with RNAFold webserver which uses the ViennaRNA package(*119*). No input options were changed and centroid output showing base pair probabilities was selected. All figures were redrawn using Inkscape (v0.91) to save space and great care was taken to match the colors representing the base-pair probabilities.

All designs for novel RNAs were checked using RNAFold to ensure that structure was identical to the original sequences.

2.3 miRNA Reporter design and Drug Screening

miRNA reporters were designed with a 2X repeat of the complementary sequence to the mature guide placed immediately downstream of ZsGreen1. NIH-3T3 cells (ATCC, Manassas, VA) grown in complete DMEM media (10% NCS, 100 U/ml

penicillin, 100 U/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids) were transduced with miRNA reporter and overexpression vectors using Lipofectamine 2000 (Thermo-Fisher Scientific). After 24 hours cultures were then supplemented with Blasticidin (10 μg/mL) and Puromycin (1 μg/mL) and selection continued for 2 weeks, where cells were split as necessary.

For drug screening NIH-3T3 cells were plated overnight in 96-well F-bottom plates coated with 1% gelatin type B (Sigma-Aldrich, St. Louis, MO). After adherence, cells were treated with 10 μM compound in DMSO in complete DMEM (DMSO reached 5%). After 24 hours cells were screened using a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA) with the plate reader attachment. LIVE/DEAD Fixable Dead Cell Stain Kit (Thermo-Fisher Scientific, Waltham, MA) was used to exclude dead cells. Data were analyzed using FlowJo v8.2 (FLOWJO LLC, Ashland, OR).

2.4 Mice

Wild-type mice C57BL/6 mice expressing CD90.1 and CD45.1 (B6.PL-Thy1a/CyJ crossed with B6.SJL-Ptprca Pepcb/BoyJ) were from The Jackson Laboratory (Bar Harbor, ME).

The floxed miR-191 (miR-191$^{f/f}$) construct was generated by PCR amplification of a 9.15 kb long arm, 900 bp short arm, and the pre-miR-191 sequence with at least 100 bp flanking sequence from BAC Clone RP23-225E15 (CHORI BACPAC, Oakland, CA)

using the high-fidelity Velocity polymerase (Bioline, Taunton, MA) and the following primers from IDT (Coralville, IA): 5′-AGT TCC GCG GCG GCT TAA TTA AGG TTA TTG GCC TTT GCT GCT-3′/5′-ATG ACC TGT AGA CTG GGA TAC TTC TC CTT GAT GT-3′ (5′ half of long arm), 5′-CCT GTT CTA GGA CTC TTC CCA GGG AAA CTC-3′/5′-ACA AGC GGC CGC AAA CCA GTC TAG TGA GAA TGA GAC G-3′ (3′ half of long arm), 5′-TCA AGT CGA CGG TGG CAG TCA GAG GCG ACG AAA AAA G-3′/5′-AGT TGA TAT CCT GCC TCT GCC TCC TGA GTA-3′ (short arm), and 5′-AAT ACC CGG GAA AAC ACC TAC TCC TTC CTA CTC AGC CCA-3′/5′-GTT ACA ATT GAT CAG TCT GGG GGT GCA CCT GAG AGA TGG-3′. Fragments were cloned into a targeting vector using the following cut sites: SacII –BclI and BclI-NotI for the 5′ and 3′ long arm sides, respectively; SalI-EcoRV for the short arm; and XmaI-MfeI for miR-191. The targeting vector was linearized with PacI, and the targeting fragment was purified following agarose gel electrophoresis and then introduced into C57BL/6N-PRX-B6N #1 embryonic stem cells (ESCs) (The Jackson Laboratory). Positive ESC clones were detected by PCR using the following primers: 5′-CGC CTT CTT GAC GAG TTC TTC TG-3′/5′-TTT CCT GTA TTC ATG AGC CCT AAC C-3′. Chimeric mice were generated by microinjecting appropriately targeted ESC clones into C57BL/6 blastocysts at the Duke Transgenic Mouse Shared Resource (Duke University, Durham, NC). A single clone produced viable pups, and correct integration of the targeting construct was confirmed by PCR amplifying a 10.98 kb fragment containing floxed miR-191 and

extending into the genome using the Ranger Polymerase (Bioline) and the following primers: 5′-GAG TGA TAA TGT CTG GTG TGC CCT G-3′/5′-TTG TGT AGC GCC AAG TGC CC-3′. Mice containing floxed miR-191 were crossed to Flpe [C57BL/6-Tg(CAG-Flpe)2Arte] mice from Taconic Farms (Hudson, NY) to eliminate the PGKneo cassette and were then backcrossed to wild-type mice to eliminate the Flpe transgene and produce miR-191$^{f/f}$ mice. miR-191$^{f/f}$ mice were crossed to Lck–Cre [B6.Cg-Tg(lck–cre)1Cwi N9] mice from Taconic Farms to delete miR-191 specifically in T cells.

Bcl-2^{YFP} animals(*120*) were a gift from Dr. You-Wen He (Duke University Medical Center) and were crossed to miR-191$^{f/f}$ and miR-191$^{f/f}$LckCre^{+} animals.

All mice were housed in specific-pathogen-free conditions. All studies were approved by the Institutional Animal Care and Use Committee at Duke University.

2.5 Cell Preparation and Immunofluorescence Analysis

Single-cell leukocyte suspensions from thymus, spleen, and peripheral lymph nodes (superficial cervical, axillary, brachial, inguinal, and mesenteric) were generated by gentle dissection, and erythrocytes were hypotonically lysed using ammonium-chloride-potassium (ACK) lysing buffer. For multicolor immunofluorescence analysis of surface proteins, viable single-cell suspensions (1 x 106) in PBS were blocked for 10 min on ice with 0.8 μg anti-CD16/CD32 (2.4G2) from Bio X Cell (West Lebanon, NH) in 100 μL. Cells were then stained for 15 min at room temperature using predetermined

optimal concentrations of mAb in FACS buffer (DPBS pH 7.4, 2 mM EDTA, 2% FCS). For analysis of intracellular proteins, cells were then washed with DPBS and fixed in DPBS containing 2% paraformaldehyde for 10 min at room temperature. Cells were washed with FACS buffer and then re-suspended in FACS buffer with 0.1% saponin (Sigma-Aldrich), held at room temperature for 10 min, and then stained with predetermined optimal concentrations of antibodies. Samples requiring methanol permeabilization (STAT5 and STAT5-pY694) were first fixed with 2% paraformaldehyde for 10 min at room temperature, were washed in FACS buffer and then re-suspended in 10% 1X DPBS/90% ice-cold methanol, held at 4°C for 10 min, and then either stored at -20°C for 24 hours or washed with FACS buffer and stained with predetermined optimal concentrations of antibodies in FACS buffer for 30 min at room temperature. If a secondary antibody was needed, cells were washed with FACS buffer and then stained with fluorophore-conjugated secondary for 15 min at room temperature. After staining and a final FACS buffer wash, cells were re-suspended in FACS buffer containing 0.5% paraformaldehyde and kept in the dark at 4°C until analysis.

Unconjugated antibodies for staining included STAT5 (3H7) from Cell Signaling Technology (Danvers, MA). Pacific Blue-, FITC-, AF-488, PE-, PerCp-Cy5.5-, PE-Cy7-, APC-, AF-647-, and APC-Cy7–conjugated antibodies for staining were as follows: CD4 (RM4-5), CD8β (YTS156.7.7), CD19 (6D5), CD25 (PC61), CD44 (IM7), L-selectin (CD62L; MEL-14), BCL-2 (BCL/10C4), and CD132 (TUGm2) from BioLegend (San Diego, CA);

STAT5-pY694 (47) from BD Biosciences; IRS1 (E-12) from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit IgG from Jackson ImmunoResearch (West Grove, PA); and goat anti-Rabbit IgG from Thermo-Fisher Scientific.

Dead cells were identified and excluded from further analysis by staining with Annexin V and 7AAD (Biolegend) staining or by staining with LIVE/DEAD Fixable Dead Cell Stain Kit (Thermo-Fisher Scientific). Single cells with the forward and side light scatter properties of lymphocytes were analyzed using a FACSCanto II flow cytometer (BD Biosciences). Background staining was assessed using isotype-matched controls or using a fluorescence minus one control. Data were analyzed using FlowJo v8.2.

2.6 Plate Bound and Cytokine Stimulation

Plates were prepared by coating the wells of a hydrophilic tissue culture plate with biotinylated poly-L-lysine in DPBS, prepared by mixing poly-L-lysine hydrobromide (Sigma-Aldrich) with EZ-link Sulfo NHS-Biotin (Thermo-Fisher Scientific). Wells were incubated at room temperature for 1 hour with orbital shaking and then washed with DPBS. Wells were then coated with a solution of 1% BSA (Sigma-Aldrich) in DPBS plus 5 μg/ml Streptavdin (Prozyme, Hayward, CA) and incubated as before and then washed with DPBS. Wells were then coated with biotinylated mAbs, which were prepared by mixing functional grade anti-CD3ε (145-2C11, 1 μg/mL) and

anti-CD28 (37.51, 0.2 μg/mL) from Bio X Cell with EZ-link Sulfo NHS-Biotin (Thermo-Fisher Scientific) according to the manufacturer's instructions. Wells were coated with a solution of 1% BSA in DPBS plus biotinylated mAbs either for 1 hour at room temperature with orbital shaking or, after 10 min of shaking, were placed at 4°C overnight. Wells were washed again with DPBS before plating cells.

$CD4^+$ or $CD8^+$ T cells were purified by FACS at the Duke Flow Cytometry Shared Resource (Duke University Medical Center, Durham, NC) or by MACS using Untouched Mouse CD4 and CD8 kits (Thermo-Fisher Scientific) according to the manufacturer's instructions. T cells were plated at 2×10^6 cells/mL in warm complete RPMI-1640 media (10% FCS and 50 μM 2-mercaptothanol, 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids) on coated plates and were incubated in a humidified 37°C incubator with 7% CO_2. For primary stimulation, T cells were given hIL-2 (Peprotech, Rocky Hill, NJ) at 5 ng/mL at plating and 1 ng/mL at each splitting for the first 72 hours. Cells receiving puromycin selection were given 3 μg/mL puromycin (Sigma-Aldrich) 24 h after infection and selection then proceeded for 48 h. Cultures were supplemented with warm, fresh complete RPMI media as needed. For all re-stimulations, live cells were isolated using Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden). For α-CD3ε/CD28 re-stimulation, cells were plated at 7.5×10^5 cells/mL in warm complete RPMI on coated plates. For IL-2

re-stimulation, cells were plated at $5x10^5$ cells/mL in U-bottom plates with 5 ng/mL IL-2 in warm, serum-free RPMI with 0.1% BSA.

2.7 Retroviral Infection

Wild-type T cells overexpressing GFP or miR-191-GFP were generated by transfection with ecotropic retrovirus containing the MSCV vector(*121*) with a Puromycin-IRES-GFP cassette(*122*). Pri-miR-191 was amplified from primary splenic T cells and was introduced downstream of the 5' long terminal repeat sequence using XhoI and EcoRI restriction sites. pBABE-puro-mouse-IRS-1-myc (pBABE-IRS1) was a gift from Ronald Kahn (Addgene plasmid # 11374)(*123*).

Plat-E cells(*124*) grown in complete DMEM media supplemented with Blasticidin (10 μg/mL) and Puromycin (1 μg/mL) were then transduced with the relevant plasmid using Lipofectamine 2000, and virus was collected 2 days later for retroviral infection of T cells. T cells were retrovirally infected after 24 h of plate-bound or 2.5 ug/mL Concavalin A (ConA; Sigma-Aldrich) stimulation by spinning at 800 x g in a centrifuge maintained at 32°C for 90 min with 10 μg/mL polybrene (Sigma-Aldrich).

2.8 Reverse Transcriptase Quantitative PCR (RT-qPCR)

$CD4^+$ and $CD8^+$ T cells and $B220^+$ B cells were purified by MACS (Thermo-Fisher Scientific). Cells were washed with DPBS and were lysed and homogenized in TRIzol

(Thermo-Fisher Scientific). Total RNA was extracted using Direct-zol (Zymo Research, Irvine, CA). cDNA was generated using the Flex cDNA synthesis kit (Quanta BioSciences Gaithersburg, MD), with the included oligodT and random primer sets used for mRNA target analysis. For miRNA targets, a polyA-tail was added using E. coli polyA polymerase (Epicentre, Madison, WI), and cDNA was then generated using a custom universal tag coupled to oligodT. After first-strand cDNA synthesis, cDNA was diluted in Tricine-EDTA for long-term storage. The qPCR reaction used SYBR Perfecta Supermix (Quanta Biosciences) and unique forward and reverse primers for mRNA targets or a unique forward primer and universal reverse primer mix for miRNA targets. Data were acquired using a LightCycler 480 II (Roche, Basel, Switzerland). Data were analyzed in Microsoft Excel 2013 (Microsoft, Redmond WA) using the geNorm method(*125*) and were plotted in Prism v5.01 (GraphPad Software, La Jolla, CA).

2.9 In Vitro Homeostatic Survival Assay

Naïve $CD8^+$ ($CD8\beta^+CD44^-CD62L^+$), central memory $CD8^+$ ($CD8\beta^+CD44^+CD62L^+$), and regulatory ($CD4^+CD25^+$) T cells were purified by FACS at the Duke Flow Cytometry Shared Resource. All cells were plated at $CD8^+$ cells were plated 5 $x10^5$ cells/mL; $CD8^+$ cells in U-bottom plates and regulatory T cells in flat-bottom plates pre-coated as in the plate bound stimulation assay described above. At plating, naïve $CD8^+$ T cells received 1 ng/mL hIL-7 (Peprotech); memory CD8+ T cells received 10 ng/mL hIL-15 (Peprotech);

and regulatory T cells received 10 ng/mL hIL-2 (Peprotech). Survival was assayed by flow cytometry 72 hours after plating.

2.10 microRNA Targeting Luciferase Assay

The full-length 3′-UTR of mouse *Irs1* (containing the miR-191 target site) was amplified from cDNA generated from mouse $CD8^+$ T cells and was cloned into the pmiRGLO dual-luciferase vector (Promega, Madison, WI) downstream of firefly luciferase. A mutated version of the *Irs1* 3′UTR was generated by replacing the miR-191 target site with the restriction enzyme site SalI by synthesizing (IDT) a fragment of the 3′UTR containing the mutation and cloning the fragment into the previously generated vector using XhoI and ApaLI sites. Each dual-luciferase reporter vector, together with a Mock or miR-191 overexpression vector, was co-transfected into NIH-3T3 cells cultured in complete DMEM media as described above using Lipofectamine 2000. Cells were incubated for 48 hours in a humidified 37°C incubator with 5% CO_2 and were then lysed. Luciferase reporter activity was determined in a dual-luciferase reporter assay (Promega) using an Infinite 200 Plate Reader (Tecan, Männedorf, Switzerland).

2.11 Statistical Analysis

All data collected are included and are shown as individual data points. The significance of differences between sample means was determined using the unpaired

Student t test, with Welch's correction for unequal variances applied where appropriate as determined using the F test.

3. Novel Tools for the Study of microRNAs

3.1 Background

Whether the goal is novel therapies or basic biological discovery, any study examining the impact of a miRNA necessitates the identification of phenotypically relevant mRNA targets. Many technological innovations have been made to enable the simultaneous identification of all members of the miRNA:mRNA regulatory network for a given condition in a cell. Technologies like HITS-CLIP(*126*) and PAR-CLIP(*127*), where active miRNA:mRNA interactions are fixed and then elucidated following high-throughput sequencing, offer powerful insight into whether a given miRNA actually targets a specific mRNA. However, such technologies require immunoprecipitation, and by necessity, large numbers of cells as input and are therefore limited to use in cell lines, which offer a limited view of networks regulating dynamic cell populations *in vivo*. The advent of current generation RNA Sequencing (RNASeq) and the adaptation of RNASeq to miRNAs(*128*) permits the generation of large datasets (e.g. The Cancer Genome Atlas (TCGA)) where, in combination with the computational prediction of miRNA targets using algorithms like TargetScan(*129, 130*), miRNA and mRNA expression levels can be correlated and complex network analyses run(*131*). Computational target prediction is becoming more reliable, and computational network analyses have accurately predicted a variety of molecular networks. Ultimately, computational methods can only offer predictions, leaving much work to be experimentally validated, which requires the

identification of phenotypes and targets driving those phenotypes as well as the manipulation of miRNA levels or function to probe those phenotypes.

3.2 Manipulation of microRNA expression

3.2.1 Modulation of overexpression using backbone switching

To begin investigating the role that a miRNA plays in either a particular phenotype or simply the relevance of a given miRNA to a specific cell type or pathway, a miRNA can either be altered in expression or functionally inhibited. Inhibition of miRNA function can make use of a variety of well-established tools (e.g. antagomiRs(*132*) or locked-nucleic acids (LNAs)(*133*)), but such tools are expensive and lack the long-term alteration permitted by retroviral overexpression.

While miRNA-decoy vectors(*134*) have been successfully utilized as a loss-of-function tool, the synthesis and cloning of such decoys as well as the additional vector elements required for interpretable results make them a much more challenging approach to probe the effect of a given miRNA relative to simple overexpression. Many vectors exist to overexpress miRNAs or shRNAs; these studies utilize the MSCV-PiG retroviral vector(*121, 122*) which has several nice features, including internal ribosomal entry sequence (IRES)-mediated bicistronic expression of both puromycin resistance (conferred by the *pac* gene, which produces a puromycin N-acetyl-transferase (PAC)) and green fluorescent protein (GFP) expression for selection, and a XhoI-EcoRI cloning

site for expression of miRNA or shRNA. To ensure proper miRNA processing, 100 bp of the relevant genomic flanking sequence was added to either end of the stem-loop miRNA sequence provided by miRBase(*135-139*). MSCV-PiG contains two eukaryotic promoters: the viral 5′ Murine Stem Cell Virus (MSCV) LTR and the murine PGK promoter, with the bicistronic selection cassette downstream of PGK. The presence of two promoters allows this vector to be used for miRNA-decoys but requires that an insulator (like the chicken β-globin FII/FIII insulator(*140*)) be cloned in between the two promoters to prevent interference. miRNA overexpression requires no such modification, but does suffer from a lack of control as to the extent of overexpression. While one could reconfigure the vector and replace PGK with other more powerful promoters that include introns for miRNA expression and are only weakly susceptible to silencing (e.g. EF1α or CAG(*141*))(*142*), such promoters are quite long, limiting the possibility of co-expressing the miRNA with any long mRNAs. Similarly, these promoters only work in one direction, which is to elevate miRNA expression.

Fortunately, miRNA expression is not only controlled by the strength of the promoter; the processing efficiency from pri- to pre- to mature miRNA is also critical for expression. Not all miRNAs are processed and expressed with the same efficiency, even when expressed in the same cluster(*108*). The root of this differing efficiency appears to lie with Drosha processing(*74*), which makes use of a variety of consensus sequences in the pri-miRNA. Thus, it seems possible to manipulate processing efficiency and thereby

the expression of a given miRNA by placing it within a different miRNA backbone. To test this theory, I used the RNAfold webserver (part of the ViennaRNA package(*119*)) to guide a transplant of the mmu-miR-191 5p and 3p sequences into the backbone of mmu-miR-148a (Figure 1A). The mmu-miR-148a backbone was selected because we had observed that overexpression of mmu-miR-148a was unusually high (Chaoran Li, Duke University Medical Center, personal communication). This backbone switch enhanced the overexpression of miR-191 from approximately 1.25 fold to 3 fold over Mock in 2B4 cells(*143*) that had been selected for stable expression with puromycin (Figure 1B), removing the possibility of transient effects. Careful analysis of the pri-mmu-miR-191 and pri-mmu-miR-148a sequences revealed that while both miRNAs have the CNNC motif 17 bases downstream of the 3′ Drosha cut-site, only pri-mmu-miR-148a has the UG 14 bases upstream of the 5′ Drosha cut-site (Figure 1A). The presence of the UG has been shown to substantially improve pri-miRNA processing efficiency(*74*), but there may be other aspects of the mmu-miR-148a backbone that lead to its enhanced efficiency. Such enhancement was not unique to mmu-miR-191, as backbone switches for mmu-miR-720 and mmu-miR-124-1 enhanced overexpression as well(Yu Wang and Shan Jiang, Duke University Medical Center, personal communication). Given the ability to enhance overexpression, it seemed possible to use a different backbone to reduce overexpression. The mmu-miR-19a backbone was selected as it showed poor overexpression ability(*108*), lacks the UG, and has shifted the CNNC motif down by a base. The mmu-miR-19a

backbone was able to reduce overexpression for mmu-miR-148a, hsa-miR-203, hsa-miR-155, hsa-miR-206, and has-miR-129-1(Xin Xu and Tao Sun, Duke University Medical Center, personal communication). Thus, through careful selection of a miRNA backbone, miRNA overexpression can be easily fine-tuned to meet a variety of experimental needs.

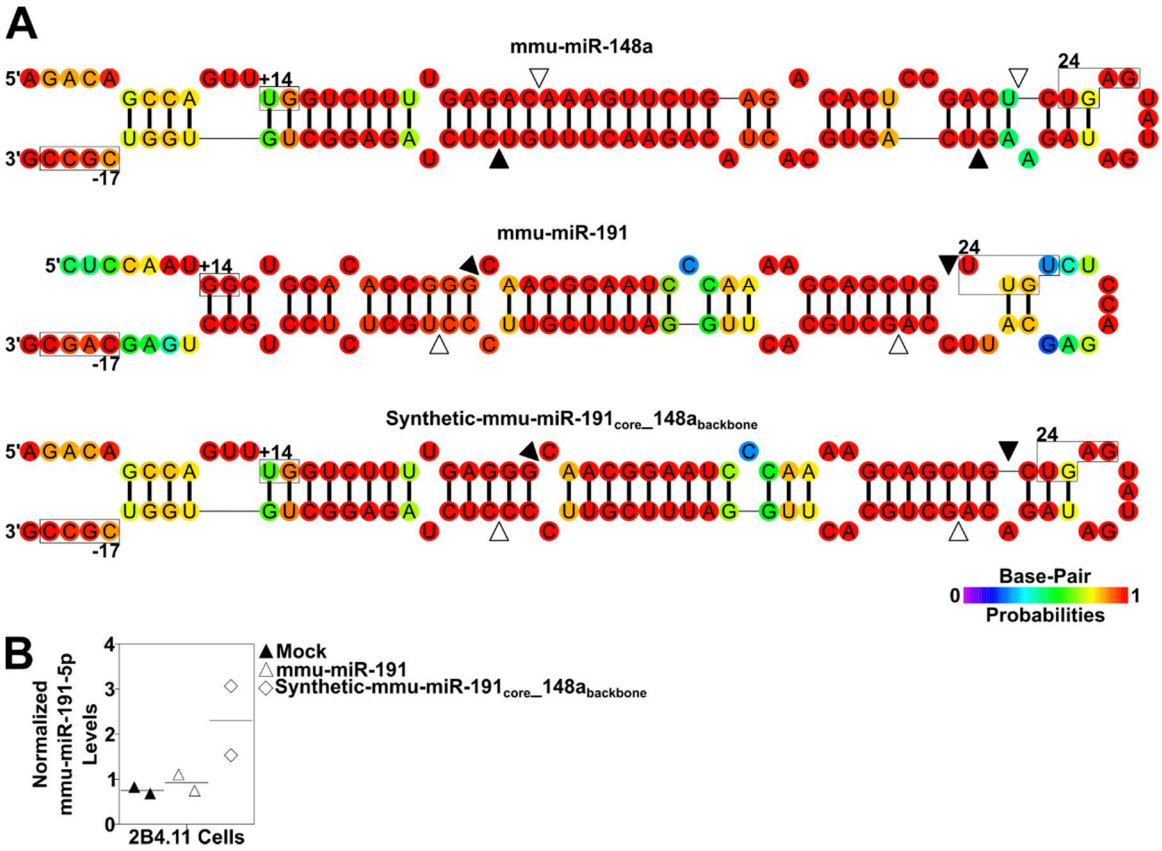

Figure 1: Placement of miR-191 into the miR-148a backbone enhances expression.

Figure 1. Placement of miR-191 into the miR-148a backbone enhances expression. *A*, Predicted secondary structures of mmu-miR-148a (top), mmu-miR-191 (middle), and synthetic-mmu-miR-191$_{core}$_148a$_{backbone}$ (bottom) were generated by RNAFold and re-drawn. Black triangles flank the sequence for the mature guide strand, while white triangles flank the passenger strand. Key sequence determinants of Drosha processing are highlighted with the base position relative to the first base of the mature 5′ strand indicated. Ideal determinants are UG at +14, UGUG at 24, and CNNC at -17. *B*, RT-qPCR data from 2B4.11 cells infected with wither Mock-GFP, miR-191-GFP, or synthetic-miR-191$_{core}$_148a$_{backbone}$-GFP retrovirus and selected with puromycin for 2 weeks. Data are from 2 independent experiments.

3.2.2 Conditional deletion of single microRNAs

Overexpression of a miRNA, particularly with the fine-tuning facilitated by backbone switching, allows for cost-effective screening of miRNA-driven phenotypes. However, to ultimately understand the significance of a particular miRNA in a biological system, the miRNA must be deleted. While much good data has come from germline miRNA knockouts, it is difficult to conclusively ascribe a phenotype to a particular cell type, particularly *in vivo*. Cell transfers are able to somewhat mitigate this caveat, but to truly isolate a study to a particular cell type requires a conditional knockout. Several systems exist for conditional deletion, including *Cre-lox*(*144, 145*) and FLP-FRT(*146*), where a site-specific recombinase (e.g. Cre or Flpe) drives recombination between pairs of *loxP* or FRT sites, respectively, allowing for a site flanked by *loxP* or FRT sites to be deleted. To make the deletion conditional, an inducible or cell-type specific promoter is used to drive recombinase expression. For example, LckCre(*147*) results in Cre recombinase expression driven by the proximal Lck promoter, which begins at the DN2 stage of T cell development(*17*) and is therefore specific in expression to T cells. With the appropriate promoter to ensure conditional deletion, all which is required is the appropriate placement of the recombination sites.

In this study, both FLP-FRT and *Cre-lox* systems were used. Flpe recombinase, driven by the CAG promoter to ensure ubiquitous expression, was used to delete the neomycin selection cassette, which was important for selection of the integrated

targeting cassette in the embryonic stem cells (ESCs). Cre recombinase was used to delete the miRNA of interest and the FRT "scar" (i.e. the FRT remaining after Flpe recombinase action). The placement of the *loxP* and FRT sites were critical to ensure that miRNA expression was not altered by the presence of the targeting cassette. So, as for miRNA overexpression, any modifications were placed 100 bp away from the miRNA's stem-loop sequence as given in miRBase. This strategy permitted the creation of floxed mmu-miR-191 (miR-191$^{f/f}$) and, upon crossing to mice expressing LckCre (LckCre^{+}), resulted in specific deletion of the mmu-miR-191 locus in CD4^{+} and CD8^{+} T cells, but not B cells (Figure 2B). Concomitant reductions in mmu-miR-191-5p levels were observed in CD4^{+} and CD8^{+} T cells from 191$^{f/f}$LckCre^{+} mice (Figure 2C). Notably, mmu-miR-191-5p levels only decrease when LckCre is present, meaning that the introduction of *loxP* sites did not interfere with miRNA processing. Given these results, it seemed likely that similar design principles would allow for the conditional deletion of miRNAs that reside within a cluster. Each miRNA within a cluster has a unique function, and some miRNAs even act at odds with others in the cluster(*108*). Enabling single miRNA elimination from within a cluster would enable more thorough dissections of function and thereby permit better decisions about therapeutic alterations to miRNA activity. Within the cluster, a miRNA will share Drosha processing regions with its neighbor(s). To permit conditional deletion of a single miRNA within the cluster while leaving other members intact, the shared Drosha processing region was duplicated, with one copy present

within the *loxP* sites to permit normal expression of the targeted miRNA and the second copy outside the *loxP* sites next to the relevant miRNA neighbor. Example insert designs for two members of the mmu-miR-17-92 cluster, mmu-miR-18a and mmu-miR-92a-1, are shown to indicate how miRNAs on the interior and exterior of a cluster are handled (Figures 2D-E), respectively. The same design paradigm enabled the deletion of mmu-miR-23a from the mmu-miR-23a~27a~24-2 cluster without altering mmu-miR-27a or mmu-miR-24-2 expression(*107*). Thus, successful conditional deletion of single miRNAs, even those in clusters, is accomplished through careful design where Drosha processing sites are left intact.

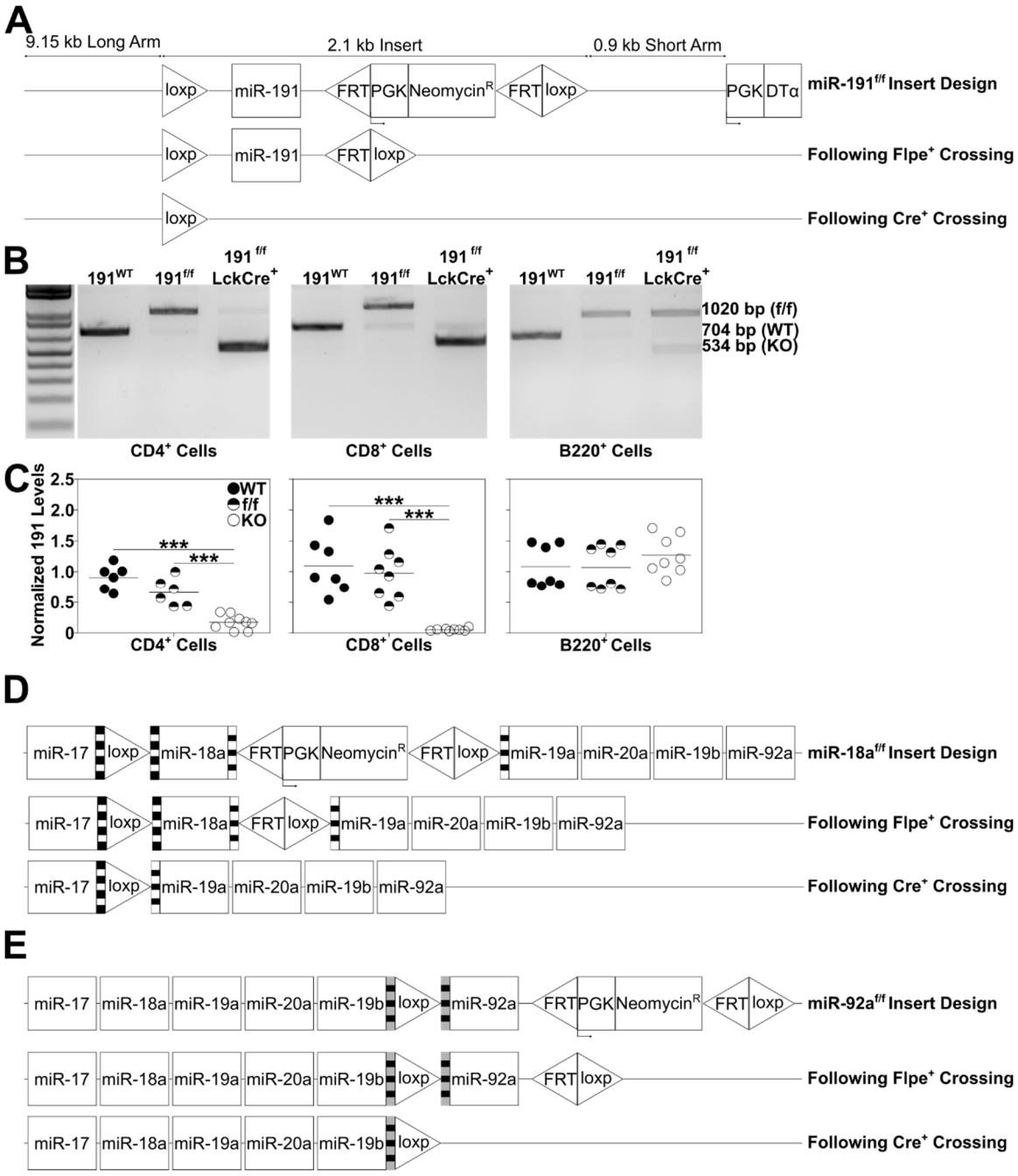

Figure 2: Conditional deletion of miRNAs.

Figure 2. Conditional deletion of miRNAs.

A, Design of miR-191$^{f/f}$ construct. A loxP site is placed upstream and a FRT-flanked neo cassette is placed downstream, followed by another loxP site. Following Flpe action, the neo cassette is deleted, but miR-191 is intact. Only Cre recombinase action removes miR-191. *B*, PCR of the miR-191 locus from MACS-purified LN B220$^+$ B cells (left panel), CD4$^+$ T cells (middle panel), and CD8$^+$ T cells (right panel), from animals of the indicated genotypes. A ladder is shown with bands ranging from 100 to 2000 bp. The wild-type miR-191 band is 704 bp, the floxed miR-191 band is 1020 bp, and the knockout band is 534 bp. Data are representative from 2 independent experiments. *C*, RT-qPCR for miR-191 expression in MACS-purified LN B220$^+$ B cells (left panel), CD4$^+$ T cells (middle panel), and CD8$^+$ T cells (right panel) from animals of the indicated genotypes. Expression levels were normalized using geNorm. Data are from 2 independent experiments; n = 6-7 per group. Differences in group means were determined by the unpaired Student's t test: ***$p < 0.001$. *D*, Design for the conditional deletion of miR-18a from the miR-17-92 cluster. The Drosha processing regions (hatched boxes) between miR-18a and miR-17 and miR-19a are duplicated, with one copy placed outside the loxP sites. *E*, Design for the conditional deletion of miR-92a from the miR-17-92 cluster. The Drosha processing region (hatched boxes) between miR-92a and miR-19b is duplicated, with one copy placed outside the loxP sites.

3.3 Drugs that alter microRNA expression and/or function

Altering miRNA expression through genetic manipulation is an important research tool, but the current molecular tools that make such manipulation possible pose many dangers in the clinic. While sequence-specific miRNA inhibitors like antogomiRs and LNAs hold great promise for anti-miRNA therapy, they have not been approved for clinical use. Screening vast libraries of small molecule compounds held by pharmaceutical companies for alterations to miRNA expression and/or function offers a mechanism to both better understand the mechanism of action for certain drugs and to uncover potentially valuable therapeutics. Given the sheer number of compounds available for screening, the ideal screening system would be high-throughput, robust, and as cost-effective as possible. This study developed a screening platform based on high-throughput flow cytometric screening of drug-treated cell lines expressing miRNA activity tracking constructs.

Tracking miRNA activity requires a vector with two promoters: one promoter is responsible for the production of the selection marker, while the second promoter drives expression of the fluorophore with miRNA target sites in its 3' UTR. The MSCV backbone was used with several notable modifications: first, to prevent promoter interference, a 2X repeat of the FII/III insulator was placed between the upstream product and the PGK promoter ; second, the SV40 poly-A terminator was added just downstream of *pac* to ensure polyadenylation of the resultant mRNA; third, the *pac*-

IRES-*gfp* cassette was removed and replaced with ZsGreen1(*148*) with a 3′ UTR containing a 2X repeat of the complementary sequence with a 6-base spacer for a given miRNA (Figure 3A). To minimize the baseline fluorescence of such a miRNA activity reporter, the reporter was added to NIH-3T3 cells overexpressing the relevant miRNA using another modified MSCV plasmid where the *pac*-IRES-*gfp* cassette was removed and replaced with a blasticidin-S deaminase (*bsr*)-porcine teschovirus-1 2A (P2A,(*149, 150*))-mTagBFP2 (mBFP2,(*151*)) cassette (Figure 3B). Following the stable integration of both reporter and expression plasmids ensured by selection with puromycin and blasticidin-S, cell lines reporting the activity of mmu-miR-19b-3p, mmu-miR-142-5p, and mmu-miR-142-3p showed variable expression of ZsGreen1 (Figure 3C). Such variability, while most likely reflective of differing endogenous levels of the miRNAs examined, demands that cell lines with high ZsGreen1 be further selected using FACS. To demonstrate that the system functioned as expected (i.e. that ZsGreen1 expression could be induced following repression of miRNA activity), mmu-miR-19b reporter cells were treated with aurintricarboxylic acid (ATA), a known inhibitor of protein-nucleic acid interactions(*152*). Treatment with ATA resulted in significantly enhanced ZsGreen1 expression (Figure 3D). Thus, miRNA activity trackers can be used in a flow-cytometric screening assay for drugs that alter miRNA expression and/or function.

To further test the system, in a collaboration with Dr. Jiyong Hong, a set of 3058 compounds were screened against the mmu-miR-19b-3p reporter line. The mmu-miR-

19b-3p reporter was selected for the primary screen as it has several strong and well-characterized T cell differentiation phenotypes that could be tested post-validation. High-throughput screening of the compounds yielded 3 putative hits: idarubicin, duanorubicin, and merbromin. These three compounds were then put through a validation screen where, in addition to re-screening against the mmu-miR-19b-3p reporter, they were also tested against wild-type NIH-3T3 cells and the mmu-miR-142-5p and -3p reporters. Screening against wild-type cells determines whether the compounds are eliciting a false positive while testing against mmu-miR-142-5p and -3p reporters assesses whether the compound works specifically on miR-19b or has a general miRNA activity suppression effect. Unfortunately, none of these compounds passed the validation screening. Indeed, all of them failed because they all act as fluorophores in the same channel as ZsGreen1 (Figure 3E). Ultimately, the screening of this small set of compounds yielded no validated compounds, a frequent occurrence for even larger sets of compounds (Jiyong Hong, Duke University, personal communication). However, the experiment confirmed that the miRNA activity reporter system can be used to quickly and cheaply screen for drugs that alter miRNA expression and/or activity and that the validation screen is able to eliminate false positives. Future work screening for suppressors of miRNA activity would benefit from the selection of alternative fluorophores to reduce the initial false positive rate (e.g. replace ZsGreen1 with iRFP(*153*)).

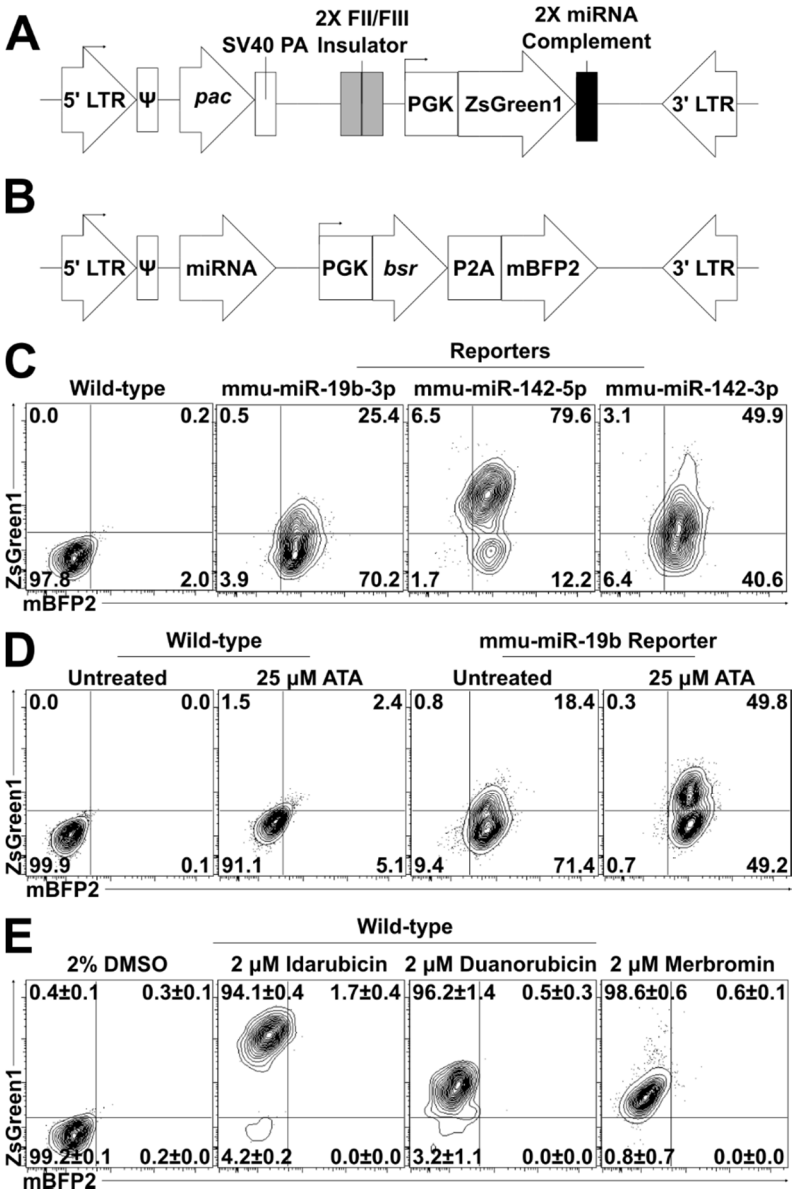

Figure 3: Generation of miRNA activity reporters for small molecule screening.

Figure 3. Generation of miRNA activity reporters for small molecule screening.

A, Linear representation of a fragment from the MSCV vector showing the design for the miRNA reporter. ZsGreen1 has a 2X miRNA complement sequence in its 3'UTR. *B*, Linear representation of a fragment from the MSCV vector showing the design a new miRNA overexpression and selection cassette. *C*, Contour plot showing the expression of ZsGreen1 (activity reporter) and mBFP2 (miRNA overexpression) for generated reporter lines after 2 weeks of selection with Puromycin and Blasticidin-S. *D*, Positive control for miRNA activity reporting. Inhibition of miR-19b activity after 24 exposure to ATA results in ZsGreen1 expression. *E*, Validation screening in wild-type cells of putative hits from screening with the miR-19b reporter.

3.4 Conclusions

These collective data offer new tools for the study of miRNA biology and function. Placing mmu-miR-191 into the mmu-miR-148a backbone enhanced mmu-miR-191-5p expression approximately 2.5 fold compared to the endogenous backbone (Figures 1A-B). Such enhancements likely derive from a key motif in that is present pri-mmu-miR-148a and absent from pri-mmu-miR-191 (Figure 1C). Consideration of the pri-miRNA is also important for the design of conditional miRNA knockouts. $191^{f/f}$ mice were generated in a manner in which all genetic modifications were placed at least 100 bp away from the stem-loop structure provided by miRBase (Figure 2A). $191^{f/f}$ mice were then bred with $LckCre^{+}$ mice to generate mice lacking mmu-miR-191 conditionally in T cells. Indeed, specific deletion of the miR-191 locus was observed in $CD4^{+}$ and $CD8^{+}$ T cells, but not B cells (Figure 2B), and concomitant reductions in mmu-miR-191 transcript were observed in $CD4^{+}$ and $CD8^{+}$ T cells from $191^{f/f}LckCre^{+}$ mice (Figure 2C). This strategy was expanded to permit the selective deletion of single miRNAs from within a cluster by duplicating Drosha processing regions (Figures 2D-E) and was validated using deletion of mmu-miR-23a. To enable the identification of potentially therapeutically viable miRNA function and/or expression modulators, a high-throughput flow-cytometry-based screening system utilizing miRNA activity reporters was tested and validated following a screen of over 3000 compounds (Figure 3). Thus, several novel and useful tools have been added to the miRNA toolbox.

4. The role of miR-191 in T Cell Survival at Homeostasis

Much of the following text, with notable exceptions, was modestly adapted from the original manuscript, "microRNA miR-191 Supports T Cell Survival Following Common Gamma Chain Signaling", which is currently in submission.

4.1 Background

The life-long maintenance of naïve and memory T cell pools is critical for productive adaptive immune responses, so there has been much investigation into mechanisms to enhance T cell homeostasis. Some of this work has been fairly radical, such as attempts to reverse thymic involution(*154*) in order to keep naïve cell input high. While such approaches are interesting, their long-term viability and utility in the clinic are still to be determined. Other work has focused on understanding the critical factors that control T cell survival at homeostasis, with much work focused on the factors that cause dramatic cell loss, as in the case of SCID(*66*). While much has been learned about the gross control of the critical cytokine receptors and downstream signaling molecules whose loss results in SCID, these factors are, fortunately, rarely mutated in the human population. It seems likely that there are other factors that play important roles in fine-tuning the expression or function of these critical pro-survival factors. These modulators may offer better targets for manipulation, as they are less likely to collapse the adaptive immune system, and the identification of such modulators may also offer insight into

minor immunodeficiencies and may serve as biomarkers for the selection of more personalized therapies (e.g. combination therapies to boost vaccine efficacy).

miRNAs are a powerful tool to probe for novel modulators of homeostatic regulators and may also prove useful in therapies targeting such modulators. In the current study, miRNA expression was examined across the hematopoietic system, revealing a highly expressed yet uncharacterized miRNA that was then screened for its role in T cell development and function. The study revealed that the miRNA targets a novel gene, which plays a role in modulating pro-survival cytokine signals, opening the field toward understanding the mechanisms that fine-tune immune homeostasis.

4.2 Results

4.2.1 miR-191 is highly expressed across the hematopoietic lineage and other tissues

A recent study that quantified miRNA expression among immune cell subsets revealed the presence of select miRNA species with high expression across T and B lymphocyte differentiation pathways(*128*). The consistently elevated expression of these miRNAs among all lymphocytes suggests that these miRNAs play a fundamental role in lymphocyte biology. While the majority of these highly expressed miRNAs belong to the well-studied let-7 family, one miRNA remains uncharacterized: miR-191 (Figure 4).

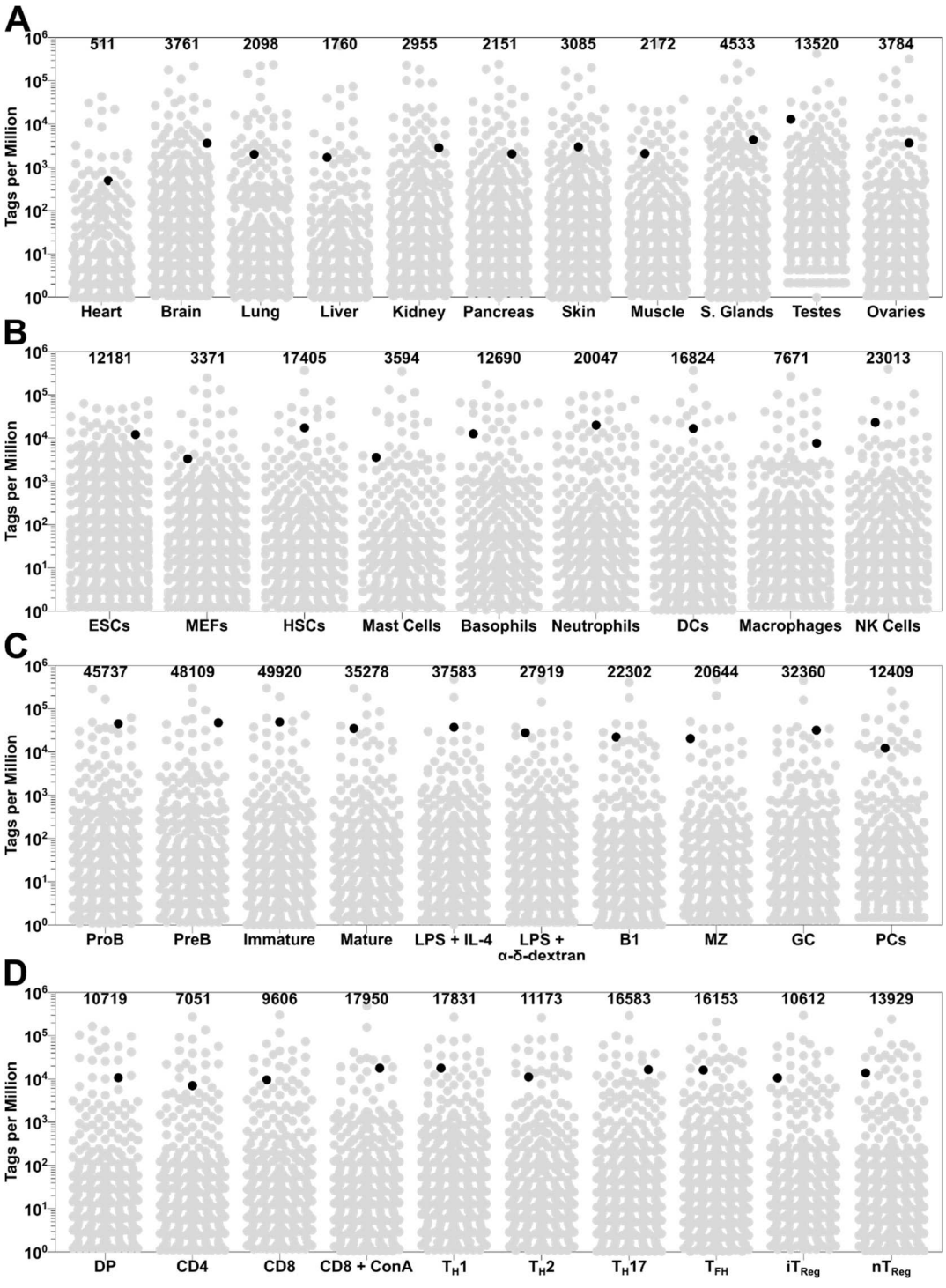

Figure 4: miR-191, and uncharacterized miRNA, is highly expressed in lymphocytes.

Figure 4. miR-191, and uncharacterized miRNA, is highly expressed in lymphocytes. Plots of reads from microsequencing of small RNAs for various T cell subsets from supplemental data provided in ref.(*128*). Each miRNA with greater than 1 tag per million (TPM) in a given sample is plotted as a gray dot. miR-191 is plotted as a black dot and the TPM value for miR-191 is given for each cell type plotted in (A) non-hematopoietic tissues, (B) stem and innate immune cells, (C) B lymphocytes, and (D) T lymphocytes.

4.2.2 miR-191 promotes T cell survival following TCR stimulation

To determine the significance of miR-191 expression in T lymphocytes, wild-type murine $CD4^+$ and $CD8^+$ T cells were retrovirally induced to overexpress miR-191. Both $CD4^+$ and $CD8^+$ T cells overexpressing miR-191 were protected from activation-induced cell death (AICD) following re-stimulation with plate-bound α-CD3ε/CD28 during in vitro culture (Figure 5A-B); no significant difference in proliferation was observed (Figure 5C-D).

The strong protection from AICD following miR-191 overexpression indicates a profound role for miR-191 in T cell survival. Whether miR-191 was essential for T cell development, survival, or function was determined using mice with T cell-intrinsic miR-191 deficiency: miR-$191^{f/f}$LckCre$^+$ mice (Figures 2A-C). miR-$191^{f/f}$LckCre$^+$ mice were viable, fertile, and exhibited no gross physical abnormalities. Following stimulation and re-stimulation through the TCR, $CD8^+$, but not $CD4^+$, T cells lacking miR-191 underwent progressively accelerated cell death over time (18-26%; Figures 6A-B). Similar to miR-191 overexpression, proliferation was unaffected by miR-191 depletion (Figures 6C-D). miR-191 expression therefore promotes T cell survival, and, in its absence, T cells die more rapidly following TCR stimulation.

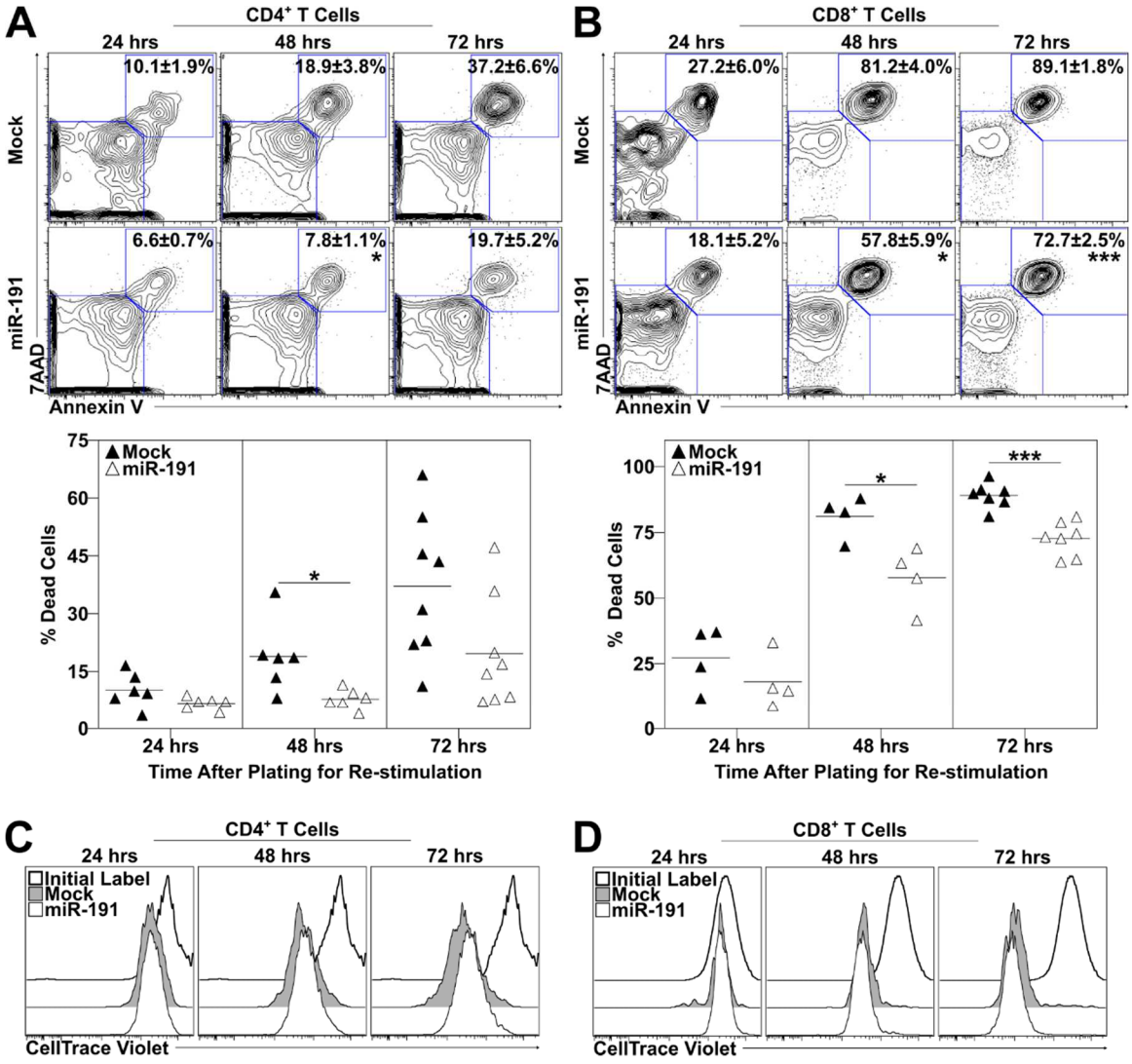

Figure 5: miR-191 enhances T cell survival.

Figure 5. miR-191 enhances T cell survival.

A-D, $CD4^+$ and $CD8^+$ T cells were purified by MACS, infected with Mock-GFP or miR-191-GFP retrovirus, re-stimulated for 24 hours post-infection with plate-bound α-CD3ε/CD28, and analyzed at the indicated times post-plating by flow cytometry. (*A-B*) Annexin V and 7AAD staining of GFP^+CD4^+ and GFP^+CD8^+ T cells. Top panels are representative contour plots. Bottom panels are summary data. (*C-D*) Representative MFI plots for CellTrace Violet levels in live, GFP^+ cells. Data derive from 2-3 independent experiments; n = 4-8 per group. Differences in group means were determined by the unpaired Student's t test: *$p < 0.05$, ***$p < 0.001$.

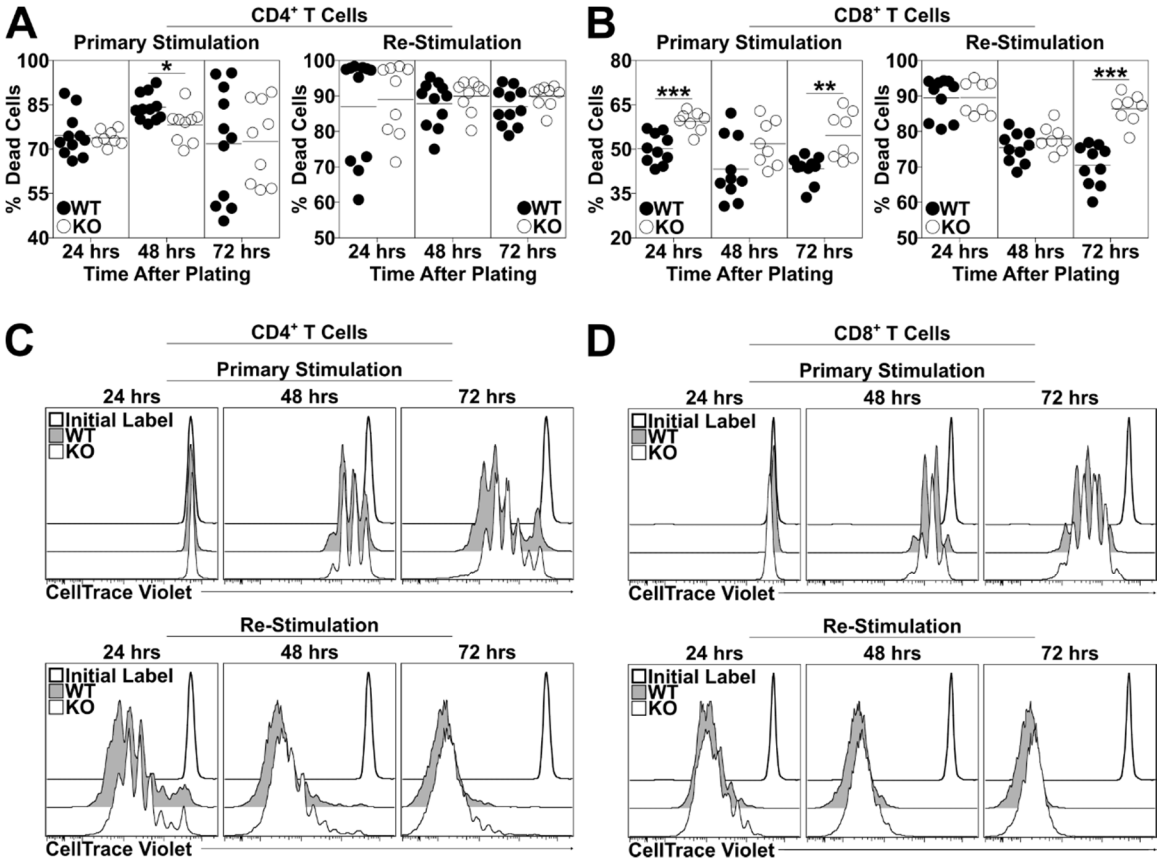

Figure 6: Loss of miR-191 in T cells impairs their survival following stimulation *in vitro*.

Figure 6. Loss of miR-191 in CD8$^+$ T cells impairs their survival following stimulation *in vitro*.

A-D, CD4$^+$ and CD8$^+$ T cells were purified by MACS or FACS, stimulated with plate-bound α-CD3ε/CD28 for 24 hours, and analyzed at the indicated times post-plating by flow cytometry. (*A-B*) Summary of cell death following primary stimulation (left panel) and re-stimulation (right panel). (*C-D*) Representative MFI plots for CellTrace Violet levels in live cells following primary stimulation (top panel) and re-stimulation (bottom panel). Data are from 3 independent experiments; n = 4-7 per group. Differences in group means were determined by the unpaired Student's t test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

4.2.3 miR-191 deficiency leads to peripheral T cell loss

T cell development and survival in vivo was then evaluated in miR-191-deficient mice. T cell-intrinsic miR-191 deficiency in 8 week-old adult mice led to a consistent and significant loss of peripheral $CD4^+$ and $CD8^+$ T cell numbers (30±6% and 49±3%, respectively, Figure 7A). This deficiency did not recover as mice aged, as 30 week-old aged mice had similar reductions in $CD4^+$ and $CD8^+$ T cells (34±8% and 45±11%, respectively, Figure 7A). T_{Regs} were also significantly reduced in number in the absence of miR-191 in young adult and aged mice (42±7% and 49±2%, respectively, Figure 7B). T_{Regs} were modestly reduced by proportion, but this did not alter homeostatic proportions of naïve ($CD44^-CD62L^+$) or activated ($CD44^+$ $CD62L^-$) $CD4^+$ or $CD8^+$ T cells (Figures 7C-D). Reductions in naïve and memory T cell numbers in these unchallenged animals were proportional to the overall reduction in T cell cellularity. $CD4^+$, $CD8^+$, and T_{Reg} cell loss was also observed in the spleens of miR-191-deficient mice (data not shown), although the losses were less pronounced.

Whether the loss of peripheral T cell numbers was reflective of gross defects in thymic T cell development was evaluated in miR-191-deficient mice. There were no significant alterations to the proportions of thymic T cell subpopulations, but young adult mice showed a consistent reduction in overall thymic cellularity stemming from losses in the $CD4^+CD8^+$ (DP) and CD4 SP compartments (29±10%, 23±2% respectively, Figure 8A). Thymic T_{Reg} frequencies were not altered in the absence of miR-191, though

total numbers were marginally reduced at rates proportional to the observed reduction in overall thymic cellularity (Figure 8B). The reduction in DP cell numbers was preceded by modest reductions in the proportion and number of DN2 cells (47±13%, Figure 8C). Thus, in the absence of miR-191, T cell development in the thymus is grossly normal.

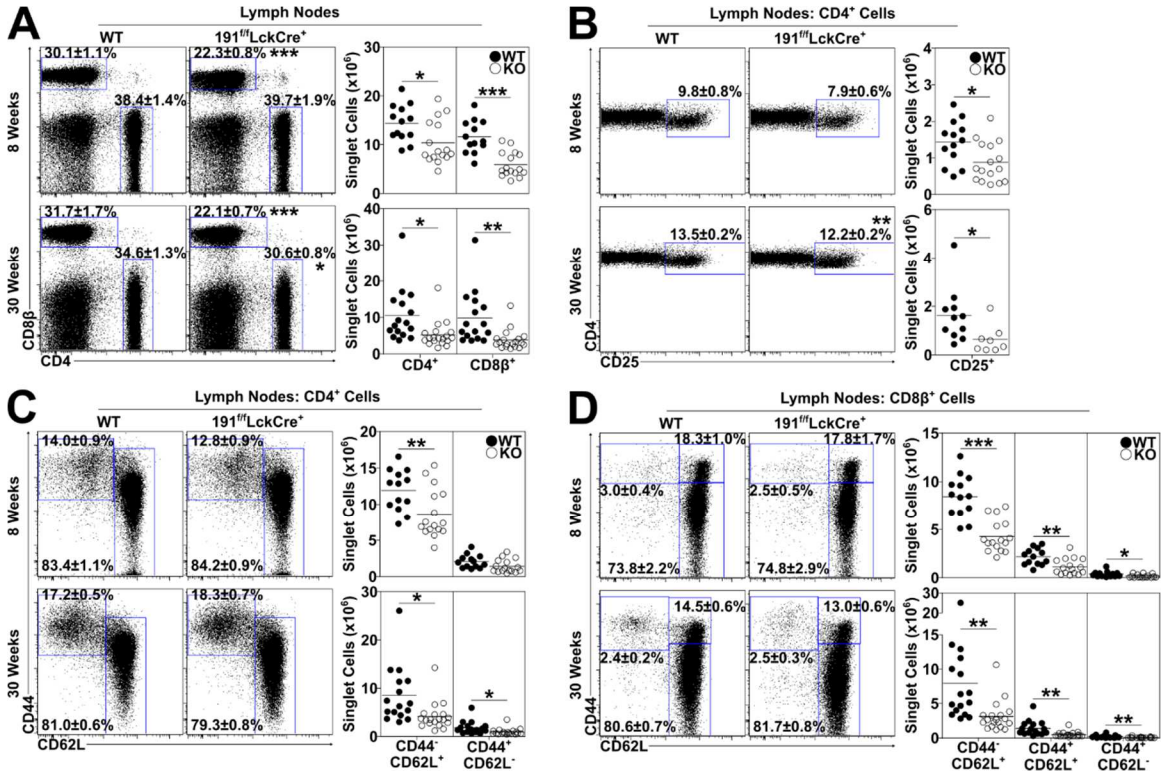

Figure 7: miR-191 supports peripheral T cell survival at homeostasis.

Figure 7. miR-191 supports peripheral T cell survival at homeostasis.

All panels have representative dot plots (left panels) and a summary of cell numbers for the specified populations (right panels) from 8-week-old (top panels) and 30-week-old (bottom panels) animals. Populations examined were (*A*) Peripheral Lymphocytes, (*B*) Peripheral Regulatory T Cells, (*C*) Peripheral $CD4^+$ T cells, and (*D*) Peripheral $CD8^+$ T cells. Data are from 2-4 independent experiments; n = 8-19 per group. Differences in group means were determined by the unpaired Student's t test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

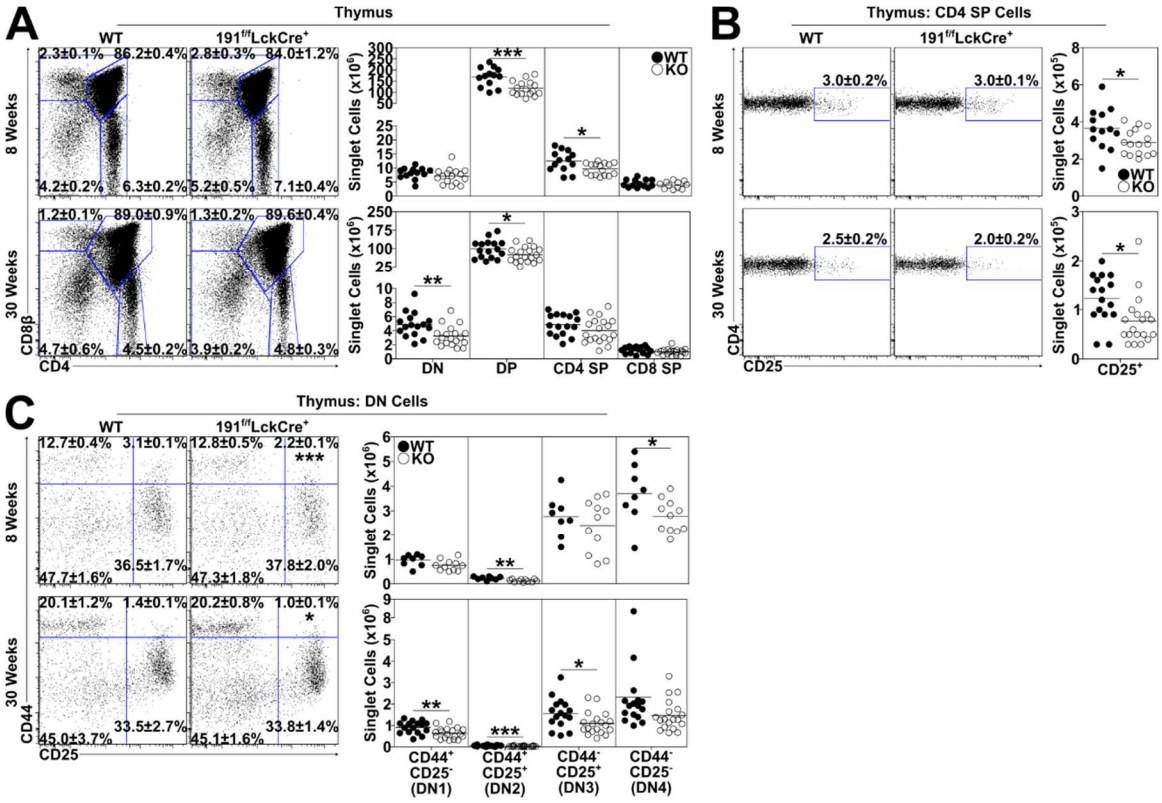

Figure 8: Loss of miR-191 during DN2 modestly impacts T cell development.

Figure 8. Loss of miR-191 during DN2 modestly impacts T cell development.

All panels have representative dot plots (left panels) and a summary of cell numbers for the specified populations (right panels) from 8-week-old (top panels) and 30-week-old (bottom panels) animals. Populations examined were (*A*) Thymocytes, (*B*) Regulatory T cells in the Thymus, and (*C*) Double Negative Thymocytes. Data are from 4 independent experiments; n = 8-19 per group. Differences in group means were determined by the unpaired Student's t test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

4.2.4 miR-191 enables cytokine-driven homeostatic survival

Peripheral T cell maintenance relies on tonic TCR signaling and appropriate cytokine signaling, and diminished signaling reduces cell survival in peripheral lymphoid tissues. To address whether peripheral T cell loss resulted from insufficient cytokine-driven homeostatic maintenance, naïve ($CD44^{-}CD62L^{+}$) and central memory ($CD44^{+}CD62L^{+}$) $CD8^{+}$ T cells and T_{Regs} were isolated and cultured in vitro with the appropriate homeostatic cytokines required for the survival of these subsets (IL-7, IL-15, and TCR plus IL-2, respectively). For each of these populations, the loss of miR-191 resulted in increased cell death (21%, 71%, and 30%, respectively, Figures 9A-C).

As the IL-2, IL-7, and IL-15 receptors require CD132, CD132 expression was evaluated among CD4+ and CD8+ T cells directly ex vivo. CD132 levels were equivalent among $CD4^{+}$ and $CD8^{+}$ T cells with and without miR-191 (Figure 9D). Downstream of CD132, the IL-2, IL-7, and IL-15, signaling pathways converge at the activation of the transcription factor STAT5. Tyrosine (Y) 694 phosphorylation is critical to activate STAT5 and initiate transcriptional programs for STAT5-dependent homeostatic survival, particularly for $CD8^{+}$ T cells and T_{Regs}(*155, 156*). While STAT5 protein levels are equivalent regardless of the presence of miR-191 (Figure 9E), both early and peak phosphorylation of STAT5 on Y694 is significantly reduced in $CD8^{+}$ cells lacking miR-191 (20-40% reductions, Figure 9F). miR-191 therefore facilitates homeostatic survival following cytokine signaling that relies on STAT5 tyrosine phosphorylation.

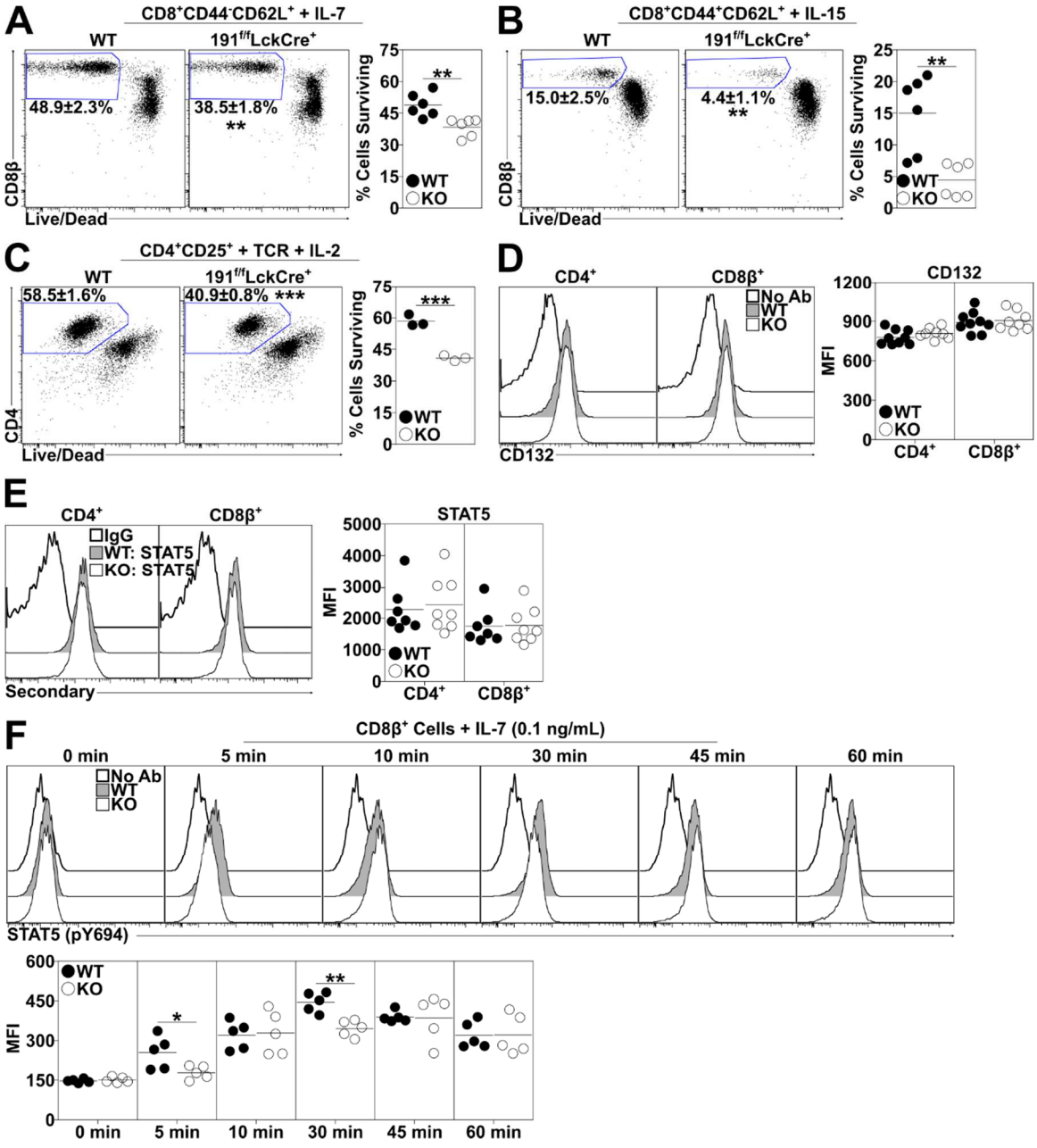

Figure 9: miR-191 promotes homeostatic STAT5-dependent cytokine signaling.

Figure 9. miR-191 promotes homeostatic STAT5-dependent cytokine signaling.

A-C, Sorted $CD44^{-}CD62L^{+}$ (*A*) and $CD44^{+}CD62L^{+}$ (*B*) $CD8^{+}$ cells, and $CD4^{+}CD25^{+}$(*C*) cells were cultured *in vitro* with the homeostatic cytokines most important for the survival of these subsets (IL-7, IL-15, and TCR plus IL-2, respectively). Representative dot plots (left panels) and summary plots (right panels) are shown. Data are from one to two independent experiments; n = 3-6 per group. *D-E*, Representative MFI plots (left panels) and summary plots (right panels) for CD132 (*D*) and STAT5 (*E*) staining in splenic $CD4^{+}$ and $CD8\beta^{+}$ cells. Data are from 2 independent experiments; n = 7-9 per group. *F*, MACS-isolated $CD8^{+}$ T cells were stimulated *ex vivo* with IL-7, and pSTAT5 levels were assessed via flow cytometry at the indicated time points. Representative MFI plots (left panel) and summary data (right panel) are shown. Data from two independent experiments; n = 5 per group. Differences in group means were determined by the unpaired Student's t test: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

4.2.5 miR-191 targets the scaffolding molecule Irs1

The impaired phosphorylation response downstream of cytokine signaling did not result from deficiencies in total STAT5 or CD132, suggesting that miR-191 specifically regulates a different gene or gene set related to this pathway. Phenotypically relevant targets of miR-191 were predicted computationally using the Target Scan algorithm(*157*). Humans and mice share 86 predicted targets for miR-191. RT-qPCR screening for these targets in $CD4^+$ T cells from wild-type and miR-$191^{f/f}$LckCre$^+$ animals yielded a single gene consistently detectable in T cells and with elevated expression in cells lacking miR-191: insulin receptor substrate 1 (*Irs1*). In both human and mouse, the 3′ UTR of (mRNA) *IRS1* contains one miR-191 target site (Figure 10A), though the 3′UTR of (mRNA) *IRS1* is not highly conserved. Similarly, other species, including *Canus lupus familiaris*, *Bos taurus*, and *Rattus norvegicus*, contain miR-191 target sites in the 3′ UTR of (mRNA) *Irs1* (data not shown), suggesting functional conservation of miR-191 targeting (mRNA) *Irs1*. IRS1 is a scaffolding protein that brings p85 and Grb2 to the insulin receptor(*158, 159*). IRS1 in human T cells co-precipitates with both JAK1 and JAK3, and this association is enriched during the course of cytokine signaling downstream of CD132(*160*). To determine whether miR-191 targets (mRNA) *Irs1* in murine T cells, intracellular IRS1 protein levels were measured. While no changes were observed for non-T cells (data not shown), $CD4^+$ and $CD8\beta^+$ T cells lacking miR-191 had increased IRS1 protein (24% and 38%, $p < 0.01$ and $p < 0.05$, respectively, Figure 10B). Irs1

transcript levels were also modestly upregulated in T cells lacking miR-191 (Figure 10C). Further, analysis of the 33 cell types within TCGA, revealed a significant negative correlation between miR-191 and (mRNA) *IRS1* expression in human cells (Figure 10D). To demonstrate directly that miR-191 can bind to the 3'UTR of (mRNA) *Irs1* and block protein synthesis, a 3'UTR luciferase assay was performed. Overexpression of miR-191 resulted in downregulation of firefly luciferase activity (21%, Figure 10E) when the full-length 3'UTR of (mRNA) *Irs1* was cloned downstream; luciferase activity was restored with mutation of the miR-191 target site. These data show that miR-191 targets (mRNA) *Irs1* directly. To determine whether elevated IRS1 levels enhance T cell death, wild-type $CD8^+$ T cells were retrovirally induced to overexpress IRS1. $CD8^+$ T cells were then cultured in serum-free media containing IL-2 to determine the rate of cytokine-induced survival. Cells overexpressing IRS1 (62% increase in IRS1 levels, Figure 10F) showed a concomitant 67% decrease in survival relative to cells expressing the Mock vector (Figure 10G). miR-191 therefore targets (mRNA) *Irs1* and increased IRS1 levels induce cell death following stimulation.

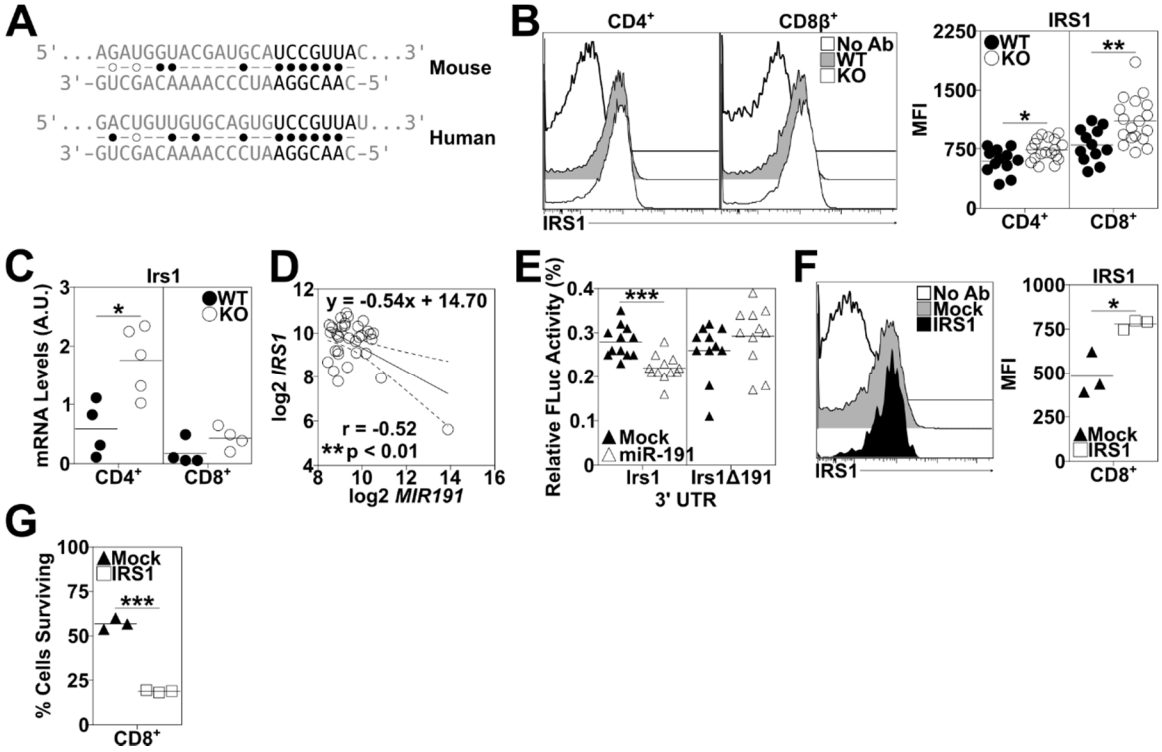

Figure 10: miR-191 targets Irs1.

Figure 10. miR-191 targets Irs1.

A, Alignment of miR-191 (lower strand) with its target site in the 3'UTR of *Irs1* transcript (upper strand) for both mice and humans. *B*, IRS1 staining by flow cytometry in splenic $CD4^+$ and $CD8\beta^+$ cells. Representative MFI plots (left panels) and summary data (right panel) are shown. Data from 2 independent experiments; n = 12-18 per group. *C*, RT-qPCR for *Irs1* transcript expression in MACS-purified LN $CD4^+$ and $CD8^+$ cells. Expression levels were normalized using geNorm. Data from one to two independent experiments. *D*, Firefly luciferase activity in NIH-3T3 cells transfected with Mock-GFP or miR-191-GFP plasmids with pmiRGLO containing either wild-type or miR-191 target site deleted versions of the *Irs1* 3'UTR downstream of firefly luciferase. Data were normalized to internal Renilla luciferase expression. Data from 3 independent experiments; n = 11-12 per group. *E*, Correlation between *MIR191* expression and *IRS1* expression as determined by RNASeq in the TCGA dataset. All samples with both miRNA and mRNA data from each of the 33 tumor types were selected. log2(mean) values were calculated within each sample and then plotted. Linear regression was performed to identify the correlation. *F-G*, $CD8^+$ T cells were purified by MACS, infected with Mock-GFP or pBABE-IRS1 retrovirus, puromycin-selected, re-stimulated with IL-2 for 24 hours and then analyzed for IRS1 expression (*F*), and for survival (*G*) by flow cytometry. Data from a single experiment; n = 3 per group. Differences in group means were determined by the unpaired Student's t test: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

4.2.6 A modulator of the miR-191-deficiency phenotype

To determine the exact mechanism through which impaired STAT5 phosphorylation led to enhanced T cell death, miR-191$^{f/f}$ and miR-191$^{f/f}$LckCre^{+} mice were crossed to mice expressing the Bcl-2^{YFP} BAC transgene(*120*). These reporter mice allow for an assessment of *Bcl-2* levels throughout development and at homeostasis. Intriguingly, examination of the lymphoid tissues revealed that miR-191$^{f/f}$LckCre^{+} Bcl-2^{YFP} mice show a greatly exacerbated phenotype relative to miR-191$^{f/f}$LckCre^{+} mice, with dramatic loss of thymic and lymph node cellularity (78±5% and 45±7%, respectively, Figure 11A). DP, CD4 SP, CD8 SP, and thymic T_{Reg} numbers were all reduced (84±4%, 86±3%, 78±7%, and 75±3%, respectively, Figures 11B-C). Preceding these losses, there was a severe block at DN3 (Figure 11D). In the peripheral lymph nodes, CD4^{+} and CD8^{+} T cell numbers were dramatically reduced (83±3% and 87±2% respectively, Figure 11E). Peripheral T_{Reg} numbers (as assessed by CD4^{+}FOXP3^{+} staining) were reduced (75±4%), but their proportion was increased (Figure 11F), presumably in response to the dramatic activation of CD4^{+} and CD8^{+} T cells (Figures 11G-H).

To further assess the exacerbated phenotype seen in these mice, the expression of Bcl-2^{YFP} and BCL-2 was examined. While Bcl-2^{YFP} levels were markedly reduced throughout development and in the peripheral lymph nodes, BCL-2 levels were normal or even increased, with the exception of peripheral T_{Regs}, where both Bcl-2^{YFP} and BCL-2 were reduced (Figures 12A-B). Discordant expression of YFP and BCL-2 protein

suggests that a novel disconnect occurs in the miR-191$^{f/f}$LckCre^{+} Bcl-2^{YFP} mice. When CD8+ T cells from these animals were stimulated *in vitro*, miR-191-deficient cells continued to exhibit decreased Bcl-2^{YFP} expression and concomitantly showed enhanced cell death, with Bcl-2^{YFP} and death both partially rescued by the addition of IL-2 (Figures 12C-D).

These data suggest the presence of a modulator of the miR-191-deficiency phenotype, one that does not create any observable phenotype when miR-191 is present. To discover the identity of the synergistic modulator, both thermal-asymmetric interlaced (TAIL)-PCR(*161, 162*) and Splinkerette PCR(*163*) reactions were designed and run. Unfortunately, sequencing results were consistent with multiple-tandem transgene insertions so the exact integration site was not identified.

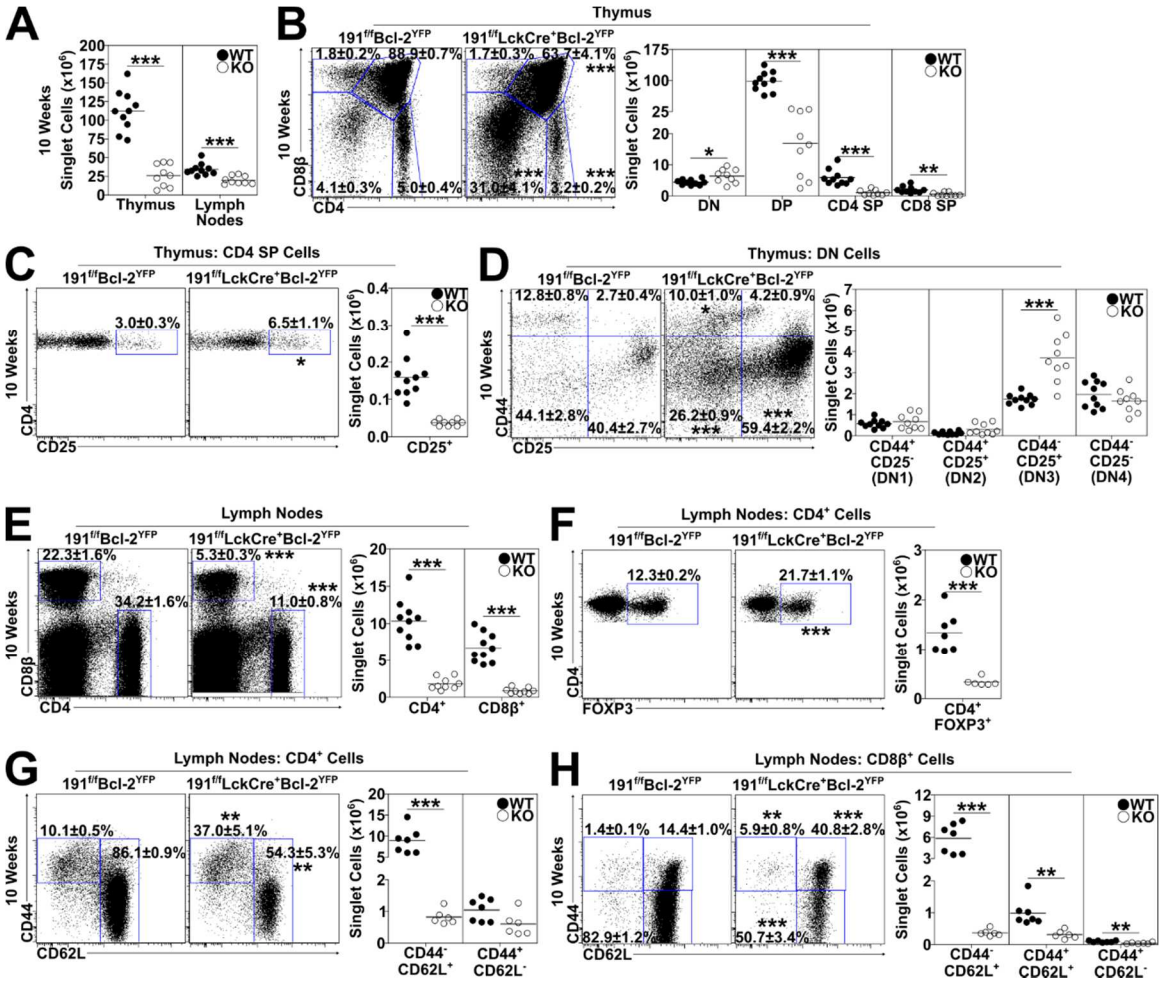

Figure 11: Loss of miR-191 in the presence of Bcl-2^{YFP} excaberates the miR-191-deficiency phenotype.

Figure 11. Loss of miR-191 in the presence of Bcl-2^{YFP} exacerbates the miR-191-deficiency phenotype.

A, Summary of cell numbers for the thymus and peripheral lymph nodes. *B-H*, All panels have representative dot plots (left panels) and a summary of cell numbers for the specified populations (right panels) from 10-week-old mice. Populations examined were (*B*) Thymocytes, (*C*) Regulatory T cells in the Thymus, (*D*) Double Negative Thymocytes, (*E*) Peripheral Lymphocytes, (*F*) Peripheral Regulatory T Cells, (*G*) Peripheral CD4$^+$ T cells, and (*H*) Peripheral CD8$^+$ T cells. Data are from 3-4 independent experiments; n = 6-10 per group. Differences in group means were determined by the unpaired Student's t test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

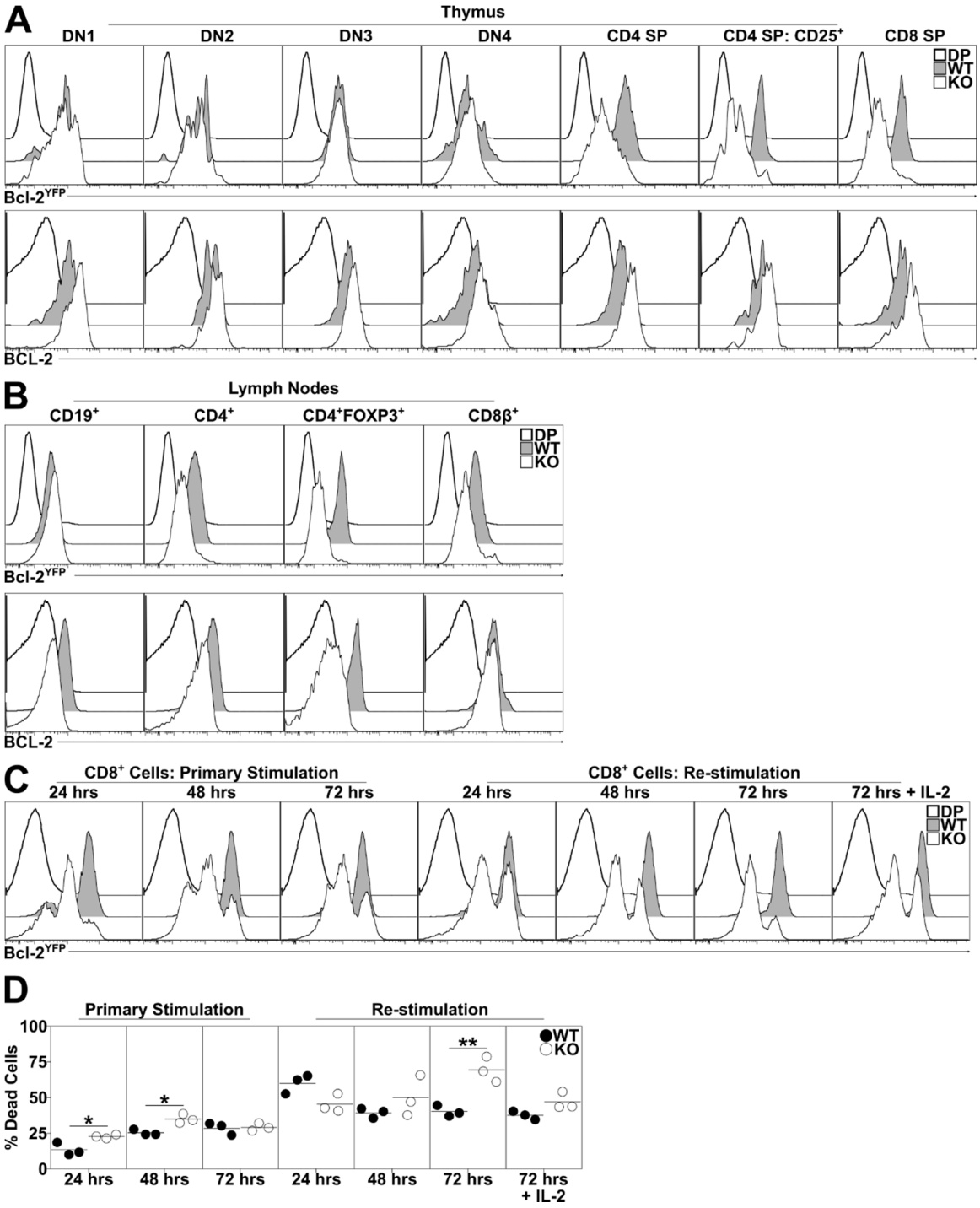

Figure 12: Loss of miR-191 reduces Bcl-2YFP expression, but not BCL-2 levels.

Figure 12. Loss of miR-191 reduces Bcl-2^{YFP} expression, but not BCL-2 levels.

A-C, MFI plots for Bcl-2^{YFP} (upper panels) and BCL-2 protein (lower panels) expression for key populations in the (*A*) thymus, (*B*) peripheral lymph nodes, and (*C*) during *in vitro* culture of CD8$^+$ T cells with TCR stimulation and re-stimulation. Wild-type DP cells were used as negative staining controls as this population does not express BCL2. Data are from a single experiment and are representative of 2-3 animals per genotype. *D*, CD8$^+$ T cells were purified by MACS stimulated with plate-bound α-CD3ε/CD28 for 24 hours, and analyzed at the indicated times post-plating by flow cytometry. Sample given IL-2 received it at the beginning of re-stimulation. A summary dot plot of cell death is shown following primary stimulation (left panels) and re-stimulation (right panels). Data are from a single experiment; n = 3 per group. Differences in group means were determined by the unpaired Student's t test: *$p < 0.05$, **$p < 0.01$.

4.3 Conclusions

These collective data demonstrate that miR-191 expression supports T cell survival at homeostasis. When miR-191 is overexpressed, it protects T cells from AICD (Figure 5); in its absence, $CD8^+$ T cells are more susceptible to AICD (Figure 6). In both cases, T cell proliferation is unaffected, highlighting a particular role for miR-191 in supporting survival. Loss of miR-191 resulted in 30% and 50% decreases in the total number of $CD4^+$ and $CD8^+$ T cell in the peripheral lymph nodes, respectively (Figure 7). T_{Regs} were also reduced by 40%, but this loss had no impact on the homeostatic activation of either $CD4^+$ or $CD8^+$ cells. These peripheral changes could not be attributed to gross defects in thymic development, as loss of miR-191 at DN2 had only a modest impact on T cell development. Similarly, a minor reduction in DP cell numbers was observed, but no alteration to the proportion of key thymic subsets occurred (Figure 8).

Given the particular impact on $CD8^+$ T cells and T_{Regs}, it seemed likely that in the absence of miR-191, there was defective homeostatic signaling through the γ_c. A direct test of this hypothesis using an *in vitro* culture with limiting levels of cytokine revealed that miR-191-deficient cells were had insufficient responses to these cytokine signals (Figure 9). Further analysis revealed that while CD132 and total STAT5 levels were normal in the absence of miR-191, miR-191-deficient $CD8^+$ T cells had impaired phosphorylation of STAT5 in response to IL-7. STAT5 is a known mediator of pro-survival cytokine signals, so this defect suggested a mechanism through which miR-191-

deficiency resulted in enhanced cell death. However, STAT5 is not a target of miR-191, and miR-191 cannot alter phosphorylation directly. To identify the target of miR-191 that resulted in altered STAT5 signaling and reduced survival, a computational search followed by a RT-qPCR screen was conducted. This screen generated a single hit, the scaffolding protein *Irs1*, which is known to interact with CD132-containing cytokine receptors. IRS1 protein levels were up-regulated in miR-191-deficient T cells, and a 3'UTR luciferase assay demonstrated that (mRNA) *Irs1* is a direct target of miR-191 (Figure 10). Further, overexpression of IRS1 resulted in enhanced T cell death in culture with limiting amounts of IL-2, confirming that elevated IRS1 expression directly contributes to cell death. These data all suggest a model in which the loss of miR-191 results in defective homeostatic cytokine signaling due to the up-regulation of IRS1 and subsequent impairment in STAT5 phosphorylation. The modest defects in pro-survival cytokine signaling result in the establishment of a new peripheral equilibrium, with a reduction in T cell numbers in response to limiting amounts of pro-survival cytokines.

This new equilibrium, however, is tenuous. Experiments with the Bcl-2^{YFP} reporter animals revealed a modulator of unknown identity that synergizes with the loss of miR-191, resulting in exacerbated developmental defects and peripheral T cell losses (Figure 11). Further examination of cells expressing the reporter revealed that loss of miR-191 resulted in a disconnect between YFP expression and BCL2 protein expression (Figure 12). However, both YFP expression and death could be rescued *in vitro* with the

addition of IL-2, suggesting that the signaling pathway remains at least partially intact. Future studies are needed to elucidate the identity of the synergistic modulator and to further define the mechanism(s) through which miR-191 controls T cell homeostasis.

5. Discussion

5.1 Novel Tools for the Study of microRNAs

The data presented in Chapter 3 describe a series of novel tools for the manipulation of miRNA expression and for the identification of drugs that inhibit miRNA function.

5.1.1 Manipulation of microRNA expression

5.1.1.1 Modulation of overexpression using backbone switching

miRNA backbone switching provides enhanced flexibility for the overexpression of miRNAs. Overexpression levels can be fine-tuned to permit more physiologically relevant assays, but can also be pushed to extremes to exacerbate phenotypes and test the miRNA regulation network under great stress. Careful analysis of the expression levels permitted by particular backbones may also reveal novel sequences and secondary structures that are important for miRNA processing.

The enhanced miRNA expression seen with the mmu-miR-148a backbone suggests that it may serve as a starting point for the next generation of shRNAs. The current generation of shRNAs are based on the hsa-miR-30a backbone, which has almost complete pairing through the stem, as do shRNAs based on this design (Figure 13). However, there is no requirement for shRNAs to have almost complete pairing throughout the stem; indeed, complete pairing creates several disadvantages, such as stem-loop structures that are difficult to synthesize and sequence. Although efforts have

been made to ease cloning of hsa-miR-30a-based shRNAs (e.g. PCR and Linker Cloning designs)(*164*), the resulting hairpins are still difficult to grow in bacteria without mutation and are also difficult to sequence for confirmation that the shRNAs lack mutations. The mmu-miR-148a backbone avoids these issues; the structure of the miRNA has many bulges that permit easier synthesis and therefore enhanced cloning, which makes this backbone less toxic to bacteria and therefore less likely to be mutated and easier to sequence to confirm that the generated construct is mutation-free. However, the mmu-miR-148a backbone does have a drawback in that the secondary structure, with its bulges, is not amenable to all possible insert sequences. Further complicating the issue is that many of the shRNA designs currently available were selected based on clonability and were not computationally selected based on generating a shRNA with sufficient knockdown capabilities and limited off-target effects. Fortunately, such complications have not proven difficult to work around, and with further modification of the mmu-miR-148a backbone to enhance Dicer processing (Figure 13), this new generation of shRNAs should offer easier cloning and even better knockdown due to higher expression of the guide strand.

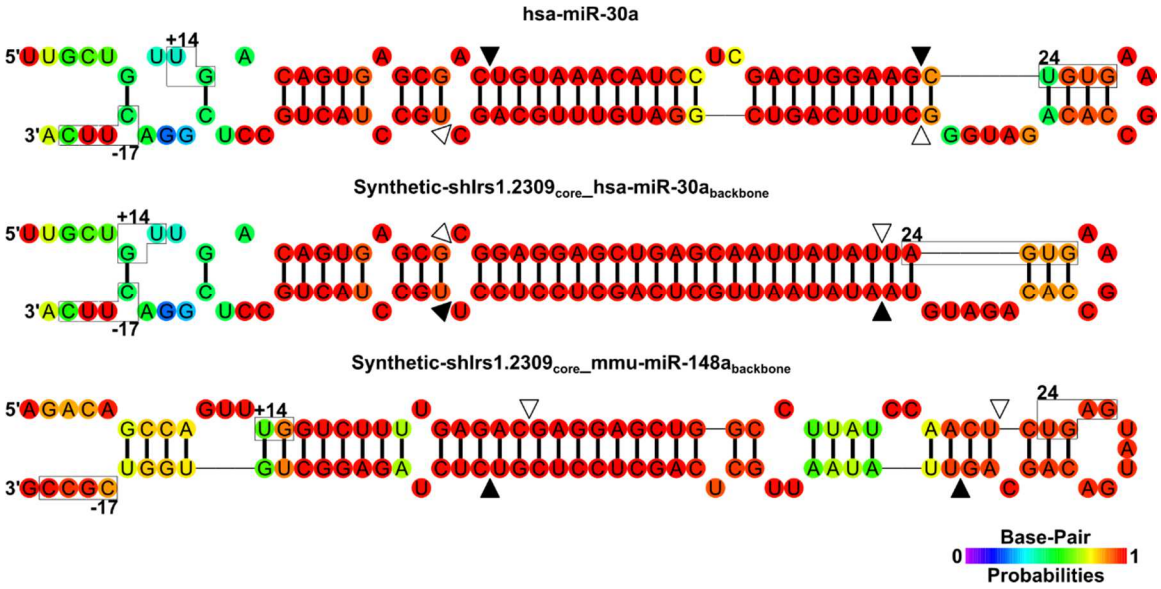

Figure 13: Placement of miR-191 into the miR-148a backbone enhances expression.

Figure 13. Placement of miR-191 into the miR-148a backbone enhances expression. *A*, Predicted secondary structures of hsa-miR-130a (top), synthetic-shIrs1.2309$_{core}$_miR-30$_{backbone}$ (middle), and synthetic-shIrs1.2309$_{core}$_miR-148a$_{backbone}$ (bottom) were generated by RNAFold and re-drawn. The sequence of the miR-148a backbone was altered to restrict movement within the loop to ensure the post-loop bulge is always exactly 2 bases from the 5′ end of the guide. Black triangles flank the sequence for the mature guide strand, while white triangles flank the passenger strand. Key sequence determinants of Drosha processing are highlighted with the base position relative to the first base of the mature 5′ strand indicated. Ideal determinants are UG at +14, UGUG at 24, and CNNC at -17.

5.1.1.2 Conditional deletion of single microRNAs

Although germline deletion of single miRNAs has revealed many informative immune phenotypes, it is difficult to ascribe such phenotypes solely to one cell type, given the interconnectedness of the immune system. Conditional knockouts provide a viable alternative to germline deletion because, by design, conditional knockouts isolate a genetic deletion to a particular cell type and/or to a particular time, with timing being highly desirable if one wishes to avoid developmental phenotypes. The data shown for conditional deletion of mmu-miR-191 (Figures 2A-C) and for mmu-miR-23a (ref. ((*107*))) demonstrate that the design principles described in Chapter 3 allow for the truly conditional deletion of single miRNAs, even when those miRNAs reside within a cluster. Future studies of miRNA clusters can benefit from these design principles as experiments can then be designed to distinguish the individual functions of clustered miRNAs.

5.1.2 Drugs that alter microRNA expression and/or function

While previous efforts have generated miRNA activity reporters, those reporters used bioluminescence (i.e. luciferase activity) as a readout(*165*). Luciferase activity has a large dynamic range and permits *in vivo* imaging, but the required substrate reagents (e.g. D-luciferine and colenterazine) are expensive and therefore not ideal reagents for large-scale screening systems. The fluorescent reporters described in Chapter 3 are much more cost-effective and could be modified for *in vivo* use by swapping ZsGreen1 for the

recently described near-infrared fluorescent protein iRFP(*153*). Additionally, a multi-color flow cytometric-based screening system is high-throughput and permits the additional assessment of molecule toxicity. Thus, although the initial screen of over 3000 compounds did not yield a novel miRNA inhibitor, it did demonstrate the feasibility of the system for high-throughput screening and the power of the validation assay to eliminate false positives.

5.2 The role of miR-191 in T Cell Survival at Homeostasis

The data presented in Chapter 4 demonstrate that the previously uncharacterized miRNA miR-191 supports the homeostatic maintenance of the cellular immune system. miR-191 was shown to support cytokine-dependent naïve, memory, and regulatory T cell survival at homeostasis by controlling the levels of IRS1 and thereby the activation kinetics of STAT5.

5.2.1 Overexpression of IRS1 modulates γ_c cytokine signals

IRS1 was initially described as the principal substrate for signal transduction downstream of the insulin receptor(*166*). Signaling through the insulin receptor phosphorylates critical tyrosines on IRS1, resulting in the recruitment and activation of various src homology 2 (SH2) domain-containing proteins, including the p85 subunit of phosphoinositide-3 kinase (PI3 kinase)(*158*) and GRB2(*159*). An early study in human T cells found that IRS1 associates with JAK1 and JAK3 in the absence of cytokine, and that

the addition of IL-2 or IL-4 increases this association(*160*). Further, this same study revealed tyrosine phosphorylation of IRS1 following IL-2, IL-4, IL-7, and IL-15 and also that cytokine signaling induces p85 PI3 kinase association with IRS1. Later experiments demonstrated that IL-7 signaling in human thymocytes drives increased association between IRS1 and JAK1, JAK3, and p85(*167*). These data support a model in which IRS1 positions at the cytokine receptor by interacting with JAK1 and JAK3. Following cytokine signaling, IRS1 activates p85 and GRB2, driving both PI3 kinase and MAPK signaling. Indeed, through its pleckstrin homology (PH) domain, IRS1 is sufficient to target PI3 kinase to AKT(*168*). PI3 kinase-mediated mTOR activation results in hyperphosphorylation of N-terminal serine residues on IRS1. This hyperphosphorylation then leads to IRS1 associating with FBW8 (part of the cullin RING E3 ubiquitin ligase 7 complex), resulting in poly-ubiquitination and proteasomal degradation of IRS1(*169*), thereby self-regulating the signaling cascade by preventing continued upstream signal input.

Enhanced expression of IRS1 results in defective phosphorylation of STAT5 following IL-7 signaling (Figure 9F). Given the known interaction partners for IRS1, there are least two mutually-exclusive models that can explain this data: a general deficiency model in which all signals downstream of the cytokine receptor are disrupted due to sequestration of JAK1 and JAK3 away from the receptor (Figure 14A), and an altered signaling model where the wild-type ratio of downstream signaling pathways

becomes skewed with enhanced localization of p85 and GRB2 at the receptor (Figure 14B). To directly assess which of the two models is valid requires a determination of the relative association of JAK1 and JAK3 with the cytokine receptor following manipulation of IRS1 levels. The simplest experimental system takes advantage of 32D Clone 3 (32Dcl3) cells, a myeloid cell line derived from long-term bone marrow cultures of C3H/HeJ mice(*170*), because this cell line lacks IRS1 expression (*171*). 32Dcl3 cells express CD132, so the introduction of IRS1 permits IL-4 signaling. Previous studies in which Fms-like tyrosine kinase 3 (FLT-3; CD135) was introduced into 32Dcl3 also confirmed that these cells express STAT5(*172*). Thus, it would be possible to introduce CD127 or CD122 into 32Dcl3 cells to permit IL-7 or IL-2 and IL-15 signaling, respectively. In order to determine the association of JAK1 and JAK3 with the cytokine receptor, the introduced CD127 or CD122 could be tagged with the small Twin-Strep-tag®(*173*) enabling isolation of the tagged protein in conditions mild enough to preserve large protein complexes; an expression vector has already been designed (Figure 14C). Western blotting could then be used to determine the association of various molecules with the tagged receptor in the presence or absence of IRS1. Loss of JAK1 and/or JAK3 association with the cytokine receptor following the introduction of IRS1 would suggest that the general deficiency model is correct, while loss of STAT5 association with the cytokine receptor would indicate that the altered signaling model is correct. Successful execution of these studies in 32Dcl3 cells could prompt additional experiments in

primary cells, although these studies would require the generation of animals with tagged cytokine receptors. Primary experiments afford the opportunity to gauge how modest alterations to IRS1 levels impact the signaling downstream of CD132-containing cytokine receptors. Should the tag interfere with signaling or the experimental conditions not permit isolation of key components of the complex, a proximity ligation assay (PLA) could be used(*174, 175*). The PLA assay relies on DNA tags attached to antibodies against the two proteins of interest. If the antibodies are close enough to interact, the DNA tags can bind each other and, with the introduction of connector oligonucleotides, will form a circle that can be amplified by rolling circle amplification. The amplified circles are then detected via the addition of a fluorescent tag, allowing the protein-protein interaction to be visualized *in situ*. PLA is a powerful tool but depends heavily on the availability of antibodies to the proteins of interest, items that are currently not widely available for IRS1. Either the pull-down or PLA assays could provide direct evidence for how IRS1 modulates the assembly of the cytokine receptor complex.

Direct evidence for IRS1-driven alterations in JAK1/3 or STAT5 associations with the cytokine receptor will help distinguish between the two proposed models. However, there are many effects downstream of the receptor complex that permit the assessment of the functional consequences of IRS1 on CD132-containing cytokine receptor signaling. Both IL-2 and IL-15 signal through trimeric complexes, which have higher affinity for

the cytokine, but the actual signaling takes place through two shared components: CD122 and CD132. CD122 and CD132 have no inherent kinase activity themselves and rely on their association with JAK1 and JAK3, respectively. Following CD122 binding extracellular cytokine, CD132, which is already bound to JAK3, is able to bind the cytokine and concomitantly associates with CD122, thereby brining JAK1 and JAK3 into close proximity. JAK1 and JAK3 then undergo mutual phosphorylation, thus permitting phosphorylation of critical tyrosine residues on CD122, enabling SH2 domain-containing protein binding and subsequent tyrosine phosphorylation of those proteins by JAK1/3. There are several major pathways activated by JAK1/3 in association with CD122: the activation of STATs, particularly STAT5, which drives both proliferative and anti-apoptotic responses through the direct regulation of a number of genes, including *Cdk6* and *Bcl2*(*65*); PI3 kinase signaling, through activation of p85, which can drive anti-apoptotic signaling through activation of RHOA(*176*) or IKZF3(*177*) and promote proliferation through activation of AKT(*178, 179*); the p42/44 mitogen-activated protein kinase (MAPK) signaling cascade through Ras family activation either by PI3 kinase or by the GRB2-SOS complex(*180-182*) to promote proliferation; and the p38MAPK/p54 c-Jun N-terminal kinase (JNK) cascade via activation of JAK3-associated PYK2(*183*), which drives proliferation and stress responses. Of note, there is data to suggest that a proline-rich region in the membrane proximal domain of CD132 provides an uncharacterized signal that leads to *Bcl-2* induction(*184, 185*) in the absence of JAK3, possibly through the

activation of LCK by an unidentified phosphatase, and subsequent activation of PI3 kinase(*186, 187*).

IL-7 signaling is quite similar to IL-2/-15 signaling, except that CD127 is used in place of CD122. IL-7 has been shown to activate the following: STAT5(*188*); PI3 kinase(*189, 190*), although several groups have shown that detection of AKT phosphorylation requires prolonged (~24 hours) IL-7 stimulation(*191-193*); p38MAPK(*194*); and LCK(*188, 195*). Many of these pathways are expected to function in the same manner as signals mediated through CD122, although there is a study that suggests IL-7-induced AKT activation does not require the intracellular portion of CD127, but direct evidence is not provided(*196*).

Clearly much work remains, both to describe the factors that associate with the cytokine receptors under various conditions and the transcriptional and biochemical outcomes of that signaling. However, given the known binding partners of IRS1, several predictions can be made based on the general deficiency and altered signaling models. IL-7, IL-2, and IL-15 signaling should elicit phosphorylation of AKT, LCK, p38MAPK, and ERK1/2. An experiment similar to the one performed in Figure 9F could examine the phosphorylation of these proteins following stimulation of 32Dcl3 cells in the presence or absence of IRS1. Impaired or enhanced phosphorylation would suggest the general deficiency model or the altered signaling model, respectively. It is also possible that some phosphorylation events would be impaired, while others are normal or enhanced.

Such data would demand new model; for example, if LCK phosphorylation were normal while the other signaling molecules were impaired, it would suggest that p85 and GRB2 are limiting factors, and enhanced expression of IRS1 results in empty IRS1 complexes associating with the cytokine receptor(s), preventing STAT5, PI3 kinase, and MAPK pathways, but not LCK. Transcript levels of STAT5 target genes (e.g. *Bcl2*) can confirm the functional consequence of altered STAT5 phosphorylation. These studies should be confirmed in primary cells, again looking for phosphorylation of AKT, LCK, p38MAPK, and ERK1/2 and STAT5 target gene transcription following cytokine stimulation of wild-type and miR-191-deficient naïve $CD8^+$ (IL-7), memory $CD8^+$ (IL-15) T cells, and T_{Regs} (IL-2). These experiments would need to be further validated with overexpression of IRS1 in wild-type cells or shRNA-mediated knockdown of IRS1 in miR-191-deficient cells to link the expression of IRS1 directly to these altered phosphorylation events.

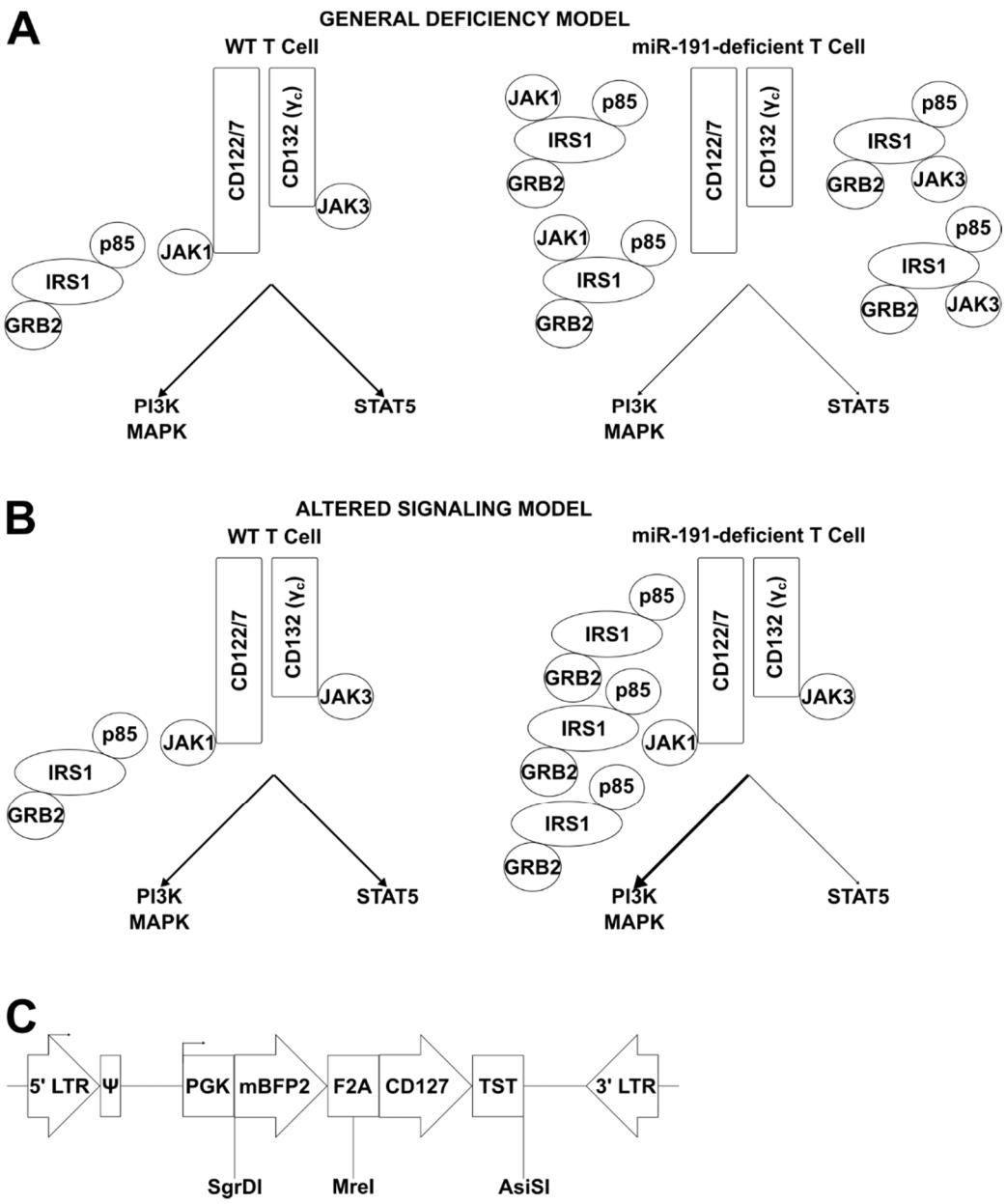

Figure 14: Models for the role of IRS1 in γ_c cytokine signaling.

FIGURE 14. Models for the role of IRS1 in γ_c cytokine signaling.

A, The General Deficiency Model: IRS1 binds JAK1 and JAK3 and prevents their associations with the cytokine receptor, hindering all downstream signals. *B*, The Altered Signaling Model: IRS1 binding to JAK1/3 at the cytokine receptor prevents STAT5 binding and subsequent phosphorylation, but promotes PI3K and MAPK signals. *C*, Linear representation of a fragment from the MSCV vector showing the design for tagged CD127. Cut sites indicated were selected based on their relative rarity in the human and mouse transcriptomes, thus this vector should be ideal for the expression of many tagged proteins.

5.2.2 Loss of miR-191 impairs T cell homeostasis

Understanding the full-extent to which cytokine signals are altered sets the stage for future studies to examine the mechanism(s) through which the loss of miR-191 results in defective T cell homeostasis. The data presented in Figures 7-8 show diminished naïve, regulatory, and memory cell numbers, and that these losses stem from defective cytokine signaling, which is at least partly attributable to deficient STAT5 phosphorylation. STAT5 is an important regulator of survival downstream of cytokine signaling. Mice lacking the entire Stat5 locus (STAT5-deficient) exhibit almost complete perinatal lethality, with the rare surviving mice exhibiting SCID-type symptoms and a near absence of thymocytes, in addition to dramatic reductions in T, B, and NK cells in the periphery (*197*). In the present study, proportionately normal thymic development, but a 30% decrease in thymic cellularity and 40-50% losses in regulatory and $CD8^+$ T cells in the peripheral lymph nodes were observed. These data very closely resemble early studies of "STAT5-deficient" mice in which the targeting strategy actually resulted in N-terminally truncated but partially functional STAT5 (STAT5ΔN; refs. (*155, 198*)). STAT5ΔN mice have grossly normal thymic development, but exhibit 50% reductions in thymocyte numbers(*199*) and have preferential loss of $CD8^+$ and T_{Regs} in the periphery(*155, 156*). Similarly, the inhibition of STAT5 tetramer formation leads to preferential loss of $CD8^+$ T cells in the periphery and fewer functional regulatory cells (*65*). Thus, modest perturbations in STAT5 signaling lead to significant defects in T cell

survival, and miR-191 regulation of IRS1 downstream of cytokine signaling is a key rheostat of homeostatic STAT5 signaling.

However, if the altered signaling model is correct, a question is raised: if pathways other than STAT5 are important for pro-survival signaling and these pathways are up-regulated, why do we still see reduced T cell numbers? There are several possible answers that still fit within the model and all focus on the fact that survival is driven by a complex network of interactions, many of which are designed to regulate mitochondrial integrity. Glucose metabolism is one mechanism of maintaining mitochondrial integrity and homeostasis(*200*). AKT activation following IL-7 engagement is known to positively regulate the expression of the glucose transporter GLUT1 on the surface of T cells(*193, 201*). GLUT1 levels should be compared for wild-type and miR-191-deficient T cells both directly *ex vivo* and *in vitro* to ensure GLUT1 up-regulation is normal or enhanced in the absence of miR-191. If not, impaired glucose metabolism could help explain the observed T cell loss and would suggest a novel role for STAT5 in supporting glucose metabolism, or perhaps more likely, that a novel target of miR-191 impedes the up-regulation of GLUT1.

If glucose metabolism is not impaired, another explanation lies in the regulation of the Bcl-2 family members. Briefly, the Bcl-2 family consists of both anti-apoptotic members (e.g. BCL-2, BCL-XL, and MCL-1) and pro-apoptotic members (e.g. BAX, BAK, BID, BAD, and BIM). The anti-apoptotic members bind the pro-apoptotic members and

prevent their activation, resulting in the disruption of mitochondrial integrity, release of cytochourome c, caspase activation, and apoptosis. The various anti-apoptotic members have different expression patterns and therefore variable importance in T development and activation. MCL-1 is important at all stages of T cell development(*202, 203*), while BCL-XL is mostly important in activated cells(*204*) and has a minor role in development(*205*), and BCL-2 is important in DN and mature resting cells(*206, 207*). Recent work has shown that both IL-2 and IL-7 signals promote the expression of all anti-apoptotic members, with STAT5 playing a dominant role in their expression(*208*). However, there are differences in the degree to which various γ_c cytokines up-regulate anti-apoptotic proteins, a process dependent on whether the T cell is $CD4^+$ or $CD8^+$ and whether it is a naïve or memory cell(*209*); whether such differences reflect inherent differences in the signaling pathways downstream of the different receptors or whether it is a consequence of differential receptor expression remains to be investigated. The anti-apoptotic members only represent half of the equation, as γ_c cytokine signals have also been demonstrated to suppress the functions of the pro-apoptic members as follows: AKT activation leads to serine phosphorylation of BAD, preventing its mitochondrial translocation(*210, 211*); MAPK activation induces serine phosphorylation of BIM, reducing its apoptotic activity and association with BAX(*212*), while AKT phosphorylates FKHOUR-L1, thereby preventing transcription of *Bim*(*213*); and IL-7 has been shown to suppress the expression of BAX(*214*). These observations highlight that

the prevention of apoptosis is as much about the up-regulation of anti-apoptosis factors as the suppression of pro-apoptosis factors and also suggest a model in which STAT5 plays a more dominant role in the former and the other pathways control the latter. This suggests a model in which the T cell loss observed in miR-191-deficient T cells is due to loss of BCL-2 and/or MCL1 expression despite decreased BAD, BIM, and/or BAX function. The expression and phosphorylation status of these factors needs to be examined to define the exact death mechanism(s) at play in the absence of miR-191. Unfortunately, the data from the Bcl-2^{YFP} animals (Figures 11-12) do not offer any insight; while Bcl-2 YFP expression is dramatically downregulated with the loss of miR-191, BCL-2 levels are normal. While different half-lives for YFP and BCL2 or stabilization of BCL-2 by IRS1(*215*) could explain this observation, neither explains the difference in survival, though perhaps measuring MCL-1 levels could clarify this issue. Identification of the modulation factor will also likely prove interesting. Continued efforts to discover the integration site can make use of the fact that these animals are agouti, despite being back-crossed to C57BL/6 for more than 10 generations, which suggests an integration near the coat color locus. There are a small number of animals that screen positive for YFP via PCR but show no protein. These animals may not express the tandem repeats of the transgene and should be examined with Splinkerette PCR. Presumably, the modulation factor plays a role in the expression of a key pro-survival molecule whose loss can be compensated for in otherwise wild-type hosts, but the loss of miR-191 pushes

the system past a tipping point, resulting in the dramatic cell losses observed. If the general deficiency model is correct, T cell homeostasis is impaired likely due to a deficiency in key pro-survival signals, so it is only the degree to which these signals are impaired that determines the T cell loss.

Whichever model is correct, future work should explore whether enhanced IRS1 expression leads to defective cytokine-driven proliferation. Although no alterations to proliferation were observed (Figures 5-6), those experiments were performed in the context of TCR stimulation. Given that naïve $CD8^+$ T cells will proliferate in response to IL-2 or the combination of IL-7 and IL-15(*65*), so the treatment of wild-type and miR-191-deficient naïve $CD8^+$ T cells with these cytokines directly *ex vivo* and would reveal potentially significant differences in proliferation. If proliferation is still normal, it suggests that there is either a difference in the relative thresholds of signaling required to drive proliferation versus survival or the presence of an additional pathway that drives proliferation but is one unaltered by the increase in IRS1. Future studies might also investigate whether defective cytokine signaling alters the TCR repertoire or TCR responsiveness of the surviving T cells. Recent work has suggested that TCR affinity for self-peptide:MHC, as assessed by CD5 expression, inversely correlates with IL-7 dependence for homeostatic proliferation (i.e. cells with higher CD5 expression require less IL-7 to undergo homeostatic proliferation and vice versa)(*53*). In miR-$191^{f/f}$LckCre$^+$ or miR-$191^{f/f}$CD4Cre$^+$ animals, it is possible that defective IL-7 signaling has selected for

T cells with higher CD5 expression. Such selection would have serious implications for the diversity of the TCR repertoire and therefore the ability of miR-191-deficient T cells to combat infections, perhaps even altering the effectiveness of the primary or memory responses(*42*). Together, such findings have powerful implications for the ability of patients with modest perturbations in T cell homeostasis to effectively fight disease and respond to vaccination.

5.2.3 Future studies: Other roles for miR-191

5.2.3.1 miR-191 in effector T cells

Without a firm understanding of the mechanism(s) at play following the loss of miR-191 it is difficult to anticipate what role(s) miR-191 may play in effector T cell differentiation and/or function. TCR signaling appears to be intact, and miR-191-deficient cells do not exhibit defects in TCR-induced proliferation (Figures 6C-D), but IRS1 may interact with more than CD132-containing receptors) Preliminary experiments testing the impact of miR-191-deficiency on the phosphorylation of other STAT molecules through different cytokine signaling pathways would provide an excellent idea of what other facets of T cell biology alterations in miR-191 expression are likely to impact. The IL-4 receptor makes a particularly interesting target as it is able to bind IRS1 directly(*216*). Given the relatively modest defect in the phosphorylation of STAT5 observed downstream of IL-7 signaling, it seems likely that *in vitro* differentiation assays, where exogenous cytokines are added at saturation levels, will not be

appropriate for testing the impact of miR-191-deficiency on differentiation. It may be possible to use limiting levels of cytokines of interest and still perform *in vitro* assays, but it may take some time to optimize the system to see adequate levels of differentiation in wild-type cells so that comparisons can be made. Differentiation in the context of infection may prove useful, although the experimental design will have to ensure equal numbers of wild-type and miR-191-deficient T cells going into the assay to rule out differences caused by defective homeostasis. It seems likely that miR-191-deficient T cells will experience enhanced cell death and will not receive adequate signaling to enter the memory compartment, which would only add to the deficiency in memory T cells. Infections or immunizations using antigens with well-established tetramers would enable clear tracking of effector cell expansion, memory formation, and memory maintenance, thus teasing apart the role(s) of miR-191 in this complicated process, and perhaps enabling the discovery of novel factors.

5.2.3.2 miR-191 in other hematopoietic cells

The data presented in these studies evaluated the specific role that miR-191 plays in T cell development and function using a conditional deletion system. Future studies should also explore the role of miR-191 in other immune cell types by crossing the miR-191$^{f/f}$ mice to mice with different conditional Cre expression. miR-191 expression data (Figure 4) revealed that, while most hematopoietic cells are interesting targets, B cells and NK cells may prove most interesting as they have particularly high expression of

miR-191 and are both known to require γ_c cytokine signals for development and/or function. B cells are known to require IL-7 for their development, but do not appear to require γ_c cytokines for homeostasis. However, they do respond to IL-4 and IL-21 during antibody responses and it would be interesting to see how the loss of miR-191 alters antibody responses.

5.2.3.3 Potential therapeutic value of altering miR-191 expression

The data presented in Chapter 4 demonstrate that the manipulation of miR-191 alters immune homeostasis without leading to overt immunodeficiency or autoimmunity. As the loss of miR-191 and the resultant overexpression of IRS1 leaves T cells with an increased susceptibility to cell death, antagomiR-type treatments to suppress the function of miR-191 or drugs that mimic or enhance IRS1 function may serve as an effective kill switch, permitting the elimination of therapeutic T cells after the completion of their effector functions. The transfer of allogenic T cells to eliminate tumor cells following immune-ablating irradiation would benefit from such a kill switch to prevent graft versus host disease. Chimeric antigen receptor (CAR) expressing T cells may also benefit from a post-effector kill switch to reduce the risk of autoimmunity. Pilot studies using transfers of T cells from miR-191$^{f/f}$LckCre^{+} will provide evidence for the therapeutic potential of an IRS1-mediated kill switch. With demonstration of efficacy, follow-up work should focus on therapeutically relevant and feasible methods for miR-191 and/or IRS1 manipulation.

It will also be important to determine whether therapeutic ablation of miR-191 can ameliorate ongoing autoimmune disease. Current treatments for autoimmune disease often focus on generalized corticosteroid-mediated suppression of T and/or B lymphocytes, or on the specific, antibody-mediation ablation of these cells. Such therapies, while often effective to slow the course of disease, are not curative and leave patients immunocompromised. It is possible that ablation of miR-191 can slow the course of disease while leaving the patients immunocompetent. The mouse model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), is a good model to test the suitability of miR-191 ablation for therapeutic treatment of autoimmunity. EAE is inducible, has a well-defined disease course and positive control therapies (e.g. prednisone), and is T cell-driven. A pilot study should compare the effect of positive control therapy versus miR-191 antogomiR treatment on relapse frequency and severity in proteolipid protein ($PLP_{139-151}$)/CFA-induced EAE in female SJL mice.

Suppression of miR-191 does not represent the only possible therapeutic manipulation. Knowing that up-regulation of miR-191 enhances T cell survival suggestions that promoting miR-191 expression or inhibiting IRS1 function may prove useful to therapeutically maintain both naïve and memory T cells in older individuals. Such enhanced maintenance would permit improved responses to vaccination against novel pathogens or the sustained ability to fight those previously encountered. Transfers of antigen specific naïve and memory T cells overexpressing miR-191 (e.g. with a

lentiviral construct) would allow for an evaluation of whether enhanced miR-191 expression improves the maintenance and immune responses in aged recipients.

While success in therapeutic models would offer much hope for treating patients, great caution must be exercised, as it is clear that the new homeostatic equilibrium achieved with the ablation of miR-191 is tenuous, and other subtle alterations can collapse the cellular immune system. Thus, future studies must work to identify and characterize the factors that synergize with miR-191 to control T cell homeostasis and to understand how these factors work together to control homeostatic equilibrium. Such understanding will prove invaluable in screening patients for novel, minor immunodeficiencies and for tailoring immunomodulatory therapies to best meet a patient's therapeutic needs while promoting healthy immune function throughout the life of the individual.

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Biography

I was born in Dallas, Texas on December 2nd, 1986 to Matthew and Suzanne Lykken, and I have two younger siblings: Sara and Karl. I graduated from Wheaton North High School in Wheaton, Illinois in June 2005. I then attended Pomona College in Claremont, California where I studied Molecular Biology and graduated cum laude with a Bachelor of Arts in May 2009. I proceeded to enter the Immunology program at Duke University in Durham, North Carolina. I spent the next few years studying the role of microRNAs in T cell biology with Dr. Qi-Jing Li. While working towards my Ph.D., I met and married my wife, Jacquie, who received her Ph.D. in Immunology from Duke University in 2015. We currently reside in Durham, North Carolina with our dog, Molly. I defended my dissertation on June 28th, 2015 with the faculty of the Duke University Department of Immunology.

I have published or submitted for publication the following manuscripts:

1. Lykken EA and Li QJ. microRNA miR-191 Supports T Cell Survival Following Common Gamma Chain Signaling. In revision at *J. Biol. Chem.*

2. Xu X, Zhang Y, Jasper J, Lykken E, Alexander P, Markowitz G, McDonnell D, Li QJ, and Wang XF. MiR-148a functions to suppress metastasis and serves as a prognostic indicator in triple-negative breast cancer. *Oncotarget* **7**(15): 20381-20394 (2016).

3. Zhang B, Liu SQ, Li C, Lykken E, Jiang S, Wong E, Gong Z, Tao Z, Zhu B, Wan Y, and Li QJ. MicroRNA-23a Curbs Necrosis during Early T Cell Activation by Enforcing Intracellular Reactive Oxygen Species Equilibrium. *Immunity* **44**(3): 568-581 (2016).

4. Li C, Jiang S, Liu SQ, Lykken E, Zhao LT, Sevilla J, Zhu B, and Li QJ. MeCP2 enforces Foxp3 expression to promote regulatory T cells' resilience to inflammation. *Proc. Natl. Acad. Sci. USA* **111**(27): E2807-E2816 (2014).

5. Jiang S, Li C, McRae G, Lykken E, Sevilla J, Liu SQ, Wan Y, and Li QJ. MeCP2 reinforces STAT3 signaling and the generation of effector CD4+ T cells by promoting miR-124-mediated suppression of SOCS5. *Sci. Signal.* **7**(316): ra25 (2014).

6. Jiang S, Li C, Olive V, Lykken E, Feng F, Sevilla J, Wan Y, He L, and Li QJ. Molecular dissection of the miR-17-92 cluster's critical dual roles in promoting Th1 responses and preventing inducible Treg differentiation. *Blood* **118**(20): 5487-5497 (2011).

7. Lykken EA and Li QJ. microRNAs at the regulatory frontier: an investigation into how microRNAs impact the development and effector functions of CD4 T cells. *Immunol. Res.* **49**(1-3): 87-96 (2011).

I received the following fellowships and academic honors while at Duke University:

1. AAI Southeastern Immunology Symposium, AAI Young Investigator Award, Duke University, Durham, NC (June 2016)

2. RIKEN Center for Integrative Medical Sciences Summer Program and International Symposium on Immunology Chosen Participant and Best Presentation Award Winner, RIKEN Institute, Yokohama, Japan (June 2015)

3. Conference Travel Fellowship Award Recipient, Duke University, Durham, NC (May 2015)

4. Duke University Scholars Fellowship, Duke University, Durham, NC (2009-2013)

5. James B. Duke Scholarship, Duke University, Durham, NC (2009-2013)

6. Duke Medical Center Chancellor's Scholarship, Duke University, Durham, NC (2009)

7. NIH Ruth L. Kirschstein National Research Service (T32) Award Recipient, Duke University, Durham, NC (September 2009 – August 2011)