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Asymmetric T Cell Division and the Self-renewal of Specific Immunity

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Abstract
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ASYMMETRIC T CELL DIVISION AND THE SELF-RENEWAL OF SPECIFIC IMMUNITY

Maria L. Ciocca

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Dedication

To my mother. For reading to me everyday.

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ABSTRACT

Asymmetric T Cell Division and the Self-renewal of Specific Immunity

Maria L. Ciocca

Steven L. Reiner

During clonal selection of a T cell in response to infection of a host with an invasive pathogen, the host must respond by producing at least two required and disparate cell populations – one that is responsible for controlling the current infection and another that is required to retain the T cell clone for protection against future insults. This diversity within the T cell response may be generated through the use of asymmetric cell division. How T cells may use asymmetric division and to what extent this molecular process plays a role in adaptive immunity is not well understood. Here we suggest that asymmetric division during the initial T cell response segregates proteins by a unique mechanism that involves unequal degradation of a fate-determinate secondary to polarized segregation of the protein degradation machinery. Furthermore, we provide data to extend the principle of asymmetric division to the memory cell response, suggesting that certain antigen-experienced lymphocytes can re-iteratively undergo this process to generate diversity when once again faced with a pathogenic challenge. Together, these results suggest highly conserved principles of stem-cell biology may be regulating the generation of diversity in the adaptive immune response both during primary and recurrent infection.

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CHAPTER 1: Introduction

Generation of a protective immune response

All living organisms encounter invasion by foreign pathogens on a daily basis. In higher organisms, two layers of protective immunity exist to respond to these external threats. In the initial, or early, phase of a reaction to a pathogen the innate immune response provides a broad, non-specific layer of protection against microbes. The recognition of highly-conserved fragments of foreign pathogens by clonally identical cells that express genetically encoded receptors allows for the rapid recognition of invasive microbes by the host (Medzhitov and Janeway, 2002). This genetic encoding of receptors that recognize commonly encountered threats allows for an immediate response by this arm of the immune system. If these mechanisms of early protection fail to clear the foreign microbe, however, the adaptive immune response becomes critical for protecting the host.

The adaptive immune response provides a unique specificity that allows for recognition of particular fragments of individual pathogens, not broad, highly-conserved fragments. Prior to infection, the host has a large and diverse set of T and B lymphocytes. Each lymphocyte harbors a unique receptor generated through V(D)J recombination and somatic hypermutation. Each lymphocyte, therefore, has the potential to recognize a distinct antigen corresponding to a fragment of microbe, or rather a fragment of non-self, that may be encountered during the host's lifetime. This broad protection against such a

diverse array of potential pathogens comes at a cost to the host. Any given invading microbe will only be recognized by a very small number of lymphocytes (<0.01%) (Janeway, 2005). During the early course of an infection, therefore, a lymphocyte with a receptor that is able to uniquely recognize the invading microbe must be recruited into an immune response by presentation of a cognate antigen by an antigen-presenting cell to that unique lymphocyte. The recruited lymphocyte then must undergo a proliferative burst in order to generate millions of cells with the identical receptor to allow for efficient control of the microbe. This clonal selection and recruitment of the T or B cell that can recognize the pathogen is critical to the protection of the host. The generation of millions of cells harboring the specific receptor is what arms the host with the capacity to fight the impending infection. Coincident with amplification, the responding lymphocyte produces a diverse set of responding progeny.

Heterogeneity and the T cell response

While a critical aspect of the adaptive immune response is the amplification and proliferation of the clonally selected cell, a hallmark of the adaptive immune response is also the generation of diverse populations of responding lymphocytes. This is highlighted by the heterogeneity generated during a T cell response, which will be the focus of the remainder of this chapter.

In response to particular infections, CD4+ (helper) T cells recruited into an immune response can generate several cell populations: Th1, Th2, Th17, and iTreg cells

can all be generated from naïve CD4+ T cells, depending on the type of threat encountered. The diversity of these helper T cell subsets is controlled by a diverse network of extracellular signals and transcriptional programs (Zhu and Paul, 2008; Reiner, 2009).

CD8+ (killer) T cells also must generate diversity during a primary immune response. During an initial response to pathogen, the host must produce at least two phenotypically and functionally distinct populations – those that are responsible for controlling the current threat (effector cells), as well as a population that will retain the host's capacity to respond to that particular pathogen (memory cells), should it be re-encountered. Whether these two populations arise from the same or different T cell clones has been difficult to establish. Recent data, however, suggests that indeed one T cell clone can give rise to both these effector and memory T cell populations (Schepers et al., 2008; Gerlach et al., 2010), and perhaps that even one cell can give rise to both CD8+ populations (Chang et al., 2007; Stemberger et al., 2007). This diversification results in at least two populations – the effector cells and memory cells. The two populations have several distinctions, and can be distinguished by their cell surface profiles as well as their transcriptional profiles. The diversification between effector and memory appears to be regulated by several fate-determining transcription factors (Pearce et al., 2003; Intlekofer et al., 2005; Joshi et al., 2007; Kallies et al., 2009; Rutishauser et al., 2009; Shin et al., 2009; Banerjee et al., 2010; Zhou et al., 2010), often in a dose dependent manor (Joshi et al., 2011). The mechanisms that aid in the generation of these two populations from a single cell are just beginning to be worked out.

As the immune response clears the pathogenic threat, the effector population is rapidly lost during the contraction phase. The cells that persist after the clearance of the infection are, by definition, memory T cells. This population provides protection to the host in the setting of secondary infections. It is likely, however, that these memory cells are present in small amounts before the contraction of the effector cell population (Kaech and Ahmed, 2001; Kaech et al., 2003). Re-encounter with pathogen results in activation of these long-lived memory cells. Memory cells, which are phenotypically distinct from naïve, or antigen inexperienced, cells can live for long periods of time in the absence of antigen, and are poised to respond should secondary infection occur. Analogous to their inexperienced predecessors, the long-lived memory cells must again produce two distinct cell populations – one to protect against the impending infection and another to perpetuate the maintenance of the T cell clone. This re-iterative nature of the T cell response is analogous to the function of an adult tissue stem cell, whereby a cell must, upon demand, produce a population of cells that will go on to become terminally differentiated progeny while simultaneously producing a population of cells to maintain the stem cell pool.

Development of an Immune Response

Much work has been done to understand the diversity generated during an immune response, but how this diversity is generated is still poorly understood. At least two models have been proposed to explain the generation of memory cells during a

primary immune response; a linear differentiation model and a divergent development model (Figure 1.1) (Gerlach et al., 2011). A linear differentiation model predicts that all the initial cells that arise after T cell recruitment into a primary immune response are effector cells. While the overwhelming majority of effector cells are lost through apoptosis during contraction, a minority survive and persist as memory cells (Wherry et al., 2003; Bannard et al., 2009). This model may suggest that memory cells would therefore arise predominately during the contraction phase (Stemberger et al., 2009). It has been suggested, however, that heterogeneity can be observed very early within the immune response, as early as 3-5 days after infection, and that discernable populations at this time have different propensity to develop into memory cells (Kaech et al., 2003). During the early response to infection, the responding cells can be segregated based on their relative levels of CD127 (Kaech et al., 2003; Huster et al., 2004), KLRG1 (Joshi et al., 2007; Sarkar et al., 2008), and CD25 (Kalia et al., 2010). Upon transfer, the cells with higher levels of CD127, lower levels of KLRG1, or lower levels of CD25 have a higher probability of surviving to become long-live memory cells with protective capacity. This early heterogeneity is suggestive of a process whereby cells need not go through an effector cell stage to become long-lived memory cells, as memory cell formation appears to be an early feature of the immune response. This favors a divergent development model.

In order for two populations to arise during an immune response, it is possible that two different naïve T cell clones are activated. In this way, intrinsic differences between the T cells or the stimulus received could result in different propensities toward

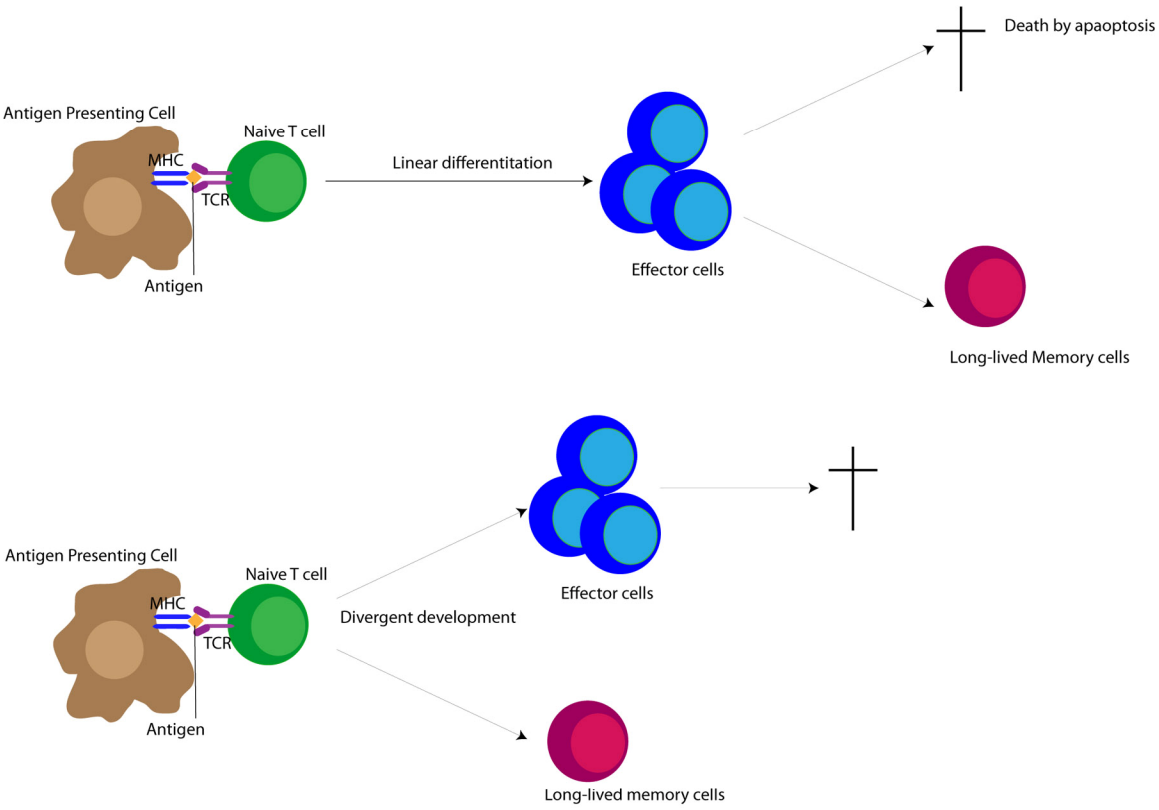

Figure 1.1. **Development of a T cell response.**
When a naïve T cell responds to an invading pathogen via presentation of antigen through an antigen-presenting cell, the stimulation of the naïve cell through the T cell receptor results in activation of that cell and triggers a developmental program. In a linear differentiation model, all cells pass through an effector cell stage. After the clonal expansion of effector cells to clear the invading pathogen, most of the cells die through apoptosis. The cells that remain after contraction become the long-lived memory cell population. In a divergent development model, however, not all cells pass through an effector cell phase. The activation of a naïve T cell, rather, results in progeny that are immediately predisposed to the long-lived memory cell fate, as well as daughters that go on to become effector cells.

effector or memory cell fates. Experiments using single cell transfer contest this theory. When a single naïve T cell is transferred to a naïve host that is subsequently infected with a pathogen, both effector and memory populations can be identified arising from that individual cell (Stemberger et al., 2007). Furthermore, barcoding experiments have also demonstrated that effector and memory cell populations can be derived from a single naïve T cell clone (Schepers et al., 2008; Gerlach et al., 2010). That a single naïve T lymphocyte can give rise to multiple T cell fates raises a fundamental question of how a single cell can yield two different daughter cell populations (Figure 1.2). Analogous to a nature versus nurture developmental pathway (Chang and Reiner, 2008), a naïve cell could divide and produce two daughter cells that are born equal but then go on to experience different environmental signals, either via proximity to a cellular niche, exposure to different inflammatory environments, or some other disparate set of signals; the cells could be nurtured to different fates in a stochastic manner. Alternatively, however, a cell could, during division, program the ensuing disparity by segregating key molecules across the plain of division, resulting in the production of two daughter cells that are distinct from one another from the moment of their generation (Figure 1.2). This process is referred to as asymmetric cell division.

Asymmetric Cell Division

The process of mitosis is often described as an equal partitioning of all cellular components between the two incipient daughter cells. While this simple description is

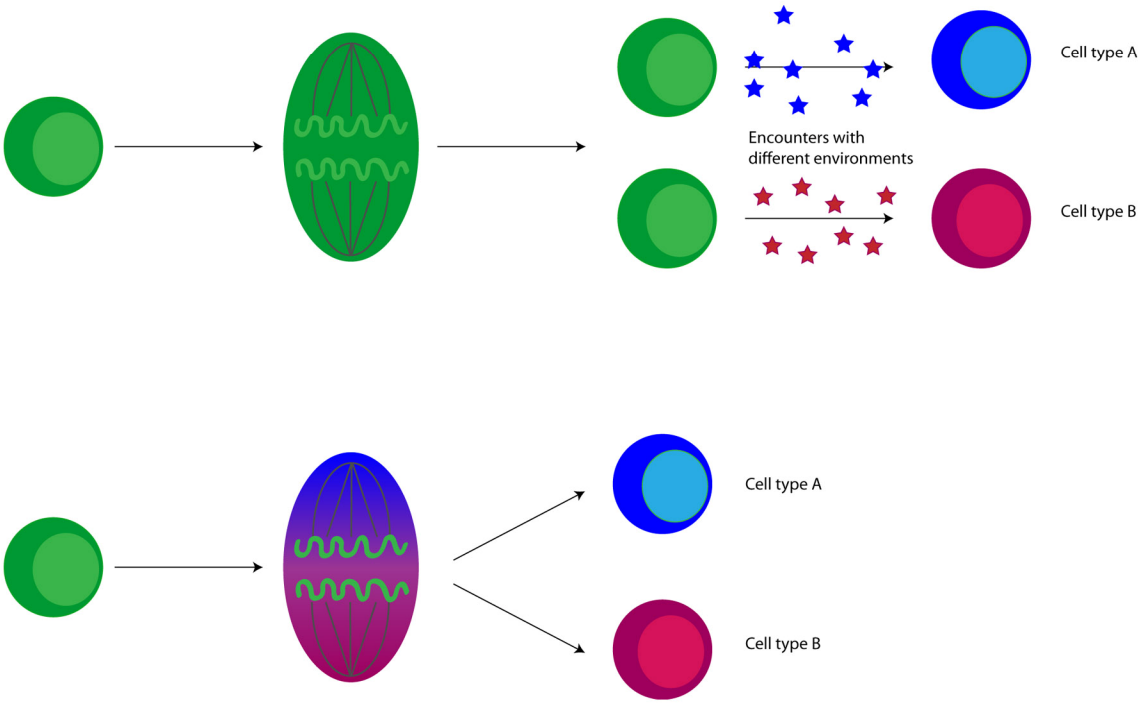

Figure 1.2. **Generating disparate cell fates from one cell.**
A single cell can give rise to two different cell populations through at least two mechanisms. A cell could divide with the result of that division being two identical daughter cells. These cells, through experiencing different extracellular environmental signals are nurtured to adopt two different cell fates (top). Alternatively, as a cell is undergoing mitosis it can program the generation of two different daughter cells by segregating key fate-determining proteins to one side of the plane of division or the other such that, as the cell progresses through cytokinesis, one daughter cell inherits more or less of particular cell components. The resulting daughter cells of this programmed asymmetric cell division are distinct from one another from the moment of their generation.

often true, in many cases there is actually unequal distribution of one or more cellular components. Asymmetric distribution can refer to cell size, cell protein content, RNA, or even DNA. This process is highly conserved and frequently encountered throughout evolution. Even the single-cell yeast, *Saccharomyces cerevisiae*, divides in an asymmetric fashion, resulting in daughter organisms of different sizes, referred to as "budding" (Chant, 1999). Many of the molecular processes that govern polarity in these lower organisms are conserved throughout evolution and utilized during asymmetric division in mammalian systems.

Much of what is known about the molecular processes that govern asymmetric division has been learned from the study of *Caenorhabditis elegans* and *Drosophila melanogaster*, both of which utilize asymmetric cell division frequently during development. Exemplifying this, the initial discovery of several of the critical proteins that govern polarity was made through the study of the *C. elegans* embryo. In a genetic screen done in this system, the first "par" (partioning defective) proteins were identified; PAR-1 through PAR-5 (Kemphues et al., 1988). Another critical member of the par protein family, atypical Protein Kinase C (PKC3, aPKC), was identified later (Tabuse et al., 1998). All of these proteins, except PAR-2, are highly conserved throughout evolution (Levitan et al., 1994; Macara, 2004). Their role is polarity, and in many cases asymmetric division, is also conserved.

The functions of the Par proteins are quite diverse. PAR-1, PAR-4 (LGL in mammalian systems), and aPKC are all serine/threonine kinases. PAR-3 (Bazooka in *D.* melanogaster) and PAR-6 are PDZ-domain containing proteins. PAR-5 (also 14-3-3ε)

contains a phospho-serine binding domain (Macara, 2004). In addition, Cdc42, a GTP-binding protein, has also been shown to be critical for asymmetric mitosis in budding yeast (Adams et al., 1990) and throughout evolution, in particular for spindle positioning (Johnson, 1999). Another set of polarity proteins was later identified; the tumor-suppressors Scribble (Bilder and Perrimon, 2000), Dlg, and Lgl (Bilder et al., 2000). All were shown to be critical for the regulation of asymmetric division in *Drosophila* (Peng et al., 2000; Bellaïche et al., 2001; Albertson and Doe, 2003) and conserved in their organization of polarity from yeast through humans.

In the *C. elegans* embryo, one of the best-understood systems of asymmetric cell division, polarity is established within the single-cell embryo utilizing the proteins described above. The single cell embryo divides asymmetrically, setting up the patterning for the rest of the worm's development, with one, larger, daughter cell destined to produce the ectodem, while the other, smaller, daughter cell produces the incipient germline as well as the mesoderm and endoderm. In this single-cell zygote, polarity is oriented based on the site of sperm entry at fertilization (Goldstein and Hird, 1996). Localization of the sperm centrosome within the oocyte results in exclusion of the proteins Par-3, Par-6 and aPKC (referred to as the Par or Par-3 complex) from surrounding cell cortex, which will become the posterior side of the developing larvae. The Par-3 complex becomes localized to the anterior side of the embryo, while Par-1 and Par-2 become enhanced on the posterior side, where the Par-3 complex is now absent (Cowan and Hyman, 2004). This establishment of polarity provides the groundwork for asymmetrical distribution of many critical fate determining cellular components. For

example, the polarity of the Par-1 kinases leads to polarized phosphorylation, and therefore polarized retention, of the fate-determinant Mex-5 (Tenlen et al., 2008; Daniels et al., 2010). Other fate determinants, such as Pie-1, are polarized through their physical association with polarity proteins (Daniels et al., 2009) and differential protein stability in the resulting daughter cells (Reese et al., 2000). Polarization occurs in the developing *Drosophila* embryo as well, and provided the initial example of mRNA polarization during asymmetric division. The activity of the protein Oskar is localized by tethering of the mRNA to the posterior side of the embryo along with restricted translation (Johnstone and Lasko, 2001). In both model systems, the polarity family of proteins provides the groundwork for polarization of these, and several more, components to be asymmetrically inherited. The unequal inheritance eventually results in varied cell fates between the daughter cells of the fertilized embryo.

In *Drosophila*, asymmetric division has been well characterized during organogenesis, as well as embryogenesis. Development of the fly brain is a well-studied model of development that relies on asymmetric division. Both the neuroblasts that derive the central nervous system and the sensory organ precursors that generate the peripheral nervous system require asymmetric cell division for accurate development. While polarity establishment and organization relies on many of the same proteins as *C. elegans*, the fate-determinants themselves and the processes by which the proteins are localized are often distinct. Many polarized proteins during *C. elegans* embryo division are found in the cytoplasm, whereas in *D. melanogaster*, many are found in association with the cell cortex (Betschinger and Knoblich, 2004).

Through the study of development of the *Drosophila* nervous system, the first asymmetrically inherited fate-determinant was discovered. Numb was the first fate determining protein to be characterized during an asymmetric division, and shown to be responsible for the differential daughter cell fates (Rhyu et al., 1994; Spana et al., 1995). It was later demonstrated that polarization of Numb relied on the same family of proteins as asymmetric division in the *C. elegans* embryo (Schober et al., 1999; Wodarz et al., 1999; 2000; Petronczki and Knoblich, 2001; Rolls et al., 2003). This highlighted the conservation of polarity across evolution.

The role of asymmetric division in development has become increasingly clear. The relationship between developmental pathways and cancer has also started to be understood, and consequently, the link between regulation of asymmetric cell division and cancer is starting to be elucidated. The first indication for a role of asymmetric division in cancer was uncovered through the study of *Drosophila* neuroblast divisions and their relation to tumor formation. Proteins that were initially demonstrated to be tumor suppressors in *Drosophila* through genetic screens (Gateff, 1978; 1994), were later found to be regulators of asymmetric cell division, including the polarity regulators Lgl and Dlg (Ohshiro et al., 2000; Peng et al., 2000), and asymmetrically inherited fate determinants, such as the adaptor protein Brat (Betschinger et al., 2006). Mutations in polarity network members, such as those that result in overexpression of aPKC, lead to over proliferation and tumor formation (Lee et al., 2006). In these, and other polarity defects, the tumor formation appears to be driven by the defect in asymmetric cell division, with the simplest explanation being that the defect in polarity results in an

excess of stem cells that continuously proliferate (Knoblich, 2010), however the exact mechanisms linking defects in asymmetric division to tumor generation have not been worked out. The link between tumor formation and asymmetric cell division is possibly conserved, given that many of the tumor suppressors linked to asymmetry defects in *Drosophila* are also known tumor suppressors in mammalian cells.

Asymmetric Cell Division in Vertebrates

Asymmetric cell division is a phenomenon that has also been observed in higher organisms, and particularly well studied in mouse. Asymmetric cell divisions have been identified in the gut (Quyn et al., 2010), mammary glands (Cicalese et al., 2009), muscle (Shinin et al., 2006), skin (Lechler and Fuchs, 2005), and the hematopoietic system (Chang et al., 2007; Wu et al., 2007; Chang et al., 2011; Barnett et al., 2012). The best studied, however, is the developmental role for asymmetric division during vertebrate neurogenesis (Götz and Huttner, 2005). In early neurogenesis, several rounds of symmetric divisions are thought to expand the stem cell pool (McConnell, 1995). This expansion is followed by asymmetric divisions that result in the generation of one cell that will form go on to form a neuron, and another that remains in the progenitor cell pool (Kornack and Rakic, 1995). The unequal partitioning of a fate determinant in higher organisms was first demonstrated by the asymmetric inheritance of Notch1 (Chenn and McConnell, 1995). The molecular mechanisms that regulate this process is vertebrates have proved elusive to understand, and hard to translate directly from our understanding

in *Drosophilla* and *C. elegans*. Although many of the same proteins appear to be involved, the regulation in vertebrates appears to be unique.

The relationship between regulation of asymmetric cell division and tumor formation does appear to have a potential parallel to the findings in *Drosopilla* described above. The link between defects in asymmetric division and tumor formation in vertebrates was first assessed in mammary stem cells, which can be cultured *in vitro* allowing for the development of "mammospheres"; unique structures derived from a single stem cell. Normal mammary stem cells in this culture system divide asymmetrically, segregating Numb to one side of the plane of division during mammosphere formation (Cicalese et al., 2009). It has been observed, however, that in at least one transgenic mouse model of breast cancer, the *ErbB2* model, the tumors contain a higher frequency of stem-cell phenotype cells, and that these cells are not able to partition Numb during division in mammosphere assays, resulting in two daughters that have higher levels of Numb signaling (Cicalese et al., 2009). Similar observations have been made in the hematopoietic system. It was observed that hematopoietic stem cells, when cultured *in vitro* were able to asymmetrically segregate Numb during mitosis. The addition of an oncogene fusion protein typically responsible for an aggressive, undifferentiated form of leukemia, resulted in an over-proliferation of the stem-cell phenotype cells as well as a loss of asymmetric mitosis. Introduction of an oncogene associated with a less-aggressive, more-differentiated form of leukemia, however, did not result in a defect in asymmetric mitosis, but rather appears to result in leukemia through promoting survival of the terminally differentiated population produced by asymmetric

division (Wu et al., 2007), suggesting that while a loss of asymmetric division may play a role in the formation of some tumors, it may not be a universal feature of oncogenesis. Finally, in the murine brain, oligodentrocytes have been demonstrated to undergo asymmetric inheritance of key fate determinants, and this property is lost in these cells in a mouse model of glioma (Sugiarto et al., 2011). Due to studies such as these, the relationship between adult tissue stem cells, "cancer stem cells", and asymmetric division has been of particular interest in the last several years (Huntly and Gilliland, 2005; Morrison and Kimble, 2006). Taken together these studies highlight the potential clinical impact of further understanding the regulation of asymmetric cell division in vertebrates. Further understanding of the molecular processes that govern asymmetric decisions will help develop treatments and therapies for cancer and possibly other diseases in the future.

Stem Cells and Adaptive Immunity

Adult tissue stem cells solve a potential paradox of long-lived organisms. Many of the cells that carry out the effector function of an organ are actually short-lived, terminally differentiated cells. This raises the issue of how to balance short-lived cell populations and homeostasis of a long-lived organism. Many tissues in mammalian systems solve this through the use of adult tissue stem cells. Unlike their functional cell counterparts in the given organ, these cells are few in number, divide infrequently, and function in maintaining homeostasis of that organ. Stem cells, therefore, are faced with the unique challenge of giving rise to two populations of cells – those that will continue

on to terminal differentiation and fulfill the effector function of the organ, and those that will repopulate the stem cell pool (Morrison and Kimble, 2006). In all the vertebrate systems discussed previously, it is the stem cell that is thought to be capable of undergoing polarized mitosis, resulting in two differentially fated daughter cells. What then, is the relation between adult tissue stem cells and the adaptive immune response?

Like other hematopoietic cells, many white blood cells have a finite life span and do not undergo proliferation in their most mature form. Rather, in response to stress, it is up-regulation of production of these cells, through mobilization of the hematopoietic stem cell, which results in expansion of their numbers. The evolutionarily youngest members of the immune response, however, are the B and T lymphocytes. These cells differ from their innate white cell counterparts in their inability to be regenerated from the hematopoietic stem cell after use. While lymphocytes are derived from HSCs, they are not clonally identical, like their innate cell counterparts. Each lymphocyte has a unique receptor that allows it to recognize a distinct piece of foreign antigen. This specificity is driven by random mutations derived during the process of maturation of the lymphocyte from the hematopoietic stem cell. Therefore, each adult lymphocyte is unique and cannot be regenerated from the hematopoietic stem cell if it were to be consumed or lost during an immune response.

Adult lymphocytes, therefore, face a similar challenge to adult-tissue stem cells (Figure 1.3). In the setting of pathogenic infection, a lymphocyte whose antigen receptor is specific for that microbe must respond by producing two populations of cells - those that will fight the current infection as well as those that will retain the memory of

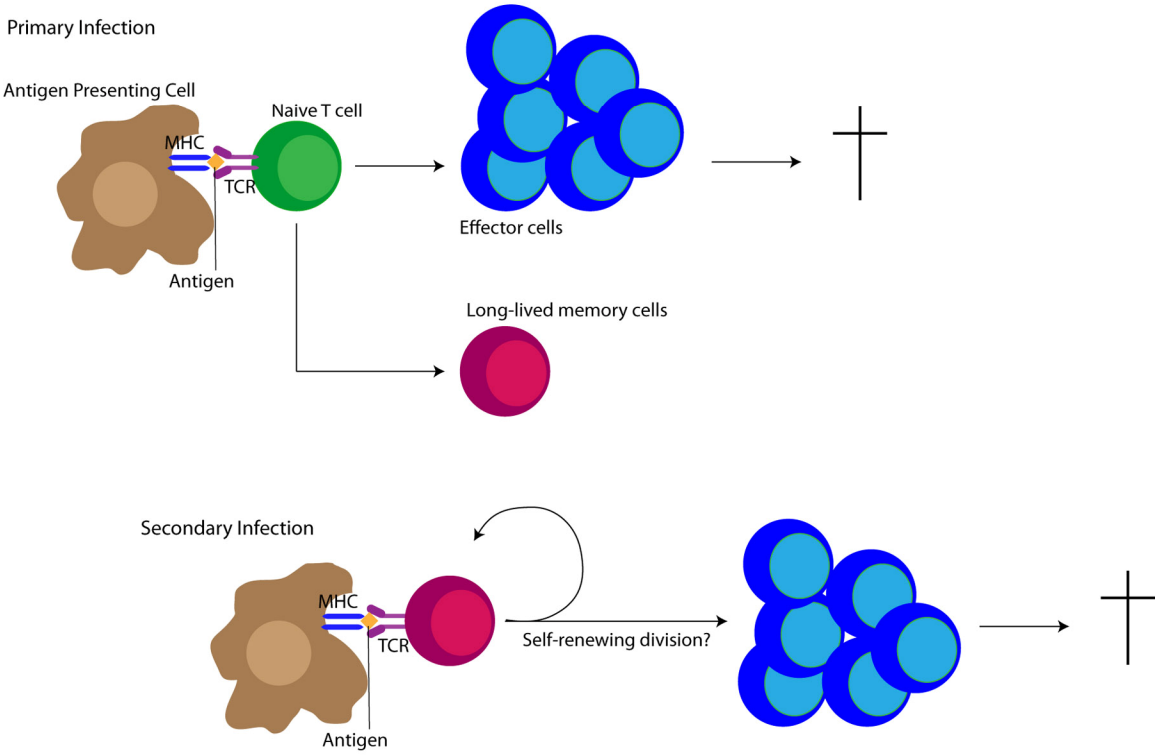

Figure 1.3. **A stem cell model of the adaptive immune response.**
During the generation of a primary immune response, a naïve T cell must produce cells that will both protect against the current pathogenic threat as well those that will remember the T cell clone to protect the host against secondary infection. The long-lived memory cell is responsible for remembering the pathogen for the life-time of the host as well as responding to re-infection by producing effector cells and maintaining the memory cell pool. This self-renewal is homologous to how adult tissue stem cells produce both cells that will undergo transit amplification and become the effector cells of that organ as well as maintain their own population.

the lymphocyte clone. These long-lived memory cells, furthermore, must be able to re-iteratively produce both effector cells in the setting of re-infection while maintaining their own population. The is reminiscent of how adult tissue stem cells must produce both the cells that will become terminally differentiated cells that carry out the function of the organ, while simultaneously repopulating their own stem cell pool.

Long-lived memory cells have been directly compared to hematopoietic stem cells, and many parallels have been recognized, including a shared transcriptional profile (Luckey et al., 2006). Within the memory T cell compartment, furthermore, cells with characteristic features of stem cells have been identified in mouse (Zhang et al., 2005), as well as in humans (Gattinoni et al., 2011).

The use of asymmetric cell division in the initiation of the adaptive immune response (Chang et al., 2007; Barnett et al., 2012), is also suggestive of a parallel between T and B lymphocytes and hematopoietic stem cells, which may use asymmetric cell division (Wu et al., 2007). Furthermore, the memory cell generated during an adaptive immune response, which shares traits with the hematopoietic stem cell, may undergo re-iterative, self-renewing asymmetric divisions during pathogenic re-challenge.

Purpose

The development of an adaptive immune response is starting to be understood. Here I have discussed what is known about how cell fate decisions are made during the initiation of an adaptive immune response, predominately in T lymphocytes, as well as

how cell fate decisions are made in other, perhaps analogous, systems. I endeavored to understand what the role and extent of asymmetric cell division is during the generation of the T cell response against foreign pathogen. First, using imaging, genetic, and molecular biochemistry techniques, I worked to understand how asymmetric cell division is utilized during a primary T cell response to pathogen to allow for the generation of fate disparity. Secondly, using genetic and imaging techniques, I worked to uncover a role of asymmetric cell division in the secondary encounter to pathogen, thereby extending the stem-cell hypothesis of adaptive immunity by elucidating another stem cell-like property of memory T lymphocytes. Collectively these studied highlight a novel role for asymmetric cell division in the generation and function of the adaptive immune response.

CHAPTER 2: Asymmetric Proteasome Segregation as a Mechanism for Unequal Partitioning of the Transcription Factor T-bet during T Lymphocyte Division[1]

Introduction

After the activation of T cells by antigen-presenting cells, the microtubule organizing center, as well as specific transmembrane and intracellular proteins, rapidly undergo reorganization toward the site of intercellular contact (Monks et al., 1998). This polarized reorganization of T cells has been characterized among naive and antigen-experienced lymphocytes stimulated in vitro (Maldonado et al., 2004; Ludford-Menting et al., 2005; Huse et al., 2006; Stinchcombe et al., 2006) and in vivo (Reichert et al., 2001; Barcia et al., 2006; Azar et al., 2010; Beuneu et al., 2010; Friedman et al., 2010). The acute polarization and redistribution of proteins subsequent to T cell activation has been suggested to regulate signal transduction and facilitate function, such as directed secretion of cytokines and cytolytic granules (Huse et al., 2006; Stinchcombe et al., 2006).

Polarized segregation of proteins may be evident several hours after activation of naive T cells (Yeh et al., 2008), and this coalescence may even persist through cell division (Chang et al., 2007). The polarized segregation of proteins during mitosis is reminiscent of an evolutionarily conserved phenomenon known as asymmetric cell division, which allows a single parent cell to give rise to two daughter cells with distinct fates (Betschinger and Knoblich, 2004; Lechler and Fuchs, 2005; Knoblich, 2008). During asymmetric division, key fate determinants are localized to one side of the plane of division, resulting in two daughter cells that inherit different amounts of critical

determinants. One such determinant in Drosophila neural stem cells is the transcription factor Prospero, which acts as a binary switch between terminal differentiation and self-renewal (Betschinger and Knoblich, 2004). It has been suggested that a T cell may undergo asymmetric division to give rise to daughter cells that are differentially fated toward the effector and memory lineages (Chang et al., 2007). It remains unknown, however, what determinants are unequally partitioned into the daughter cells of a selected T cell and how their asymmetry is mediated.

Several transcriptional regulators have been implicated in regulating fate decisions of effector and memory T cells (Intlekofer et al., 2005; Joshi et al., 2007; Kallies et al., 2009; Rutishauser et al., 2009; Shin et al., 2009). Genetic evidence suggests that the T-box transcription factor T-bet is a critical fate determinant in activated naive CD8+ T cells, promoting differentiation toward the effector fate while repressing development toward the memory fate (Intlekofer et al., 2005; Joshi et al., 2007). In activated CD4+ T cells, T-bet promotes the T helper 1 (Th1) cell fate while repressing the development of the Th2 and Th17 cell lineages (Szabo et al., 2000; 2002; Hwang et al., 2005; Lazarevic et al., 2011). Small changes in the amount of these factors can have profound influences on T cell fate (Szabo et al., 2002; Intlekofer et al., 2005; Joshi et al., 2007; Kallies et al., 2009; Rutishauser et al., 2009; Shin et al., 2009).

We now provide evidence that in activated naive T cells undergoing division, T-bet was asymmetrically partitioned between the daughter cells. Moreover, the mechanism by which T-bet asymmetry was mediated appeared to involve proteasome dependent degradation specifically during mitosis in the setting of asymmetric distribution of the degradation machinery, the proteasome. The localization of the proteasome was opposite

to that of T-bet, such that the daughter cell that received less proteasome acquired more T-bet. This reciprocal partitioning, along with the observation that T-bet asymmetry is prevented by inhibiting its proteasome-dependent degradation, indicates that the asymmetric localization of T-bet and proteasome may be related. Inhibiting the polarized segregation of the proteasome during mitosis, moreover, prevented the asymmetric partitioning of T-bet. Together, these findings suggest a mechanism of asymmetric cell division whereby asymmetric localization of the proteasome, and consequently unequal degradation of factors targeted for destruction during mitosis, yields unequal partitioning of key fate determinants to two daughter cells.

Results

Asymmetric partitioning of T-bet during T lymphocyte division

To examine the cellular distribution of T-bet, we employed a model system that has allowed us to examine T cells preparing for their first division in vivo in response to a microbe (Chang et al., 2007). Naive CD8+ T cells transgenic for the P14 T cell receptor were labeled with a fluorescent dye (CFSE) that allows determination of whether a cell has undergone division. Cells were then adoptively transferred into recipient mice that were infected 24 hr previously with recombinant Listeria monocytogenes bacteria expressing a specific gp33-41 peptide epitope (gp33-L. monocytogenes) recognized by the transgenic T cell receptor. Undivided donor T cells were isolated by flow cytometry at 36 hr after transfer and examined by confocal microscopy. Among activated cells in interphase and prophase, we observed that T-bet was localized in the nucleus (Figure 2.1A). Among cells in metaphase, we observed a substantial reduction in T-bet signal

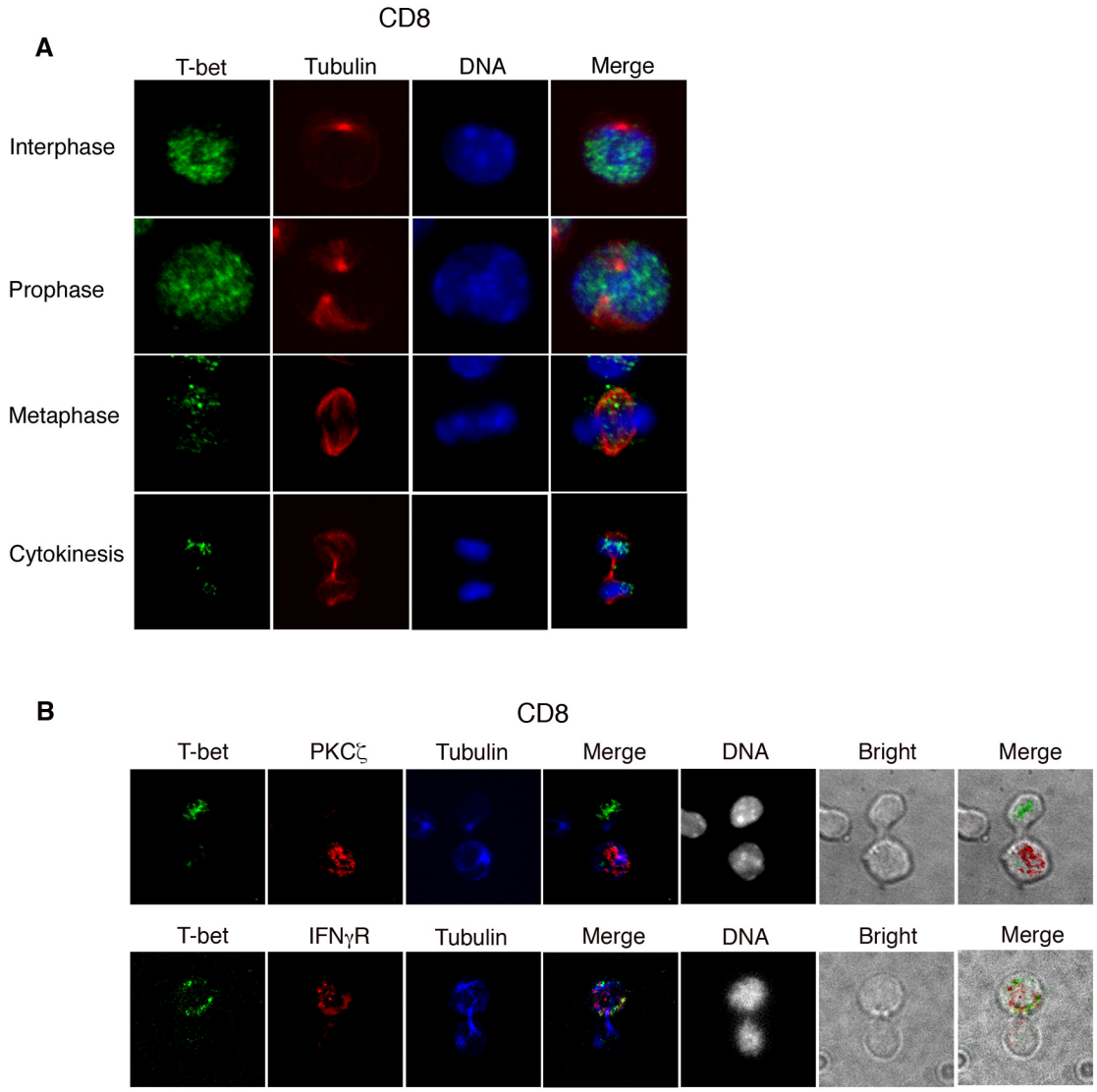

Figure 2.1. **Asymmetric partitioning of T-bet in dividing T lymphocytes.**
(A) Undivided P14 CD8+ TCR transgenic T cells were adoptively transferred into wild-type recipients infected with gp33-L. monocytogenes, harvested at 36 hr after transfer, and examined by confocal microscopy after staining for T-bet (green), b-tubulin (red), and DNA (blue). Asymmetry of T-bet inheritance was observed in 66% of cells (n = 80). (B) Undivided P14 CD8+ TCR transgenic T cells were harvested as in (A) and stained for T-bet (green), PKCζ or IFN-γR (red), β-tubulin (blue), and DNA (grayscale). In costaining experiments where both molecules were asymmetrically inherited, T-bet and the IFN-γR were inherited by the same daughter cell in 100% (n = 15) of cells, and T-bet and PKCζ were inherited by opposite daughters in 87% (n = 14) of cells. Results are representative of three separate experiments. Micrographs courtesy of John Chang.

compared to those in interphase and prophase, suggesting the possibility that T-bet was undergoing degradation prior to cell division. We also observed that among cells in metaphase, T-bet was displaced from the chromatin and localized asymmetrically on one side of the cell. The asymmetry of T-bet persisted into cytokinesis, with unequal amounts of T-bet detected in the conjoined daughter cell pairs (Figure 2.1B). Based on the preferential partitioning of T-bet into the daughter cell receiving more of the proximal cell marker interferon-g receptor (IFN-gR) and less of the distal cell marker, Protein Kinase C-zeta (PKCz) (Chang et al., 2007), the greater share of T-bet appeared to be partitioned into the putative proximal daughter cell (Figure 2.1B).

We next confirmed that the unequal amounts of T-bet protein acquired by the daughter cells during mitosis persisted after division. We have previously used flow cytometry to distinguish putative proximal and distal daughter populations on the basis of CD8 abundance (Chang et al., 2007). CFSE-labeled P14 transgenic CD8+ T cells were adoptively transferred into recipient mice that were infected 24 hr later with gp33-L. monocytogenes. At 48 hr postinfection, splenocytes were analyzed by flow cytometry. Examination of T-bet protein amounts revealed greater abundance of T-bet in the putative proximal daughter cells, which expressed higher amounts of CD8, compared to distal daughter cells (Figure 2.2A). Putative distal daughter cells had higher amounts of T-bet compared to naive cells and some undivided cells. The amounts of T-bet in the highest-expressing undivided cells appeared to be less than that present in the proximal and distal daughter cells combined, suggesting that resynthesis of T-bet in the proximal and/or distal daughter cells may follow asymmetric partitioning of pre-existing parent cell T-bet protein during mitosis. Because genetic studies have suggested that T-bet drives terminal

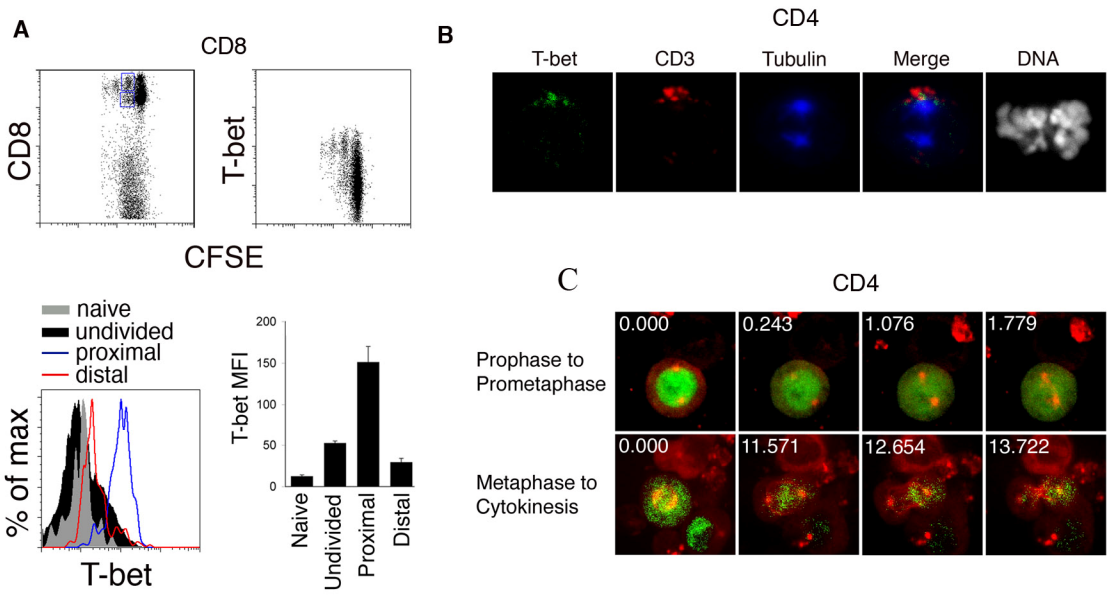

Figure 2.2. **Asymmetric distribution of T-bet in mitotic T cells.**
(A) CFSE-labeled P14 transgenic CD8+ T cells from recipients infected with gp33-L. monocytogenes were harvested 48 hr after infection, stained with antibodies to detect CD8 and T-bet, and analyzed by flow cytometry. Putative proximal and distal daughter cells (detected as the second brightest CFSE peak) were gated on the basis of CD8 expression (gates shown in top left). T-bet mean fluorescence intensity (MFI) of the gated proximal (blue histogram), distal (red histogram), naive (gray filled histogram), and undivided (black filled histogram) populations is shown. Error bars indicate standard error of the mean (SEM). (B) CD4+ T cells were activated in vitro and stained for T-bet (green), CD3 (red), β-tubulin (blue), and DNA (grayscale). In costaining experiments where both molecules were asymmetrically inherited, T-bet and CD3 were inherited by the same daughter cell in 100% (n = 16) of cells. (C) CD4+ T cells were activated in vitro and transduced at 48 hr with T-bet-GFP and cherry-alpha-tubulin fusion proteins. After 3 days, T cells were restimulated in vitro for 24 hr and imaged by time-lapse confocal microscopy. Prophase cells were imaged through prometaphase (top) and metaphase cells imaged through cytokinesis (bottom). Asymmetric T-bet partitioning by the daughter cells was observed in 68% of cells (n = 23). Relative time (in minutes) is indicated in the top left corner of each panel. Results are representative of three separate experiments. Some micrographs courtesy of John Chang.

differentiation of effector T cells while repressing self-renewal of memory CD8+ T cells (Intlekofer et al., 2007; Joshi et al., 2007), asymmetric partitioning of T-bet into the proximal daughter cell is consistent with prior evidence suggesting that the proximal daughter cell gives rise to the effector lineage while the distal daughter cell is the predecessor of the memory lineage (Chang et al., 2007).

In addition to regulating fate decisions in CD8+ T cells, T-bet plays a critical role in the fate choice of CD4+ T cells (Szabo et al., 2000). In a quantitative manner, T-bet promotes Th1 cell differentiation while repressing the development of the Th2 and Th17 cell lineages (Szabo et al., 2000; 2002; Hwang et al., 2005; Lazarevic et al., 2011). T-bet binds directly to GATA-3 and prevents it from binding to its target DNA (Hwang et al., 2005); T-bet also cooperates with the transcription factor Runx1 to inhibit the transcription of RORgt (Lazarevic et al., 2011). As with CD8+ T cells, small changes in the amount of T-bet results in profound phenotypic changes. T-bet heterozygous mice, which exhibit only a 50% reduction in T-bet protein relative to wild-type mice (Szabo et al., 2002), exhibit early and dense defects in Th1 cell development and manifest a similar degree of Th2 cell-mediated airway hyperresponsiveness as homozygous T-bet-deficient mice (Finotto et al., 2002; Szabo et al., 2002).

Because of the ability of small differences (50% or less) in the amount of T-bet to alter cell fate and function (Finotto et al., 2002; Szabo et al., 2002; Intlekofer et al., 2007; Joshi et al., 2007), we examined the first daughter cells of CD4+ T cells activated in vitro (Figure 2.3). We observed a 3.6-fold disparity in T-bet abundance between the T-bet-higher and T-bet-lower daughter cells. The T-bet disparity in the two daughter populations positively correlated with a 3.3-fold greater likelihood to express IFN-g and a

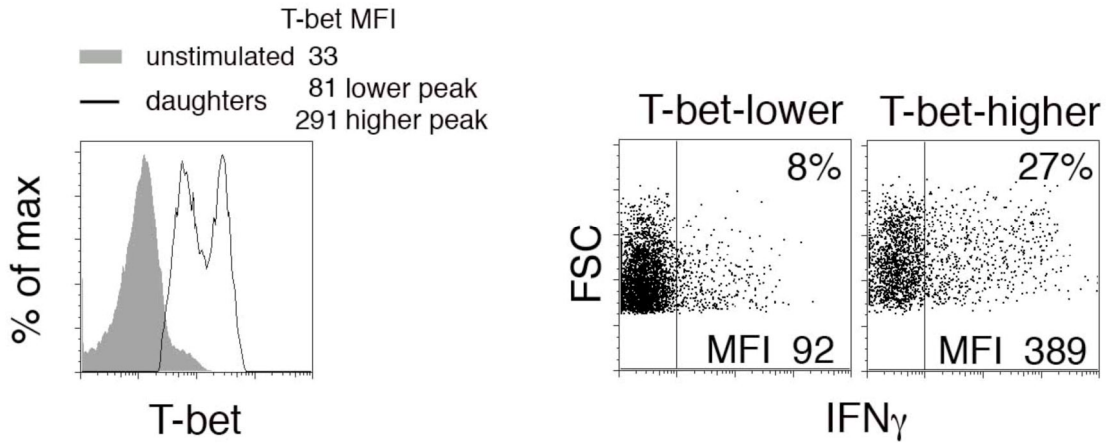

Figure 2.3. **Differential abundance of T-bet in the first CD4+ T daughter cells.** Small disparity in T-bet protein affects CD4+ T cell fate. Purified CD4+ T cells were labeled with CFSE and activated in vitro for 48h. PMA and ionomycin were added for the last 4h of culture to induce cytokine production. Unstimulated CD4+ T cells are shown as the gray filled histogram, and daughter cells (detected as the second brightest CFSE peak) are shown as the black histogram. Daughter cells were further gated on the basis of T-bet abundance, and expression of IFN-γ by daughter cells expressing higher and lower levels of T-bet is shown in the right panel. Daughter T cells exhibited a 3.6-fold disparity in T-bet abundance. This disparity in T-bet positively correlated with a 3.3-fold greater likelihood to express IFN-γ and a 4.2-fold higher level of cytokine per expressing cell.

4.2-fold more intense IFN-g signal per expressing cell (Figure 2.3). Minor disparity in the partitioning of T-bet during the first division of a CD4+ T cell could, thus, influence the subsequent fates of the daughter cells.

To examine the localization of T-bet in dividing CD4+ T cells, we developed a reductionist cell culture-based model system that recapitulated the key features of CD8+ T cell division in vivo. Naive CD4+ T cells were stimulated with immobilized anti-CD3 and anti-CD28 along with immobilized ICAM1-Fc fusion protein. This approach was taken in order to mimic a polarizing stimulus plus integrin-mediated contact because ICAM1 dependence was one of the defining features of asymmetric T cell division in vivo (Chang et al., 2007) and because immobilized ICAM1-Fc was found to be critical for asymmetric division in vitro (Figure 2.4). We observed that T-bet was asymmetrically partitioned to the side of the cell that receives more CD3 (Figure 2.2B). Because CD3 is a marker of the immune synapse (Monks et al., 1998), T-bet was partitioned to the side of the cell that is presumed to have been in contact with the stimulus, consistent with the findings in CD8+ T cells activated in vivo (Figure 2.1B).

To examine the steps leading up to T-bet asymmetry in real time, CD4+ T cells were activated and transduced with retroviruses encoding T-bet-GFP and cherry-alpha-tubulin fusion proteins. Three days later, when the transduced lymphocytes expressing fluorescent fusion proteins were no longer dividing, they were restimulated with immobilized anti-CD3 and ICAM1-Fc fusion protein. Among CD4+ T cells in interphase and prophase, we observed that T-bet was localized in the nucleus, consistent with the staining of endogenous T-bet in CD8+ T cells responding to a microbe in vivo (Figure 2.1A). During prometaphase, T-bet-GFP began to leak out of the disintegrating nuclear

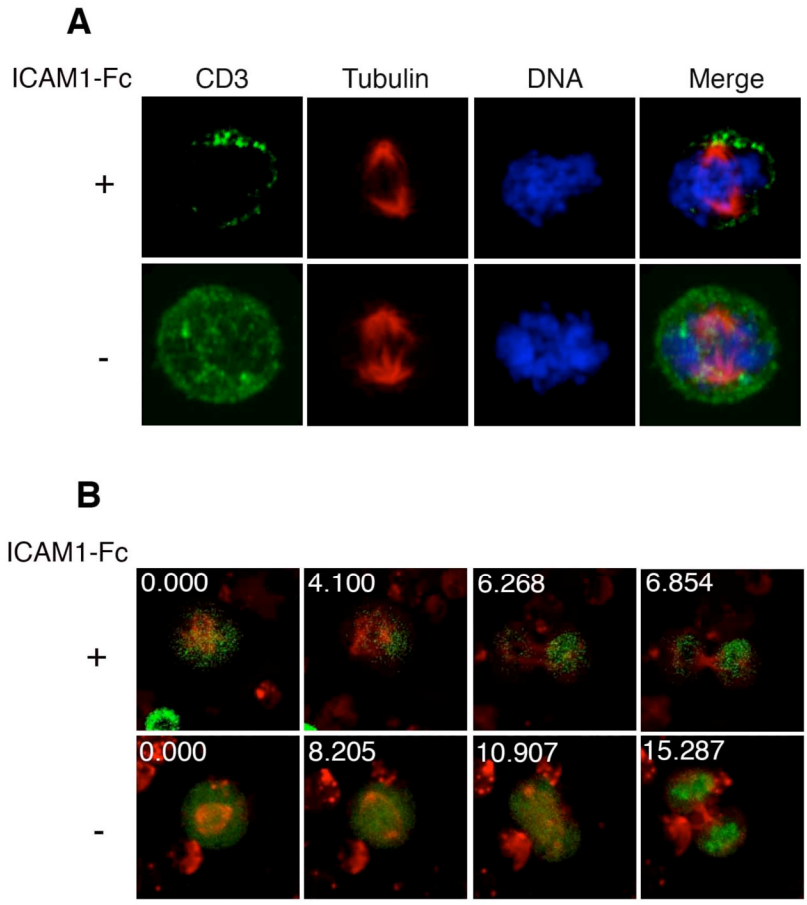

Figure 2.4. **Asymmetric partitioning in CD4+ T cells activated in vitro requires ICAM1-mediated interactions**.
(A) CD4+ T cells were activated in vitro for 28h with immobilized anti-CD3/anti-CD28 with (upper row) or without (lower row) immobilized ICAM1-Fc. Cells were stained for CD3 (green), tubulin (red), and DNA (blue), and examined by confocal microscopy. Asymmetric segregation of CD3 was observed in 80% of cells (n=15) activated with anti-CD3/28/ICAM1-Fc compared to 18% of cells (n=17) activated with anti-CD3/28 alone. $p<0.001$. (B) CD4+ T cells were activated in vitro with immobilized anti-CD3/anti-CD28 for 48h, and transduced with retroviruses encoding cherry-alpha tubulin and T-bet-GFP. Three days later, cells were restimulated in vitro using immobilized anti-CD3 with (upper row) or without (lower row) immobilized ICAM1-Fc for 24h prior to imaging. Asymmetric partitioning of T-bet into the daughter cells was observed in 74% of cells (n=19) restimulated with anti-CD3/ICAM1-Fc and in 14% of cells (n=28) stimulated with anti-CD3 alone. $p<0.001$. Relative time (in minutes) is indicated in the upper left corner of each panel. Some micrographs courtesy of John Chang.

envelope, eventually filling the cytoplasm as it became fully displaced from condensed chromatin in early metaphase (Figure 2.2C, top). The displacement of T-bet from mitotic chromatin is consistent with the reported behavior of other transcription factors during mitosis, which may be incapable of binding to highly condensed mitotic chromatin (Martínez-Balbás et al., 1995). As metaphase progressed, we observed a decrease in T-bet-GFP fluorescence (Figure 2.2C, bottom), consistent with the reduction of endogenous T-bet during metaphase in T cells dividing in vivo. Because anaphase began and the mitotic spindle began to separate, T-bet-GFP appeared to localize asymmetrically toward one side of the cell, becoming unequally inherited by the incipient daughter cells (Figure 2.2C, bottom).

T-bet undergoes proteasome-dependent degradation during mitosis

The reduction in T-bet signal observed in cells during metaphase with both static and time-lapse imaging approaches suggested that T-bet might be undergoing degradation just prior to or during its asymmetric localization. Specifically, in experiments where interphase and metaphase blasts were imaged in the same field of view, quantitation of T-bet signal revealed a greater than 90% reduction in metaphase cells compared to interphase cells (Figure 2.5A). This reduction of T-bet signal was observed in all mitotic T cells, regardless of whether T-bet was partitioned asymmetrically. By using biochemical and flow cytometric approaches, we confirmed that T-bet underwent proteasome-dependent degradation during mitosis. CD4+ or CD8+ T cells were activated in vitro and synchronized with an inhibitor of microtubule polymerization, nocodazole, to enrich for cells reversibly arrested in G2-prometaphase.

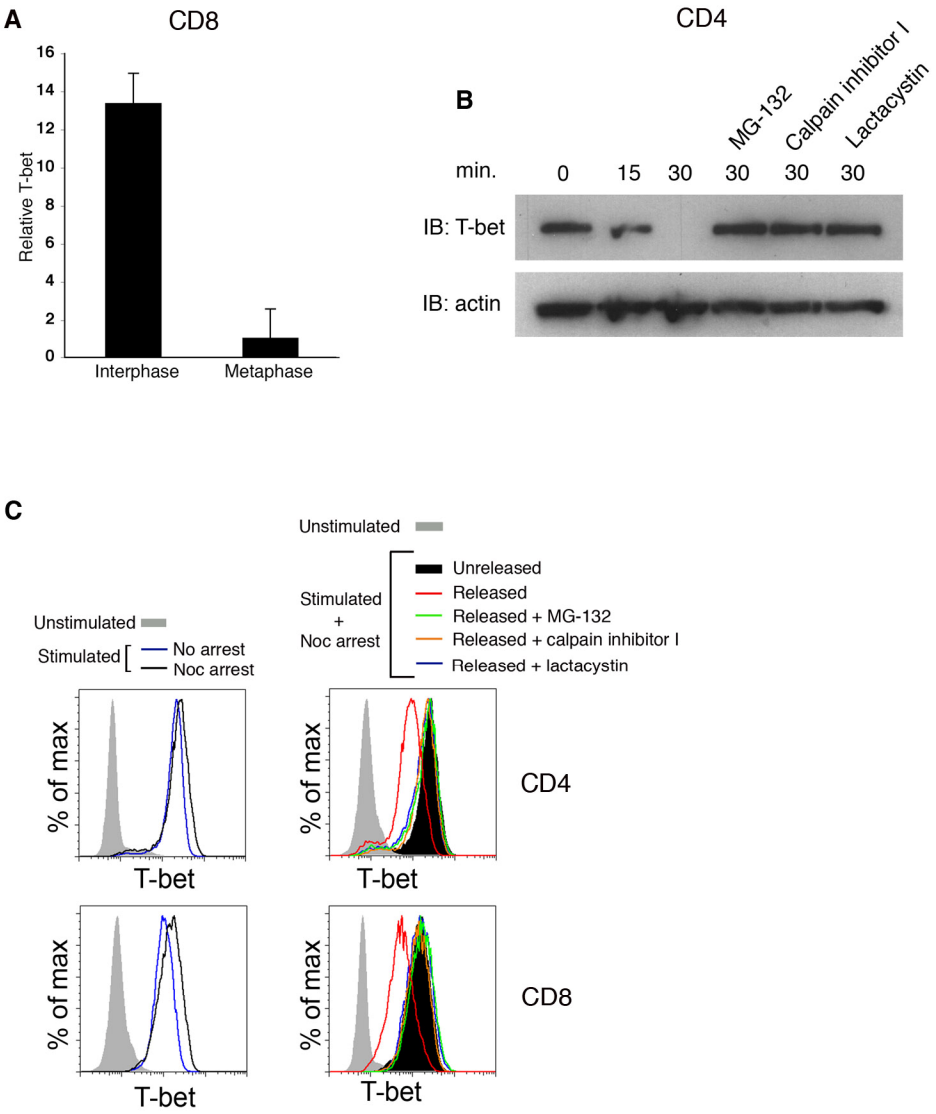

Figure 2.5. **T-bet undergoes proteasome-dependent degradation during mitosis.** (A) Quantification of T-bet signal in interphase versus metaphase T cells represented in Figure 1A. T-bet signal was compared between interphase and metaphase blasts imaged in the same field of view (n = 61). Error bars indicate SEM. (B) CD4+ T cells were activated in vitro for 24 hr and then synchronized with nocodazole. Cells were washed free of drug and cultured in vitro with or without the proteasome inhibitors MG-132, calpain inhibitor I, or lactacystin. Cell lysates were prepared at 0, 15, or 30 min after nocodazole washout. T-bet and b-actin levels were assessed by immunoblotting. (C) CD4+ or CD8+ T cells were prepared as in (B) and T-bet levels assessed by intracellular staining at 0 and 30 min after nocodazole washout. T-bet levels of unstimulated T cells and activated cells not treated with nocodazole (‘‘unstimulated’’) are also shown. Results are representative of three separate experiments. Immunoblots courtesy of John Chang.

Cells were then washed free of drug and allowed to progress into metaphase in the presence or absence of proteasome inhibitors. We observed that T-bet appeared to undergo degradation within 30 min of release from nocodazole (Figures 2.5B and 2.5C). Furthermore, the degradation of T-bet could be prevented by the addition of an inhibitor of proteasome activity (Figures 2.5B and 2.5C). Degradation of T-bet was cell cycle specific, as indicated by the fact that T-bet underwent degradation after drug washout in cells arrested in G2-M, but not in G1 or S phase (Figure 2.6).

Asymmetric localization of the proteasome during mitosis

The finding that T-bet underwent degradation specifically during M phase raised the possibility that unequal degradation during mitosis might result in asymmetric partitioning of T-bet into the daughter cells. For asymmetric degradation to occur, however, some component of the destruction process would need to be asymmetrically localized. Consistent with this prediction, examination of activated T lymphocytes dividing in vivo (Figure 2.7A) and in vitro (Figure 2.7B) revealed evidence for asymmetry in the localization of the proteasome. During interphase and prophase, the proteasome was localized throughout the cell. During metaphase, however, we observed asymmetric segregation of the proteasome on one side of the lymphocyte and unequal segregation of the proteasome into daughter cells during cytokinesis. The asymmetric localization of the proteasome, moreover, was confirmed with antibodies to two distinct proteasomal epitopes (Figure 2.8). Proteasomal asymmetry is not a general feature of cell division, however, as shown by the fact that dividing HEK293T cells exhibited equal

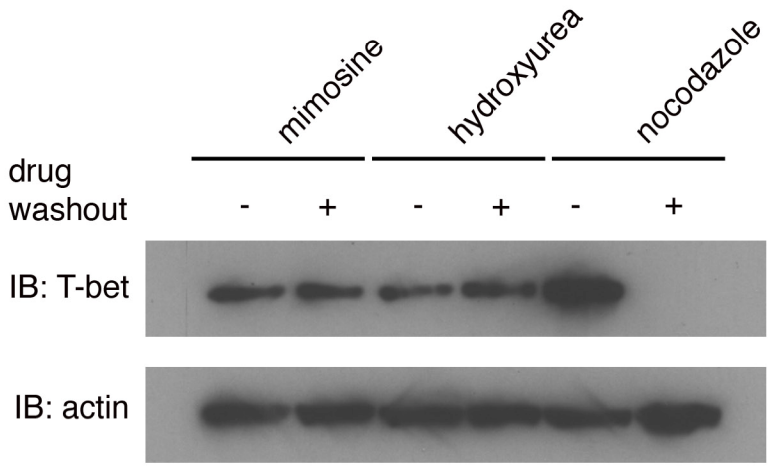

Figure 2.6. **Degradation of T-bet is cell cycle-specific.**
(A) CD4+ T cells were activated in vitro in the presence of the cell cycle inhibitors mimosine, hydroxyurea, or nocodazole, resulting in the arrest of the cells in G1, S, or G2/prometaphase, respectively. Cell lysates were prepared after 40h, or after cells were washed free of drug and cultured in vitro for an additional 30 minutes. Immunoblots done in collaboration with John Chang.

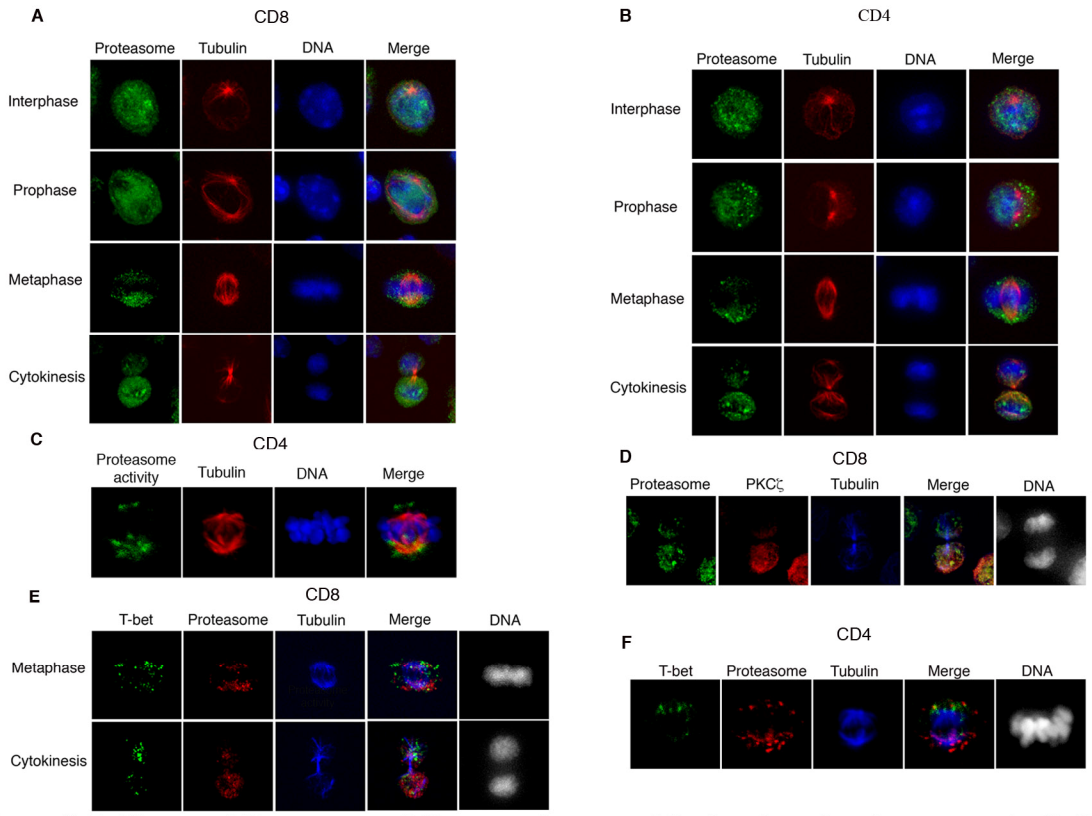

Figure 2.7. **Unequal Proteasomal Segregation as a Mechanism for Asymmetric Cell Division**

(A) Undivided P14 CD8+ TCR transgenic T cells were harvested as in Figure 2.1A and stained for the a1 chain of the proteasome 20S subunit (green), b-tubulin (red), and DNA (blue). Asymmetry of proteasome localization was observed in 62% (n = 74) of cells. (B) CD4+ T cells were activated in vitro for 28 hr and stained as in (A). Asymmetry of proteasome localization was observed in 74% (n = 125) of cells. (C) CD4+ T cells were activated in vitro for 28 hr, treated with the proteasome activity probe MVB003 for 2 hr, and stained for b-tubulin (red) and DNA (blue). Asymmetry of degradative activity was observed in 65% (n = 22) of cells. (D) Undivided P14 CD8+ TCR transgenic T cells were harvested as in Figure 1A and stained for the proteasome 20S a1 subunit (green), PKC-ζ (red), b-tubulin (blue), and DNA (grayscale). In costaining experiments where both molecules were asymmetrically inherited, proteasome and PKC-ζ were inherited by the same daughter cell in 95% of cells (n = 34). (E) Undivided P14 CD8+ TCR transgenic T cells were harvested as in Figure 1A and stained for T-bet (green), proteasome 20S a1 subunit (red), b-tubulin (blue), and DNA (grayscale). In costaining experiments where both molecules were asymmetrically inherited, proteasome and T-bet were inherited by opposite daughter cells in 90% of cells (n = 29). (F) CD4+ T cells were activated as in (B) and stained as in (E). In costaining experiments where both molecules were asymmetrically inherited, proteasome and T-bet were inherited by opposite daughter cells in 90% of cells (n = 14). Results are representative of three separate experiments. Some micrographs courtesy of John Chang.

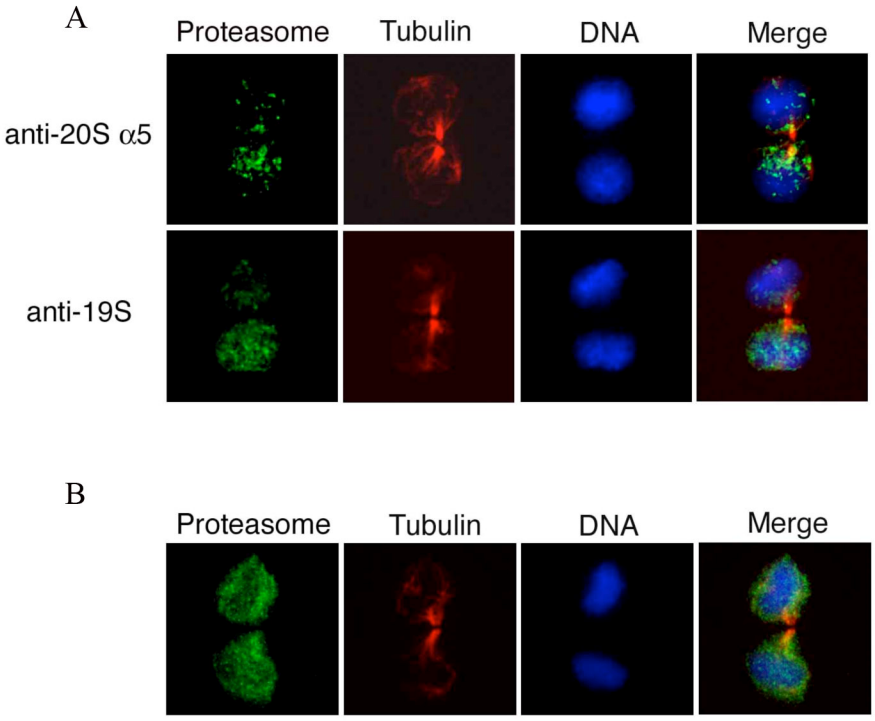

Figure 2.8. **Asymmetric proteasome localization using antibodies directed against two proteasomal epitopes.**
(A) After activation in vitro for 28h, CD4+ T cells were stained with antibodies against two distinct proteasomal epitopes (green), one against a component of the 20S core subunit and the other against a component of the 19S regulatory subunit. Cells were costained for β-tubulin (red) and DNA (blue). Asymmetry of proteasome 20S and 19S localization was observed in 63% (n=30) and 73% (n=30) of cells, respectively. (B) Symmetric proteasome localization in dividing HEK293T cells. 293T cells were examined by confocal microscopy after staining for the proteasome 20S α1 subunit (green), β-tubulin (red), and DNA (blue). Asymmetry of proteasome localization was observed in 0% of cells (n=14). Micrographs courtesy of John Chang.

segregation of the proteasome into the daughter cells (Figure 2.8).

To determine whether asymmetric localization of the proteasome was associated with differential rates of degradation within a mitotic cell, we used the proteasome activity probe MVB003, which functions as a reporter for degradative activity (Florea et al., 2010). After 24 hr of activation in vitro, T lymphocytes were incubated with the proteasome activity probe and examined by immunofluorescence microscopy. Unequal proteasome activity was observed within mitotic T lymphocytes (Figure 2.7C), suggesting that both localization and degradative activity of the proteasome were unequal during cell division. In a model wherein asymmetry of T-bet results from unequal degradation by the proteasome, the greater share of T-bet would be predicted to be partitioned into the daughter cell that receives less proteasome. Costaining experiments with activated CD8+ T lymphocytes dividing in vivo in response to microbe (Figures 2.7D and 2.7E) and activated CD4+ T lymphocytes dividing in vitro (Figure 2.7F) indicated that T-bet was partitioned asymmetrically into the daughter cell that received less proteasome.

The polarity network regulates asymmetry of the proteasome

The observation that a conserved network of polarity proteins is involved in T cell migration, polarity, and asymmetric division (Ludford-Menting et al., 2005; Chang et al., 2007; Yeh et al., 2008) raised the possibility that this conserved network might also regulate asymmetry of the proteasome. In particular, the mammalian homolog of atypical PKC (PKCz), an essential component of a complex containing the partitioning-defective (PAR) proteins Par-3 and Par-6, has been implicated in T cell function (Martin et al.,

2005). To determine whether PKCz might play a role in regulating proteasome asymmetry, we used a pharmacologic inhibitor of PKCz, the myristolated PKCz pseudosubstrate, which has been shown to inhibit its kinase activity (Sun et al., 2005). CD4+ T cells were activated in vitro and treated at 24 hr with vehicle or PKCz inhibitor. We observed that inhibition of PKCz kinase activity resulted in a loss of proteasomal asymmetry (Figure 2.9A). Consistent with these results, PKCz knockdown with a siRNA approach also resulted in a loss of proteasomal asymmetry (Figures 2.9B and 2.9C). Inhibition of PKCz, however, had no effect on PKCz localization in dividing T cells nor did it affect T-bet amounts (Figure 2.10). Together, these results suggest a role for the conserved polarity network in regulating asymmetry of the proteasome and consequently the asymmetry of T-bet.

Phosphorylation of T-bet links its degradation with its asymmetric partitioning

To further explore whether the degradation of T-bet is related to its asymmetric localization, we examined the signals regulating the degradation of T-bet. Tyrosine phosphorylation is a post-translational modification of T-bet that is thought to be critical for mediating its ability to interact with other proteins (Hwang et al., 2005). The inducible T cell kinase ITK phosphorylates T-bet at a critical tyrosine residue 525 (Hwang et al., 2005). ITK is activated and recruited to the T cell receptor by the adaptor protein SLP-76 (Hwang et al., 2005; Jordan et al., 2008). To determine whether tyrosine phosphorylation of T-bet might play a role in targeting it for mitotic degradation, we examined CD4+ and CD8+ T cells from ITK-deficient mice (Hwang et al., 2005) or from mice expressing a tyrosine-to-phenylalanine knockin mutation in SLP-76 at residue 145

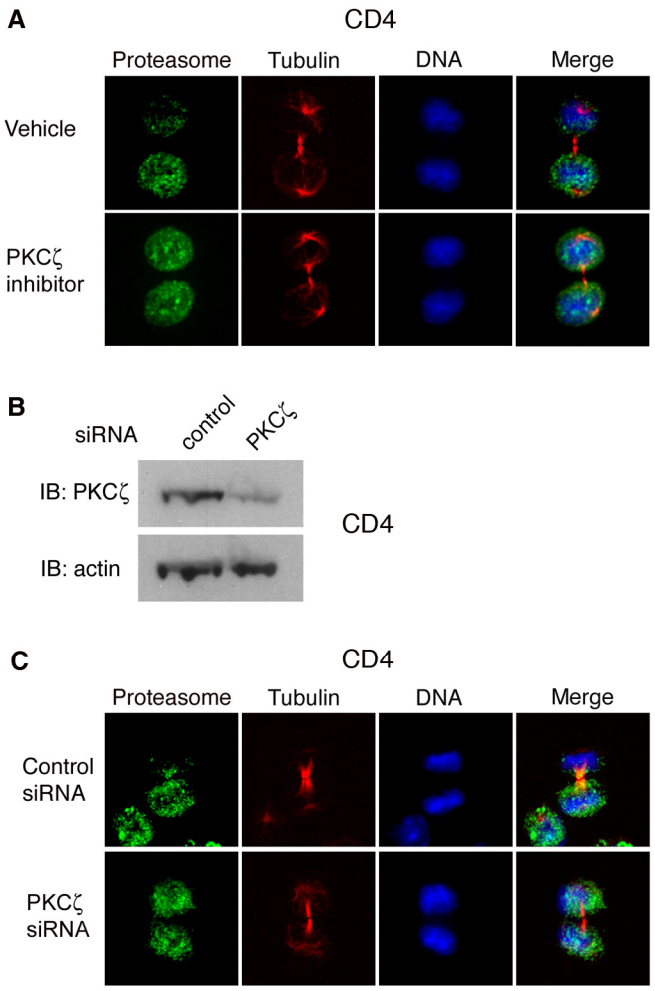

Figure 2.9. **Asymmetry of the Proteasome May Depend on the Polarity Network.** (A) CD4+ T cells were activated in vitro for 28 hr, treated for 1 hr with vehicle or a pharmacologic inhibitor of PKC-ζ, and stained for the proteasomal 20S a1 subunit (green), b-tubulin (red), and DNA (blue). Asymmetry of proteasome localization was observed in 61% (n = 25) of vehicle-treated versus 27% (n = 29) of PKC-ζ inhibitor-treated cells (p % 0.001). (B) CD4+ T cells were activated in vitro for 48 hr, and control or PKCz siRNA was introduced with electroporation. Lysates were prepared 72 hr later, and PKC-ζ and b-actin levels were assessed by immunoblotting. (C) CD4+ T cells were transfected with control or PKC-ζ siRNA as in (B). 48 hr later, cells were restimulated in vitro for 24 hr and stained with the proteasomal 20S a1 subunit (green), b-tubulin (red), and DNA (blue). Asymmetry of pro- teasome localization was observed in 63% (n = 60) of control transfected versus 32% (n = 62) of PKC-ζ siRNA-transfected cells (p % 0.001). Results are representative of two separate experiments.

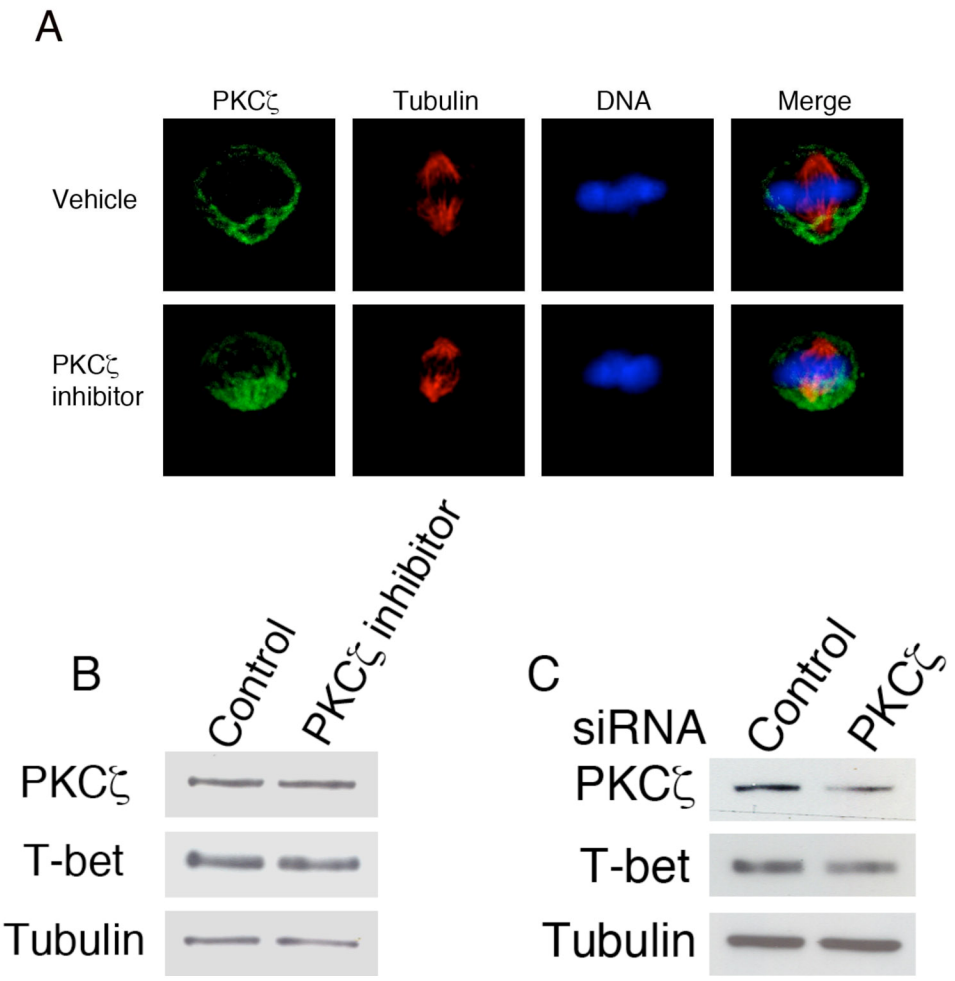

Figure 2.10. **Inhibition of PKCζ does not affect its localization within dividing T cells nor does it affect T-bet levels**.
(A) CD4+ T cells were activated in vitro for 28h, treated for 1h with vehicle (top panel) or a pharmacologic inhibitor of PKCζ (bottom panel), and stained for PKCζ (green), β-tubulin (red), and DNA (blue). (B) CD4+ T cells were activated as in (A), then treated for 1h with vehicle or a pharmacologic inhibitor of PKCζ. PKCζ, T-bet, and tubulin levels assessed by immunoblotting. (C) CD4+ T cells were activated in vitro for 28h, and control or PKCζ siRNA was introduced using electroporation. Lysates were prepared 72h later, and PKCζ, T-bet, and tubulin levels were assessed by immunoblotting.

(SLP-76 Y145F) that prevents the activation of ITK (Jordan et al., 2008). In mitotic T cells from SLP-76 Y145F or ITK-deficient mice, T-bet failed to undergo degradation (Figures 2.11A-D). We also examined cells expressing a mutation of T-bet with a tyrosine-to-phenylalanine substitution at residue 525 (Y525F-T-bet), which prevents it from undergoing phosphorylation (Hwang et al., 2005). CD4+ T cells from T-bet-deficient mice were reconstituted with either wild-type T-bet-GFP or mutant Y525F-T-bet-GFP. We observed that wild-type T-bet, but not mutant Y525F-T-bet, underwent proteasome-dependent degradation during mitosis (Figure 2.12A). Although both constructs are expressed under the control of retroviral regulatory elements, the general transcriptional inactivity of mitosis may allow us to observe T-bet protein degradation. Together these findings suggest that phosphorylation of T-bet is required for its degradation.

If degradation of T-bet is critical for its asymmetry, then defects in phosphorylation that prevent its degradation would also be predicted to disrupt its asymmetry. Mutations that prevent the phosphorylation of T-bet affect its asymmetric partitioning in vivo and in vitro. In mice infected with gp33-L. monocytogenes, CD8+ T cells harboring the SLP-76 Y145F mutation were found to exhibit a loss of T-bet asymmetry compared to wild-type cells (Figure 2.12B). To further test this hypothesis, CD4+ T cells from wild-type, ITK-deficient, and SLP-76 Y145F mice were transduced with cherry-alpha-tubulin and either wild-type T-bet-GFP or Y525F-T-bet-GFP. In contrast to wild-type cells, dividing cells from ITK-deficient and SLP-76 Y145F mice, as well as those transduced with Y525F-T-bet-GFP, could not support asymmetric partitioning of T-bet (Figures 2.12C and 2.12D). These results suggest

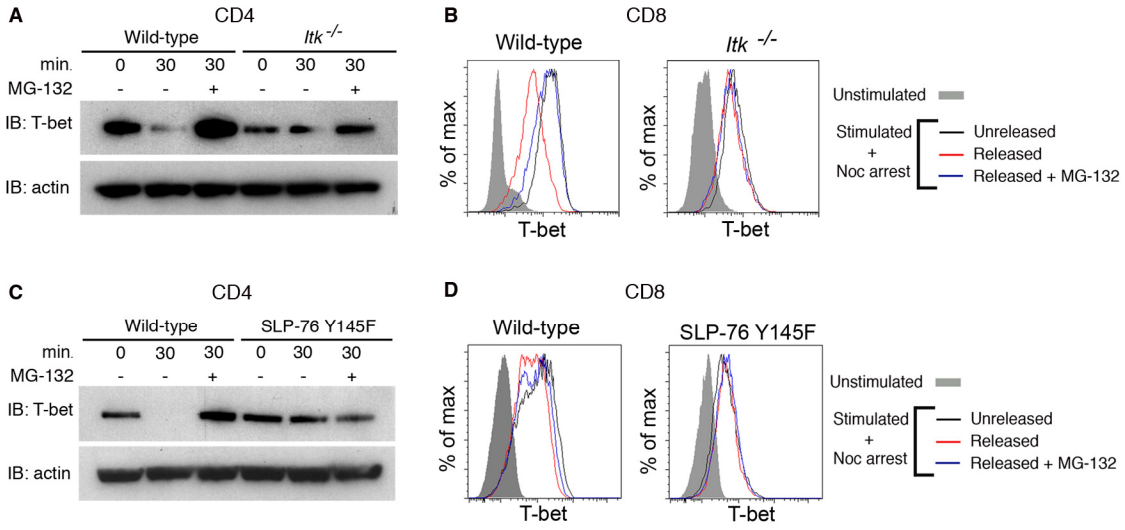

Figure 2.11. **Mutations Preventing Phosphorylation of T-bet Impair Its Proteasome-Dependent Degradation.**
(A) CD4+ T cells from wild-type or Itk-/- mice were activated in vitro and synchronized with nocodazole as in Figure 2.5B. After drug washout, cells were cultured with or without MG-132 for 30 min. Cell lysates were prepared at 0 or 30 min after drug washout. T-bet and b-actin levels were assessed by immunoblotting. (B) CD8+ T cells from wild-type or Itk-/- mice were activated as in (A) and T-bet levels were assessed by intracellular staining at 0 or 30 min after drug washout. (C and D) CD4+ (C) and CD8+ (D) T cells from wild-type and SLP-76 Y145F mice were activated and analyzed as in (A) and (B), respectively. Immunoblots courtesy of John Chang

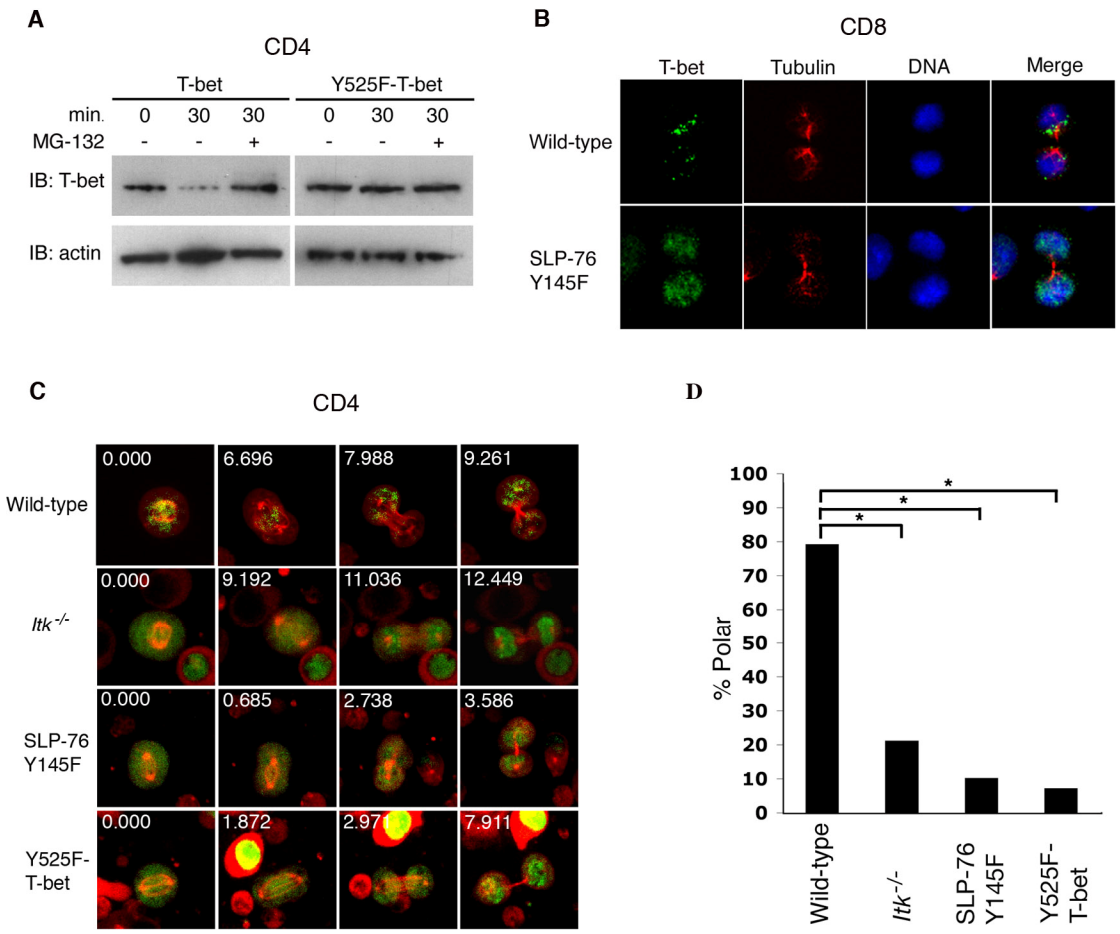

Figure 2.12. **Mutations Preventing Phosphorylation of T-bet Impair Its Asymmetric Partitioning during Mitosis.**

(A) CD4+ T cells from T-bet-deficient mice were transduced with retroviruses encoding wild-type T-bet-GFP or Y525F-T-bet-GFP. After 3 days, cells were restimulated for 24 hr and synchronized with nocodazole and analyzed as in (2.10A). (B) Wild-type or SLP-76 Y145F P14 CD8+ TCR transgenic T cells were adoptively transferred into wild-type recipients infected with gp33-L. monocytogenes, harvested at 36 hr after transfer, and stained for T-bet (green), b-tubulin (red), and DNA (blue). Asymmetric partitioning of T-bet was observed in 72% (n = 21) of wild-type versus 15% (n = 26) of SLP-76 Y145F P14 CD8+ T cells (p % 0.001). (C) CD4+ T cells from wild-type, Itk-/-, and SLP-76 Y145F mice were transduced with cherry-alpha-tubulin and either T-bet-GFP or Y525F-T-bet-GFP. Cells were imaged as in Figure 2.2C. (D) Quantification of asymmetric T cell partitioning into daughter cells represented in (C). The number of cells transduced with T-bet-GFP that were examined in each group: wild-type (46), Itk-/- (48), SLP-76 Y145F (29). The number of wild-type cells transduced with Y525F-T-bet-GFP examined was 27. *p<0.001%. Results are representative of two separate experiments. Micrographs courtesy of John Chang.

that phosphorylation of T-bet, which appears to be required for its degradation, is also necessary for its asymmetric partitioning during mitosis.

Asymmetric localization and function of the proteasome is required for T-bet asymmetry

To further establish a mechanistic link between degradation and asymmetry of T-bet, we treated mitotic T cells with the proteasome inhibitor MG-132. Inhibition of the proteasome resulted in a substantial defect in T-bet asymmetry (Figure 2.13A), suggesting that degradation of T-bet may be causally linked to its asymmetry. It remains possible, however, that preventing T-bet degradation pharmacologically or through the aforementioned genetic approaches might perturb the asymmetric partitioning of T-bet even if an alternative mechanism were responsible for T-bet asymmetry. To evaluate this possibility, CD4+ T cells from T-bet-deficient mice were simultaneously transduced with wild-type T-bet-cherry and mutant Y525F-T-bet-GFP fusions. We observed that wild-type T-bet, but not Y525F-T-bet, was asymmetrically partitioned into the daughter cells (Figure 2.13B). This finding supports the hypothesis that unequal degradation underlies T-bet asymmetry: mutant T-bet lacking the ability to be degraded is mislocalized but does not disrupt the ability of wild-type T-bet to be localized unequally, presumably by asymmetric degradation, within the same dividing cell.

If localized degradation owing to proteasomal asymmetry were responsible for T-bet asymmetry, inhibiting proteasomal asymmetry would be predicted to disrupt the asymmetric partitioning of T-bet. We observed that loss of proteasomal asymmetry resulting from inhibition of PKCz was associated with a loss in T-bet asymmetry (Figure

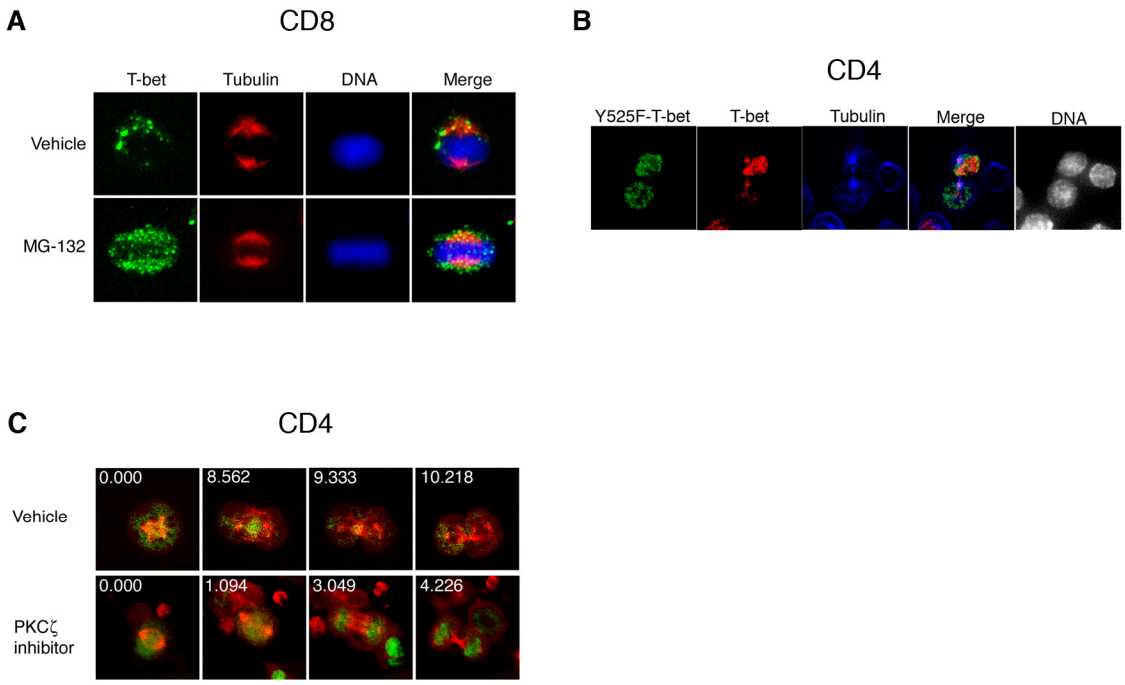

Figure 2.13. **Preventing Degradation of T-bet Disrupts the Asymmetric Partitioning of T-bet.**
(A) Undivided P14 CD8+ TCR transgenic T cells were harvested as in Figure 2.1A and cultured in vitro with vehicle or MG-132 for 4 hr prior to staining with T-bet (green), b-tubulin (red), and DNA (blue). (B) CD4+ T cells from T-bet-deficient mice were transduced with both wild-type T-bet-cherry and Y525F-T-bet-GFP, restimulated in vitro, and stained for b-tubulin (blue). Cytokinetic cells expressing both wild-type T-bet (red) and Y525F-T-bet (green) were scored. Asymmetry of wild-type T-bet and Y525F-T-bet was observed in 60% and 9% (n = 23) of cells, respectively ($p<0.001\%$). (C) CD4+ T cells were transduced as in Figure 2.2C. After 3 days, cells were restimulated for 24 hr, treated for 1 hr with vehicle (top) or PKC-ζ inhibitor (bottom), and imaged as in Figure 2.2C. Asymmetric partitioning of T-bet occurred in 82% (n = 28) of vehicle-treated versus 14% (n = 42) of PKCz inhibitor-treated cells ($p<0.001\%$). Results are representative of two separate experiments. Some micrographs courtesy of John Chang.

2.13C). It remains possible that PKCz may have a direct effect on T-bet asymmetry, in addition to influencing T-bet localization indirectly through its effect on proteasome asymmetry. Inhibiting the activity or asymmetric localization of the proteasome thus prevents unequal partitioning of T-bet. Together these results support the hypothesis that localized degradation of T-bet by virtue of proteasome asymmetry may underlie the asymmetric partitioning of T-bet.

Discussion

When a lymphocyte is engaged in an immune response, it must undergo vigorous cell division to amplify its numbers. The progeny of a selected lymphocyte must also adopt new patterns of gene expression representing the spectrum of fates of antigen-experienced cells. Whether the progeny of a single lymphocyte all adopt the same fate or whether the fates of clonally related cells differ has been difficult to establish. Recent studies with single-cell adoptive transfers and cellular barcoding have suggested the possibility that a single naive cell may give rise to progeny of heterogeneous fates (Stemberger et al., 2007; Schepers et al., 2008; Gerlach et al., 2010). Hypothetically, there are at least two different ways by which sibling cells could adopt dissimilar fates. Cells could be born identically and subsequently receive different signals from their environments, prompting them to diverge in fate. Alternatively, a single cell could unequally transmit information to its daughter cells, causing them to diverge in fate. The evolutionarily conserved process whereby two sibling cells acquire unequal shares of certain determinants is known as asymmetric cell division (Betschinger and Knoblich, 2004; Knoblich, 2008). It has been suggested that a T lymphocyte selected for an immune

response undergoes asymmetric division, enabling it to produce progeny of heterogeneous fates (Chang et al., 2007; Oliaro et al., 2010a).

In order for a lymphocyte to undergo an asymmetric division, it needs to apportion unequal shares of regulatory molecules to its daughter cells. The presence of such determinants at sufficiently high levels should promote the acquisition of one fate, whereas their relative paucity would favor adoption of an alternative fate. In activated CD8+ T cells, the transcription factor T-bet promotes the effector fate at the expense of the memory fate (Intlekofer et al., 2005; Chang et al., 2007; Joshi et al., 2007; Oliaro et al., 2010b; Pepper et al., 2011). In activated CD4+ T cells, T-bet promotes the Th1 cell fate while repressing the development of the Th2 and Th17 cell lineages (Szabo et al., 2000; 2002; Hwang et al., 2005; Lazarevic et al., 2011). These effects of T-bet are highly dose dependent, as shown by the fact that small changes (50% or less) in the abundance of T-bet protein result in profound alterations in CD8+ and CD4+ T cell fate (Finotto et al., 2002; Szabo et al., 2002; Intlekofer et al., 2005; 2007; Joshi et al., 2007). These observations suggest that seemingly small differences in T-bet abundance between the daughter cells of a T cell selected for an immune response would be predicted to influence their subsequent fates.

The present findings suggest that CD8+ and CD4+ daughter T cells that have completed their first division indeed exhibit differences in T-bet abundance. This disparity begins during the single cell stage; asymmetry of T-bet localization can be observed during mitosis and in the nascent daughter cells even prior to the completion of division. After division, asymmetric segregation of the IFN-g receptor (Chang et al., 2007) could reinforce the pre-existing differences in the amount of T-bet protein between

the daughter cells, by virtue of differential IFN-g signaling resulting in unequal T-bet mRNA expression (Lighvani et al., 2001; Afkarian et al., 2002). Together these distinct mechanisms may function to promote differences in T-bet amounts in the daughter cells. What signals instruct a dividing cell to asymmetrically apportion T-bet to its daughter cells? Our findings suggest that the tyrosine kinase ITK may be one such critical signal. ITK participates in signaling events downstream of T cell receptor ligation and has a role in developmental and differentiation pathways in T cells (Siliciano et al., 1992; Atherly et al., 2006; Berg, 2007; Gomez-Rodriguez et al., 2009). The present results suggest that another critical function for ITK is to target T-bet for proteasome-dependent degradation during mitosis. In situations where asymmetric partitioning of T-bet is defective, as in ITK-deficient T cells, the failure to exclude T-bet from the distal daughter cell might be predicted to interfere with its ability to become a memory cell. Such a prediction is consistent with recent evidence that suggests a role for ITK in CD8+ memory cell development (Smith-Garvin et al., 2010). Similarly, in a CD4+ T cell, defective T-bet asymmetry by virtue of ITK deficiency might be predicted to result in excess T-bet partitioned to a daughter cell that otherwise would have been fated toward the Th2 cell lineage, thereby precluding it from developing into a Th2 cell. This is consistent with the defect in Th2 cell differentiation that has been observed in ITK-deficient mice (Fowell et al., 1999; Schaeffer et al., 2001).

Signals that solely target T-bet for destruction might not be sufficient to mediate T-bet asymmetry. In order for T-bet to undergo asymmetric inheritance, the signals that target T-bet for proteasome-dependent degradation must seemingly be accompanied by signals that instruct the cell to segregate some component of the degradation machinery

asymmetrically during mitosis. Here we provide data suggesting that this segregated component may be the proteasome. Although the signals mediating this asymmetric segregation remain to be extensively evaluated, our initial experiments suggest that the conserved cell polarity network may be involved in mediating this effect; loss of function of a key member of this family appears to prevent the proteasome from being asymmetrically distributed. Such a mechanism could allow the polarity network, by regulating asymmetry of the degradation machinery, to influence the partitioning of fate determinants that have been targeted for destruction.

The present findings indicate that regulated destruction controlled by distinct localization of the degradation machinery may be a mechanism to allow for the asymmetric partitioning of cell fate determinants. Recent evidence has suggested that regulated degradation can also occur by virtue of polarized segregation of other components of the degradation pathway, such as ubiquitin or even ubiquitinated proteins themselves (Fuentealba et al., 2008; Narimatsu et al., 2009). In this way, distinct mechanisms regulating degradation may function to render unique transcriptional programs between the daughter cells by unequally degrading key transcriptional regulators, such as T-bet or other transcription factors that regulate T cell fate decisions. In addition, it remains possible that other proteins targeted for destruction during mitosis, such as regulators of the cell cycle, proliferation, or homeostasis, could be unequally inherited by the daughter cells because of proteasome asymmetry. Although the full extent of the disparities mediated by unequal segregation of the proteasome remains to be determined, our findings suggest that proteasome asymmetry may be a mechanism to

allow for the unequal partitioning of determinants that can influence fate and function in sibling cells.

[1] Originally published in *Immunity*, 34, Chang, J.T., Ciocca, M.L., Kinjyo, I., Palanivel, V.R., McClurkin, C.E., Dejong, C.S., Mooney, E.C., Kim, J.S., Steinel, N.C., Oliaro, J., et al., Asymmetric proteasome segregation as a mechanism for unequal partitioning of the transcription factor T-bet during T lymphocyte division, 492–504, Copyright (2011), with permission from Elsevier.

CHAPTER 3: Asymmetric memory T cell division in response to re-challenge[1]

Introduction

Adaptive immune responses require the generation of both effector T cells, responsible for controlling acute infection, and memory T cells, which enable responses to recurrent infections. Whether these two cell populations arise from the same or different naïve T cells has been controversial. Recent evidence suggests that a single cell can beget heterogeneous daughter cell populations (Chang et al., 2007; Stemberger et al., 2007; Schepers et al., 2008). Asymmetric cell division has been suggested as one potential mechanism to generate essential diversity among the progeny of a selected lymphocyte (Chang et al., 2007; Oliaro et al., 2010a; Barnett et al., 2012). Adult tissue stem cells divide asymmetrically to produce a daughter cell fated for differentiation and a daughter cell to maintain the stem cell pool (Morrison and Kimble, 2006). Here we present data to suggest that memory cells responding to re-challenge are capable of undergoing asymmetric cell division and producing two distinct populations of daughter cells that phenotypically resemble secondary effector cells versus self-renewal of the central memory cell pool. These findings further support a stem cell-like model of adaptive immunity.

Results and Discussion

Memory Cells Can Undergo Asymmetric Cell Divisions

We first generated mice containing a defined population of antigen-experienced $CD8^+$ T cells. A small number of P14 $Thy1.1^+$ T cells were transferred to naïve wild-type

mice, which were subsequently infected with $LCMV_{arm}$. After >60 days, mice were infected with *Listeria monocytogenes* expressing gp33 (LMgp33) to specifically re-challenge the GP33-specific memory $CD8^+$ T cells *in vivo*. At 42-46 hrs after re-challenge, $Thy1.1^+$ memory $CD8^+$ T cells were sorted for confocal microscopy. CD3 and the IFN-γ receptor (IFN-γR) polarize to the immunological synapse (Monks et al., 1998; Maldonado et al., 2004) and segregate asymmetrically in mitotic $CD8^+$ T cells recruited into a primary immune response, hereafter referred to as primary responding T cells (Chang et al., 2007; 2011). Using confocal microscopy, we found both of these proteins co-localized with the microtubule-organizing center (MTOC) of blasting, pre-mitotic memory cells (Figure 3.1A). In mitotic memory, CD3 and IFN-γR segregated to one side of the plane of division (Figure 3.1B). Cells with a central memory ($CD62L^{high}$) phenotype appeared more likely to exhibit mitotic asymmetry than cells with an effector memory ($CD62L^{low}$) phenotype (Figure 3.1C), which may be consistent with the suggested division of labor among memory subsets. Effector memory cells preferentially home to non-lymphoid tissues and exert immediate function at sites of pathogen re-entry without needing to divide. Central memory cells retain an intermediate state of differentiation, lymphoid migration, brisk mitotic potential and an apparent capacity to regenerate more memory cells while producing secondary effector cells (Sallusto et al., 1999; Wherry et al., 2003).

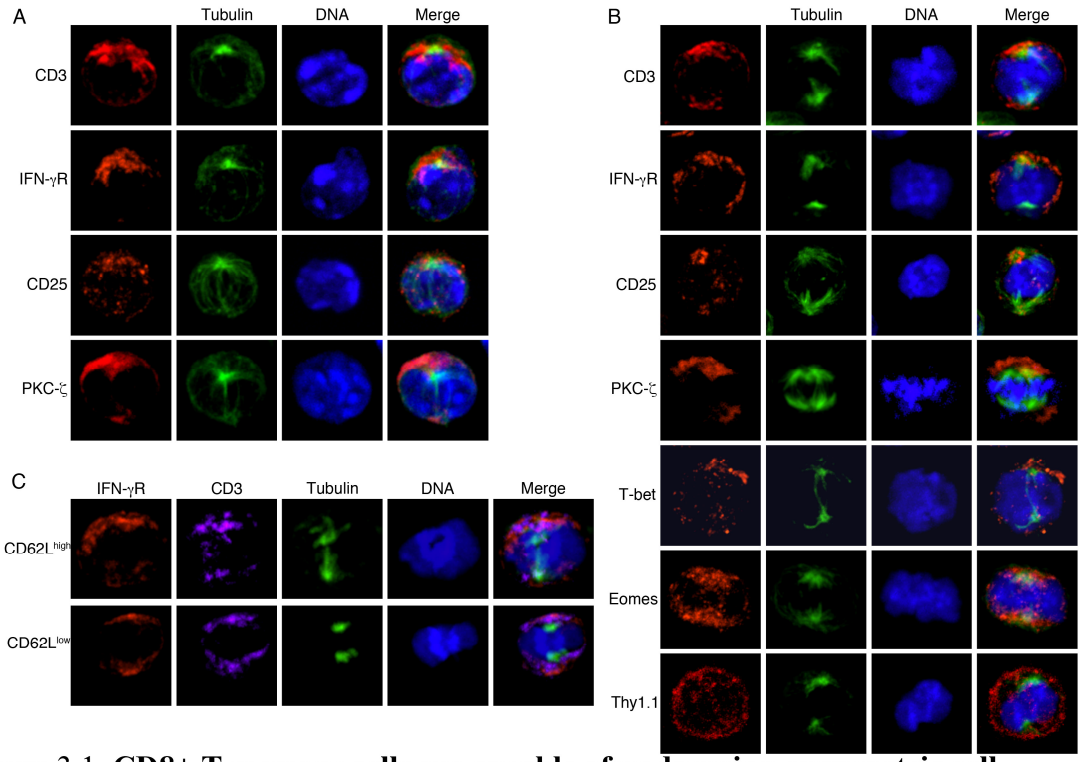

Figure 3.1. **CD8+ T memory cells are capable of undergoing asymmetric cell division.**

(A) WT mice received 5 x10^5 Thy1.1+ P14+ splenocytes and were infected with LCMVarm. At 60+ days p.i., mice were re-challenged with LMgp33. At 42-46h after re-challenge, Thy1.1+ cells were sorted from spleens. Cells were stained for CD3, IFN-γR, or CD25 (red), tubulin (green) and DNA (blue). In interphase blasts, CD3, IFN-γR, and CD25 localized to the same side of the cell as the microtubule-organizing center in 63% (n=11), 78% (n=9), and 64% (n=11) of cells, respectively. Polarity network protein, PKC-ζ (red) also localized with the MTOC in 58% (n=12) of blasts. (B) Mitotic cells beyond prophase were identified by the presence of a tubulin spindle, polarization of MTOCs to opposite sides of the cell, and condensed DNA. CD3, IFN-γR, CD25, PKC-ζ, T-bet, Eomes, and Thy1.1 were polarized in 41% (n=58, $p<0.0001$), 44% (n=56, $p<0.0001$), 46% (n=43, $p<0.0001$), 50% (n=54, $p<0.0001$), 49% of cells (n=35, $p<0.0001$), 12% of cells (n=25, $p>0.1$), and 7% (n=15, $p>0.1$) of cells, respectively, by comparison to tubulin. (C) Comparison of $CD62L^{high}$ and $CD62L^{low}$ subsets. Cells were sorted based on indicated CD62L status and stained for IFN-γR, CD3, tubulin and DNA. In one experiment, IFN-γR and CD3 were polarized in 55% and 52% of $CD62L^{high}$ cells (n=29) versus 35% and 26% of $CD62L^{low}$ cells (n=23). In a second experiment, IFN-γR and CD3 were polarized in 50% and 44% of $CD62L^{high}$ cells (n=16) versus 13% and 13% of $CD62L^{low}$ cells (n=8). Significance of the differences between CD62L subsets across both experiments was compared using a Chi-squared test. $CD62L^{high}$ cells had a greater incidence of IFN-γR and CD3 asymmetry ($p<0.05$ and $p<0.01$, respectively) than the $CD62L^{low}$ subset. Results are representative of three pooled spleens in each experiment.

Memory cells asymmetrically segregate CD25 and T-bet to the same side of the dividing cell

IL-2 is thought to play a role in the re-expansion of memory $CD8^+$ T cells during secondary infection (Williams et al., 2006; Bachmann et al., 2007). We found that the alpha chain of the IL-2 receptor, CD25 was polarized in blasting (Figure 3.1A) and mitotic (Figure 3.1B) memory $CD8^+$ T cells, as had been suggested for CD4+ T cell blasts (Maldonado et al., 2004). We also found that the transcription factor T-bet was polarized during mitosis (Figure 3.1B), as suggested for primary responding cells (Maldonado et al., 2004; Chang et al., 2011). Eomes, however, was not asymmetrically partitioned (Figure 3.1B), suggesting the two homologous transcription factors are regulated differently. Thy1.1 was also evenly distributed during mitosis, suggesting asymmetry is not a feature of all proteins during division (Figure 3.1B).

The ancestral polarity protein, protein kinase C-zeta (PKC-ζ), has been shown to have a role in T cell migration, activation, and asymmetric division of primary responding T cells (Ludford-Menting et al., 2005; Yeh et al., 2008; Chang et al., 2011), as well as T cell differentiation during an immune response (Martin et al., 2005). In pre-mitotic memory cell blasts, we found PKC-ζ polarized to the same side of the cell as the MTOC (Figure 3.2A), opposite of what was observed in primary responding T cells (Chang et al., 2007). Moreover, PKC-ζ was localized to the same side of the cell as CD3 in mitotic memory $CD8^+$ T cells (Figure 3.2B), also opposite from its localization in primary responding T cells (Chang et al., 2007; Oliaro et al., 2010a; Chang et al., 2011). PKC-ζ localized to the same side of the cell as both CD25 and T-bet (Figure 3.2B),

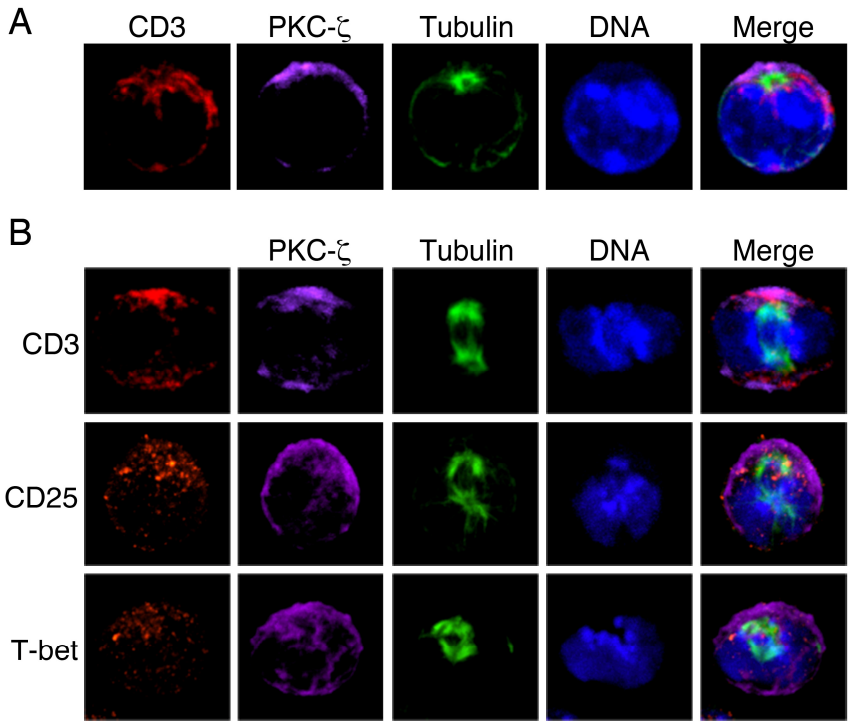

Figure 3.2. **CD25 and T-bet are polarized to the same side of a dividing memory CD8+ T cell.**
Cells were prepared for microscopy as in Figure 3.1 and stained for CD3, CD25 or T-bet (red), and PKC-ζ (purple). (A) In interphase blasts PKC-ζ, localized to the same side of the cell as CD3. In cells where both CD3 and PKC-ζ were polarized, they polarized to the same side of the cell as the MTOC in 100% of cells (n=7). (B) In mitoses, CD3, CD25 and T-bet localized to the same side of the cell as PKC-ζ in 100% (n= 27), 89% (n= 24), and 88% (n=30) of cells, respectively.

suggesting one daughter cell could inherit more CD25 and T-bet than the other daughter cell.

Why PKC-ζ localizes to the opposite side of a dividing memory $CD8^+$ T cell than has been observed in primary responding $CD8^+$ T cells is not yet clear. It has been suggested that PKC-ζ is part of a transcriptional signature shared between memory T and B cells and hematopoietic stem cells (Luckey et al., 2006). It is possible that preformed PKC-ζ protein must be segregated to the putative memory daughter of a primary responding naïve T cell in order to catalyze establishment of the memory cell fate. In re-activated memory cells, it may be unnecessary to donate greater PKC-ζ protein to maintain a less differentiated daughter if enhanced transcription of the gene encoding PKC-ζ is already an established, heritable trait of the memory parent cell. Other differences in the current model system, such as the primary challenge having been viral rather than bacterial may account for the difference in PKC-ζ localization. PKC-ζ function appeared critical for the asymmetry of T-bet (Chang et al., 2011), yet the present findings suggest that T-bet is still asymmetrically inherited in memory cells with PKC-ζ localized on the opposite side of the cell as it was in naïve cells. This suggests that the critical, T-bet-positioning activity of PKC-ζ is independent of the precise localization of PKC-ζ protein, another mammalian atypical PKC (probably PKC-λ/ι) may subserve this function, or that the mechanism for T-bet polarization is not analogous between naïve and memory T cells.

T cell division upon re-challenge yields two distinct phenotypic cell subsets

To further investigate the early phenotype of memory $CD8^+$ T cell progeny, CFSE-labeled $Thy1.1^+$ memory cells were transferred secondarily to naïve mice that were subsequently infected with LMgp33. In uninfected recipients, transferred memory cells displayed heterogeneity of CD62L expression but remained undivided, $CD25^{low}$, and T-bet^{low} (Figure 3.3A). At the earliest point at which division could be detected, first generation memory daughter cells contained differing CD25, CD62L, and T-bet levels (Figure 3.3A). $CD25^{high}$ cells had higher levels of CD8, higher side scatter (SSC), and lower CD62L levels compared to $CD25^{low}$ cells (Figure 3.4A), as has been observed in primary responding $CD8^+$ T cells (Chang et al., 2007). $CD25^{high}$ cells also contained higher amount of T-bet, (Figure 3.4A), which is consistent with the co-localization of CD25 and T-bet in mitotic memory cells (Figure 3.2). It is, therefore, possible that CD25 and T-bet may be unequally inherited during memory cell mitosis.

At slightly later times, we still detected two distinct populations of daughter cells with differential CD25 levels in the spleen (Figure 3.3A). Generally, cells that had undergone more than two rounds of division were $CD25^{high}$ (Figure 3.3A), but a population of $CD25^{low}$ cells that had undergone fewer than three divisions remained detectable (Figure 3.3A). Later generation $CD25^{high}$ cells also contained higher T-bet, lower CD62L, and higher SSC than $CD25^{low}$ cells (Figure 3.4B,C). The observed heterogeneity in daughter cells was induced specifically by antigen-driven division since memory cells transferred into uninfected *Rag1*$^{-/-}$ and wild-type recipients remained $CD25^{low}$ daughter and parent cells, respectively (Figure 3.5). These data suggest that, within the spleen, antigenic activation of memory cells results in two populations.

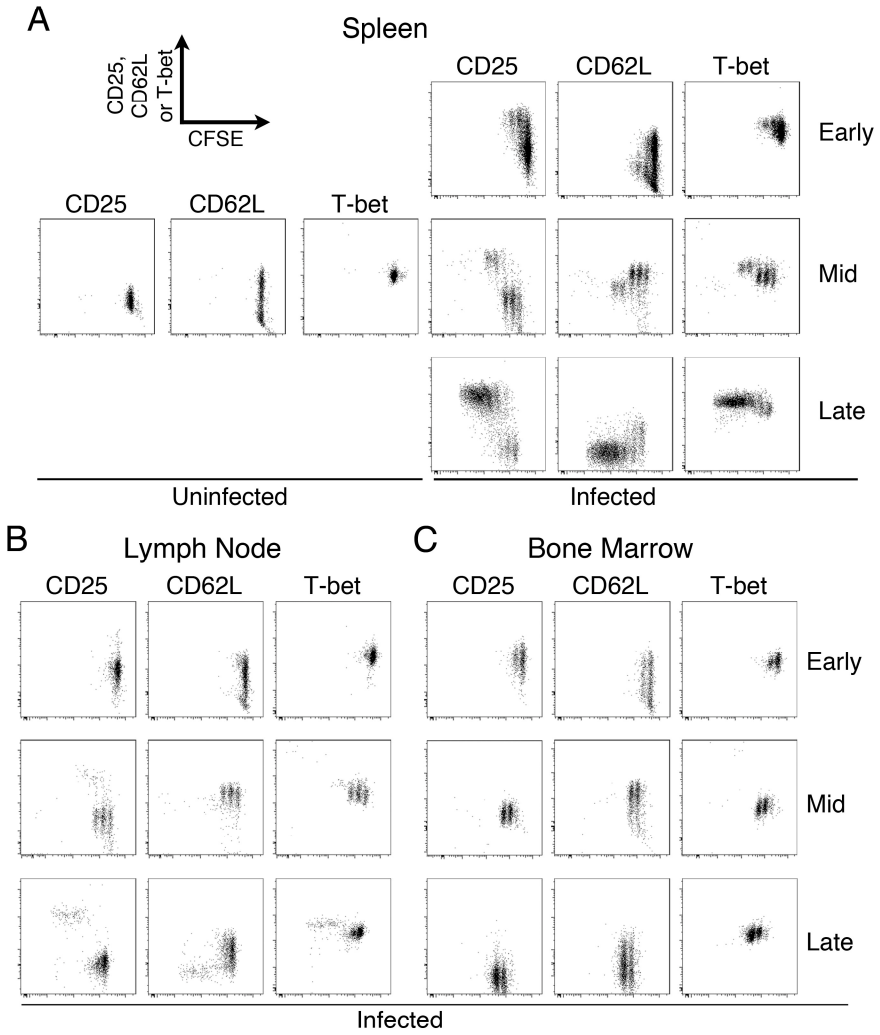

Figure 3.3. **Heterogeneity in the earliest divisions of memory CD8+ T lymphocytes upon re-challenge.**
Spleens were harvested at 60+ days after LCMV infection from mice that had received Thy1.1+ P14+ splenocytes. Cells were labeled with CFSE and transferred to naïve animals, which were then infected with LMgp33. At 42, 47, and 52 hours p.i. with LMgp33, indicated organs were analyzed. All plots are gated on Thy1.1+ CD8+ cells. (A) Response in the absence or presence of re-challenge. In uninfected recipients, transferred memory cells are undivided, $CD25^{low}$, $T\text{-}bet^{low}$, and heterogeneous for CD62L. At the site of primary antigen encounter of the re-challenge, cells can be distinguished based on CD25, CD62L and T-bet levels. (B-C) In the lymph nodes and bone marrow, fewer $CD25^{high}$ cells and divisions are evident. Results are representative of at least two independent experiments per time point.

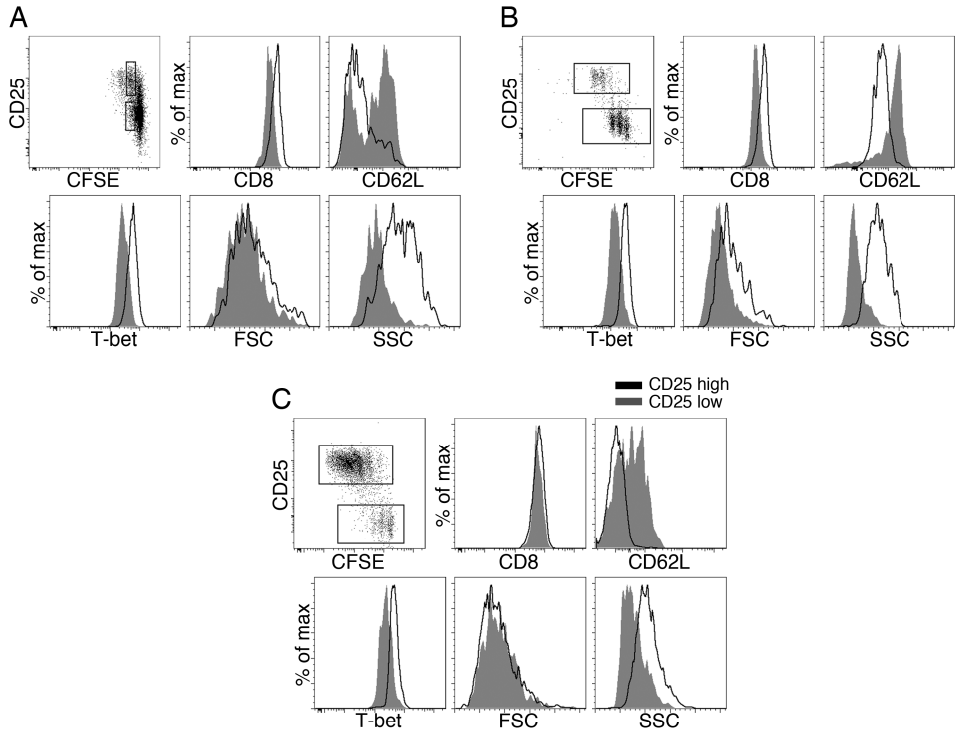

Figure 3.4. **Phenotype of early cell divisions in the memory CD8+ T cell response.** Cells from the spleen were collected as in Figure 3.3 and analyzed at various time points (42 – 52h) post infection with LMgp33. Thy1.1+ CD8+ cells are displayed. (A) Initially, two populations could be detected within the first daughter cell generation, based on differential CD25 levels. (B-C) After further division, two daughter cells populations with CD25 disparity could still be detected. $CD25^{high}$ cells are analyzed in open histograms and $CD25^{low}$ in shaded histograms. $CD25^{high}$ cells displayed higher T-bet and SSC, but lower CD62L than $CD25^{low}$ cells. Results are representative of three independent experiments.

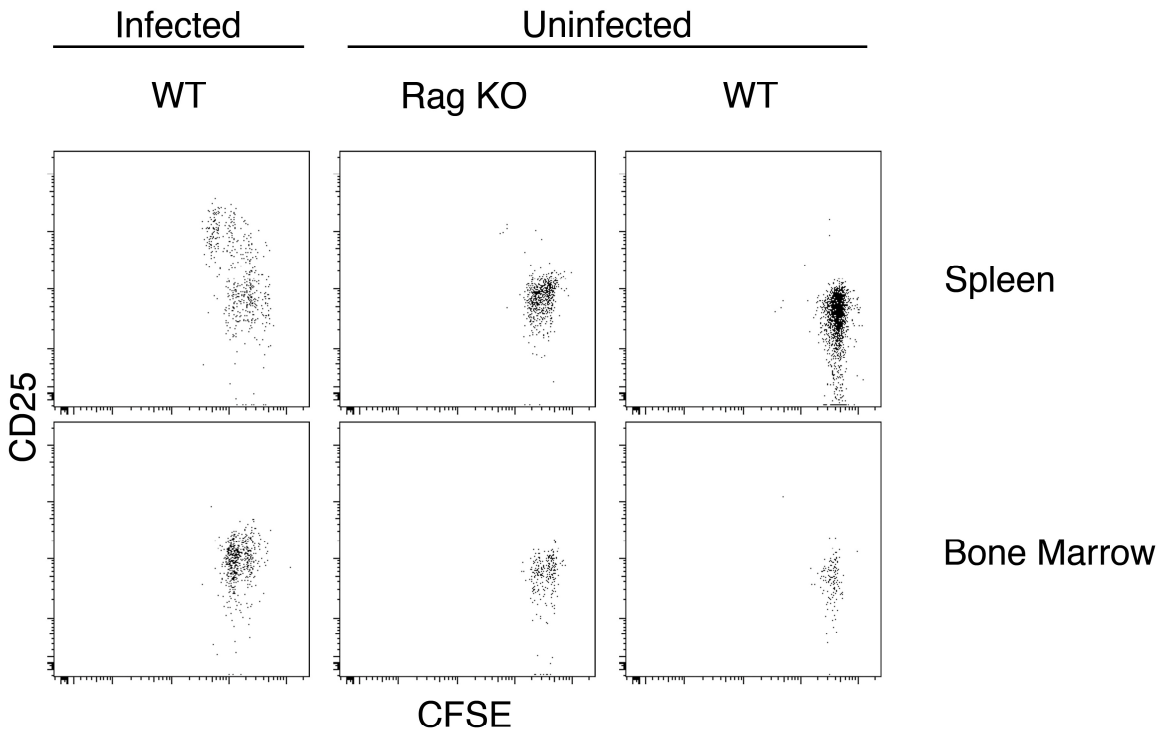

Figure 3.5. **Phenotypic heterogeneity in the early re-call response of memory CD8+ T cells is dependent on antigen activation.**
Splenocytes from mice that had received P14 Thy1.1+ cells and were subsequently infected with LCMV were harvested at >60d post infection and labeled with CFSE. Labeled cells were transferred into new WT animals subsequently infected with LMgp33 (left), into uninfected Rag1-/- animals (middle), and into uninfected WT mice (right). At 46h post cell transfer, spleen and bone marrow were analyzed. Thy1.1+ CD8+ cells are displayed. In the spleen of infected animals, $CD25^{high}$ and $CD25^{low}$ cells are detected. In Rag1-/- recipients, divided and undivided cells are $CD25^{low}$. In uninfected WT mice, cells are undivided and $CD25^{low}$. Results are representative of two independent experiments.

$CD25^{high}$, $T\text{-}bet^{high}$, $CD62L^{low}$ cells, which may represent transit amplification and differentiation of secondary effector cells. $CD25^{low}$, $T\text{-}bet^{low}$, $CD62L^{high}$ cells may represent renewal of a less-differentiated memory pool. The observed bias in the localization of $CD25^{low}$, $T\text{-}bet^{low}$, $CD62L^{high}$ daughter cells in lymph nodes (Figure 3.3B) and bone marrow (Figure 3.3C) may be consistent with their role as a regenerated memory cell pool.

The present data support a model wherein a resting memory $CD8^{+}$ T cell may up-regulate markers of effector differentiation, such as CD25 and T-bet, upon re-encountering antigen. If the re-activated memory cell is capable of asymmetric, self-renewing division, it might beget one daughter cell that contains higher levels of CD25 and T-bet, divides more, and produces the majority of the secondary effector pool. The other, self-renewing daughter cell might inherit low levels of CD25 and T-bet, which facilitates less division and differentiation, thereby replenishing a central memory reservoir. The present data do not exclude conversion of $CD25^{low}$ to $CD25^{high}$ cells, which might even be necessitated if antigen or inflammation persists. The present findings provide a mechanistic basis for how the continual selection of validated clonotypes can accommodate the two mutually opposing demands of adult stem cells, terminal differentiation and self-renewal. Understanding how the process of self-renewal is maintained in infrequent re-challenges and stressed during chronic infection may offer new strategies for immunotherapy.

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CHAPTER 4: Discussion and Future Directions

Introduction

Infections, or invasion by foreign pathogens, are encountered by all living organisms. The ability to clear these infections is critical to the long-term survival of the host. The mechanisms used by the immune system to clear the body of foreign invaders include the use of genetically encoded receptors that recognize commonly encountered pathogen signatures (non-specific immunity), as well as the highly-specific recognition of unique foreign fragments by individualized non-germline encoded receptors (specific immunity). These two arms of the immune system recognize a threat, and then respond to help clear the host of the foreign microbe. The work presented here has focused on how the specific arm of the immune system responds when faced with a pathogenic threat.

When the adaptive immune system is recruited into a response, recognition of the foreign pathogen occurs through presentation of a fragment of that pathogen, by an antigen-presenting cell, to the rare, and highly specific lymphocyte that harbors the receptor cognate to that unique piece of microbe. In order to control the ensuing infection, that lymphocyte is faced with the task of undergoing vast proliferation to amplify its numbers. While proliferation and amplification of the number of pathogen specific lymphocytes is critical, simple expansion of the population is insufficient to mount a fully competent immune response. Diversity among the cellular progeny is also crucial. Heterogeneity among the responding lymphocytes is needed in all arms of the adaptive immune response; CD4+ T cells, CD8+ T cell, and B cells. The generation of this diversity has been the main focus of my work.

Here I have provided evidence that asymmetric cell division may be utilized in the primary immune response to generate the disparate fates that are required of both CD4+ and CD8+ T cells. T-bet, a transcription factor with known roles in governing CD4+ and CD8+ fates during an immune response, is polarized during mitosis in both cell subtypes during an immune response. Furthermore, we have provided evidence of a novel mechanism of asymmetric inheritance. The degradative machinery of the cell, the proteasome, appears to be able to be polarized during mitosis, resulting in unequal capacity for degradation on one side of the plane of division. This, combined with the targeting of the putative fate determinant for destruction, results in asymmetric inheritance of the protein by virtue of it's being retained on the side of the cell with less degradative capacity. This method of generating polarity provides insight into a novel mechanism by which cells may regulate the generation of diversity. Unequal inheritance of the proteasome could result in reciprocal, unequal inheritance of any protein targeted for degradation at the time of mitosis.

Furthermore, we have suggested that the role of asymmetric division in the generation of diversity may also be utilized during a secondary, or re-call, immune response. Previous work has suggested a role for stem-cell like properties in the memory cell response. The role of asymmetric divisions in the maintenance and function of adult tissue stem cells has been characterized in several vertebrate systems. Asymmetric division of memory lymphocytes further strengthens the hypothesis that many of the rules that govern adult tissue stem cells may be regulating immune memory and secondary responses. The goal of many vaccine and other disease treatment strategies is to strengthen the quality and quantity of the memory cell response. Better understanding the

principles that govern memory cell maintenance and function will help to further the development of these technologies.

Asymmetric division as a mechanism for generating diversity

Throughout evolution, asymmetric division is utilized to generate diverse populations of cells from a single parent cell (Betschinger and Knoblich, 2004). From *C. elegans* to *Drosophila* to vertebrates, asymmetric division is used to generate cellular diversity during the development of the embryo or organ, or to maintain homeostasis. Work in model organisms, worms and flies in particular, has elucidated many of the molecular pathways that govern mitotic polarity (Gönczy, 2008).

In vertebrates, the role of asymmetric division is starting to be elucidated in several systems. Asymmetric cell division has been demonstrated to occur in several vertebrate organ systems; gut (Quyn et al., 2010), brain (Götz and Huttner, 2005), skin (Lechler and Fuchs, 2005), mammary glands (Cicalese et al., 2009), muscle (Shinin et al., 2006), and the hematopoietic system (Wu et al., 2007). In all these, the property of asymmetric division has been attributed to the stem cell compartment. Recently, a role for asymmetric division was proposed in the induction of the adaptive immune response in T cells (Chang et al., 2007), and B cells (Barnett et al., 2012). This parallel between adult tissue stem cells and mature lymphocytes highlights a common need in both populations – the necessity of generating cellular diversity within their cellular progeny. Asymmetric cell division is well suited to accomplish this task.

Generation of cellular diversity can occur through at least two mechanisms. Daughter cells can be born identical, and through their interaction with different

extracellular environments, they can stochastically become distinct from one another, resulting in different populations of cells. Alternatively, however, fate disparity can be deterministically organized during division, allowing for enhancement of cellular components to one side of the mitotic spindle, such that one incipient daughter cell inherits more or less of a fate-determining component than another. In this way, the result of the division is two daughter cells that are unique from the moment of their generation. Fate decisions of the daughter cells, therefore, are not stochastic – rather they are preprogramed, ensuring the generation of the specified cell fates. The role of extracellular signals and deterministic patterning is not mutually exclusive. The parent cell of an asymmetric division, frequently a stem cell, often exists in a specific location within the organ, referred to as a niche. The cell that remains in contact with the niche is often the one fated to retain the stem cell like fate (Losick et al., 2011). Often, however, the cell must remain in contact with the niche for this to be true, or re-initiation of contact with the niche can result in re-establishment of the stem-cell like fate (Kai and Spradling, 2003), blurring the line between stochastic and deterministic cellular decisions. The role for asymmetric mitosis is still being understood, but it appears to be necessary for the generation of fate heterogeneity in many settings.

It has been suggested that the generation of immune memory and heterogeneity in the immune response may be stochastic. It was been postulated that a naïve lymphocyte clonally expands when faced with pathogen, resulting in a dramatic increase in the number of copies of that cell. Those cells all adopt an effector cell fate, in order to participate in clearing the present infection. Once the infection is cleared, however, the large proliferative burst must be reversed – many of the now un-needed effector cells die

through apoptosis. Some cells, however, survive. These cells, adopt the memory cell fate. The new environmental setting allows this to occur in the survivor cells. The process repeats itself during secondary infection.

The potential problem with a model that relies on a stochastic decision to make a long-lived memory cell is the risk to the host. Mature lymphocytes are generated during the host's development in a manner that allows for the generation of unique, non-germline encoded receptors in each cell such that no two cells are the same. The benefit of this is that it allows the host to express antigen receptors to a vast array of potential pathogens. This broad coverage comes at a risk to the host as well, as a given adult may have only a very small number of cells that are capable of responding to a particular infection. Since a host will often become infected with the same pathogen multiple times, the lymphocyte with the capacity to respond must be used but not lost during the generation of the immune response. It becomes critical to preserve a T cell clone that has been recruited into an immune response. If the stochastic determination of preserving a T cell after an immune response were to fail or preserve a T cell clone that had somehow become altered, this would mean loss of the recognition of that antigen for that host; a mistake that could prove deadly during subsequent infections. A deterministic model of cell fate, however, allows for the generation of memory cells early within the immune response. In this way, while the host is being protected from the current infection, it is simultaneously establishing the ability to be protected from its next infection.

Polarity in the immune system

The precursor for asymmetric division is the establishment of cellular polarity. In most systems of asymmetric cell division, the cell that is undergoing the division exists in a highly polarized structure. For example, the stem-cell often exists in a niche where it borders another cell that is frequently thought to be critical for its maintenance (Losick et al., 2011). The niche, however, provides more than just survival signals to the putative stem cell. The contact between the stem cell and the surrounding environment is often polarized, for example, as in the skin (Tumbar et al., 2004). This polarity provides an important framework for the establishment of an axis along which the cell can orient itself and position the mitotic spindle such that the gradient of cellular components is perpendicular to the plane of division.

Lymphocytes are highly motile cells that can exist almost ubiquitously around the body. Their lack of a defined home and their representation as free floating in the blood stream endorses a common misconception that lymphocytes are non-polarized, round, uniform cells. This, however, may not be true. Lymphocytes, while perhaps frequently isolated from cellular contact, floating in the blood stream, have a large capacity and propensity to become polarized.

This is particularly highlighted by the contact between the recruited T cell and the antigen-presenting cell. When an antigen-presenting cell encounters a T cell whose receptor recognizes the antigen the cell is presenting, the T cell establishes a firm and long-lasting connection with that antigen-presenting cell. This connection leads to the development of the highly polarized immunological synapse. The immune synapse is dense accumulation of proteins at the site of the T cell – presenting cell interface. Several

aspects of this engagement and polarity have been shown to be critical for the generation of an effective immune response. The initiation of contact between the T cell and the antigen-presenting cell is critical for triggering activation and eventual mitosis of the T cell. The nature of that contact is also critical beyond activation. For example, in settings where the integrin ICAM is missing on the antigen presenting cell, the contact is sufficient to result in stimulation and division of the selected T cell, however, the resulting immune response is defective in the memory population (Scholer et al., 2008). Furthermore, the length and quality of the T cell contact is also important. The interaction between the T cell and the antigen-presenting cell lasts several hours, and disruption of this ensuing T cell polarity, even at very late stages, can have deleterious consequences for the immune response. In mice that are defective for only late stage polarity, the responding T cells can divide and amplify their numbers appropriately, however, the resulting response to pathogen is misappropriated, resulting is specific signaling defects in daughter cells (Yeh et al., 2008).

These and other studies raise the possibility that the maintenance of polarity during a T cell response is required for more than just the coalescence of signaling molecules to the immunological synapse resulting in activation of the cell in order to lead to division and therefore amplification. The suggestion that disrupted polarity can result in seemingly acute activation and amplification, but consequently yield a defective response, raises the possibility that polarity is more than a simple activation requirement. Polarity during the interaction between a T cell and an antigen-presenting cell appears to be critical for the generation of cell fate later in the immune response. While this could be suggestive of a scenario in which the signals required for the various cell fates are

acquired during the initial cell contact, it is also suggestive of a role for the polarity itself – the regulation and maintenance of polarity could be critical to the initial responding cell because the early and late interphase polarity could be setting up mitotic polarity. In other words, the previously studied defects in T cell polarity that result in an aberrant immune response could be in part related to ensuing defects in T cell division. This hypothesis assumes that the polarity that allows a T cell to establish an asymmetric cell division comes from the site of contact between the T cell and the antigen-presenting cell. The suggestion that several T cell components that are polarized during mitosis are components of the immunological synapse (Chang et al., 2007) supports this. Induction of polarity in the previously uniform T cell by the interaction with a cognate antigen-presenting cell would therefore be a critical step not only for activation of the T cell, but the nature of this contact would be critical for the eventual cell fate of the generated progeny.

For asymmetric division to be a mechanism for the generation of fate disparity, however, there must be specific components that are polarized, and subsequently asymmetrically inherited, that then play a critical role in the regulation of cell fate in the daughter cells. Understanding what these fate determinants are, and how they are regulated during polarization of the cell, may be critical to our ability to manipulate the response for therapeutic benefit.

Fate determinants in the T cell response

Several factors, both external and internal, have been found to be critical to the development of the heterogeneous fates of a T cell response. The finding that T cells may

undergo asymmetric cell division during the initiation of an immune response (Chang et al., 2007), predicts that during an asymmetric division critical fate-determinants would be segregated unequally between the two daughter cells. Identification of a fate determinant in T cells relied on educated hypotheses based on previous studies. An ideal fate determinant in asymmetric cell division regulates one fate at the expense of the other in a dose dependent manor. While there are many candidates for fate determinants in T cells, several transcription factors have been well studied for their role in both CD4+ and CD8+ T cell fates (Szabo et al., 2000; 2002; Pearce et al., 2003; Intlekofer et al., 2005; Joshi et al., 2007; Kallies et al., 2009; Shin et al., 2009; Banerjee et al., 2010). T-bet was of particular interest because of its dose-dependent effects (Joshi et al., 2007), and repressive role in memory formation (Intlekofer et al., 2007). T-bet, in way ways, resembled an idealized fate-determinant.

In both CD4+ and CD8+ T cell development during an immune response T-bet appears to play a formative role. It is a critical driver of the Th1 lineage (Szabo et al., 2000), at the expense of other CD4+ lineages. In CD8+ cells, T-bet is critical for promoting the effector cell fate at the expense of the central memory CD8+ T cell fate (Intlekofer et al., 2007). Now we have data that suggests that T-bet is unequally inherited between the two putative daughter cells of a CD4+ and CD8+ primary division. In this way, one daughter cell inherits more T-bet, and may be more likely to develop the Th1 or effector cell fate.

In the setting of CD8+ T cells, furthermore, this unequal inheritance of T-bet appears to be reiterative. T-bet is polarized during the initial division to one daughter. The daughter that inherits less T-bet may be predicted to be more likely to adopt the

central memory cell fate. This central memory cell, when re-challenged with pathogen, again divides asymmetrically, and again polarizes T-bet to one side of the plane of division. Furthermore, the daughter cell populations can be discerned based on their levels of T-bet in both the primary and secondary responses, further suggesting that polarization during mitosis may generate two populations of daughter cells.

In the secondary response, we also observed that, while T-bet is polarized during mitosis, the homologous transcription factor eomesodermin (Eomes) was not. Eomes contains a very high homology to T-bet (Pearce et al., 2003) and several aspects of their regulation overlap in CD8+ T cells. Eomes is critical to the development of the effector cell response (Pearce et al., 2003), but also appears to play a role in the regulation of CD8+ central memory development (Banerjee et al., 2010). Despite their similarities, these two transcription factors have disparate roles in the regulation of cell fate. These differences in cell fate may predict their different regulation during mitosis. Eomes, which appears to be required for the fates of both daughter cells, is equally inherited between the two resulting daughter cells. Due to its role in the development of both incipient fates, it cannot act as a molecular switch between the two. T-bet, however, does appear to act as a molecular switch, and is asymmetrically segregated between the two daughter cells.

Asymmetric destruction as a mechanism of asymmetric inheritance

Asymmetric division results in an unequal amount of a particular fate determinant distributed to two daughter cells. The mechanisms by which this can be achieved are widely varied. In *C. elegans* and *Drosophila*, there are several mechanisms that exist for

different fate determinants in different cells. Even during a single asymmetric cell division, several factors may be polarized to one side of the cell or the other, each with its own mechanism. One mechanism that is presented here is the unequal destruction of the fate determinant during mitosis. Unequal inheritance through unequal destruction, however, may not be a novel concept. In *C. elegans*, the fate-determining transcription factor, SKN-1, is polarized to one of the daughter cells resulting from the initial division of a fertilized embryo. The regulation of SKN-1 polarization was found to be dependent on the novel protein, EEL-1. Through homology, EEL-1 was predicted to be a HECT E3 ligase. Indeed, further analysis revealed that EEL-1 interacts with the C-terminus of SKN-1 through its N terminus, as would be expected for an E3 ligase-substrate interaction. In addition, the ubiquitin-ligase domain for EEL-1 is critical for the degradation and localization of SKN-1 (Page et al., 2007). Taken together these results suggest that SKN-1 polarization is regulated, specifically at the one-to-two cell stage embryo, by ubiquitin-mediated degradation. Furthermore, the loss of SKN-1 degradation through knockdown of EEL-1 resulted in a highly specific defect – only the asymmetry of SKN-1 was disrupted in this system. Other known asymmetrically inherited proteins continued to be asymmetrically inherited. In many ways, this story is similar to our findings in T-cells. When the ability of the cell to degrade T-bet is disrupted, either through genetic manipulation of the signaling pathway that results in T-bet being susceptible for degradation, or through manipulation of the proteasome, T-bet asymmetry is specifically lost. The asymmetry of other markers, however, was preserved.

In addition to the role for degradation in *C. elegans* asymmetric cell division presented above, other examples of ubiquitin-mediated degradation playing a regulatory

role in cell polarity and asymmetric cell division have been discovered. For example, in mammalian systems it has recently been demonstrated that members of the Smurf family of HECT E3 ubiquitin ligases play critical roles in the establishment of planar cell polarity (Narimatsu et al., 2009). Furthermore, it has been suggested that several proteins marked for degradation by the presence of ubiquitination may be inherited asymmetrically in human embryonic stem cells undergoing self-renewing divisions in culture (Fuentealba et al., 2008). These are other examples of the potentially unexplored role for proteasome dependent degradation in asymmetric cell division. The hypothesis that proteasome-regulated degradation plays a conserved role in asymmetric inheritance of proteins during mitosis is an attractive one because of the potential for the cell to target several proteins to be distributed unequally between the two resulting daughters while only spending the energy to target and maintain one cellular entity in a polarized fashion.

The question they arises as to how the cell localizes the proteasome to only one side of the cell. Is the entire 26S entity moved to one pole? Is the 20S core ubiquitously expressed and only the 19S regulatory cap localized to one side? Is the proteasome formed on only one side of the cell? While all these possibilities are feasible, it seems unlikely that only one component of the 26S proteasome, the core or the cap, is being localized to one side, since staining for either component of the complex in T cells showed a polarized distribution. One hypothesis then, is that the proteasome is being nucleated on one side of the cell, and the presence of an enhanced amount of the proteins that lead to proteasome formation on one side of the cell results in the presence of more active proteasome on that side. This would allow for a more conventional regulation of the localization of the proteasome: perhaps a nucleating factor, a single protein, could be

polarized in a manor parallel to other asymmetrically inherited fate determinants, for example, through an association with one of the protein complexes that polarizes during an asymmetric division, such as the Par-complex (Par-3, Par-6 and aPKC).

Is the canonical Par complex conserved in T lymphocytes? While one of the human homologues to aPKC, PKC-□has been show to localize to the putative distal daughter in primary responding T lymphocytes (Chang et al., 2007), neither Par-3 nor Par-6 localization has been studied. The polarity complex containing Scribble, however, which is frequently observed on the opposite side of the cell from the Par complex (Knoblich, 2001) has been preliminarily studied in T cells, with Scribble localizing to the opposite side of the cell from PKC-□ (Chang et al., 2007). Future work needs to be done to understand the function of the polarity network in T cells, as well the meachanistic link between the polarity network and the localization of the proteasome.

Regulation of proteasome localization during asymmetric cell division is a potentially attractive hypothesis for regulation of cell fate during division across multiple species and evolution. Polarization of the proteasome may regulate cell fate through unequal destruction of specific cell fate determinants (T-bet in T cells, Perhaps SKN-1 in *C. elegans*) in the two incipient daughter cells. The logic, however, that the entire 26S proteasome would be polarized to account for unequal inheritance of one fate-determining factor is perhaps surprising. More logical may be that the polarization of the proteasome is set up to establish a broad switch in cell fate between the two resulting daughter cells. While only one target of proteasome dependent degradation during mitosis has been identified in these studies, the full effects of proteasome polarization have not been studied. For example, in T cells, large networks of transcription factors and

other proteins balance cell fate decisions. It is possible that T-bet is one of many factors targeted for degradation during mitosis, and the proteasome is regulating not one, but several fate-determinants at a time. By polarizing one component, therefore, the cell can generate large changes in daughter cell fate by targeting various proteins for degradation.

Memory T cells and a Stem-cell model of immunity

The regulation and role of asymmetric cell division in the immune system is just beginning to be understood. The understanding of asymmetric cell division in the immune response started with observations of the phenomenon in T cells (Chang et al., 2007) and B cells (Barnett et al., 2012). Here we've identified a possible mechanism by which asymmetric cell division may regulate fate disparity and fate decisions during the primary (Chapter II) and secondary (Chapter III) immune responses. The role of asymmetric cell division in the regulation of fate decisions is reminiscent of the role of polarized divisions in many adult tissue stem cell systems (Knoblich, 2001). Several groups have noted this parallel between adult tissue stem cells and the immune system. In particular, early work demonstrated the subsets of memory CD8+ T cells may function as stem cells in the immune system of mice (Zhang et al., 2005), a finding that was later duplicated in humans (Gattinoni et al., 2011), and perhaps applies to other T cell subsets (Muranski et al., 2011).

The hypothesis that the adaptive immune system functions under a stem-cell or stem-cell like model requires an understanding of the singular nature of the immune system. One of the adaptive immune systems greatest strengths is that it provides the host protection against a wide variety of foreign pathogens. The mechanism by which this

diversity is generated, however, comes at a price to the host. Since random genetic recombination and mutation are used to generate the wide diversity of receptors in lymphocytes, no single lymphocyte can be re-created in the host. Each T and B cell generated is highly specific and unique. The adaptive immune system, therefore, is well designed to protect the host against a broad array of potential pathogens, but only once per pathogen in each host's lifetime. Hosts, however, often encounter the same pathogen multiple times. Lifelong protection, therefore, requires preservation of the singular, unique, and non re-creatable clone. In this way, the preserved clone will be able to respond in the chance of re-exposure to pathogen.

Adult tissue stem cells are filling similar demands as memory lymphocytes; they are preserving a particular genetic state of development in order to maintain an organ. They must maintain their population for the lifetime of the host in order to sustain the organ they supply. While most organs face a constant low-level demand for regeneration, the possibility of acute regeneration is also common. For example, in the hematopoietic system, there is a constant low-level of loss and repopulation of the blood lineages as terminally differentiated cells such as red blood cells and neutrophils die and need to be replaced to maintain homeostasis. Situations arise, however, when the production of these cells needs to be up regulated acutely, for example during an infection when the demand for neutrophils increases or during hemorrhage when the loss of blood results in a more global deficit. In these situations, signals are relayed to the hematopoietic stem cell that result in up-regulation of production of terminally differentiated progeny. This is similar to a situation when re-encounter with a pathogen might result in the need for mobilization and proliferation of a memory lymphocyte.

The basic biological need is not the only similarity between adult tissue stem cells and lymphocytes. Genomic analysis has revealed shared transcriptional profiles of memory T and B cells with hematopoietic stem cells (Luckey et al., 2006), for example. Another parallel may be the use of asymmetric division to establish fate disparity among daughter cells. In several systems of adult-tissue organ homeostasis, asymmetric cell division has been described. Examples range from the *Drosophila* testes (Yamashita et al., 2005) to several vertebrate systems including skin (Lechler and Fuchs, 2005), muscle (Shinin et al., 2006), gut (Quyn et al., 2010), and the hematopoietic system (Wu et al., 2007). The use of asymmetric cell division appears to be a critical feature of almost all known stem cell populations, highlighting a potential link between adult lymphocytes and stem cells.

Beyond the ability, and perhaps requirement, to divide asymmetrically, several other parallels between lymphocytes and stem cells exist. Stem cells frequently exist in highly specific regions of the organ they foster, referred to as stem cell niches (Losick et al., 2011). Similar properties may be true to memory lymphocytes. Memory cells, specifically central memory cells, have been proposed to preferentially localize to a niche within the bone marrow (Mazo et al., 2005). The various functional and phenotypic parallels between adult tissue stem cells, specifically hematopoietic stem cells, and long-live memory cells highlight the logic behind a hypothesis for stem-cell properties governing aspects of the immune response. If the immune system is indeed governed by some of the same principles as adult tissue stem cells, this will provide novel mechanisms by which memory formation, maintenance, and function can potentially be modulated.

This opens up new avenues for therapeutic intervention in several fields from vaccine development to cancer therapies.

Future Directions

The role of asymmetric cell division on fate and function in the immune system is just beginning to be understood. The data presented here represents advances in an understanding of how asymmetric cell division may be used to modulate fate of daughter cells (Chapter II) and how asymmetric cell division may be repeatedly utilized to ensure fate disparity and maintenance of a T cell clone (Chapter III). There is much left to understand regarding both the mechanisms of fate disparity and the generalizability of asymmetric cell division in the immune system. Future work will hopefully help expand the principles elucidated here and allow for a better understanding of how the immune system as a whole regulates cell fate decisions and maintains life-long protection of the host, which will help enhance technologies in many areas.

One area of continued investigation is the role of asymmetric division in the regulation of fate disparity in primary responding T cells. While studies presented here outline one potential mechanism by which asymmetric division may regulate cell fate in primary responding T cells (Chapter II), there are likely many more factors. While we have provided evidence that T-bet is asymmetrically inherited through its polarized destruction by the proteasome, the full repercussions of proteasomal asymmetry have not been studied. Future work may focus on understanding the complete role of proteasome polarity during primary T cell division. Large-scale protein analysis is a potentially useful mechanism for uncovering the multiple changes that ensue following polarized

proteasome inheritance. In primary CD8+ T cells responding to microbes *in vivo*, daughter cells can be sorted into CD8 high and low subsets (Chang et al., 2007), thought to represent the two daughters of an asymmetric division. As technologies allow more cells to be captured in these experiments and protein analysis can be performed on increasingly small numbers of cells, assays such as mass spectrometry can allow the content of these two populations to be compared to each other, and to activated naïve cells. Studies such as these will help to uncover protein signatures of these cells, providing further avenues of study regarding other factors that may be asymmetrically inherited during division. Furthermore, identification of ubiquitinated proteins in responding T cells, perhaps again through proteomics approaches, may also provide a mechanism by which determination of the molecular signature of proteasomal asymmetry could be understood. As mentioned above, proteasome asymmetry possibly regulates asymmetric destruction of several proteins, rather than just one. Identification of methods that allow for unbiased determination of which proteins are asymmetrically segregated will allow for rapid advances in understanding how fate disparity is regulated in T lymphocytes responding to a primary challenge. Also, if proteasome asymmetry is an evolutionarily conserved mechanism of generating fate disparity, these approaches will be able to be applied to areas of study beyond the immune system.

While we have provided evidence to suggest that asymmetric division does occur in the immune response both during primary and secondary responses, the biological importance of this phenomenon has been unexplored. Future studies should include perturbation of the molecular process of asymmetric cell division, either entirely or partially, and determination of the impact on the overall immune response. For example,

genetic manipulation of the polarity network of proteins through targeted deletion is possible. T cell specific, or perhaps activation specific deletion of one or several of these highly conserved proteins may lead to defect in cellular asymmetry, as is suggested by the siRNA and chemical inhibitor to PKC-ζ studies presented previously (Chapter II). The use of genetic deletions will allow for the study of these cells during an intact immune response. These studies may answer several questions: Is asymmetric cell division critical for generating cell fate during an immune response? Are the daughter cells of a response sufficiently plastic or exposed to a diverse set of signals such that the required cellular fates can be recapitulated even in the absence of cellular asymmetry? If global asymmetry is perturbed, can the responding cells be skewed toward one fate or another depending on the mechanism of perturbation? In other words, will manipulation of asymmetry allow for the ability to direct the immune response towards one particular cell fate over another? Can cells be forced to develop a memory cell fate or an effector cell fate? Can CD4+ T cells be skewed *in vivo* towards one particular subset? If manipulations like these are possible, they might allow for large advances in vaccine development and the treatment of a whole host of immune mediated diseases, from asthma to cancer.

Summary

At the time this work began, the understanding of the role and impact of asymmetric cell division in the immune system was just beginning to be understood. It was known that asymmetric division was an observable phenomenon, but an understanding of how it contributed to fate disparity and its generality within the immune

response was not clear. Based on the findings presented here, the role of asymmetric division is becoming increasingly clear. A putative model for how protein disparity in daughters could regulate cell fate in the immune response through the unequal inheritance of at least one critical fate-determining protein, T-bet, is suggested. Furthermore, the role of asymmetric division is now extended to the memory cell response, highlighting the re-iterative, stem-cell like nature of adaptive immunity. Manipulation of cellular asymmetry would have an impact on not only the development of a primary response, but perhaps also secondary responses. Furthermore, an enhanced understanding of the regulation of memory cells, and how they are similar and different from adult tissue stem cells, will help enhance our ability to develop technologies that permit enhanced memory cell generation. This could represent a major advance in the field of vaccine development. While there are many aspects of asymmetric division to still be explored within the immune response, these findings should help further future efforts at understanding the disparity of fate in the adaptive immune response.

APPENDIX: Experimental Proceedures

Mice

All animal work was done in accordance with Institutional Animal Care and Use Guidelines of the University of Pennsylvania. All mice were housed in specific-pathogen-free conditions prior to use. Wild-type C57BL/6 and P14 TCR transgenic mice recognizing LCMV peptide gp33-41/Db were used; generation of Tbx21-/- (T-bet-null) mice has been previously described (Intlekofer et al., 2005). The generation of mice expressing a tyrosine-to-phenylalanine knockin mutation in SLP-76 at residue 145 (Y145F) has been described (Jordan et al., 2008). SLP-76 Y145F P14 TCR transgenic mice were generated by breeding P14 TCR transgenic mice with SLP-76 Y145F mice. Itk-/- mice have been described (Liu et al., 1998). Adoptive transfers and infectious challenges with gp33-Listeria monocytogenes were performed as previously reported (Chang et al., 2007).

T Lymphocyte Confocal Microscopy

Immunofluorescence of T cells was performed as previously described (Chang et al., 2007) with the following antibodies: anti-b-tubulin (Sigma); anti-T-bet, anti-CD3e, anti-Eomes (eBioscience); anti-a-tubulin, anti-PKCz, anti-proteasome 20S a1, anti-proteasome 20S a5, anti-proteasome 19S (Abcam); anti-IFNgR-biotin (BD Bioscience); anti-CD25 (BioLegend); and anti-mouse and anti-rat Alexa Fluor 488, anti-mouse, anti-rabbit, and anti-rat Alexa Fluor 568, anti-rat and anti-rabbit Alexa Fluor 647, and streptavidin-conjugated Alexa Fluor 647 (Invitrogen). Hoechst 33258 (Invitrogen) was

used to detect DNA, or ProLong Gold with DAPI (Invitrogen) was used to both label DNA and mount coverslips on glass slides. The proteasome activity probe MVB003 has been previously described (Florea et al., 2010).

Acquisition and Analysis of T Lymphocyte Confocal Microscopy

Mitotic cells were selected for analysis based on the appearance of tubulin staining; cells undergoing cytokinesis were identified by dual nuclei and pronounced cytoplasmic cleft by brightfield, and then, secondarily, the morphology of the other fluorescence channels was revealed. Acquisition of image stacks was performed as previously reported (Chang et al., 2007). The volume of 3D pixels (voxels) containing the designated receptor fluorescence was quantified within each hemisphere of mitotic cells or within each nascent daughter in cytokinetic cells with Volocity (Improvision) software. In mitotic cells, the two hemispheres were delineated with the pattern of tubulin fluorescence to define the poles of the mitotic spindle, with the equator bisecting the line connecting the two poles. In cytokinetic cells, the two nascent daughters were delineated via the pattern of tubulin fluorescence to define the border of each daughter cell. Receptor enrichment in one hemisphere or in one nascent daughter cell greater than 1.5-fold compared to the other hemisphere or daughter cell was considered polarized. All images are depicted with pseudo-colors. In cells labeled with CFSE, the ‘‘true’’ green channel occupied by CFSE fluorescence was not shown. In such cells, antitubulin staining was detected with Alexa Fluor 488, which could be resolved in the green channel because of its enhanced brightness relative to CFSE.

Cell Culture

CD8+ or CD4+ T cells were purified with the CD8+ or CD4+ T Cell Isolation Kit (Miltenyi), respectively. For microscopy experiments, naive cells were activated in vitro with immobilized anti-CD3/anti-CD28 and immobilized recombinant ICAM1-Fc fusion protein (R&D Systems), and previously activated cells were restimulated in vitro with immobilized anti-CD3 and immobilized ICAM1-Fc. In certain experiments, cells from T-bet-deficient mice were simultaneously transduced with both wild-type T-bet-cherry and Y525F-T-bet-GFP. In some experiments, after 28 hr of activation, cells were incubated with the proteasome activity probe MVB003 (5 mM) for 2 hr prior to harvesting cells for immunofluorescence studies. In certain experiments, an inhibitor of PKCz, the myristoylated PKCz pseudosubstrate (10 mM) (Invitrogen), was added to cells 28 hr after activation for 2 hr prior to harvesting cells for immunofluorescence studies. For biochemistry experiments, naive cells were activated in vitro with immobilized anti-CD3 and anti-CD28, and previously activated cells were restimulated in vitro with immobilized anti-CD3. Nocodazole (1 mM) (Sigma) was added after 24 hr of stimulation to reversibly synchronize the cells in G2/prometaphase. After 12–16 hr of nocodazole arrest, cells were washed free of nocodazole and then cultured in media alone or with MG-132 (10 mM) (Calbiochem), calpain inhibitor I (100 nM), or lactacystin (100 nM) (Sigma). In other experiments, cells were activated in vitro with immobilized anti-CD3 and anti-CD28 in the presence of mimosine (300 mM), hydroxyurea (200 mM) (Sigma), or nocodazole. After 40 hr, cells were washed free of drug and cultured in media for an additional 30 min.

Statistical Analysis

For Chapter II, Asymmetry of cells was summarized as proportions and compared with chi-square or Fisher's exact test, as appropriate. All statistical tests were two-tailed. p values of <0.05 were considered significant. For Chapter II, cells were divided in halves along the equatorial plane relative to the two poles of the mitotic spindle. Fluorescence of a specific protein was calculated for each half, and the ratio between halves was compared to the ratio of tubulin fluorescence. Distribution of protein in a cell was designated asymmetric if its ratio was 2 standard deviations greater than the ratio for tubulin. Each cell was designated either asymmetric or symmetric, resulting in binary data. Chi-squared tests were used to compare the frequency of asymmetry between different experimental groups and/or molecules. P values <0.05 were considered significant.

Retroviral Constructs

The cherry-alpha-tubulin fusion construct has been previously described (Day et al., 2009). Generation of the MIGR and T-bet-MIGR construct has been described (Mullen et al., 2002). For T-bet-C-terminal-GFP or T-bet- C-terminal-cherry fusion constructs, PCR was performed with Pfx polymerase (Invitrogen) with a forward primer including a BglII site (50-ATGACAGATCTCC ACCATGGGCATCGTGGAGC-30). For T-bet-GFP, the reverse primer was designed by omitting the stop codon and adding an EcoRI site for in-frame fusion to GFP (50-ATGACAGAATTCTGTTGGGAAA ATAATTATAAAACTGGC CTTC-30). For T-bet-cherry, the reverse primer was 50 - ATGACAGAATTCGTTGGGAAAATAATTATAAAACTGGCCTTC-30 . The PCR

product was digested by BglII and EcoRI (New England Biolabs) and fused in-frame with Cherry in the MIGR retrovirus vector (Shu et al., 2006). The Y525F mutation was introduced with the following primers: forward 50-ATGACAGATCTCCA CCATGGGCATCGTGGAGC-30 and reverse 50 –ATGACAGAATTCT GTTGGGAAAATAATTAAAAAACTGGCCTT-30.

Immunoblotting and Immunoprecipitations

Cell lysates were prepared in 1% NP40 lysis buffer with the following additives: 0.1 M DTT (Roche), protease inhibitor cocktail, sodium vanadate (10 mM), NaF (10 mM), and PMSF (10 mM) (Sigma). Protein was prepared for SDS-PAGE followed by transfer to nitrocellulose membrane. Immunoblotting was performed with the following antibodies: rabbit anti-PKCz, anti-tubulin-HRP (Abcam), mouse anti-T-bet (eBioscience), anti-mouse or anti-rabbit-HRP (Cell Signaling), and b-actin-HRP (Sigma).

RNA Interference

CD4+ T cells were purified and stimulated in vitro with immobilized anti-CD3/ anti-CD28 for 48 hr prior to electroporation with control or PKCz ON-TARGET SMARTpool siRNA (Thermo Scientific) with a ECM830 Squarewave Electropo- rator (BTX). Pulses were performed for 10 ms at 190 mV. 48 hr after electropo- ration, cells were analyzed by immunoblotting or restimulated for microscopy studies.

Flow Cytometry

Adoptive transfers and infectious challenges with gp33-Listeria monocyto- genes were performed as previously reported (Chang et al., 2007). Splenocytes were stained with anti-T-bet-Alexa Fluor 647 (BD Bioscience) or T-bet-eFluor 660 (eBioscience) and anti-CD8 PE (BD Bioscience) and analyzed on a FACS Calibur (BD Bioscience).

Adoptive transfers and infectious challenges

Splenocytes (5x105) from naïve P14 TCR-transgenic mice harboring the Thy1.1+ allele were transferred intravenously (i.v.) into non-irradiated C57BL/6 (Thy1.2+) recipients that were subsequently infected intraperitoneally (i.p.) with 2x105 plaque-forming units of LCMV Armstrong (LCMVarm) strain, which is cleared by d8 post infection (p.i.). For microscopy experiments, mice at day 60+ p.i. were infected i.v. with 5x103 colony forming units of recombinant Listeria monocytogenes expressing gp33-41 (LMgp33). At 44-46h p.i., P14 CD8+ memory cells were harvested from infected mice by sorting Thy1.1+ cells from the spleen. For flow cytometric analysis, spleens were harvested from mice day 60+ p.i. with LCMV. 2.5x107 carboxyfluorescein succinimidyl ester (CFSE) labeled splenocytes were transferred i.v. to naïve mice. One day after transfer, secondary recipients were infected i.v. with LMgp33. 42 – 52h after infection, single cell suspensions were stained with indicated antibodies.

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