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In Vivo Genetic Fate Mapping of T-cell Receptor Signal Strength

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In Vivo Genetic Fate Mapping of T-cell Receptor Signal Strength

by

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DISSERTATION

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Dedications

To my wife (and our family).

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I performed all the molecular biology, ES cell targeting, and Southern blot screening that lead to the successful creation of the 3373 3SCS reporter mice used in all subsequent experiments, summarized in Fig 2-1**.** Mark produced the HTNC-Cre, and assisted in the characterization of the reporter alleles.

***In Vivo* Genetic Fate Mapping of T-cell Receptor Signal Strength**

Stephen A. Chmura

Abstract

Following acute infection, rare naive T-cells expand in number, differentiate into effector cells, and acquire functions to combat the pathogen. Resolution of the response results in the death of a majority of the effector cells and survival of a small population of memory T-cells with properties that contribute to increased protection from reinfection. Many T-cell intrinsic and extrinsic signals have been found to enhance or detract from effector and memory cell differentiation. It has been postulated that strength of antigen-receptor signals influences subsequent fate of naive T-cells during the effector to memory transition, however, the nature of these signals and mechanisms of action are unclear.

An approach was taken to genetically mark populations of T-cells in mice that differed in the amount of antigen-receptor stimulation. By using this method with the well characterized acute lymphocytic choriomeningitis virus (LCMV) model of infection, the fate of antigen-specific CD4+ effector T-cells generated as a result of different amounts of TCR signal strength was determined in vivo. Our findings provide in vivo evidence in support of a deterministic model of CD4+

memory generation following acute viral infection where programming events driven in part by the strength of initial antigen-receptor stimulation influence the fate of responding CD4+ cells during the transition to memory.

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Chapter 1

Introduction

A Model T-cell Response

Following their generation in the thymus, naive T-cells circulate throughout the body's blood and lymph systems continuously scanning antigen presenting cells (APCs), typically dendritic cells (DCs), within secondary lymphoid tissues. Upon recognition of foreign peptides displayed by DCs, the naive T-cell becomes activated. T-cell activation is dependent upon the T-cell integrating many extracellular signals including antigen density, costimulation, and local cytokine milieu (Jenkins et al., 2001). These signals contribute to T-cell activation and expansion, along with qualitatively influencing T-cell fate during the effector response (Kaech and Wherry, 2007; Williams and Bevan, 2007).

Upon sufficient activation, the single T-cell goes through an enormous expansion phase concomitant with chromatin remodeling and changes in gene expression (Figure 1-1) (Ansel et al., 2003; Blattman et al., 2002; Butz and Bevan, 1998; Whitmire et al., 2006). During this period, the resulting T-cell effector population acquires effector functions including secretion of cytokines and expression of effector proteins and migratory receptors. Following the effector phase, the lymphocyte population will contract in size leaving behind a

small population of memory lymphocytes capable of long term survival (Macleod et al., 2010; McKinstry et al., 2008; van Leeuwen et al., 2009).

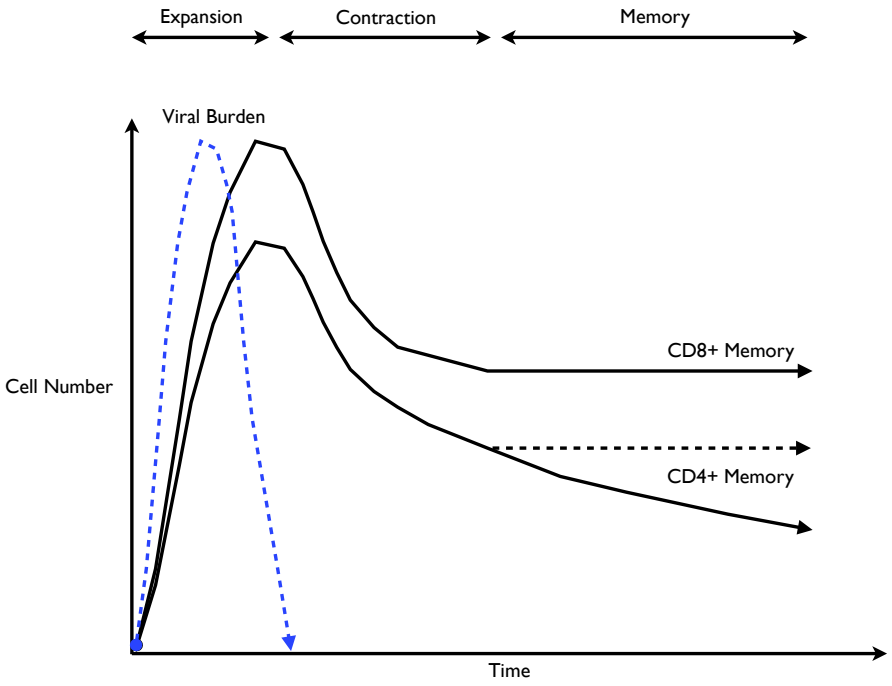

FIGURE 1-1. The T-cell Response Following Acute Infection. Recognition of viral peptides by the lymphocyte compartment leads to expansion and acquisition of effector functions to combat the viral infection. The effector phase is followed by contraction of the responding lymphocytes populations, resulting in a relatively stable and long lasting memory population of CD4+ and CD8+ T-cells. The kinetics and magnitude of expansion of responding CD4+ and CD8 lymphocyte populations may differ, but generally CD8+ T-cells exhibit greater expansion than CD4+ T-cells. CD4+ memory T-cells may decline in number. Adapted from Kaech et al., 2002.

Memory T-cells

The generation of memory lymphocytes is a hallmark of the adaptive immune system. Memory lymphocytes are capable of protecting the host by producing a more robust response following reinfection with a previously encountered pathogen. Increased protection is a result of an increase in the frequency of differentiated, antigen-reactive cells advantageously localized within the host (Masopust et al., 2001; Reinhardt et al., 2001; Sallusto et al., 1999). A significant lag period occurs following primary infection within the naive host as rare antigen-specific lymphocytes recognize, proliferate and differentiate following infection. During this period of time, the pathogen may replicate, damage tissue, and spread to secondary hosts. Increased frequencies of antigen reactive memory lymphocytes generate effector responses more rapidly by minimizing time required to expand and control the infection (Homann et al., 2001; MacLeod et al., 2009a).

Responses by memory lymphocytes have been shown to be of greater quality than naive cells with the same antigen specificity. Memory lymphocytes have undergone long-lasting changes to their chromatin structure, enabling effector responses to be generated more effectively, with transcriptional machinery poised on promoters, ready for action (Ansel et al., 2003; Whitmire et al., 2008b). Memory lymphocytes may be found in both lymphoid and non-lymphoid tissues. Their localization within non-lymphoid tissues often places them on the frontline of protection against reinfection, minimizing the time required for pathogen recognition and reactivation (Masopust et al., 2001;

Reinhardt et al., 2001). For these reasons, the deliberate generation of memory lymphocytes is a primary goal of successful vaccination programs. Therefore, studies investigating biological mechanisms that influence the generation of long-lasting pathogen-specific memory populations from rare naive lymphocytes, have significant importance.

Infection activates the host's defenses against foreign pathogens by expanding rare pathogen-specific naive CD4+ and CD8+ T-cells along with B-lymphocytes. Once amplified in number, pathogen-specific CD8+ T-cells typically clear intracellular infections via direct cell cytotoxic mechanisms and secretion of cytokines. Pathogen-specific B-cells protect the host from extracellular pathogens by generating antibodies that neutralize the pathogen, facilitate its uptake by macrophages, or target it for destruction by the complement pathway. Although these responses may be generated without CD4+ T-cell help, their overall quality and longevity may be greatly increased with concomitant CD4+ T-cell activation (Janssen et al., 2003; McHeyzer-Williams and McHeyzer-Williams, 2005; Shedlock and Shen, 2003; Sun and Bevan, 2003). CD4+ T-cells can provide help to both CD8+ T-cell and B-cell responses through the secretion of effector cytokines and by direct cell-cell contact (Fazilleau et al., 2009a; Janssen et al., 2005; Williams et al., 2006). The ability to modulate both CD8+ T-cell and B-cell responses positions the CD4+ T-cell at a pivotal position during an effective immune response. Thus, studies performed to uncover and better understand biological mechanisms involved in CD4+ T-cell activation, function and maintenance have great potential to influence T- and B-cell immunity and

findings from these studies may contribute to vaccine development and improvement of immunotherapeutic approaches for treatment of cancer.

Whereas these stated beneficial properties of CD4+ T-cells have largely been attributed to effector CD4+ T-cell populations, increasing evidence supports the notion that memory CD4+ T-cells provide protection during reinfection with intracellular bacteria (Flynn et al., 1993), worms (Anthony et al., 2006), and during viral infections (Liang et al., 1994; MacLeod et al., 2009b; Swain et al., 2006). Data from influenza studies suggest a T-cell-based vaccine against internal virus-derived peptides may be more beneficial to preventing infection than viral particle surface antigens targeted by B-cells that seasonally change (Swain et al., 2006). Memory CD4+ T-cells may provide greater help than naive T-cells to memory B-cell populations as a result of their decreased dependence on costimulation and ability to respond to an array of APCs (Croft et al., 1994). Thus, a greater understanding of the nature and mechanism of signals that influence CD4+ T-cell fate resulting in long lasting memory cell populations and protection are of great significance.

Fate Determining Signals

The influential signals that contribute to naive T-cell activation and fate determination during an immune response will be discussed in detail below. These signals can broadly be categorized into two groups; intrinsic signals related to the TCR structure and affinity for antigen, and extrinsic signals encountered by the T-cell during and following activation upon interacting with

antigen and APCs. It is becoming clearer that the impact a particular signal has on cellular fate may vary between CD4+ and CD8+ T-cell lineages. These differences likely reflect discrepancies in properties and requirements between the two lineages for effector and memory fate determination, and will be highlighted where appropriate.

Intrinsic Fate Determining Factors

The T-cell Receptor

It has been nearly thirty years since the discovery of the TCR and elucidation of the combinatorial mechanism by which a host generates a diverse TCR repertoire within the naive T-cell populations (Brack et al., 1978; Davis et al., 1984; Kappler et al., 1983). Naive T-cells express antigen receptors (T-cell receptors or TCRs) with a broad range of specificities (Davis and Bjorkman, 1988) (Bousso and Kourilsky, 1999). It has been estimated that the potential number of unique TCRs capable of being generated approaches $1x10^{15}$ in the mouse (Davis and Bjorkman, 1988) and $1x10^{18}$ in humans (Janeway, 2005). Given that an adult mouse may have $1x10^{8}$ total naive T-cell lymphocytes at any time, it is theoretically possible that every naive T-cell expresses a unique TCR. Studies quantifying TCR diversity of the naive T-cell population, by estimating the product of all possible contributors to TCR diversity (Arstila et al., 1999; Bousso and Kourilsky, 1999), by titration of TCR transgenic T-cells (Blattman et al., 2002; Whitmire et al., 2006), and most recently by direct observation following tetramer-enrichment (Moon et al., 2007) have shown only a very small subset, in the

range of 10 to 3000 cells per animal, are capable of recognizing a particular foreign peptide epitope in the context of self-MHC, leading to activation and expansion. This small number of potentially reactive T-cells makes it difficult to study early antigen-specific activation events, prior to the enormous expansion phase that then allows for reliable detection and characterization of antigen-specific cells with conventional reagents and functional assays.

Furthermore, studying antigen-specific populations of T-lymphocytes has been relatively difficult, as individual TCR-peptide-MHC interactions, with affinities estimated on the order of $1x10^{-6}$ M (Alam et al., 1996), are quite weak compared to antigen-receptor interactions found within the B-cell lineage that may range in affinity from $1x10^{-4}$ - $1x10^{-14}$ M (Steward and Steensgaard, 1983). Identification of antigen-specific T-cell populations also relies on knowledge of complex tripartite interactions between the TCR, processed antigenic peptide, and MHC protein (Sant and Yewdell, 2003). Unique *in vivo* systems have been employed to gain insight into how the broad diversity within the naive repertoire may influence effector fate.

Certain strains of mice generate cellular responses to foreign antigens dominated by lymphocyte repertoires of limited TCRα- and β-chain diversity. McHeyzer-Williams and Davis took advantage of such a system, the anti-pigeon cytochrome c (PCC) CD4+ T-cell response in I-E^k expressing mice, to characterize the antigen-specific response following immunization in the context of a normal polyclonal T-cell repertoire (McHeyzer-Williams and Davis, 1995). By analyzing TCR α- and β-chain expression, along with single cell analysis of TCR

third complimentary determining region (CDR3) sequences, responding T-cells were found to express unique lengths and amino acids sequences within the CDR3 of the TCR. Analysis of multiple TCR-pMHC protein crystal structures have shown the CDR3 loop of the TCR is juxtaposed to the displayed peptide within MHC and important in determining peptide specificity and repertoire diversity (Garcia et al., 1998). These unique CDR3 lengths and sequences found within the responding population of PCC-reactive cells *in vivo* were previously observed in cell lines and hybridomas of PCC-reactive CD4+ clones, indicating their significance in PCC antigen-recognition (Hedrick et al., 1988). Interestingly, certain PCC-specific sequences were consistently found at the peak of the response and could be grouped into hierarchies with respect to abundance. These hierarchies of abundance remained constant during the contraction of the primary effector population and during the expansion of secondary effector cells following challenge. This suggested that these CDR3 sequences were being selected with antigen and played a role in determining clonal abundance during a response. Similar results characterizing the TCR repertoire by means of Vα and Vβ chain expression, PCR analysis and spectratyping of CDR3 lengths within CD8+ clones (Casanova et al., 1991), during infection or immunization (Bousso et al., 1998; Busch and Pamer, 1999b; Butz and Bevan, 1998), and by tetramer-reagent studies (Altman et al., 1996; Homann et al., 2001; Murali-Krishna et al., 1998), have revealed that the TCR is the primary checkpoint for expansion and certain TCRs within the antigen-

reactive population selectively undergo expansion based upon their unique TCR structures.

Comparisons of TCR-repertoires between effector T-cells and subsequent memory populations have shown relative conservation of TCR Vβ chain usage following bacterial (Busch et al., 1998a; Busch et al., 1998b) and viral infections (Blattman et al., 2000; Lin and Welsh, 1998; Sourdive et al., 1998). These data suggested that a stochastic mechanism within the pool of effector cells selected for memory cells (i.e. strict stochastic selection would predict relative conservation in receptor diversity between effector and memory populations because particular TCRs would not be favored). Equally important, it also suggested that the signals that influence the expansion of naive T-cells play a significant role in memory cell determination due to this apparent lack of selection. Such signals included those related to TCR-affinity for pMHC complexes.

TCR-peptide-MHC affinity and avidity

Thymic selection of lymphocyte precursors shares many similarities to peripheral T-cell activation including the importance of TCR-peptide-APC interactions influencing subsequent fates. An avidity model has been proposed to explain how structural diversity in TCRs within the population of preselected thymocytes may lead to divergent fates during thymocyte development; namely progression to the naive lymphocyte pool or programmed cell death (Ashton-Rickardt et al., 1994; Hogquist et al., 1994). In this model, both the TCR affinity

for antigen as well as the amount of antigen influenced cell fate. In general, very strong or very weak TCR affinities for selecting peptides led to cell death, and intermediate affinities led to maturation. Furthermore, the small differences in affinities quantitatively measured in soluble TCR-peptide-MHC interactions along with small differences in the amount of selecting antigen that were necessary to change a pro-life selection event into a pro-death selection event suggest this mechanism is quite sensitive (Alam et al., 1996; Ashton-Rickardt et al., 1994; Hogquist et al., 1994; Savage and Davis, 2001). Similar data measuring TCR affinities for peptides in mature T-cells have been shown to predict functional responses in mature peripheral T-cells (Lyons et al., 1996; Matsui et al., 1994). Thus, TCR affinities may shape T-cell responses and influence cell fate *in vivo*.

Experiments carried out by Savage and Davis using tetramer reagents to measure affinity and kinetic differences within responding T-cell populations provided evidence in support of quantitative differences in TCR affinities determining fate *in vivo* (Savage et al., 1999). Here it was found that the effective affinities of the responding CD4+ MCC/I-E^k-reactive population were higher in secondary responders than in primary effectors T-cell. T-cells expressing TCRs with slow off-rates (and long half-lives) preferentially survived the primary response and contributed at a higher frequency to secondary responses than low affinity clones. This was most likely the result of retention of unique T-cells during the contraction phase of the primary response, or selective expansion of these T-cells during the secondary response (Savage et al., 1999). Similar “affinity maturation” has also been shown during bacterial infection in

CD8+ cells (Busch and Pamer, 1999a; Busch et al., 1998a). These results indicated TCR-affinities for antigen peptides influenced which naive T-cell clones contributed to the expanding populations of effector cells, which in turn had long lasting effects on the generation of memory CD4+ and CD8+ T-cells.

Detailed characterization of affinity-maturation within responding T-cell populations during early time points, prior to the peak of expansion, was lacking due to the small numbers of cells one could isolate in an antigen-specific manner for analysis. To decipher the importance of TCR affinity and off-rate to clonal selection of CD4+ T-cells early during an immune response, McHeyzer-Williams performed an intriguing experiment using two separate TCR-β chain transgenic animals (Malherbe et al., 2004). These two transgenic animals incorporated two separate TCR β-chains (2B4-β and 5CC7-β) that were found to be enriched within PCC responses in B10.BR mice following immunization. Mice expressing these TCR β-chains had pre-immune repertoires that were enriched for PCC-specificity, yet, were polyclonal. This approach allowed for selection of preferred clones to occur normally following PCC immunization. Notably, individual PCC-reactive clones were present at a high enough frequency in the pre-immune repertoire that allowed for direct measurement of off-rate and affinity with tetramer reagents; previously experimentally impossible.

By comparing the pre-immune repertoires of the 5CC7-β- and 2B4-β-expressing mice with those generated in the same mice as a result of PCC immunization, exhaustively examining CDR3 sequence, affinity for tetramer, TCR off-rates, and Vα-chain usage, it was found that CD4+ T-cells with strong

affinities for antigen were enriched within the effector population as a result of selective expansion of these clones. Although enrichment for high affinity clones was not as apparent in the 5CC7-β model, likely as a result of the generation of pre-immune cells with generally high PCC-I-E^k affinities, immunized 2B4-β mice showed recruitment of nearly all PCC-reactive clones early during the response, and then selective loss of clones with lower affinities for antigen during expansion. These data are consistent with two affinity thresholds existing; a quantitatively lower affinity threshold that allowed recruitment into the response, and a higher affinity threshold that when achieved allowed full expansion. Both contributed to shaping the immune response *in vivo* following immunization, and thus were important in determining fate of responding CD4+ T-cells.

CD8+ T-cells were found to undergo similar affinity-based selection during an immune response *in vivo*. Using characterized altered-peptide ligands (APLs), single point mutations within the antigenic peptide that alter the affinity for transgenic OT-I TCRs, Bevan and colleagues generated a library of APL-containing bacteria that could be used to compare the responses of a transferred transgenic CD8+ T-cell population to peptides of differing affinity, under controlled inflammatory conditions (Zehn et al., 2009). Similar to McHeyzer-Williams' finding within the CD4+ lineage (Malherbe et al., 2004), CD8+ responses were determined by TCR affinity for antigenic peptide. High affinity APLs generated larger populations of effector T-cell during the peak of the response, and low affinity APLs generated relatively smaller effector cell populations. These differences in effector population sizes had lasting effects in the memory phase.

Importantly, low affinity APLs were still able to generate memory cells, although at decreased frequency compared to infection with high affinity APLs. Kinetic analyses of the responses indicated low affinity APLs generated effectors that stopped expanding earlier in the response, compared to high affinity APLs. This may have been due to early migration into peripheral tissues. Surprisingly, all memory cells, independent of the APLs used for their generation, were able to survive for months, and proliferate and expand in number upon secondary challenge. Thus, TCR affinity within the CD8+ lineage contributed more to the overall size and magnitude of the response, and less in determining long-term cellular fate.

Signal Strength

Studies manipulating the strength of TCR stimulation have tried to decipher the underlying mechanisms that determined fate as a result of quantitatively different amounts of TCR signalling. Correlations between high TCR affinities with subsequent cell fate in the thymus and during peripheral activation suggested these quantitative differences may have induced various amounts of signalling within the T-cell (Daniels et al., 2006; Malherbe et al., 2004; Savage et al., 1999; Savage and Davis, 2001; Zehn et al., 2009). Initial *in vitro* findings by Iezzi and Lanzavecchia have shown extended periods (40 hours) of T-cell-APC interactions are necessary for naive CD4+ T-cells to undergo expansion. Costimulation by B7-CD28 interactions played a role in activation by

effectively lowering antigen-concentrations necessary for expansion (Iezzi et al., 1998). Strong *in vitro* stimulation of CD4+ cells by antigen-receptor cross-linking antibodies also led to greater fitness upon adoptive transfer of *in vitro*-activated CD4+ cells into host mice than weak stimulation (Gett et al., 2003). These strong stimulatory conditions correlated with increased expression of anti-apoptotic family member proteins (Bcl/xL, and Bcl-2) and increased expression of gamma-chain cytokine receptors (IL-2, -7, and -15). Therefore, at least within the CD4+ lineage, strong and/or extended periods of *in vitro* activation promoted greater fitness than weak and/or short periods of activation (Lanzavecchia and Sallusto, 2002).

In contrast to these findings, studies by Schoenberger revealed that CD8+ T-cells only needed 2 hours of antigen stimulation *in vitro* in order to expand and acquire cytotoxic effector function (van Stipdonk et al., 2001). By using transgenic TCR CD8+ T-cells and adherent artificial APCs, temporal requirements of CD8+ activation were investigated in an *in vitro* system that allowed tight control of T-cell-peptide-MHC interactions by physically separating T-cells and APCs (Schoenberger et al., 1998). Interestingly, culture times greater than 2 hours did not effectively increase the amount of division, nor the quality of cytotoxic responses. Thus CD8+ T-cells may differ from CD4+ by requiring short periods of stimulation for effector differentiation.

In vivo studies seeking to correlate the amount of TCR stimulation with *in vivo* cell fate by controlling the dose of antigen or duration of infection found similar discrepancies in the requirements for expansion and differentiation

between the CD4+ and CD8+ lineages. CD8+ transgenic T-cells only required a short period of stimulation by bacteria for expansion (Mercado et al., 2000), and when responding to high, intermediate, and low dose bacterial challenge, only differed in the relative numbers of effectors generated, with high antigen challenge resulting in greater number of effectors than low antigen challenge. All conditions exhibited equal proliferation, effector function, and survival (Badovinac et al., 2007; Kaech and Ahmed, 2001; Mercado et al., 2000; Prlic et al., 2007). These findings led to the notion that CD8+ T-cells have an "auto-pilot" program for expansion and differentiation, with minimal reliance on further TCR signals to regulate fate (Bevan and Fink, 2001).

In contrast, CD4+ T-cell may require longer periods of antigen-dependent stimulation in order to fully differentiate into effectors and subsequent memory cells. Removing stimulatory peptide in dendritic cells through the use of a tetracycline-responsive promoter during an ongoing response led to immediate cessation of expansion by the responding CD4+ T-cell population (Obst et al., 2005). The observations of TCR repertoire skewing to higher-avidity clones within primary and secondary responding CD4+ T-cell populations, described earlier, suggest antigen may be influencing this selection process throughout expansion *in vivo* (Malherbe et al., 2004; Savage et al., 1999). Increasing the precursor frequency of antigen-reactive clones, and therefore increasing antigen competition, by adoptive transfer of transgenic CD4+ T-cells resulted in deficient effector function and memory cell generation following viral infection (Blair and Lefrançois, 2007). Intravital imaging of CD4+ T-cell activation *in vivo* have noted

antigen dependent serial contacts between T-cell and DC that may have modulated subsequent fate during expansion and differentiation *(Celli et al., 2005)*. In all, the duration and dose of antigen may have greater downstream effects within the CD4+ lineage during activation, expansion, and differentiation, than the CD8+ lineage.

Models of Activation and Differentiation

Discrepancies between the requirement for antigen-dependent stimulation driving activation, expansion, and memory cell generation have led to multiple models (Figure 1-2) (Kaech and Wherry, 2007).

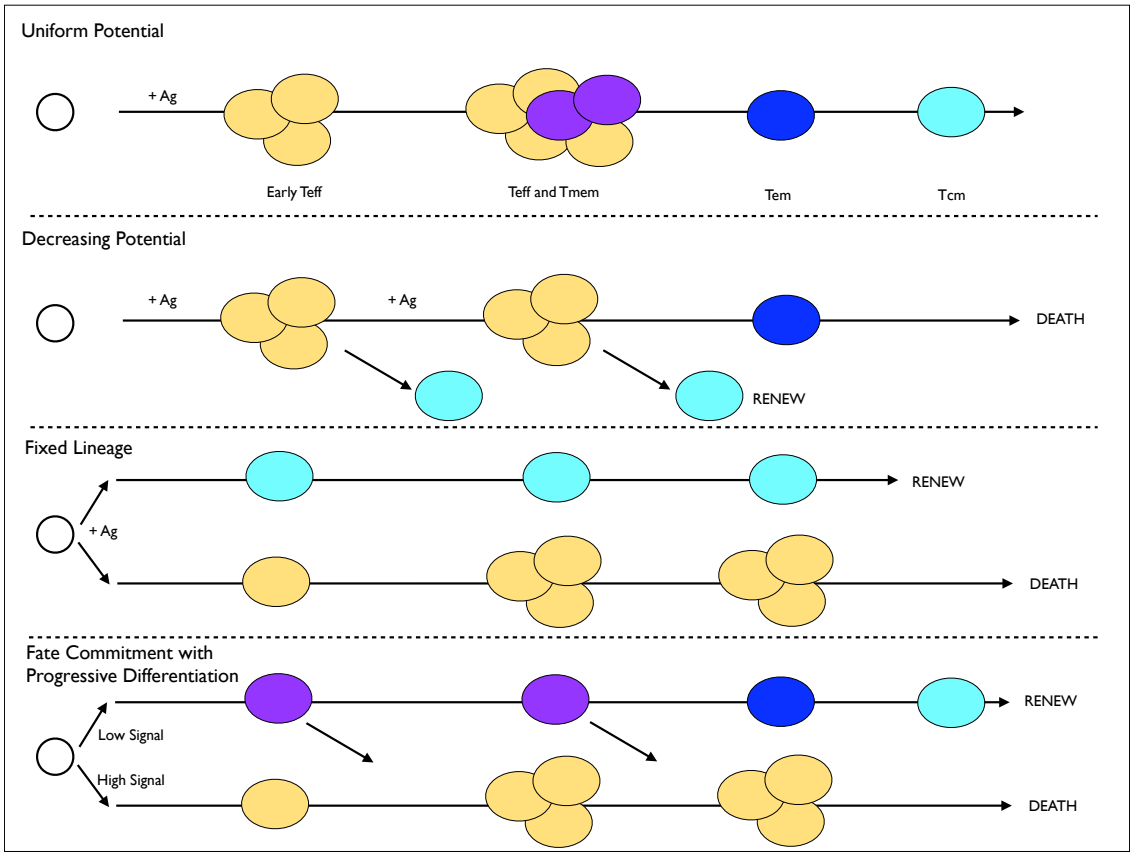

FIGURE 1-2. Models of T-cell Fate Determination. 1. Uniform potential - Antigen expands a naive cell (white) into early and late effector (Teff) cells (yellow) with equal memory potential (purple) to enter long-lived memory pool. Effector memory (Tem, blue) cells give rise to central memory cells (Tcm, aqua) with self-renewal properties. 2. Decreasing potential - Antigen drives more differentiated states which result in terminal Tem cells. Short amounts of stimulation favor Tcm generation. 3. Fixed lineage - Antigen stimulation results in the development of two separate lineages. Memory cells may bypass Teff differentiation. 4. Fate commitment with progressive differentiation - Strength of signal dictates fate early during activation. Low and high stimulatory signals favor memory and effector potential, respectively. Cells with memory potential may give rise to Teff cells. (adapted from Kaech and Wherry, 2007)

The uniform potential model postulates the pool of effector cells, derived from antigen expanded naive T-cells, have equal potential to differentiate into long lived effector-like memory (Tem) cells and central memory cells (Tcm) with self-renewal capabilities. Competition for extrinsic survival factors limit the number of effector cells surviving contraction (Freitas and Rocha, 2000). This relatively simple uniform potential model does not explain the functional and phenotypic heterogeneity within the cell populations, including IL-7 receptor expressing memory-cell precursors within the CD8+ effector cell pool.

The decreasing potential model consists of antigen stimulation driving more differentiated states within the responding T-cell populations. Relatively strong or serial stimulation may lead to terminal differentiation into effector-like memory cells or death, whereas relative short amounts of stimulation favor the generation of central memory cells with self-renewal capabilities (Sallusto et al., 2004).

The fixed lineage model posits initial antigen activation leads to the generation of separate lineages, one of memory fate, and one of effector cell fate. The fixed lineage model may be useful in explaining the heterogeneity within responding populations, in particular the observations of memory precursor cells within effector populations (Kaech et al., 2003) and the possibility of asymmetric cell division during activation dictating fate (Chang et al., 2007). However, recent discoveries showing memory cells may be derived from cells with effector function are inconsistent with predictions from a strict interpretation of the fixed lineage model (Bannard et al., 2009; Harrington et al., 2008).

The fourth proposed model, fate commitment with progressive differentiation, is a hybrid model of the previous three. Here, the strength of stimulation during activation dictates subsequent fate, with strong and weak stimuli resulting in effector and memory fate, respectively. However, one important property in this model is the ability for the memory fated cells to retain some effector properties, allowing for the generation of effector cells in later stages of the response from cells with memory potential. This model takes into account signal strength as a fate determining factor, along with the observed heterogeneity within the responding population of T-cells. Furthermore, the short amounts of stimulation necessary for an autopilot CD8+ memory program to be initiated is consistent with this model (Bevan and Fink, 2001; Kaech and Ahmed, 2001; Mercado et al., 2000; van Stipdonk et al., 2001).

These models have largely been proposed to explain phenomena within the CD8+ lineage. CD4+ T-cells, however, showed differences in the requirements needed for activation, expansion, and differentiation (Gett et al., 2003; Obst et al., 2005). Phenotypic characteristics of CD8+ memory precursors, including IL-7 receptor expression have not been shown to be as relevant within CD4+ T-cell differentiation (Kaech et al., 2003; Lees and Farber, 2010b). Recent studies by Williams and Bevan suggested CD4+ memory fate may be programmed during a response, and therefore CD4+ memory precursor cells likely exist, but are yet undistinguishable from terminal effector cells (Williams et al., 2008). Some studies suggest that CD4+ memory cell fate may have been determined by the relative strength of TCR, as CD4+ cells destined

for apoptosis appeared to have had lower functionally avidity compared to endogenous effector cells within the same host that survive. Functional avidity informs on the amount of antigen required for a normalized functional response. If cell population A has twice the functional avidity compared to cell population B, population A needs half the antigen to generate a comparable functional response. Thus, although a fair amount of information regarding T-cell memory differentiation has been investigated regarding the role of the TCR receptor in determining fate within the CD8+ lineage, the intrinsic differences that exist between CD4+ and CD8+ T-cells necessitates additional studies focused on TCR signal strength and CD4+ T-cell fate.

Extrinsic Fate Determining Factors

Along with TCR-derived signals, other factors including cytokine signalling and the inflammatory environment have been shown to modulate and influence fate in responding T-cell populations. IL-2-derived signals during priming impacted memory cell formation in CD4+ and CD8+ lineages (Dooms et al., 2007; Kalia et al., 2010; Pipkin et al., 2010; Williams et al., 2006). Within the CD8+ lineage, IL-2 has been shown to enhance the programming of CD8+ memory cells. Surprisingly, the lack of IL-2 signalling had little effect on primary CD8+ effector expansion and function, but was apparent during secondary expansion of CD8+ memory cells upon rechallenge (Williams et al., 2006). The effect of IL-2 on responding CD8+ T-cells may also have been dependent upon the amount of cytokine signalling. High degrees of IL-2 signalling appeared to

have promoted terminal effector memory cells at the expense of central memory cells with self-renewal capacity (Kalia et al., 2010; Pipkin et al., 2010). Within the CD4+ lineage, IL-2 signals were shown to be necessary for re-expression of the IL-7 receptor, which resulted in the generation of CD4+ T-cells with a long-term survival advantage (Dooms and Abbas, 2006; Dooms et al., 2004).

Along with IL-2, high levels of IL-12 signalling affected memory T-cell programming, possibly by modulating the expression of influential transcription factors within CD8+ T-cells (Joshi et al., 2007). CD4+ T-cells may compete for other cytokine signals, like interferon-γ, during expansion because CD4+ T-cells that were able to respond to interferon-γ expression were more likely to have survived into the memory phase(Whitmire et al., 2007). Thus, cytokines have been shown to modulate and shape effector and memory populations and cytokine-derived signals may have been important early during a response to program memory cell fate.

Genetic Fate Mapping Studies of T-cell Responses

The technique of marking complex cellular systems with long lasting dyes has been used to study the fate of groups or single cells during developmental processes (Vogt, 1929). Genetic targeting techniques in the mouse have allowed for the generation of transgenic strains expressing site-specific recombinases (SSRs) and indicator alleles. When used in conjunction, recombinase expression has been shown to induce the expression of reporter proteins to non-invasively mark cells within the embryo and adult mouse (Branda and Dymecki,

2004; Legue and Joyner, 2010). Because this approach was genetic, the indicator allele and state of the allele were inherited by all daughter cells creating a "fate map" (Figure 1-3). All daughter cells derived from the original marked cell expressed the reporter protein. Such an approach has been used in immunology to study the fate of cells having expressed functional proteins during an immune response (Bannard et al., 2009; Jacob and Baltimore, 1999).

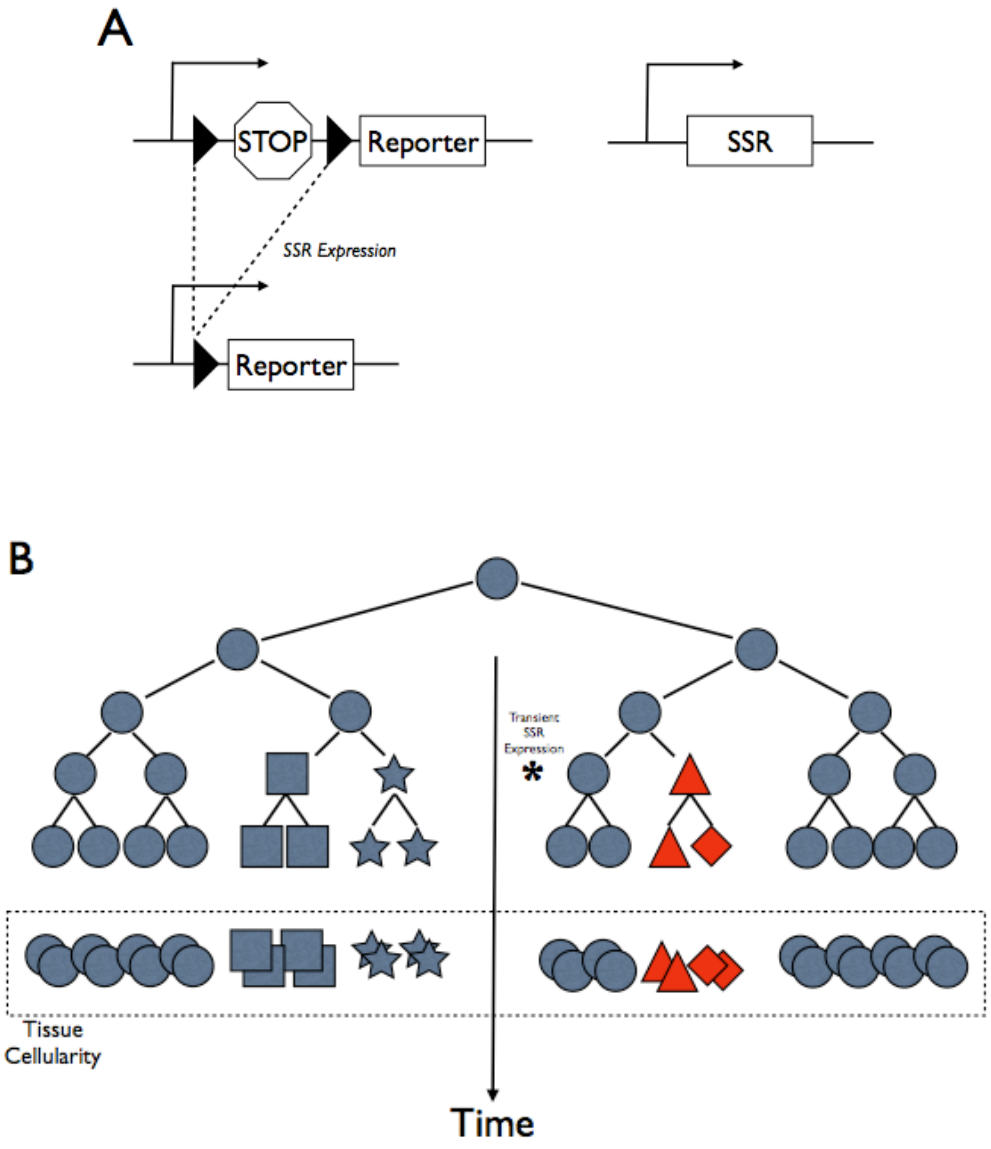

FIGURE 1-3. A Genetic Fate Mapping Scheme. A. Conventional genetic fate mapping methods use two separate transgenic alleles, an indicator allele (left) and a site-specific recombinase (SSR) allele (right). The indicator allele does not express reporter protein prior to Cre recombinase expression due to the placement of a STOP element upstream of reporter transcription. Following SSR expression, the STOP element, flanked by recombinase target sites (black triangles), is removed, resulting in reporter protein expression. B. Complex cellular systems (dashed rectangle) may be composed of many differentiated cell types (circles and polygons) derived from progenitor cells. Transient expression of an SSR during development (*) results in the stable marking of SSR-expressing progenitors with reporter protein (red triangle). All subsequent daughter cells inherit the induced reporter allowing for the characterization of cellular fate with time. (Adapted from Branda and Dymecki, 2004)

By using a double transgenic mouse strain carrying a truncated human *GZM-B* promoter Cre-recombinase expressing allele and a T-cell-specific indicator allele (CD2 promoter-driven placental alkaline phosphatase, CD2-PLAP) Jacob and Baltimore showed granzyme-b-expressing CD4+ and CD8+ T-cells could be marked following immunization and viral infection, respectively, *in vivo* (Jacob and Baltimore, 1999). These marked effector cell populations could be identified by flow cytometry and immunohistochemistry and tracked over time as the immune response subsided and entered the memory phase. This was due to the stable expression of the PLAP reporter protein from the recombined indicator allele. Identification and characterization of these effector and memory cells could be done without any prior knowledge of TCR specificities and pathogen-derived peptide epitopes because the marking process was based on effector function and employed biological relevant promoters to drive recombinase expression. Although this version (see below) of a method to mark granzyme-B expression proved to be imprecise (only 8% of the CD8+ T-cells recombined reporter at the peak of the effector phase following LCMV infection, and no naturally arising memory-phenotype $CD44^{hi}$ CD8+ T-cells expressed reporter. This may have been due to the use of a truncated human granzyme-B promoter), the potential to study immune responses using a genetic fate mapping approach became evident as the method provided a robust means to investigate complex multicellular systems over time, in a non-invasive manner *in vivo*.

An improved version of a *gzmB-cre* allele was developed recently to address the role of asymmetric cell division in memory cell generation (Bannard

et al., 2009). The model of asymmetric cell division implicates the orientation of the T-cell with APC in influencing subsequent effector and memory cell potential. The proximal cell generated following the first cell division had experienced greater TCR stimulation than the distal cell (as most of the TCRs are at the T-cell/ APC interface) and was destined to become an effector cell. The distal cell was destined to contribute to the memory cell population. The original study that proposed asymmetric cell division as a relevant process controlling fate during an immune response characterized the proximal cell to also have greater expression of granzyme-B than the distal cell (i.e. effector-like) (Chang et al., 2007). Thus, the fate of the granzyme-B-expressing daughter cell following the first division should be effector-like. This prediction was tested using an improved *granzyme-B-cre* transgene to map the fate of granzyme-B-expressing cells generated early during a response.

A bacterial artificial chromosome (BAC)-derived granzyme B transgene was used in the approach to drive a ligand-inducible form of Cre recombinase with an eYFP indicator allele. The BAC incorporated more endogenous regulatory elements of the *gzmB* locus to reliably control Cre expression. Following transfer of a single reporter cell and infection, it was definitively shown that CD8+ T-cells derived from a single precursor that had expressed granzyme-B (marked by eYFP) contributed to the memory cell population (Bannard et al., 2009). The improved transgenic system did not mark all granzyme-B-expressing cells. The system only marked those cells, mostly CD8+ T-cells, producing granzyme-B within the first 4 days of infection during the pulse of Cre ligand.

This left late-arriving T-cells and expanding T-cell clones that may have acquired granzyme-B-competent signals during expansion after day 4 unmarked. However, those T-cells that were marked by eYFP, presumably early differentiators of high levels of granzyme-B expression, contributed to nearly one third of the antigen-specific response in the effector and memory phases. These marked memory cells were further capable of secondary expansion indicating that previous granzyme-B expression during the effector phase had little bearing on memory cell fate with regards to proliferative capacity.

These data question the notion that during asymmetric cell division, the daughter cell expressing more granzyme-B expression is fated for effector-like fate (Chang et al., 2007). The granzyme-B mapping approach likely marked the granzyme-B/effector-fated daughter cell during the first division along with its progeny during expansion, although direct observation of the granzyme-B expressing daughter cell following the first division was not done. Because these marked cells contributed to the memory population and exhibited proliferative potential upon rechallenge, granzyme-B expression was independent of memory cell potential. However, along with marking the granzyme-B expressing cell of the first cell division, the reporter marked any cell during the four days of expansion that expressed granzyme-B. This may have included memory-fated cells with some effector-like properties (consistent with the fate commitment with progressive differentiation model by Kaech, pg.16). Data from Chang and Reiner actually showed that memory-fated cells (those cells found distal to the APC and with low expression of granzyme-B) had equal protective effect upon transfer and

early challenge versus effector-fated cells, thus supporting this notion. Therefore, the findings that granzyme-B-expressing cells contributed to the memory compartment does not rule out the influence of asymmetric cell division in initiating fates during an immune response; however they do suggest granzyme-B expression alone is likely not a faithful marker to delineate effector and memory lineages in this model.

The study by Bannard et al. did highlight the strength of a genetic fate mapping approach in uncovering the complex lineage relationships that developed during a immune response. First, cells were indelibly marked *in vivo* with little manipulation of the host, preserving lymphoid architecture. Second, the use and choice of a biologically relevant genetic locus, *gzmB*, to control the marking event showcased the relative precision and flexibility which was afforded by this method. The endogenous mouse *gzmB* regulatory elements drove *cre* expression which allowed for conclusions to be made regarding *gzmB* activity *in vivo*. Furthermore, *gzmB* was chosen specifically to test the hypothesis that *gzmB* expression was unique to effector-cell fates, however any locus potentially could be engineered to test a similar (or different) hypothesis. Thus genetic fate mapping approaches provide great flexibility along with a degree of precision to studies investigating complex cellular systems *in vivo*.

The Problem under Investigation

Given the evidence, introduced above, for strength of TCR stimulation influencing T-cell fate, there exists a lack of information regarding the nature of

these processes within the CD4+ T-cell lineage. The Mathis and Benoist groups have shown the duration of antigen presentation in mice influenced CD4+ activation, with long durations of presentation necessary for full expansion of CD4+ T-cells (Obst et al., 2005). However their approach involved expression of an agonist peptide within the DC compartment, which surprisingly drove expansion of transferred T-cells without the need for inflammation. Such settings more closely resemble that of a toleragenic response rather than one elicited from pathogen infection (Bluestone et al., 2007).

Williams and Bevan observed a correlation between effector CD4+ T-cell functional avidity and fate during contraction of the response in mice(Williams et al., 2008). Effector clones, derived from transferred transgenic TCR-expressing T-cells expanded with bacterial infection, displayed lower functional avidity compared to the endogenous responders. These transferred cells subsequently underwent apoptosis during the transition to memory. Surprisingly, the same TCR-bearing clonal population derived from viral infection was able to survive contraction, and in these settings the transferred population's functional avidity was greater than that displayed following bacterial infection, and on par with that of the endogenous response. One interpretation of these data could be increased functional avidity was a response to pro-memory signals earlier during activation, and not the causal mechanism because transgenic T-cells with a fixed TCR (such as those used in this study) are able to undergo functional avidity maturation (Slifka and Whitton, 2001). Studies tracing the fates of activated

CD4+ T-cells derived from various amounts of TCR stimulation may directly shed light on the role of TCR signal strength and *in vivo* fate.

We sought to understand the nature of TCR-derived signal strength and the consequences of these signals during CD4+ immune responses *in vivo*. An approach to genetically mark antigen-specific CD4+ T-cell during the expansion phase of the response, based upon the amount of TCR stimulation received during activation, would allow for the determination of their subsequent fate throughout the response. This approach would rely on the use of a Cre-recombinase allele whose expression is induced within the CD4+ lineage as a result of T-cell activation and is proportional to the amount of TCR stimulation. The *cre* allele would also need to be transiently induced following activation, thereby only allowing for recombinase expression (and reporter recombination) during T-cell activation and not at later time points during the response. With these criteria in mind, *Ox40/Ox40-cre* was chosen (Klinger et al., 2009).

The ability to mark separate populations of antigen-specific T-cells as a result of different amounts of recombinase expression (on the basis of TCR stimulation strength) would require an indicator allele conducive to flow cytometric analyses. Furthermore, an indicator allele capable of discriminating between populations of cells having expressed different amounts of recombinase may be beneficial for our purposes of studying TCR signal strength. Chapter 2 presents the methods of generation of novel reporter alleles in mice capable of performing such duties *in vivo*. Integral to the utility of these new reporter alleles is their ability to adopt an intermediate state, thereby capable of discriminating

between multiple populations of cells that differed in amount of recombinase expression. Two separate alleles were generated with different sensitivities to recombinase activity allowing for the use of a broader range of *cre* alleles with disparate intrinsic expression. Results are shown indicating these alleles work well and as predicted *in vitro* with Cre expression plasmids and recombinant Cre protein, and *in vivo* with *Ox40-cre* mice.

In chapter 3, we present data utilizing one of the above new *Cre* reporter alleles with *Ox40/Ox40-cre* mice. We show CD4+ antigen-specific effector cells generated as a result of differing amounts of TCR stimulation during acute LCMV Armstrong infection have distinct fates. CD4+ effector T-cells generated from relatively strong pMHC interactions, uniquely marked by the reporter allele and Ox40-cre, preferentially survive contraction and were found at high frequencies within the stable memory cell population.

In chapter 4, based on our findings we propose a simple model for CD4+ antigen-dependent differentiation and memory cell generation based on our findings and discuss the significance and implications of our studies. This chapter proposes potential follow-up experiments to our studies that may be informative in further advancing our understanding of TCR signal strength and cell fate. This includes discussion of preliminary data generated using a CD69-cre mice which may be found in the appendix.

Chapter 2

Reporter Alleles that Inform on Differences in Cre-Recombinase Expression

Abstract

Alleles that express reporters after Cre recombination allow for fate-mapping studies when used in combination with appropriate *cre* alleles. In this study, we describe two fluorescent reporter alleles that differentially mark populations of cells as a function of their level of expression of Cre recombinase. Mice carrying these alleles were generated and used to demonstrate the usefulness of the reporter alleles for informing on prior Cre recombinase expression in lymphocytes. The alleles expand the range of genetic tools available for understanding how differences in gene expression result in divergent developmental fates during the development and differentiation of lymphocytes and other cells.

Introduction

Throughout embryogenesis, cells adopt different fates as a consequence of signaling in response to secreted, matrix-associated, or cell-bound factors. For a class of factors that includes morphogens, individual cell fate depends both on factor identity and how much of it the cell encounters (Ashe and Briscoe, 2006). It follows, therefore, that cells of different but related lineages should be distinguishable from one another on the basis of how much signaling they experienced from individual morphogen receptors during their development and the transcriptional output of genes that were induced by this signaling. Analytical procedures that inform on the gene expression history of cells are therefore of great interest for understanding the basis of complex lineage commitment processes.

The major developmental morphogens (members of the Wnt, Hedgehog, and TGF-β families) have important functions in diverse aspects of hematopoiesis (Campbell et al., 2008; Crompton et al., 2007). As in other developing systems, some of these functions may rely on the establishment of morphogen gradients with fate again being at least partially determined by quantitative differences in morphogen receptor signaling. In the adaptive immune system, there is also evidence that graded signaling through Ag receptors can instruct cell fate in a related fashion. For T cells, this includes the stage at which cells commit to the α/β versus γ/δ lineages (Haks et al., 2005; Hayes et al., 2005; Hayes and Love, 2006), whereas for B cells, it includes the commitment of cells to the B-1 versus follicular/marginal zone fates (Cariappa and Pillai, 2002;

Lam and Rajewsky, 1999). Other possible, if more controversial, examples include the commitment of thymocytes to the CD4 versus CD8 lineages(Itano et al., 1996; Singer et al., 2008) and the adoption of the Th1 fate over the Th2 fate (Badou et al., 2001; Iezzi et al., 1999; Jorritsma et al., 2003; Malherbe et al., 2000; Tao et al., 1997).

Controversy in determining the signficance of signal strength in the control of lineage choice derives in part from difficulties associated with manipulating signaling *in vivo* in a fashion that avoids the possibility of artifact [e.g., due to complicating effects of premature loss or gain of signaling, prolonged or constitutive signaling when extinguishing it is a critical part of regulation(Singer et al., 2008), or simply overexpression outside of the normal range experienced by cells]. Cells actively undergoing commitment are also typically short-lived intermediates within minority populations, and such cells often lack markers that unambiguously define them as those that are experiencing the critical signaling processes. It can sometimes be difficult, therefore, to redirect commitment experimentally in a fashion that can be reliably interpreted or to identify and study cells undergoing commitment events.

An alternative approach to studying lineage commitment depends on marking cells that undergo specific experiences during commitment (Zinyk et al., 1998). If the mark once imparted on the cells is stable, then the fate of the cells can be traced and correlated to the commitment experiences they had. In its most sophisticated form, this type of approach can inform on highly specific experiences that occur in cells in narrowly defined periods of their differentiation.

Fate mapping of this sort has been widely used and has shed light on diverse commitment processes (Joyner and Zervas, 2006).

We have designed a new fate-mapping scheme that is intended to provide an enhanced perspective on differences in gene expression that occur during commitment events *in vivo*. This scheme involves use of the Cre recombinase and novel reporter alleles that can adopt different states as a function of recombinase concentration in cells. In this study, we describe the design and properties of the reporter alleles and establish the foundation for their use in mice. We demonstrate that the alleles perform as expected *in vivo* by showing they can discriminate populations of T cells that differ in their prior expression of a gene (*Tnfrsf4*) that is variably induced as a function of TCR signaling. The study makes clear the properties of the new reporter alleles and their potential benefits for the analysis of cell fate decisions within and beyond the immune system.

Materials and Methods

Generation of gene targeting vectors

The Stop element in the reporter alleles was generated by inserting a PGK-puro gene (Ramirez-Solis et al., 1995) just after the 59 *loxP* site of an existing Stop element (Lakso et al., 1992) that had been modified such that the 39 splice acceptor (SA) sequence was replaced with an SV40 polyadenylation sequence. An adenovirus SA sequence was isolated from pSA-bgeo (Friedrich and Soriano, 1991) and inserted immediately upstream of this. DNA fragments containing the tdTomato (Shaner et al., 2004) and human CD2 (hCD2) open reading frames (ORFs) were generated by PCR and inserted downstream of the Stop element. An internal ribosome entry site (IRES)-enhanced GFP (eGFP) element (derived from pIGCN21) (Lee et al., 2001) was flanked by recombinase recognition sites (FRT, 5172, and 3373) by conventional ligation with fragments taken from plasmids containing these sites. The modified IRES-eGFP was then cloned downstream of either SA-Stop-tdTomato or SA-Stop-hCD2. SV40 polyadenylation sequences were added downstream of the eGFP or hCD2 ORFs, and, finally, the fully assembled reporter inserts were cloned into a polylinker inserted into the XbaI site of a modified form of pRosa26-1.

Embryonic stem cell culture, gene targeting, and generation of mice

E14 mouse embryonic stem (ES) cells (Nichols et al., 1990) were cultured and transfected using standard conditions. The cells were cultured in Glasgow MEM supplemented with penicillin, streptomycin, L-glutamine, 2-ME, sodium pyruvate, nonessential amino acids (all from Invitrogen, Carlsbad, CA), 15% FCS

(Cambrex, East Rutherford, NJ), and 0.3% LIF-containing supernatant. Cells were cultured on gelatinized tissue culture-treated plates without feeder cells. The targeting vector (25 mg DNA linearized with AscI) was electroporated into 2 3 107 E14 ES cells in PBS (Invitrogen) using cuvettes with a 0.4-cm electrode gap and a Bio-Rad Genepulser II set at 250 V and 500 mF (Bio-Rad, Hercules, CA). Puromycin (2 mg/ml) selection was imposed after 2 d, and single ES cell colonies were picked after an additional 7–9 d in selection. The colonies were expanded in 96-well plates, after which one-half of the culture was frozen down, whereas the other half was used for isolation of DNA. The DNA was screened by Southern blot using the mini-Southern procedure (Ramirezsolis et al., 1992) and a radiolabeled XhoI fragment of the *ROSA26* locus from the pRosa26 plasmid (Zambrowicz et al., 1997a). Mice were generated from targeted ES cells by microinjection of the ES cells into C57BL/6x(C57BL/6xDBA/2 F1) eight-cell embryos using a laser-assisted technique (Poueymirou et al., 2007). Germline transmission was accomplished by breeding chimeric males to C57BL/6 females. Mice were used between 6 and 10 wk of age for experiments. All mice were maintained and bred under specific pathogen-free conditions under the approval of the University of California, San Francisco, Institutional Animal Care and Use Committee (San Francisco, CA).

Transient transfections and use of cell-permeant Cre

For transient transfections, targeted E14 cells were trypsinized, washed, and resuspended at 2.5 3 107/ml. A total of 40 mg circular DNA was mixed with 0.8

ml cells, after which the suspension was transferred to a cuvette (0.4-cm electrode gap) and electroporated at 250 V and 950 mF. To purify cells exhibiting a partially recombined phenotype, targeted ES cell clones were transfected with pMC-Cre(Gu et al., 1993), incubated for 5 d, and then flow-sorted using a BD FACSAria flow cytometer (BD Biosciences, San Jose, CA). After further expansion, the sorted tdTomato+ eGFP+ cells were transfected with a Cre-estrogen receptor (ER) plasmid (Metzger et al., 1995) and a human CD2 expression vector as a tracer for transfected cells. Twenty-four hours later, the cells were incubated with varying concentrations of 4-hydroxy- tamoxifen for 24 h. After an additional 24 h of culture in the absence of 4- hydroxy-tamoxifen, the cells were analyzed for human CD2 and reporter expression by flow cytometry. Cell-permeant Cre was prepared and purified as described (Peitz et al., 2002) and added to targeted ES cell clones for 4 to 5 h in Glasgow MEM without serum or antibiotics. The cells were then washed before incubation in normal medium for an additional 48 or 72 hours prior to analysis by flow cytometry.

Flow cytometry and immunofluorescence

Conjugated Abs were purchased from BD Biosciences and eBioscience (San Diego, CA). Single-cell suspensions were prepared from mouse spleens using 45-μm cell strainers (BD Falcon, BD Biosciences) and PBS containing BSA (0.3% w/v). T cell stimulations were performed with purified anti-CD3ε (clone 145-2C11) and plate-bound goat anti-Armenian hamster IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) with or without purified anti-

mouse CD28 (clone 37.51, BD Biosciences). Cells were washed by centrifugation and then incubated on ice for 30 min with saturating concentrations of Abs specific for cell surface molecules (B220, CD4, CD8, CD25, and CD44). DAPI (0.3 mM) was used for live-dead cell discrimination. Cells were analyzed using either BD LSRII or FACSCalibur flow cytometers (BD Biosciences). All data were acquired using BD FACSDiva software (BD Biosciences) and further analyzed with FlowJo software (TreeStar, Ashland, OR). Spleens or lymph nodes were embedded in 4% low-melting agarose/PBS, and 150–250-μm sections were cut in cold PBS with a Vibratome (Vibratome, Bannockburn, IL) at high amplitude and slow speed with a blade angle of 25–28˚, mounted onto slides, and imaged. Fixed tissues were prepared by incubating spleen quarters or entire lymph nodes in 2–4% (w/v) paraformaldehyde/PBS at room temperature for 1–3 h. Tissues were washed briefly in PBS, floated on cold 30% (w/v) sucrose, frozen in OCT (Sakura Finetek, Torrance, CA) and stored at -80˚C until sectioning on a Leica CM3050 S cryostat (Leica Microsystems, Deerfield, IL). eGFP was detected in fixed samples using a rabbit anti-GFP antisera (Novus Biologicals, Littleton, CO), followed by a biotinylated donkey anti-rabbit F(ab')2 Ab (Jackson ImmunoResearch) and a tyramide signal amplification-based FITC detection system (PerkinElmer, Waltham MA). Confocal imaging was performed using a modified Axiovert 200M microscope (Zeiss, Oberkochen, Germany) equipped with a spinning-disk confocal scanner (Yokogawa, Sugar Land, TX) with a 403/1.3 NA oil immersion objective and an iXon EMCCD camera from Andor (South Windsor, CT). Data collection and processing were performed using

MetaMorph software with the “Scan Slide” drop-in application (Molecular Devices, Downingtown, PA).

Results

loxP sites are 34 bp in length and are composed of two inverted repeats flanking a central spacer sequence (Sternberg and Hamilton, 1981) (Fig. 2-1A). Mutations introduced into the spacer or repeat sequences do not necessarily prevent recombination, but they can affect both the efficiency and specificity of recombination. Some mutant *loxP* sites recombine efficiently with the native *loxP* sequence, whereas others will only recombine with *loxP* sites that are similarly mutated (Hoess et al., 1986; Lee and Saito, 1998).

We selected two mutant *loxP* sites as the basis of a novel reporter allele design. A sensitive *in vitro* recombination assay previously established that these two sites do not recombine detectably with native *loxP* sites (Lee and Saito, 1998). The sites also show reduced homotypic recombination efficiencies relative to *loxP*: 10% and 30% of native efficiency for 3373 and 5172, respectively (Fig. 2-1A). We designed two novel reporter alleles (one incorporating 3373 sites and the other 5172 sites) based on a successful design used previously by many laboratories. A conventional component of the design was the use of the Gt(ROSA)26Sor locus (Zambrowicz et al., 1997b) (referred to in this study as *ROSA26*) as a site into which the reporter elements would be inserted. This locus was attractive because it is transcriptionally active in most cell types in the mouse, and it shows useful expression levels in hematopoietic lineages (Mao et al., 2001; Srinivas et al., 2001). A second conventional component of the design was the placement of a *loxP*-flanked disruption (commonly referred to as a Stop element) (Lakso et al., 1992; Srinivas et al., 2001) within an intron of the

ROSA26 locus immediately downstream of a strong SA sequence. The purpose of this element was to terminate transcription such that any reporter ORFs appended to it would not be expressed, unless the element was first removed by Cre recombination.

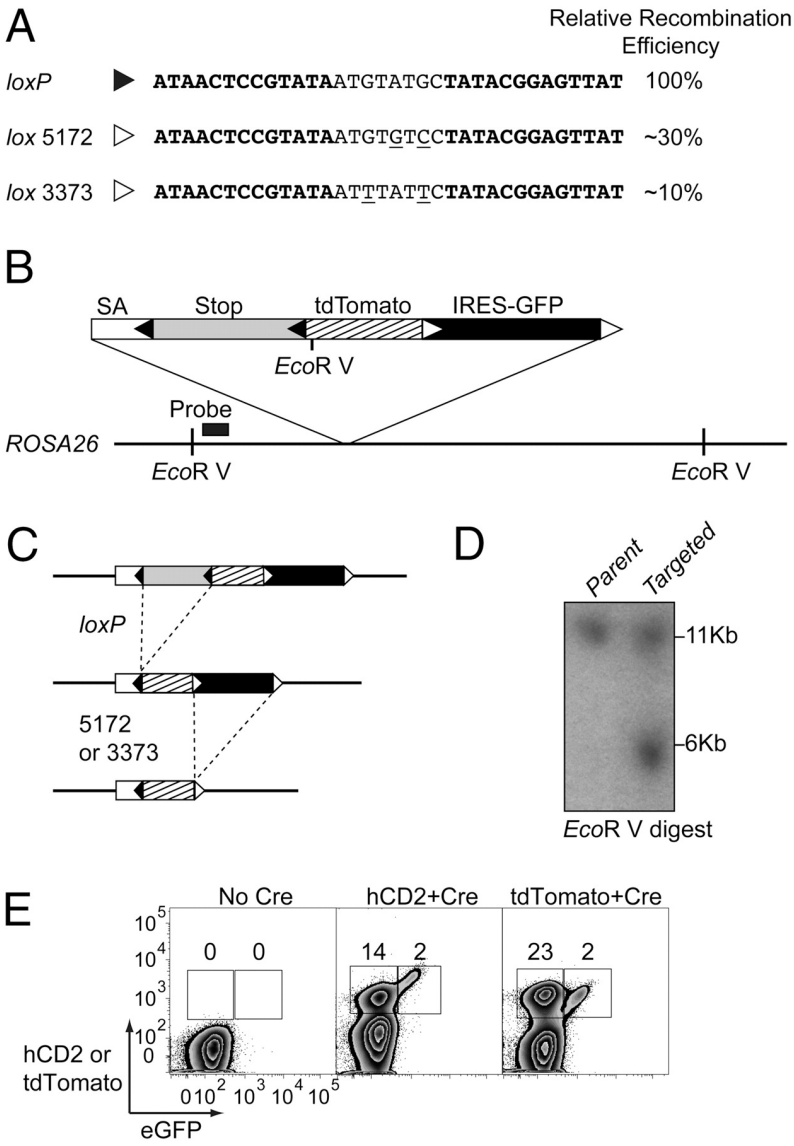

FIGURE 2-1. Design and Cre-dependent activation of reporter alleles. A, Nucleotide sequences of wild-type and mutant *loxP* sites and their relative recombination efficiency as measured *in vitro* (Lee and Saito, 1998). Mutations in the central spacer region are underlined. B, Design of the reporter alleles generated by gene targeting at the *ROSA26* locus in mouse ES cells. C, Diagram of states adopted by the reporter alleles due to Cre recombination at the *loxP* sites (top) followed by 5172 or 3373 sites (bottom). The partially recombined state (middle) results in expression of both tdTomato and eGFP (red and green fluorescence), whereas fully recombined alleles (bottom) express only tdTomato (red fluorescence only). D, Southern blot of EcoRV-digested genomic DNA from a representative ES cell clone and its parent hybridized with the probe shown in B. E, Expression of reporters (hCD2 or tdTomato, and eGFP) in gene-targeted ES cells carrying the hCD2 (middle panel) or tdTomato (right panel) forms of the reporter allele after transient transfection with a Cre expression vector. Percentages of cells falling within the marked fluorescence gates are shown.

The two reporter ORFs we chose were those that encoded the tdTomato variant of dsRed (Shaner et al., 2004) and eGFP (Yang et al., 1996). A human CD2 cDNA was also used in place of the tdTomato gene in alternative versions of the reporter alleles. We placed an IRES between the two reporter ORFs to allow for their cotranslation (Fig. 2-1B). tdTomato and eGFP were favored as reporter proteins because they can be readily detected and discriminated by flow cytometry and fluorescence microscopy.

The most important aspect of the reporter design was the placement of mutant Cre recombination sites (3373 or 5172) (Lee and Saito, 1998) around the IRES-eGFP element. This was done so that the IRES-eGFP could be selectively excised from the reporter allele with an efficiency that was reduced relative to excision of the upstream *loxP*-flanked Stop element. The most favored recombination event in cells expressing Cre, therefore, would be loss of the Stop element resulting in gain of both eGFP and tdTomato expression (Fig. 2-1C). Loss of eGFP would then occur in some but not all of the tdTomato+ cells, and the fraction of them undergoing this event would be a function of the amount of Cre recombinase they expressed (Lin et al., 2004; Peitz et al., 2002). Reporter phenotype should therefore reflect the amount of Cre expressed by a population of cells with tdTomato+eGFP+ cells expressing lower levels of Cre than tdTomato +eGFP- cells. Because 3373 sites are recombined less efficiently than 5172 sites (Lee and Saito, 1998), loss of eGFP should occur with lower efficiency in populations carrying the 3373 reporter allele than in cells carrying the 5172 allele.

The *ROSA26* locus was mutated in mouse ES cells (Fig. 2-1D) by gene targeting using targeting vectors that carried the indicated reporter configurations (Fig. 2-1 B). Initial versions of the reporter alleles incorporating a dsRed ORF upstream of the IRES-eGFP failed to generate detectable red fluorescence in targeted ES cells following transient Cre expression, even though we could readily detect eGFP in the transfected populations. To correct this problem, we replaced the dsRed ORF with ORFs encoding either the tdTomato variant of dsRed (Shaner et al., 2004) or human CD2. Targeted ES cells carrying these alternative versions of the alleles showed Cre-dependent expression of both eGFP and tdTomato or human CD2 (Fig. 2-1 E). These preliminary experiments established that the reporter alleles were functional in ES cells, so additional experiments were performed to analyze their properties in more detail.

We performed a series of experiments to explore the relationship between Cre recombinase expression levels and reporter recombination status. These involved various strategies to titrate Cre activity in the cells, of which the most robust proved to be treatment with varying doses of Tat-Cre, which is a form of the recombinase that crosses the membrane because it contains a short N-terminal extension from the HIV Tat protein (Peitz et al., 2002).

Both reporter alleles (3373 and 5173) were recombined in treated ES cells in a Tat-Cre dose-dependent fashion (Fig. 2-2 A). A key observation was that at low doses of Cre, cells that had undergone full recombination of the reporter alleles were less common than at higher doses of Cre. That is, the ratio of single-positive cells (SP; tdTomato+eGFP- cells carrying fully recombined alleles) to

double-positive cells (DP; tdTomato+eGFP+ carrying partially recombined alleles) was lower at the low doses of Cre than at the high doses. Incubation of Tat-Cre with cells carrying a variant of the reporter allele lacking the mutant *loxP* sites rendered the cells positive for both tdTomato and eGFP (Fig. 2-2 B), showing that the tdTomato+ eGFP- phenotype depended on Cre recombination. A second important observation was that the frequency of cells carrying fully recombined forms of the 5172 reporter allele was higher than that of cells carrying fully recombined forms of the 3373 allele at all concentrations of Cre (Fig. 2-2 C). Both observations were consistent with the anticipated behavior of the alleles based on the fact that both of the mutant *loxP* sites are recombined less efficiently by Cre than native *loxP* and that 3373 shows one-third the efficiency of 5172 (Lee and Saito, 1998).

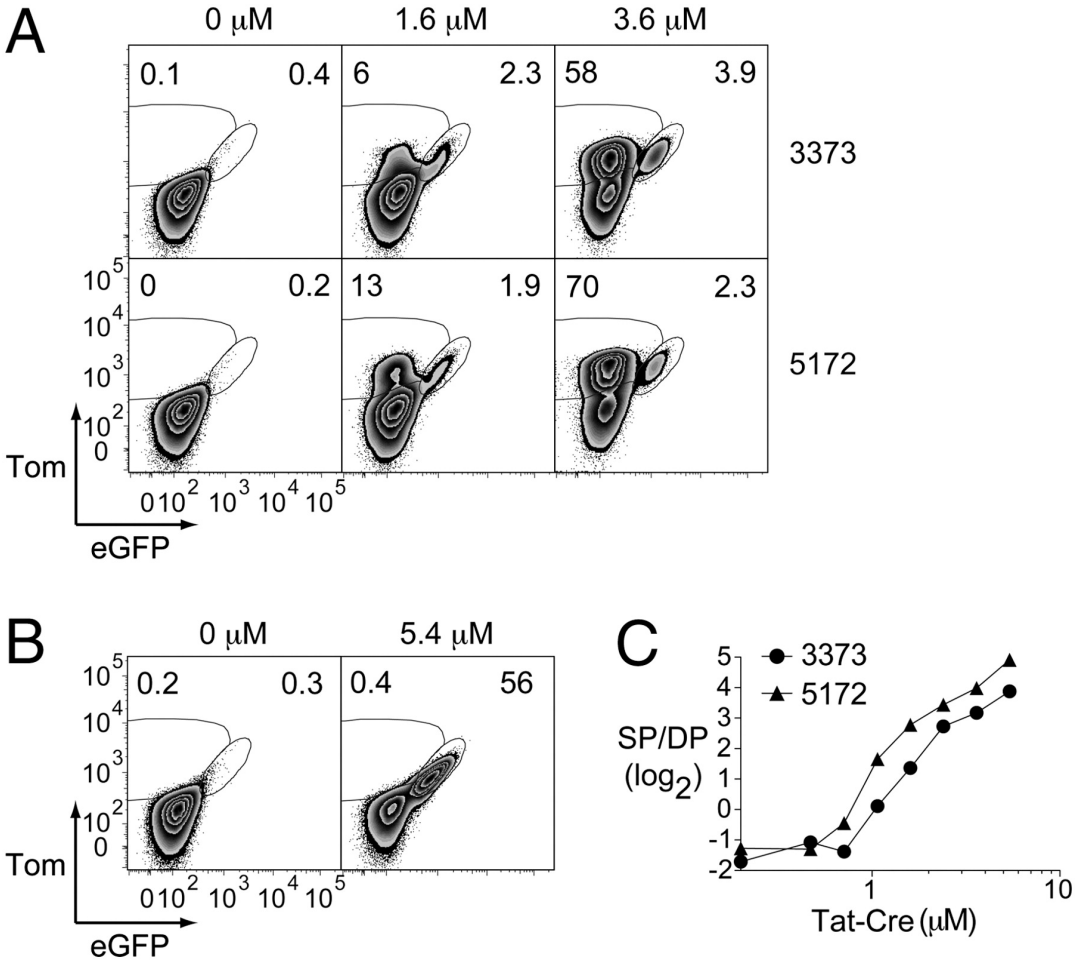

FIGURE 2-2. Sensitivity of the reporter alleles to Cre recombination. A, ES cell clones carrying the 3373 or 5172 reporter alleles were incubated with varying concentrations of Tat-Cre protein for 5 h, washed, and then returned to culture for 48 h before analysis by flow cytometry. The plots show relative expression of tdTomato and eGFP in the cells post-exposure to the indicated concentrations of Tat-Cre. Numbers on the plots refer to the percentages of cells in the marked fluorescence gates. B, Loss of eGFP from tdTomato+ cells depends on Cre recombinase-dependent excision of the IRES-eGFP element from the reporter alleles and is not observed when the element is not flanked by Cre recognition sites. Cells were treated and analyzed as in A. C, Graph showing the ratio of cells (carrying either the 3373 or 5172 reporter alleles) with fully (tdTomato +eGFP–, SP) versus partially (tdTomato+eGFP+, DP) recombined alleles (SP/DP) as a function of Tat-Cre concentration. Cells were treated and analyzed as in A. Statistical significance: p = 0.03, calculated by Student t test.

To focus specifically on the efficiency of recombination that was dependent on 3373 or 5172 sites, we sorted tdTomato+eGFP+ cells from clones of targeted ES cells that had been transiently transfected with a Cre expression vector. These sorted cells were ~95% pure and retained the partially recombined reporter phenotype during prolonged culture (Fig. 2-3A). We treated these cells transiently with varying doses of Tat-Cre and then cultured them before analysis by flow cytometry. Loss of eGFP occurred in the cells in a Cre recombinase dose-dependent fashion (Fig. 2-3A), and it was more prevalent in cells carrying the 5172 allele than in those carrying the 3373 allele (Fig. 2-3B). Similar results were obtained when Cre was delivered to the cells by other means, including transient transfection of a vector expressing a Cre-ER fusion protein followed by induction of Cre activity by treatment of the cells with varying doses of 4-hydroxy-tamoxifen (Metzger et al., 1995) (Fig. 2-S1). Thus, as above, these data showed that the reporter alleles behaved in the expected fashion and that the ratio of cells carrying fully recombined alleles to those carrying partially recombined alleles (SP/DP) informs on how much Cre recombinase the cells expressed.

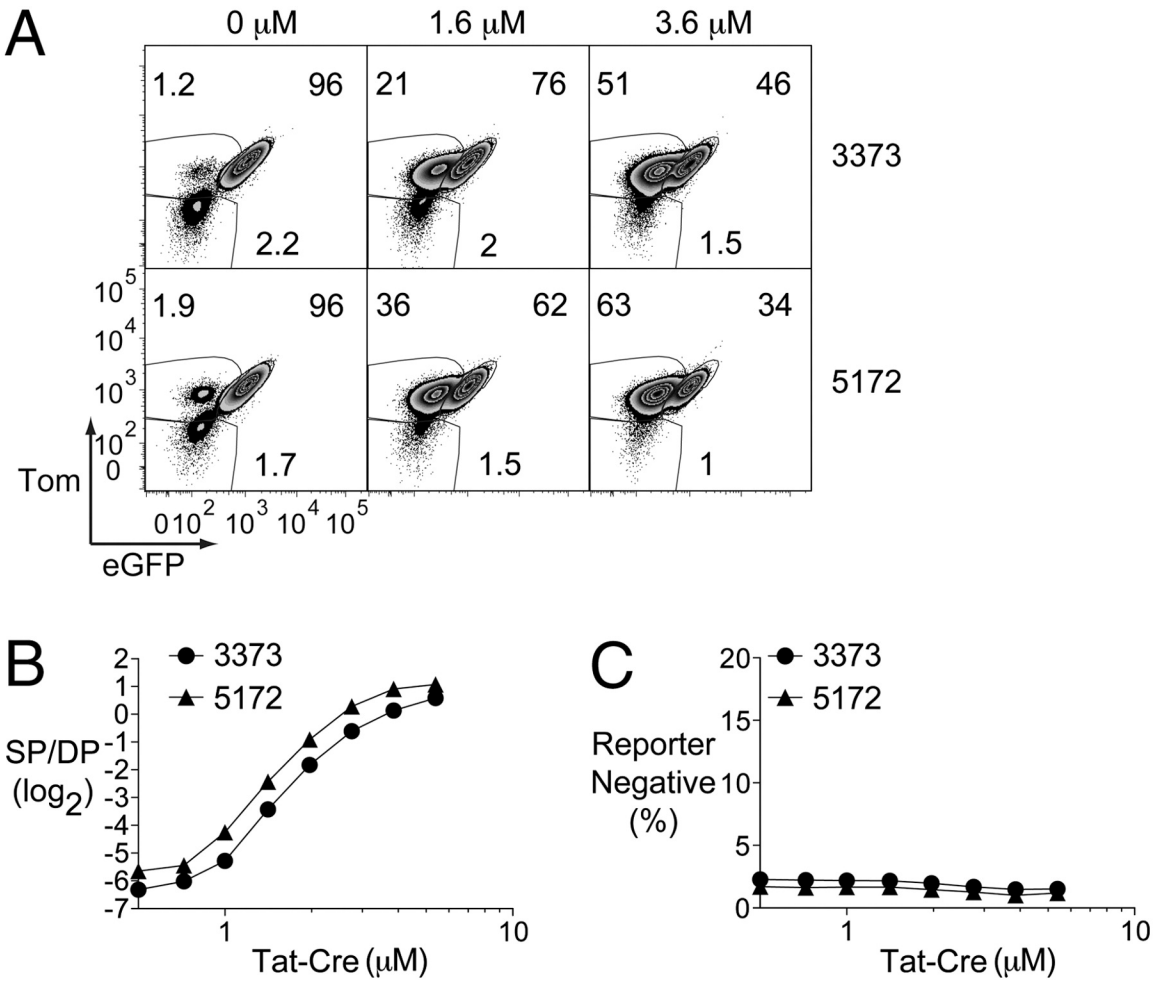

FIGURE 2-3. Cre recombinase-dependent excision of IRES-eGFP from cells carrying partially recombined reporter alleles. A, ES cell clones with partially recombined alleles (3373 or 5172) were generated by sorting tdTomato+eGFP+ cells from cultures of cells that had been transiently transfected with a Cre recombinase expression vector. The cells were subsequently treated with varying concentrations of Tat-Cre before analysis by flow cytometry. The plots show relative expression of tdTomato and eGFP in the cells post-exposure to the indicated concentrations of Tat-Cre. Numbers on the plots refer to the percentages of cells in the marked fluorescence gates. B, Graph showing the ratio of cells (carrying either the 3373 or 5172 reporter alleles) exhibiting the fully recombined phenotype (tdTomato+eGFP–) versus the partially recombined phenotype (tdTomato+eGFP+) as a function of Tat-Cre concentration (post-treatment and analysis as in A). Statistical significance: $p = 0.02$, calculated by Student t test. C, Frequency of tdTomato–eGFP– cells in cultures treated as in A.

In vitro recombination data predicted that the reporter alleles would adopt one of only two possible recombined states because *loxP* sites do not recombine detectably with either 3373 or 5172 sites. It was nonetheless of interest to determine whether such unfavored recombination might be prevalent in the context of the reporter constructs stably integrated into the genome of ES cells. The 3373 and 5172 sites were inserted into the constructs in inverted orientation relative to the upstream *loxP* sites, so heterotypic recombination would result in inversion of the DNA between the involved sites. Of six possible inversion events (four without prior excision of the Stop element and two with it), two had the potential to allow for reporter expression (eGFP without tdTomato), whereas four would result in an absence of reporter expression. We detected no cells with a tdTomato-eGFP+ phenotype following Cre recombination in cells carrying either of the two reporters (Figs. 2-2A, 2-3A, 2-S1A). Moreover, when Tat-Cre was added to the flow-sorted tdTomato+eGFP+ cells, we noted no accumulation of cells with a tdTomato-eGFP- phenotype (Fig. 2-3C) such as would be expected if unfavored recombination events occurred at a significant frequency. Thus, heterotypic recombination is not a prevalent event, and the reporter alleles behave as predicted.

We generated lines of mice with the ES cells carrying the tdTomato forms of the reporter alleles (Poueymirou et al., 2007). Lymphocytes from these mice responded to treatment with Tat-Cre by expressing tdTomato and eGFP in a similar fashion to what we had observed in the ES cells. Specifically, the ratio of fully to partially recombined alleles (SP/DP) increased as a function of Cre

concentration, and the 5172 allele was more readily recombined than the 3373 allele (Fig. 2-4A).

To determine whether the reporter alleles would respond to concentrations of Cre that are useful experimentally, we crossed the reporter mice to *Ox40-cre* mice. These mice express Cre from the native *Tnfrsf4* locus and show persistent high expression of the recombinase in CD4+ regulatory T cells, transient high expression in precursors of CD4+ memory T cells, and transient weak expression in a subpopulation of precursors of CD8+ memory T cells and the thymic precursors of a small subpopulation of naive T cells (Klinger et al., 2009). Ox40-cre recombined the reporter alleles in a fashion that reflected the relative levels of Cre expression in the different types of T cells. Specifically, populations that expressed high amounts of Cre (CD4+ regulatory and memory T cells) showed the highest ratios of fully to partially recombined reporter alleles (Fig. 2-4B, 2-4C). By contrast, fully recombined alleles were less prevalent in naive and CD8+ T cells, which expressed lower levels of the recombinase (Fig. 2-4B, 2-4C).

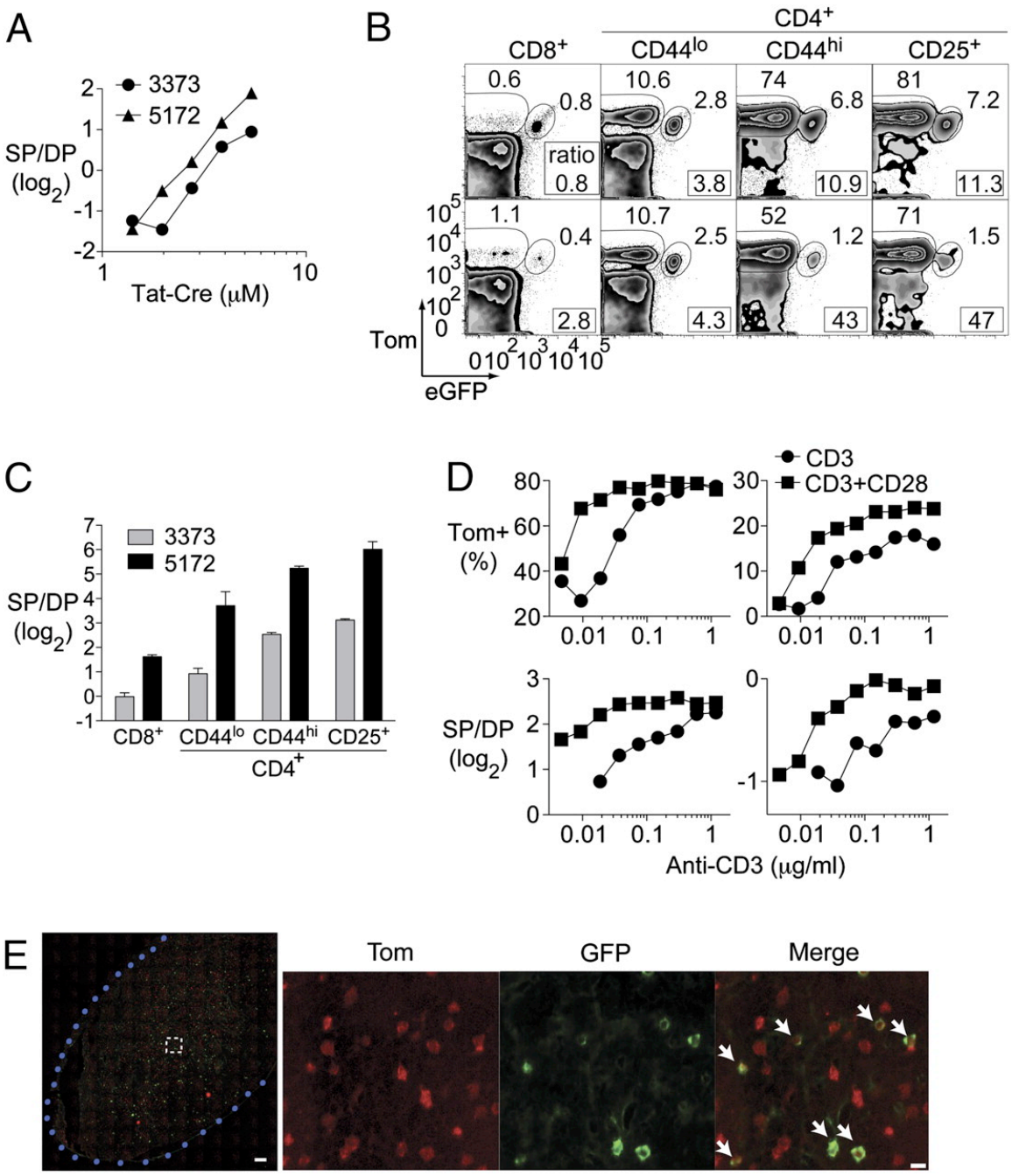

FIGURE 2-4. Behavior of the reporter alleles in cells from mice created with targeted ES cells. A, Pooled spleen and lymph node cells (from mice carrying either the 3373 or 5172 reporter allele) were incubated with varying concentrations of Tat-Cre protein for 4 h. The cells were washed and then returned to culture for 48 h before analysis by flow cytometry. The graph shows the ratio of B220+ cells exhibiting the fully recombined phenotype (tdTomato +eGFP–) versus the partially recombined phenotype (tdTomato+eGFP+) as a function of Tat-Cre concentration. Statistical significance: p = 0.05, calculated by Student t test. B, The plots show representative flow cytometry data of different

types of T cells from *Ox40-cre* mice carrying the two reporter alleles (results from both the 3373 and 5172 reporter alleles are shown in the upper and lower panels, respectively). Percentages of cells expressing tdTomato with or without eGFP are indicated next to the relevant gates. The ratios of cells carrying the alleles in the fully or partially recombined state (SP/DP) are shown in the boxes at bottom right of each plot. C, A graph showing the mean (and SE) of recombination ratios in T cells from *Ox40-cre* mice carrying either of the two reporter alleles (three mice per group) calculated as in B. D, *Ox40-cre* CD4+ (left panels) or CD8+ (right panels) T cells carrying the 3373 reporter allele were stimulated with anti-CD3 in the presence (squares) or absence (circles) of anti-CD28. The graphs at top show the frequency of reporter-positive cells (i.e., tdTomato+ cells) in the cultures after 48 h of stimulation. The graphs at bottom show the ratios of T cells exhibiting the fully recombined versus the partially recombined phenotypes (SP/DP) at the same 48-h time point. E, Reporter expression in a single fixed popliteal lymph node (left panel) from an *Ox40-cre* mouse containing the 3373 reporter allele. Original magnification x40. Scale bar, 80 μM. The capsule of the lymph node is outlined in blue circles, and a dashed box highlights the magnified area of interest. The three smaller images at right show tdTomato (left panel) and eGFP (middle panel) expression in the magnified area. A merged overlay is shown at the extreme right with tdTomato+eGFP+ DP lymphocytes marked with arrows. Scale bar, 8 μM.

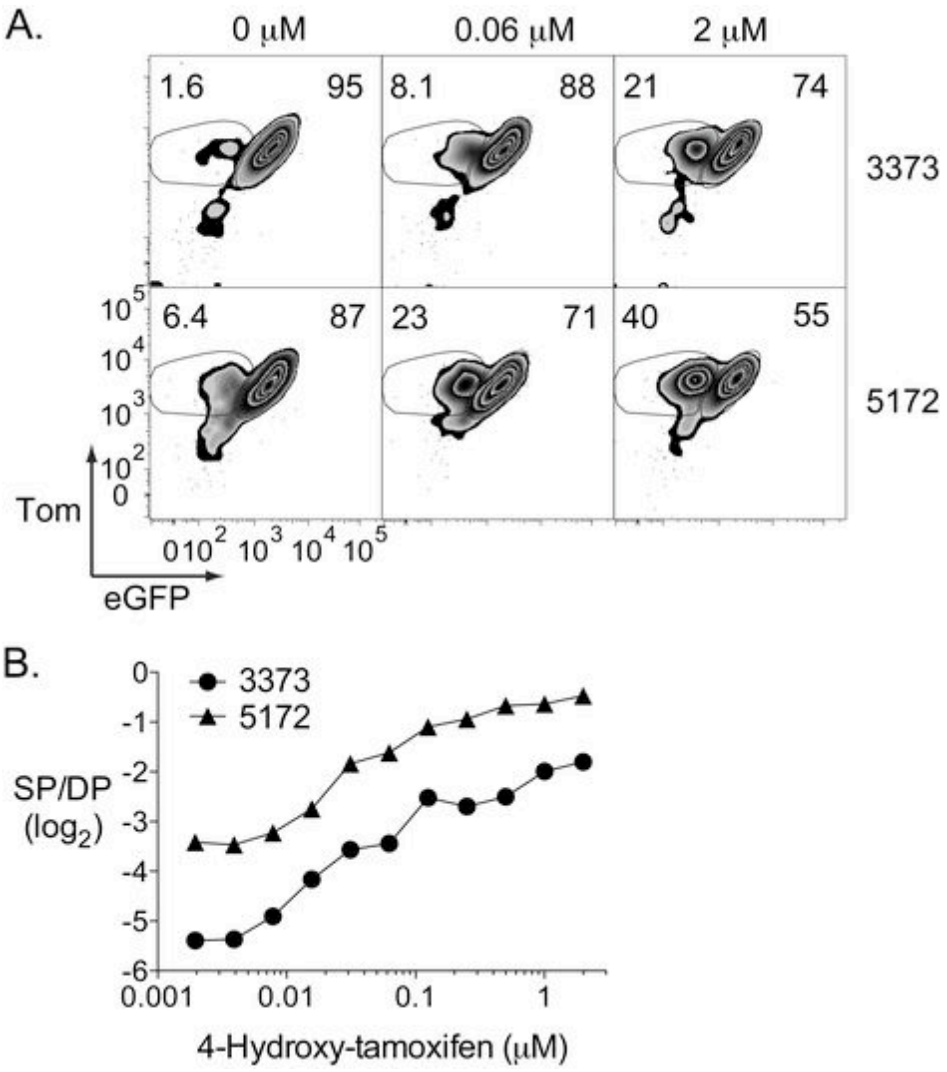

FIGURE 2-S1. Recombination of the reporter alleles in response to expression and induction of Cre-ER. (A) ES cell clones carrying the 5172 or 3373 alleles were transiently cotransfected with an expression vector encoding Cre-ER(Metzger et al., 1995) and human CD2. Cre recombination was induced by incubation with varying concentrations of 4-hydroxy-tamoxifen for 24 hours after transfection. The cells were then incubated for 72 hours before analysis by flow cytometry. The plots show expression of tdTomato and eGFP on cells that were gated for human CD2 expression as a transfection marker. Numbers on the plots refer to the percentages of cells in the marked fluorescence gates. (B) Graph showing the ratio of cells (carrying either the 3373 or 5172 reporter alleles) exhibiting the fully recombined phenotype (tdTomato+eGFP–) versus the partially recombined phenotype (tdTomato+eGFP+) as a function of 4-hydroxy-tamoxifen concentration (after treatment and analysis as in A). Statistical significance, p=0.0002, calculated by Student's t- test.

Cre expression levels should be elevated in mice that are homozygous for *Ox40-cre* relative to heterozygous mice. This predicts that reporter-positive cells should be more numerous and recombination ratios higher in T cells from the former than in those from the latter. Lymphocytes from mice homozygous for the *Ox40-cre* allele do no express OX40, and this would be expected to compromise the survival of some T cells (such as CD4+ memory T cells)(Rogers et al., 2001). Despite this, however, we found increases in reporter-positive T cells in the homozygous mice (Fig. 2-S2A), and, associated with this, there were increases in the SP/DP recombination ratios (Supplemental Fig. 2-2B).

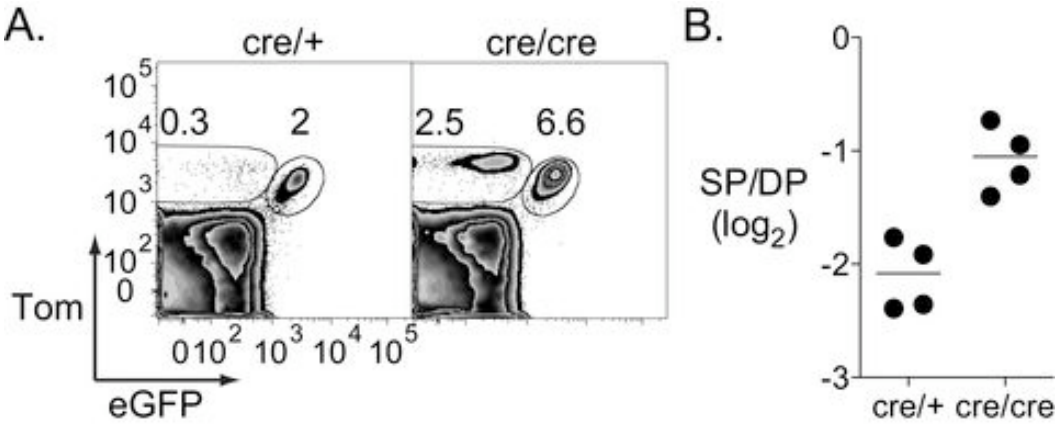

FIGURE 2-S2. A two-fold increase in the number of Cre recombinase alleles increases the frequency of cells exhibiting the fully recombined reporter phenotype. (A) Reporter activity in peripheral blood CD4+CD25-CD44^{low} lymphocytes from either heterozygous *Ox40-cre*/+ (left) or homozygous *Ox40-cre/Ox40-cre* (right) mice as assessed by flow cytometry. Numbers above the gates indicate the frequency of cells with fully or partially recombined alleles. (B) The graph shows the ratios of fully to partially recombined alleles from 4 mice of each genotype. The data are representative of two independent experiments. p=0.009, calculated by Student's t-test.

T cells were purified from *Ox40-cre* mice and stimulated *in vitro* with an anti-CD3 Ab. Induction of OX40/*Ox40-cre* in this context occurs as a function of anti-CD3 concentration (Klinger et al., 2009) and is potentiated by costimulatory signaling delivered by inclusion of an anti-CD28 Ab. Consistent with this, we found a higher frequency of cells carrying fully recombined reporter alleles in cultures treated with high amounts of anti-CD3 compared with those treated with low amounts or in cultures that received costimulation compared with those that did not (Fig. 2-4D). Cumulatively, therefore, we conclude that the reporter alleles behave as expected and can be used to inform on differences in the levels of Cre expressed by populations of cells *in vivo*.

As a final test of the utility of the alleles, we determined whether they could be detected by fluorescence confocal microscopy in sections of lymphoid tissue from *Ox40-cre* reporter-positive mice. Whereas the tdTomato and eGFP moieties could be readily detected in fresh tissue sections (Fig. 2-S3), eGFP fluorescence was typically low and became undetectable by confocal microscopy upon routine fixation. This problem could be overcome by use of fluorescently labeled anti-GFP antisera, and, using this approach, we could easily distinguish lymphocytes carrying fully recombined reporter alleles from those with partially recombined alleles in frozen sections (Fig. 2-4E). These data show that the alleles can be used for determining the localization of cells that differ in their reporter expression and thus gene expression history.

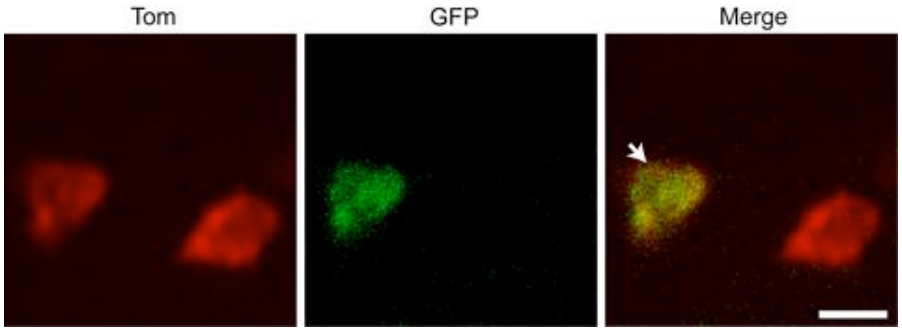

FIGURE 2-S3. Reporter fluorescence in fresh tissue sections. The images show tdTomato (left), eGFP (middle) and merged fluorescence (right) from a section of popliteal lymph node. A tdTomato/eGFP double positive lymphocyte is marked in the overlay with an arrow. Scale bar = 8μM.

Discussion

Reporter alleles that inform on Cre activity in cells are typically designed to transition between inactive and active states (Lobe et al., 1999; Mao et al., 1999; Srinivas et al., 2001). The active state arises when Cre excises a *loxP*-flanked element from the allele, rendering the cell positive for persistent expression of a reporter protein from a downstream ORF. Populations of cells that express the reporter have previously expressed Cre, whereas those that do not are enriched for cells that never expressed Cre or expressed insufficient levels of it for recombination to occur efficiently. Reporters that have been used in this sort of context include β-galactosidase, eGFP and variants of eGFP, placental alkaline phosphatase, and wheat-germ agglutinin (Lobe et al., 1999; Mao et al., 2001; Mao et al., 1999; Novak et al., 2000; Soriano, 1999; Srinivas et al., 2001).

The reporters we have described in this paper include the core components of the conventional design and thus they can be used for all of the same purposes. They also include an additional component that can be used to discriminate between populations of cells that differ in how much Cre they previously expressed. Thus, instead of two possible configurations (i.e., inactive versus active), the reporters are capable of adopting three states (i.e., inactive and two distinct active states) depending on the amount of Cre expressed in cells. If Cre expression is governed in a physiologically significant fashion (e.g., by elements taken from a developmentally regulated gene), then the reporter alleles can be used to discriminate populations that have different gene expression histories. This type of discrimination can be exploited in the study of

lineage commitment decisions because it allows for cell fate to be correlated with prior gene expression levels.

To analyze the performance of the reporter alleles, we employed various strategies to deliver graded amounts of Cre recombinase activity to the targeted ES cells. These included transient transfection of expression vectors encoding Cre or transient transfection (or retroviral transduction) of vectors encoding Cre-ER(Metzger et al., 1995) followed by induction of Cre activity with 4-hydroxy-tamoxifen. Relative to these, the cell-permeant Tat-Cre protein (Peitz et al., 2002) provided the highest efficiency Cre recombination and the greatest experimental dynamic range. Using Tat-Cre, we could show that the penetrance of complete recombination at the reporter alleles increased as a function of Cre recombinase activity, and, consistent with our expectations from *in vitro* recombination assays (Lee and Saito, 1998), we found that recombination of 3373 sites occurred less efficiently than that of 5172 sites. Complete recombination of the 3373 reporter allele therefore depended on higher levels of Cre activity in cells than the 5172 allele.

The characteristics of the reporter alleles just summarized make clear their value for informing on prior Cre expression in cells, and thus, as argued above, their potential applicability to the study of lineage commitment decisions in which fate determination is a function of quantitative differences in signaling and downstream gene expression. The decision to generate two types of alleles, one involving 3373 sites and the other 5172 sites, was made with two related considerations in mind. The first was that for any given Cre expression level from

a *cre* allele, the population of cells carrying a reporter in its partially recombined configuration would be larger with the 3373 allele than with the 5172 allele. If expression of the *cre* allele was induced differentially as part of a differentiation process, then correlations between reporter state and differentiated phenotype would likely be higher with one allele than with the other. This is simply because the partially recombined versus fully recombined states would more closely align with differentiated phenotypes in one case than in the other. The second related consideration was that different levels of Cre will be expressed in cells depending on what *cre* allele has been used. In general, the 3373 reporter allele is likely to be more useful than the 5172 allele when Cre expression is high because the penetrance of complete recombination in affected populations of cells would approach 100% with the 5172 allele (and thus the allele would not discriminate different populations of Cre-expressing cells). When Cre expression is lower, the potential utility of the 5172 allele would increase.

Cre recombination approached 100% penetrance in regulatory T cells in *Ox40-cre* mice because these cells express the *Ox40* gene in a constitutive fashion. Recombination penetrance was lower in other types of cells that express *Ox40* transiently and/or at submaximal levels. These characteristics allowed us to examine the behavior of the two reporter alleles in mice as a function of differing Cre expression levels. Our analysis included an examination of reporter allele recombination in cells that had been stimulated *in vitro* with anti-CD3ε (to induce OX40 expression). In all cases, the recombination status of the alleles correlated well with the known prior/ongoing expression of the *Ox40* gene in the populations

under analysis. Furthermore, the two alleles (3373 and 5172) consistently differed in their sensitivity to Cre levels as expected, and consequently, they discriminated different subpopulations of cells (i.e., the relative frequencies of cells with fully versus partially recombined alleles were different in the two types of mice for all lineages examined). The results of these experiments therefore substantiate the rationale behind making the two types of alleles, while also providing a clear demonstration of their utility for lineage marking.

In addition to providing information about absolute levels of Cre expression, the new reporter alleles are also expected to inform on differences in the duration of Cre expression in cells. That is, the probability that they will adopt the fully recombined state will increase the longer the cells stay positive for expression of Cre, even if they only express low levels of the recombinase. This aspect will limit the utility of the alleles in discriminating between effects caused by transient high versus prolonged low gene expression, and this must be borne in mind when considering their usefulness for addressing specific biological problems. It is also important to consider that even though the reporter alleles inform on prior Cre activity in single cells, they were nonetheless designed for the analysis of populations of cells rather than single cells. For a given subsaturating level of Cre, a population of cells may be highly enriched for a particular recombination outcome (i.e., full or partial recombination of a reporter allele), but it is expected that some cells within it will harbor alternative outcomes. The extent to which this is the case will differ for the two alleles as a function of Cre expression levels as discussed above. Despite these potential limitations, however, it seems likely that

the alleles will provide useful information in many settings and that mice carrying them will be beneficial when studying the activity of multiple cre alleles expressed in a broad range of cell types and developing tissues.

Chapter 3

Genetic Fate Mapping of Anti-Viral CD4+ T-cell Responses *In Vivo*

Abstract

A genetic fate mapping approach is taken to determine the fate of effector CD4+ T-cells generated following variable amounts of antigen receptor stimulation during naive T-cell activation. This approach utilizes the 3373 3SCS Cre-reporter allele, developed and characterized in chapter 2, with *Ox40-cre* mice. By marking CD4+ effector T-cell on the basis of TCR signal strength and by tracking their fate throughout an anti-viral response, we find different amounts of antigen-receptor stimulation during activation lead to separate cellular fates. Specifically, effector cells generated as a result of relatively strong antigen receptor stimulation accumulate within the antigen-specific memory population. Preferential enrichment for these effector cells within the responding population takes place during the contraction phase as a result of differential susceptibility to apoptosis and is independent of immediate effector function at the peak of the response. This study provides *in vivo* evidence for a programmed model of CD4+ memory T-cell generation following acute viral infection where early programming events, driven in part by TCR signal strength, influence the fate of effector CD4+ cells during the transition to memory.

Introduction

During acute infection with a pathogen, naive T-cells are activated by encounter with specialized antigen presenting cells (APCs). Such activation occurs in secondary lymphoid tissues and involves the integration of signals from the T-cell antigen receptor (TCR) and both costimulatory and cytokine receptors. Activated cells proliferate extensively and ultimately differentiate into effector cells with distinct functions(Jenkins et al., 2001; Kaech and Wherry, 2007; Williams and Bevan, 2007). Strikingly, the majority of effector cells are short-lived and will undergo apoptosis when the pathogen has been cleared leaving behind a small population of long-lasting memory cells to protect the host against future infections with the same pathogen (Seder and Ahmed, 2003; van Leeuwen et al., 2009; Williams and Bevan, 2007)).

CD4+ T-cells play a central role in both adaptive and innate immune responses. They are critical for germinal center reactions, promoting class switching and affinity maturation (Fazilleau et al., 2007; McHeyzer-Williams et al., 2009). They are similarly important for optimal responses by CD8+ T cells, promoting memory cell survival and secondary proliferation upon reinfection (Janssen et al., 2005; Janssen et al., 2003; Khanolkar et al., 2007; Shedlock and Shen, 2003; Sun and Bevan, 2003; Sun et al., 2004). CD4+ T cells also provide help to macrophages inducing them to increase their bactericidal activity (Dalton et al., 1993) or differentiate (Gordon, 2003). In addition to these indirect effects on immunity, CD4+ T-cells have limited cytotoxic capacity, which allows them to contribute directly to the clearance of pathogen-infected cells under certain

circumstances (Jellison et al., 2005). Thus CD4+ T-cells are key to generating effective and long-lasting immunity.

Increasing evidence supports the notion that like their naïve precursors, memory CD4+ T-cells are similarly crucial for protection during reinfection with intracellular bacteria (Flynn et al., 1993), parasites (Anthony et al., 2006; Mohrs et al., 2005), and during viral infections (Liang et al., 1994; MacLeod et al., 2009a; Swain et al., 2006). As is true of CD8+ memory T cells, CD4+ memory T cells make more rapid and effective responses than naïve T cells of the same specificity for antigen (Kaech and Wherry, 2007; Lees and Farber, 2010a; Williams and Bevan, 2007). Augmented sensitivity in initiating responses is due in part to increased expression of signalling proteins within the TCR-signalling pathway (Slifka and Whitton, 2001), decreased requirements for costimulatory pathways and professional APCs (Croft et al., 1994), and alterations in chromatin at cytokine loci (Ansel et al., 2006). Memory lymphocytes are located within lymphoid and non-lymphoid tissues (Masopust et al., 2001; Reinhardt et al., 2001) at higher frequencies than naive T-cells of the same specificity (Homann et al., 2001; Murali-Krishna et al., 1998; Rees et al., 1999) and are capable of long-term survival (Hammarlund et al., 2003; Homann et al., 2001; Surh and Sprent, 2008). Understanding the mechanisms influencing memory T-cell generation and function is of considerable importance, with the goal of enhancing memory cell function following immunization in mind.

The amount of antigen and duration of antigen presentation during priming have been shown to influence the fate of responding T-cells, with varying effects

on CD4+ or CD8+ lymphocyte populations. CD8+ T-cells can be fully committed to clonal expansion and differentiation following relatively short encounters with APCs presenting peptide. Similarly, they can also differentiate into functional effector cells following very brief exposure to pathogens *in vivo* (Kaech and Ahmed, 2001; Mercado et al., 2000; van Stipdonk et al., 2001). CD4+ T-cells, on the other hand, require longer periods of interaction with antigen to become fully activated. Studies that increase the precursor frequency of antigen-reactive cells (Blair and Lefrançois, 2007; Foulds and Shen, 2006), decrease the dose of infectious agents (Foulds et al., 2002), or control the duration of antigen presentation (Obst et al., 2005) show CD4+ T-cell expansion and differentiation benefit from extended interactions with antigen and increases in antigen dose. Such data suggest that the generation of memory CD4+ and CD8+ T cells is inhibited by increased competition for antigen during priming.

TCR affinities for pMHC complexes influence and shape the developing effector and memory populations. Selection for CD4+ T-cell clones expressing TCRs of relatively high affinity occurs during the course of primary and secondary expansion although many, if not all, peptide-reactive clones are recruited early into the response (Busch et al., 1998a; Malherbe et al., 2004; Savage et al., 1999). There may exist an upper limit for beneficial pMHC affinities as selection for T-cells with the highest affinity is not absolute (Malherbe et al., 2004). Within the CD8+ lineage, low affinity interactions lead to less expansion and earlier migration into peripheral tissues, whereas high affinity interactions lead to greater expansion and delayed migration out of lymphoid

tissues (Zehn et al., 2009). This that suggests TCR affinities for antigen may direct other aspects of a response other than recruitment into a response, including clonal size and migratory behavior. Although the importance of TCR-pMHC interactions in effector and memory cell differentiation are apparent, additional studies are necessary to better define the significance of TCR stimulation strength and *in vivo* fate, in particular within the setting of a normal polyclonal repertoire.

The process by which effector T-cells populations contract and give rise to a stable memory population remains unclear. In one model, memory T-cells result from stochastic selection of effector cells. In this model all effector cells have equal potential to become memory cells, however events during contraction, possibly related to competition for antigen-receptor interactions or cytokine signals, lead to the majority of effector cells dying from apoptosis (Freitas and Rocha, 2000). Stochastic selection predicts that there should be conservation of antigen receptor diversity between effector and memory populations, i.e., memory cells should be selected independently of the binding properties of their TCRs. Diverse studies have provided support for this model (Busch et al., 1998a)(Bousso et al., 1998; Bousso and Kourilsky, 1999; Malherbe et al., 2004).

In a second model of memory T cell development, effector and memory cells are the product of separate lineages, programmed by distinct TCR signals, costimulation and the surrounding inflammatory environment during activation (Kaech and Ahmed, 2001; Williams et al., 2006). Support for this model comes

from the presence of CD8+ memory cell precursors within effector T-cell populations that preferentially give rise to long-lived memory cells, and the finding that short periods of stimulation are sufficient to drive CD8+ effector and memory cell differentiation (Kaech and Ahmed, 2001; Kaech et al., 2003; Kaech and Wherry, 2007; Mercado et al., 2000; van Stipdonk et al., 2001). Although CD4+ memory precursors have not been identified as of yet (Lees and Farber, 2010b), and the CD4+ lineage appears to be more dependent on prolonged antigen stimulation for activation (Obst et al., 2005), CD4+ effector cells are capable of transitioning to memory-like cells *in vitro* by default (McKinstry et al., 2007; McKinstry et al., 2008) and transgenic CD4+ effector cells with high functional avidity appear to preferentially survive contraction (Williams et al., 2008). Thus, memory cell fate may be programmed early during the response, possibly as a result of strong TCR signals.

To investigate the role of strength of the initial TCR-pMHC interaction on CD4+ memory T-cell generation, we developed an approach to differentially mark T-cells *in vivo* with fluorescent proteins as a function of the TCR signal strength. Our approach depends on a 3-state Cre-sensitive (3373 3SCS) allele, developed and characterized in chapter 2, and a Cre-recombinase allele that is expressed selectively during T cell activation (*Ox40-cre)* in a fashion that reflects the amount of T-cell receptor stimulation delivered to the cell (Klinger et al., 2010). Using this combination of alleles we have followed the fate of antigen-specific CD4+ T-cells during acute LCMV infection.

We found that CD4+ effector T-cells generated as a result of strong TCR signal strength at the time of priming accumulated within the stable memory cell population where they were found at high frequencies. The relative abundance of these cells within the CD4+ memory T-cell population was a result of decreased susceptibility to apoptosis during contraction of the effector response and did not correlate with immediate effector function at the peak of the response. This study provides *in vivo* evidence for a deterministic model of CD4+ memory T-cell generation following acute viral infection where early programming events driven in part by TCR signal strength influence the fate of effector CD4+ cells during the transition to memory.

Materials and Methods

Mice, Adoptive Transfers and LCMV Infections

3373 3-state Cre sensitive (3373 3SCS) and *Ox40-cre* mice have been described previously (Klinger et al., 2009). Briefly, 3373 3SCS mice allow for bicistronic expression of tdTomato and eGFP proteins from the *Rosa26* locus following Cre recombinase expression (Soriano, 1999). Further delineation of cells with relatively high versus low expression of Cre can occur due to the presence of mutant 3373 *loxP* sites flanking the IRES-eGFP element(Lee and Saito, 1998). These reporter mice, referred to herein as “3373 mice”, were generated in E14 ES cells and maintained on the 129P2/Ola parental strain background. Intercrossing 3373 3SCS reporter mice with B6 *Ox40-cre* mice generated 129P2.B6(F1) "Ox40-cre/3373" experimental mice. Mice were infected at 6-8 weeks of age by intraperitoneal injection with $2x10^5$ pfu of LCMV-Armstrong in PBS. Single-cell suspensions of splenocytes were generated following ammonium chloride lysis and filtered through nylon mesh. Peritoneal cells were collected following injection of 10mL cold PBS into the peritoneal cavity with an 19G needle. Blood samples were collected in EDTA-containing PBS at indicated times via submandibular puncture, and at memory time points where stated. Splenocytes from Ox40-cre/3373 Smarta TCR mice specific for a peptide derived from the glycoprotein of LCMV comprising amino acids 61-80 (GLNGPDIYKGVYQFKSVEFD) were generated on mixed 129P2/B6 background and used in *in vitro* experiments (Oxenius et al., 1998). Adoptive transfer studies were performed with purified CD4+ T-cells from Smarta TCR-expressing Ox40-

cre/3373 129P2.B6(F1) mice with compatible 129P2/B6(F1) hosts. Small numbers, 2-5x10^3, of Smarta Ox40-cre/3373 cells were transferred one day prior to infection into hosts by retroorbital injection, seeding experimental mice with 200 - 500 naive Tg CD4+ cells, comparable to endogenous frequencies (Whitmire et al., 2006) assuming 10% seeding efficiency (Williams et al., 2008). All animals and infections were performed according to approved UCSF IACUC protocols.

Flow Cytometry

All stains for surface antigens were performed using standard procedures in cold PBS/0.3% BSA (Holmes et al., 2001) at saturating concentrations of antibodies (BDBiosciences, Biolegend, EBiosciences). GP66-APC tetramers composed of I-A^b-gp66-77 (DIYKGVYQFKSV) biotin monomers complexed with streptavidin-APC identify CD4+ T-cells specific for the major B6 epitope, a peptide derived from the LCMV glycoprotein containing amino acids 66-80 and were obtained from the NIH Tetramer Facility. Tetramer stains were performed prior to surface staining for 2 hours at 18-20° C in RPMI supplemented with 2% fetal calf serum. Ex vivo cytokine production was assessed by culturing splenocytes in 96-well plates with varying concentrations of gp66-80 peptide (DIYKGVYQFKSVEFD) at 37° C for 6 hours in complete medium. One hour following the start of incubation, Brefeldin A or GolgiPlug was added according to the manufacturer's recommended dose (BD Biosciences). Cell surface staining was performed on ice, followed by fixation in 4% p-formaldehyde (EM Scientific) for 5 min at 37° C,

quenched with FCS, and stored on ice overnight or permeabilized in BD PermWash on ice for 20 minutes (BD Biosciences). Incubation with conjugated anti-mouse cytokine antibodies in BD PermWash followed for an additional hour on ice. Cells were washed and analyzed immediately. Annexin V staining was performed following manufacturer's procedure (BDBiosciences). All flow cytometry data were collected on LSRii instruments equipped with a yellow-green lasers for optimal tdTomato excitation using FACSDiva acquisition software. Cells were sorted on an AriaIII with a yellow-green laser. All analysis was performed using FACSDiva (BD Biosciences) or FlowJo software (Treestar).

In vitro Stimulations

All stimulations involved culture in RPMI-10%FCS-1xPenicillin/Streptamycin/ 2mM L-glutamine with 50uM 2-mercaptoethanol (Invitrogen). TCR-transgenic CD4+ cells were stimulated with irradiated splenocytes and varying amounts of gp66-80 peptide. In some experiments, day 3 *in vitro* cultures were washed and necrotic cells/debris were removed by density centrifugation. Viable cells were further cultured for 7 days in fresh complete medium before analysis(McKinstry et al., 2007). In some experiments, splenocytes were serum-starved for 15 minutes at 37°C degrees,followed by stimulation with IL-7 (5ng/mL) for 15 minutes in complete medium at 37°C. Cells were fixed immediately with 4% p-formaldehyde for 5 min and then permeabilized and stained for intracellular p-STAT5 as described (Perez et al., 2005).

Results

In vitro Stimulation of CD4+ T-cells

We have previously described the properties of a three-state Cre-sensitive reporter allele (3373 3SCS). This allele marks cells as a function of the amount of Cre-recombinase they have previously expressed [(Klinger et al., 2010) and Figure 3-1 A-B]. Cells harboring the 3373 3SCS allele that have not expressed Cre carry the reporter allele in an unrecombined state and thus do not express any reporter proteins (R-N; reporter-negative phenotype). If cells express the recombinase, they can undergo partial or complete recombination at the reporter allele. Partial recombination results in the expression of two reporter proteins (tdTomato and GFP; the Red-Green [R-G] phenotype) whereas full recombination results only in expression of one reporter (tdTomato; the Red-only [R] phenotype). Consistent with expectations, cells adopt the R phenotype following high expression of the Cre recombinase (Klinger et al., 2010). Moreover, the ratios of cells exhibiting distinct reporter phenotypes (i.e. R:R-G, R-G:R-N, or R:R-N) could be used to inform on relative amounts of Cre activity experienced by populations (Klinger et al., 2010). In this study, we have combined the 3373 3SCS reporter allele with the *Ox40-cre* allele, which expresses the recombinase in a fashion that reflects the normal expression of the OX40 protein.

OX40 is transiently expressed on TCR transgenic naive T-cells following *in vitro* stimulation, and it reaches maximal levels within 48-72 hours, decreasing thereafter to near background levels by 5 days (Gramaglia et al., 2000;

Gramaglia et al., 1998). We have shown that naive CD4+ T-cells from Ox40-cre/3373 mice stimulated *in vitro* with antigen-receptor antibodies recombine the 3373 reporter allele and that increases in receptor stimulation resulted in the generation of a greater number of R and R-G marked cells than R-N marked cells, as predicted (Klinger et al., 2010). Thus, these results from antibody stimulated Ox40-cre/3373 T-cells demonstrated that the Ox40-cre/3373 reporter may by useful in marking T-cells on the basis of the amount of antigen stimulation they experience. T-cells generated from relatively strong stimulatory conditions were marked by reporter differently (favored generation of the R state) than T-cells marked as a result of relatively weak conditions. Thus, populations of T-cells could be discriminated by reporter on the basis of the amount of receptor stimulation (Klinger et al., 2010).

To establish the effects of strong versus weak antigen-specific stimulation on Ox40-cre/3373 reporter recombination, naive CD4+ splenocytes from Ox40-cre/3373 mice expressing the transgenic CD4+ Smarta TCR were stimulated *in vitro* with varying amounts of agonist peptide. The Smarta TCR is specific for a peptide from the LCMV glycoprotein (residues 61-80) presented by I-A^b(Oxenius et al., 1998). Stimulation of Ox40-cre/3373 Smarta splenocytes led to reporter recombination within 72 hours (Figure 3-1C). Increasing the amount of peptide added to stimulation cultures resulted in increasing amounts of reporter allele recombination (total R and R-G cells, Figure 3-1C) along with increased ratios of cells in more recombined reporter states (R:R-G,R-G:R-N, and R:R-N Figure 3-1C-D). Therefore, R and R-G CD4+ T-cells are preferentially generated

following relatively strong TCR stimulation, whereas R-N cells are a result of weaker stimulation.

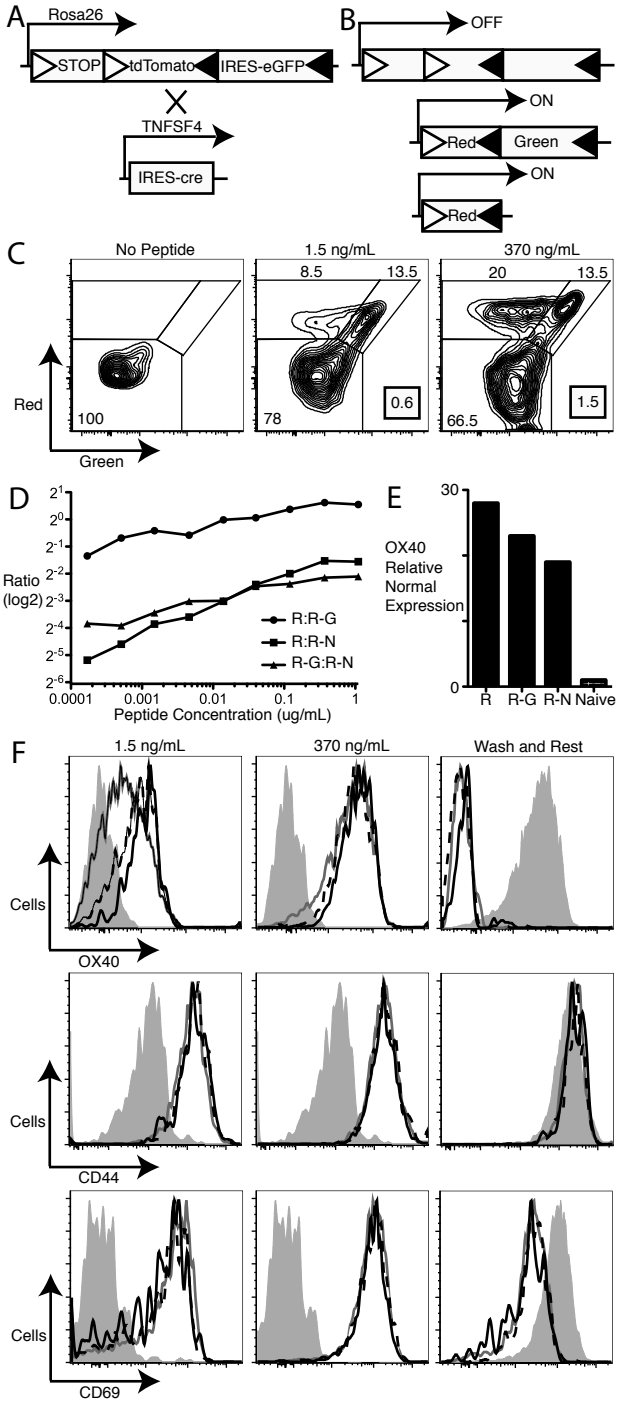

FIGURE 3-1. Ox40-cre/3373 T-cells are Marked *In Vitro* as a Function of Strength of TCR Stimulation. A. Experimental T-cells and mice are double transgenic for the 3373 3-state Cre sensitive (3SCS) reporter allele (top) and *Ox40-cre* allele(bottom). The 3373 3SCS reporter allele consists of a floxed (white triangles) STOP element upstream of a tdTomato ORF followed by a mutant 3373 *loxP* (black triangles) flanked IRES eGFP ORF. The reporter is targeted to the endogenous *ROSA26* locus. The *Ox40-cre* allele consists of an

IRES-cre ORF and is targeted to the endogenous *Tnfrsf4* locus. B. The three states of the 3373 reporter include the reporter negative (R-N) "off" state (top), an intermediate Red-Green (R-G) "on" state (center) and a terminal Red-only (R) "on" state (bottom). C. Smarta Ox40-cre/3373 splenocytes were stimulated with 0, 1.5, and 370 ng/mL agonist peptide for 72 hour *in vitro* and analyzed for reporter induction (tdTomato y-axis, eGFP x-axis). Events displayed are gated on total CD4+ cells. Numerical values show the percent of gated events in depicted R-N, R-G, and R gates, as shown on contour plots. Boxed numerical values display R:R-G ratios. D. Informative reporter ratios are depicted following stimulation as in (C) with various amounts of agonist peptide. Y-axis = log2 scale. E. Relative *Ox40* transcript levels were determined by qPCR in sorted unstimulated and stimulated reporter populations as in (C). Normalized to unstimulated Smarta control = 1. HPRT was used as the relative expression control. F. (left, center) Cell surface expression of OX40 (top), CD44 (center) and CD69 (bottom) following peptide stimulation as in (C). Red-only = solid black line, Red-Green = dashed black line, Reporter Negative = dark gray line, naive Smarta = filled histogram. (right) Stimulated Smarta cells were washed and rested for 4 additional days before analysis of cell surface expression. Filled histogram = surface expression of indicated activation marker on total CD4+ T-cells stimulated as in (C) for 3 days.

Cre protein is expressed using a Mengo IRES targeted to the endogenous *Tnfrsf4* locus in Ox40-cre/3373 mice (Klinger et al., 2009). To determine whether OX40 protein expression levels correlated well with the R, R-G, and R-N populations in a predictable manner, Ox40-cre/3373 Smarta cells were stimulated and analyzed for both reporter status and cell surface OX40 expression. Compared to unstimulated controls, nearly all Smarta cells had elevated levels of surface OX40 protein. Importantly, and as predicted, R cells had the highest levels of OX40 protein; R-G cells had intermediate levels, particularly at sub-saturating conditions; and R-N cells had the lowest surface levels of OX40 (Figure 3-1F, top). Examination of *Ox40* transcript levels from similarly stimulated and sorted R, R-G, and R-N Ox40-cre/3373 Smarta cells showed R cells contained the greatest amount of *Ox40* transcript, R-N cell contained the least, and R-G cells had intermediate levels (Figure 3-1E). These results were consistent with the predicted behavior of the Ox40-cre/3373 reporter, where cells that had expressed the highest, intermediate, and lowest amounts of OX40 (and Ox40-cre) were marked by the R, R-G, and R-N states, respectively.

We found equally high expression of CD44 and CD69 on all reporter populations (Figure 3-1F, center and bottom). Furthermore, following activation and *in vitro* rest, all reporter populations shared low surface expression of OX40 and CD69, but high CD44 expression, consistent with all reporter populations having undergone sufficient activation resulting in stable high expression of the effector/memory marker CD44 (Figure 3-1F, right). Thus, *in vitro* antigen

stimulation of naive T-cells from Ox40-cre/3373 mice led to recombination of reporter on the basis of the amount of TCR stimulation. R cells were generated at greater frequencies with strong TCR stimulation than with weak TCR stimulation. Therefore, the reporter state adopted by an activated CD4+ T-cell informed on the relative strength of TCR stimulation. Experiments were performed using Ox40-cre/3373 mice to investigate the *in vivo* fate of effector CD4+ T-cells generated as a result of differing amounts of TCR stimulation following acute infection.

Longitudinal Analysis of Infected Ox40-cre/3373 Mice

LCMV-Armstrong infection of Ox40-cre/3373 mice resulted in the generation of an appreciable number of anti-viral CD4+ effector T-cells. Following expansion, the majority of these antigen-specific effector CD4+ T-cells die (Homann et al., 2001). Longitudinal blood analysis of LCMV-Armstrong infected Ox40-cre/3373 mice was performed to determine the fate of effector CD4+ T-cells generated following acute infection and marked by the relative strength of TCR stimulation. At the peak of infection (day 8), R and R-N CD4+ tetramer-reactive cells were found in equal numbers in blood, with R-G cells present in smaller numbers (Figure 3-2 A). After day 8, all CD4+ tetramer-reactive reporter populations contracted independent of reporter state. During contraction of the response, however, R CD4+ tetramer-reactive cells increased in relative proportion, and R-N CD4+ cells decreased in relative proportion, within the total tetramer-reactive population. R-G cells contracted but made up a

constant portion of the tetramer-reactive population. These changes in the relative contribution of R and R-N effector cells to the total tetramer-reactive population stabilized between day 18 and day 28. After day 28, the frequencies of all three reporter populations remained constant for at least 180 days. This resulted in R tetramer-reactive cells contributing to the majority of antigen-specific cells found at later time points (day 40-180, Figure 3-2 B). Thus, R CD4+ effector cells appear to be enriched for cells with memory cell fate.

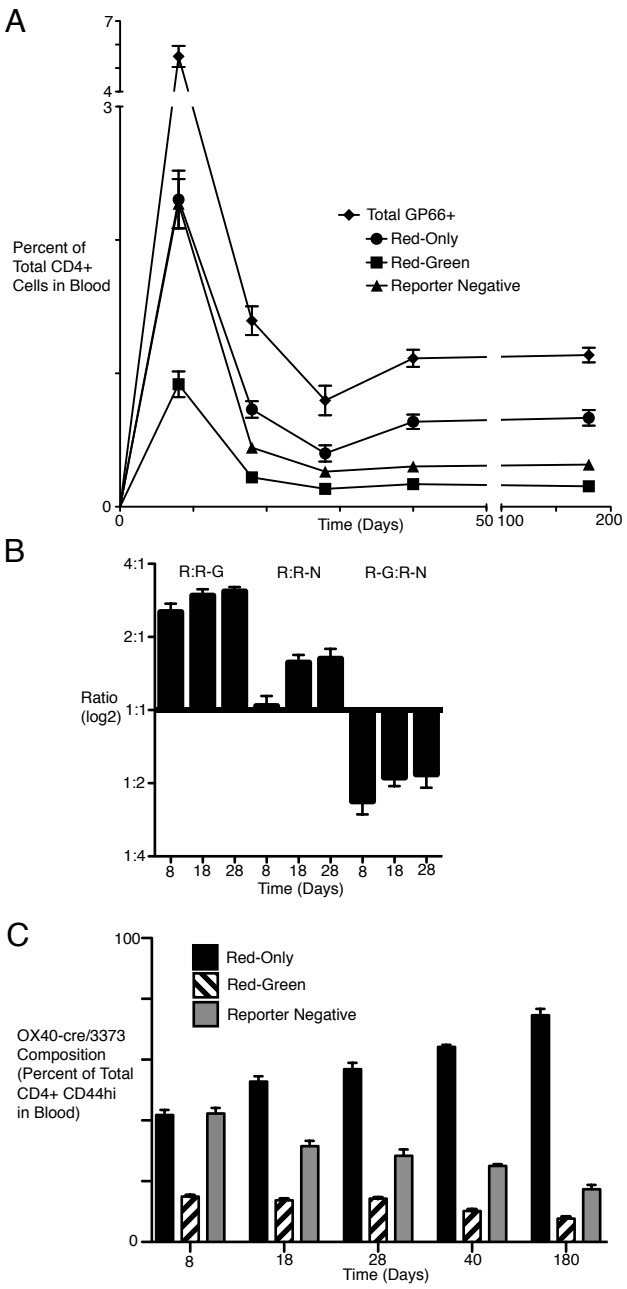

FIGURE 3-2. During the effector-to-memory transition, TCR signal strength correlates with fate. A. Longitudinal analysis of blood from LCMV infected Ox40-cre/3373 mice. Percent of tetramer-reactive CD4+ cells within total blood CD4+ population shown (diamonds) along with each individual tetramer-reactive reporter population (Red-only = circle, Red-Green = square, and reporter negative = triangle). B. Ratio of frequency (log2 scale) of individual reporter populations as indicated within tetramer-reactive CD4+ population at various times following infection. Data represents mean value of cohort +/- SEM. C. Percent contribution of individual reporter populations (Red-only, Red-Green, and reporter negative = black, diagonal and gray bars, respectively) within total CD4+ $CD44^{hi}$ CD25- memory-phenotype cells following LCMV infection as in (A). Data

from one of three independent experiments, with three to ten mice per cohort. Data represents mean value of cohort +/- SEM.

Reporter expression was analyzed within the CD4+ CD44^{hi} memory-phenotype (MP) population to determine whether similar changes in the frequencies of R, R-G, and R-N occurred within this compartment following infection. Here, R CD4+ MP cells increased their relative abundance within the CD4+ MP population, and R-N MP cells decreased their relative abundance, similar to the antigen-specific responses. Unlike the antigen-specific responses, however, the relative proportions of MP reporter populations never stabilized during the entire time of the experiment (180 days) (Figure 3-2 C). The R CD4+ MP cells constantly grew in relative frequency such that at 180 days post-infection, nearly 80% percent of all MP CD4+ T-cells expressed the R reporter state. Therefore, the MP CD4+ compartment displayed similar kinetics to the antigen-specific response early during the response, but differed later with constant increases in the proportion of MP CD4+ R cells.

Enrichment for R antigen-specific CD4+ T-cells over time was not a result of low recombinase activity in R-N effector cells as shown by adoptive transfer of R-N effector cells into congenically marked infected hosts. A very low rate of conversion was seen in these settings (Figure 3-S1). These findings were consistent with the Cre recombination event being dependent upon transient *Ox40* transcriptional activity as a result of acute TCR-dependent stimulation prior to day 8 (Figure 3-S1, Figure 3-1). These data also suggest the changes we observed in relative frequencies of R and R-N antigen-specific cells within the total antigen-specific population likely reflected differences in the intrinsic fate of R and R-N cells during contraction, as opposed to an artifact of low rates of

Ox40-cre/3373 reporter recombination from persistent antigen or low grade infection.

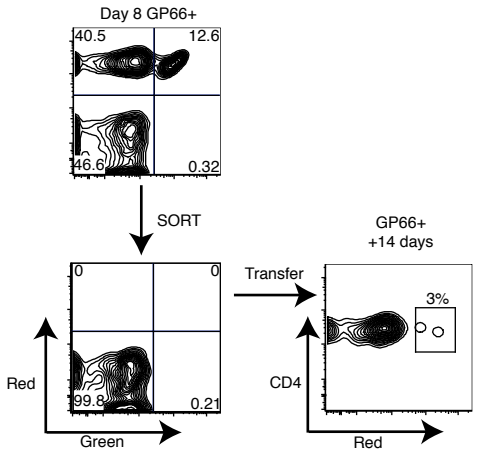

FIGURE 3-S1: Enrichment for Red-only antigen-specific cells following effector phase is not a function of low recombinase expression and activity in reporter negative populations. (left) Reporter negative antigen-specific splenocytes from day 8 LCMV infected Ox40-cre/3373/CD45.2+ were sorted to high purity and transferred into LCMV infected CD45.1+ hosts. (right) Two weeks following transfer, reporter recombination was measured in antigen-reactive donor population. Gated on live donor CD44^{hi} CD4+ gp66+ events. Percent reporter induction (all Red+ cells) is depicted numerically. Note the change in RN contribution to the gp66+ population between day 8 and day 18 is 22%, nearly eight-fold higher than background recombination, shown here.

Multiple mechanisms may have accounted for the accumulation of R antigen-specific cells following infection. These included greater proliferation within the R effector cell compartment than R-G and R-N cells, greater death within the R-N effector cell compartment than R and R-G cells, and/or migration of R-N effector cells to other tissues during the effector to memory transition. By determining the frequency of cycling effector cells and by comparing reporter status within effector populations from spleen and peritoneal cavity, we showed differential proliferation and migration between the effector reporter populations did not contribute to the observed preferential accumulation of R cells (Figure 3-S2).

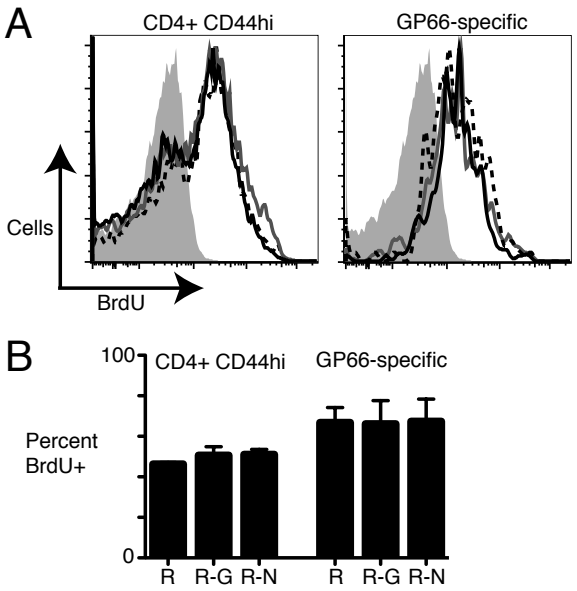

FIGURE 3-S2. Effector cells have similar frequencies of cycling cells. A. Histograms depicting BrdU uptake are shown in total CD4+ CD44^{hi} (left) and tetramer-reactive splenocytes from infected and BrdU injected Ox40-cre/3373 mice, as stated in materials and methods. Red-only = solid black line, Red-Green = dashed black line, reporter negative = dark gray line, no BrdU = filled histogram B. Quantitation of BrdU(+) events in (A) depicting average and SEM from 3 individual infected mice. Representative of two individual experiments.

Apoptosis of Effector T-cells

To determine whether differences in apoptosis within the CD4+ effector reporter populations contributed to the observed enrichment for R cells during the transition to memory, splenocytes from infected Ox40-cre/3373 mice were cultured *in vitro* for short periods of time and assayed for Annexin V reactivity, a marker for pre-apoptotic effector cells (Wang et al., 2004; Wang et al., 2003). *In vitro* culture resulted in a fraction of all CD4+ antigen-specific reporter populations binding Annexin V (Figure 3-3). Significant differences were observed between the three reporter populations. R and R-G effector cells showed similar frequencies of Annexin V reactive cells, but at significantly lower frequencies than R-N cells(Figure 3-3 B). This was consistent with R effectors being partially resistant to apoptosis during the contraction phase of the response. The increases in Annexin V reactivity within the effector populations could not be inhibited by culturing in IL-7-containing medium (Figure 3-3 B), although surface expression levels of IL-7 receptor (CD127) on the three reporter populations were equally elevated compared to naive CD4+ T-cells (data not shown). All three reporter populations were able to respond to this essential survival cytokine, as assayed by phosphorylation of STAT5 (Figure 3-3C). These data were consistent with the R-N CD4+ effector T-cell population being enriched for cells more likely to undergo apoptosis during the transition to memory. The inability to inhibit apoptosis within these populations with exogenous IL-7 suggested cell death was programmed, and increased levels of cell death correlated with low TCR signal strength earlier in the response. Thus, these data

are consistent with the notion that CD4+ memory cell fate was programmed early during an acute infection as a result of strong TCR signal strength.

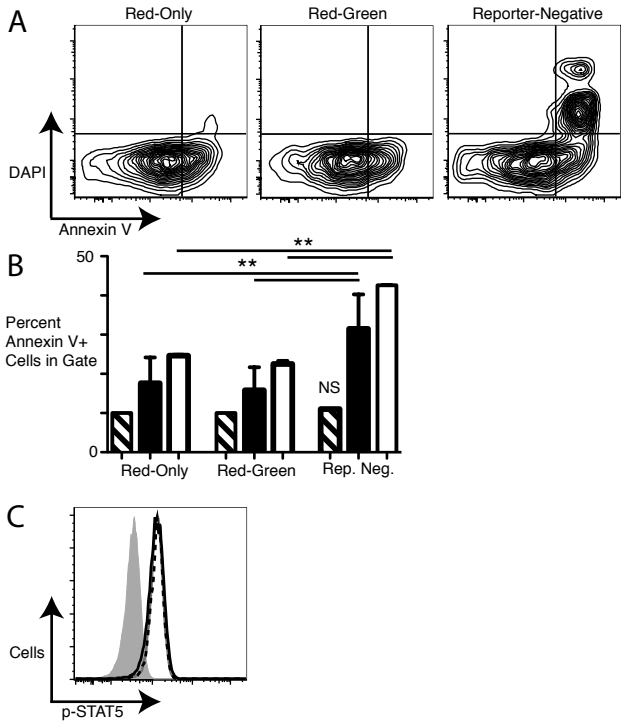

FIGURE 3-3. **Weak TCR signal strength programs apoptosis.** A. Splenocytes from Ox40-cre/3373 mice infected 13 days prior were cultured *in vitro* and analyzed for Annexin V reactivity and DAPI inclusion. Events shown gated on I-A(b)-gp66+ CD4+ CD44^{hi} CD25(-) cells. B. Quantitation of Annexin V(+) events in (A) stained directly ex vivo (cross-hatched), or following culture with(empty bars) and without (filled bars) exogenous IL-7. Graphs display means and SEM of 4 individual infected mice. NS = Not significant. ** P<0.01 by repeated measures ANOVA with Tukey post-test for significance. C. Intracellular phospho-STAT5 expression levels are shown from *in vitro* cultured and IL-7-stimulated antigen-specific CD4+ effector cells from infected mice (see materials and methods). Solid Black Line= Red-Only, Dashed Black Line= Red-Green, Solid Gray Line= Reporter Negative, naive CD4+ = filled histogram.

Effector Cell Phenotype and Function

Some studies have suggested expression of certain surface markers, like elevated expression of CD43, informed on resistance to cell death and memory cell fate in CD4+ T-cell populations (He and Bevan, 1999). In some systems, IFN-gamma secreting effector cells effectively contributed to the memory cell population (Harrington et al., 2008). In other systems this was not observed (Wu et al., 2002). To determine whether surface expression of activation markers within the effector population correlated with memory cell potential (i.e. R-marked effector cells), CD4+ effector T-cells were identified in single cell suspensions from spleen and peritoneal exudate eight days following infection by their high expression of CD44 and reactivity towards gp66-I-A^b, and characterized by surface marker expression. Splenic and peritoneal tetramer-reactive populations showed similar composition of R, R-G, and R-N cells (Figure 3-4A). Cell surface analysis of splenic populations showed that all three reporter populations shared similar elevated expression of CD44 and CD43 and low expression of OX40 and CD25 (Figure 3-4 B). Thus, phenotypically, the three reporter effector T-cells population appeared similar.

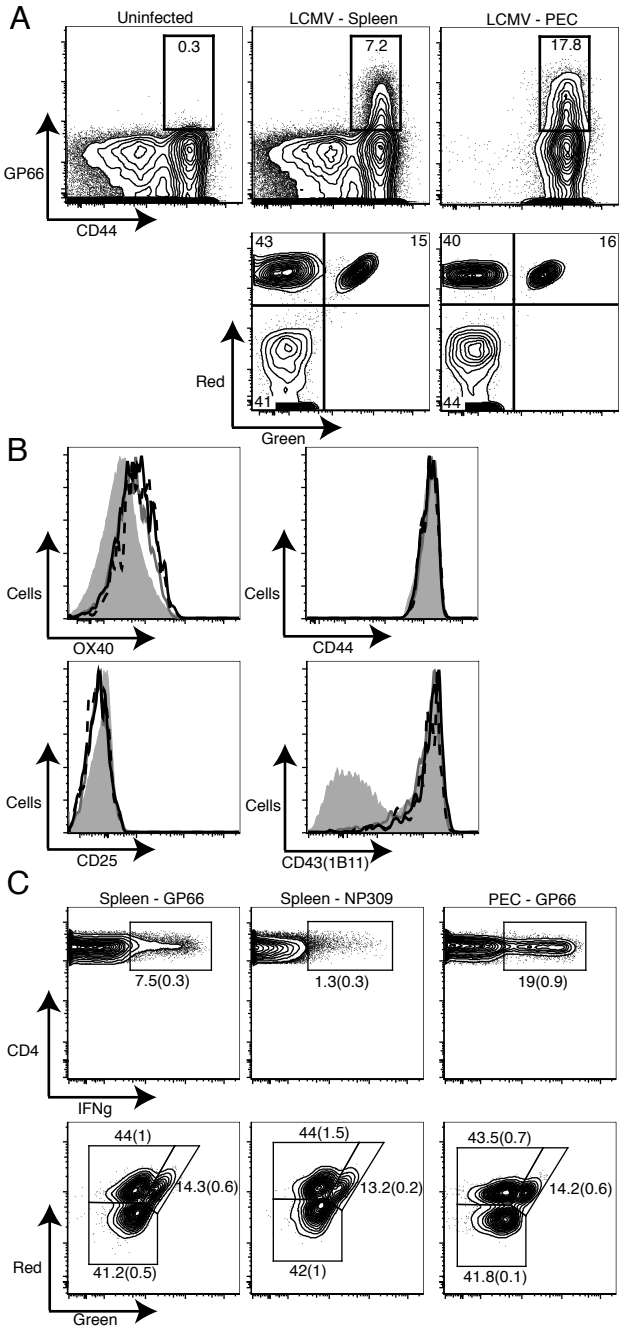

FIGURE 3-4: Endogenous antigen-specific CD4+ effector T-cells marked by the Ox40-cre/3373 reporter are phenotypically and functionally similar at the peak of the anti-LCMV response. A. (Top) Contour plots (5% probability) displaying CD44 expression and GP66-tetramer reactivity of splenocytes (center) and peritoneal exudate cells (PEC, right) from uninfected (left) and day 8 LCMV-Arm infected (center, right) Ox40-cre/3373 mice. Events shown are gated on total live CD4(+) CD25(-) cells. Numerical values represent the percent of tetramer-reactive cells within the region of total live CD4(+) CD25(-) $CD44^{hi}$ events. (Bottom) Ox40-cre/3373 reporter composition of tetramer-reactive

events are shown. Numerical values indicate the percent of total tetramer-reactive events within each reporter quadrant. Data is representative of a single infected mouse from at least 5 experiments with 2-6 mice per experiment. B. Histograms depict Red-only (solid black), Red-Green (dashed black), and reporter negative (solid gray line) cell surface expression analysis of indicated activation markers on tetramer-reactive splenocytes from day 8 infected Ox40-cre/3373 mice gated as in (A). Filled histograms show total CD4+ $CD44^{hi}$ populations for comparison. C. (Top) Intracellular expression of IFNg by CD4+ T-cells from indicated tissues as in (A) following *in vitro* peptide stimulation. Contour plots are gated on CD4+ CD25(-) $CD44^{hi}$ cells. Numerical values indicate the average(SEM) percent of cytokine expressing events gated as in (A). (Bottom) Cytokine-expressing populations, gated above, are further analyzed for Ox40-cre/3373 composition. Numerical values indicate average(SEM) percent of gated cells within each region shown. Data is from one of at least three experiments with 2-4 mice per experiment.

LCMV-Armstrong infection generated a robust Th1-type response in CD4+ T-cells, characterized by IFN-gamma cytokine expression. To determine if IFN-gamma secretion by effector cell populations correlated with memory cell fate, intracellular cytokine expression was assayed within splenic and peritoneal cavity CD4+ effector T-cell populations following 6 hours of *in vitro* peptide stimulations. Incubation with the dominant CD4+ restricted peptide epitope gp66-80, and subdominant peptide NP309 (spleen only), resulted in detection of IFN-gamma-expressing cells within all three reporter populations from both tissues. The frequency of cytokine expressing cells in each reporter population was similar to the frequency of tetramer-reactive cells within each reporter population (Figure 3-4 C). Multicytokine expressing cells (IFN-γ, TNF-α, IL-2) were also found at similar frequencies within all three populations (data not shown). These data revealed no correlation between CD4+ memory cell fate (i.e. R effector cells) with effector function as detected by ex vivo IFN-gamma secretion.

Monoclonal T-cell Studies

TCR affinities found within the population of antigen-specific responding clones may be a primary contributor to TCR signal strength and memory cell fate (Malherbe et al., 2004; Savage et al., 1999). High TCR affinities for antigenic peptide likely would produce strong TCR signal strength and therefore increased memory potential. To investigate the role of non-TCR affinity-based mechanisms in influencing TCR signal strength and effector cell fate, we fixed the TCR within a population of LCMV- specific CD4+ T-cells. Ox40-cre/3373/Smarta TCR

expressing cells (CD45.1+) were adoptively transferred in small numbers into congenically marked host mice (CD45.2+), infected, and analyzed. In this setting, all responding transferred cells had equal affinity for antigen.

Ox40-cre/3373 Smarta T-cells generated effector responses that largely resembled those made by endogenous CD4+ T-cells. They expanded to a similar extent as endogenous responses and were found at similar frequencies in the spleen and peritoneal cavity (Figure 3-5A). The proportion of R and R-G Smarta effector cells generated within the total donor population were lower compared to normal polyclonal responses (Figure 3-5B vs Figure 3-4 A), however all transgenic T-cells expressed equally high levels of CD44 (Figure 3-5 C) at the peak of the response, consistent with complete activation and effector cell differentiation. Longitudinal analysis of infected hosts showed similar enrichment for R effector cells, and to a lesser degree R-G cells, during the transition to memory between days 8 and 28 following infection (Figure 3-4 D). These data were consistent with strong TCR signal strength programming CD4+ effector cells with memory cell fate, and weak signals programming cell death. Furthermore, differences in TCR-pMHC affinities within the population of responding CD4+ clones did not exclusively program these fates *in vivo*.

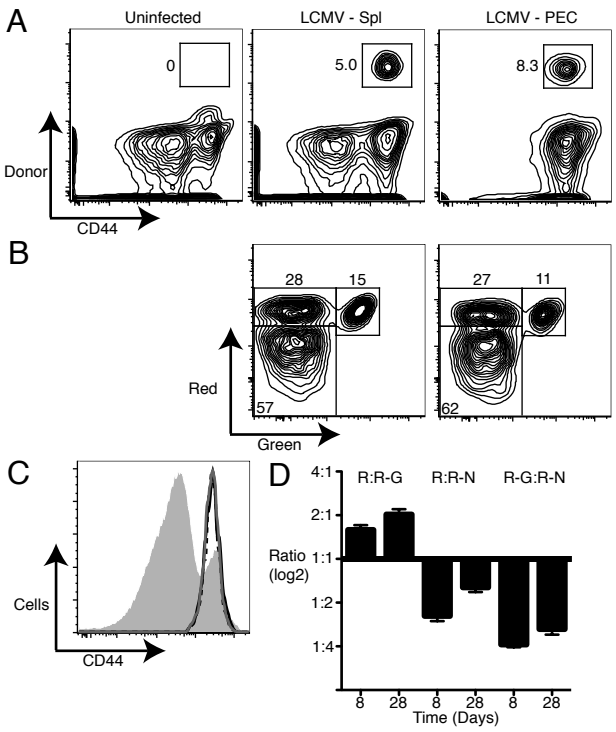

FIGURE 3-5. Differences in TCR affinity do not exclusively program memory cell fate. A. Smarta Ox40-cre/3373 T-cells expand and are found in spleen and peritoneal cavity at increased frquency compared to uninfected control (left). Events gated on total CD4+ events, with values indicating percent within gate. B. Donor Smarta cells with drawn region in (A) are analyzed for Ox40-cre/3373 reporter phenotype. Precent of total donor Smarta cells within gate is displayed numerically. C. Levels of CD44 surface expression is shown from spleen cells in (B). Red-only = solid black line, Red-Green = dashed black line, Reporter Negative = dark gray line, total CD4+ T-cells = filled histogram. D. Indicated ratios of the frequency of Red-only (R), Red-Green (R-G), and Reporter Negative (R-N) found with donor Smarta populations from peripheral blood collected following infection at indicated times. Events shown are gated as in (B). Average ratios are displayed in graphs with SEM from cohorts of 6-10 mice.

Discussion

In this report we present an approach that allows for three separate populations of CD4+ T-cells to be discriminated on the basis of strength of TCR stimulation. Our approach employed a genetic method for marking these separate populations allowing for long term studies of cellular fate *in vivo*. Using this approach we were able to gain insight into the fate of effector CD4+ T-cells generated following relatively strong and weak TCR signals during the transition to memory following acute viral infection. Specifically, we found that effector CD4+ T-cell generated as a result of strong TCR stimulation were programmed with a survival advantage that becomes apparent during contraction of the response. These findings were consistent with a deterministic model of CD4+ memory T-cell generation where effector T-cells consisted of mixtures of effector cells with separate fates. Additionally, the separate fates of these effector cells appeared to have been determined earlier during the response and programmed by the relative strength of TCR stimulation. Thus, like CD8+ T-cell responses, CD4+ responses appear to share properties consistent with a programmed model of memory T-cell generation.

The lineage relationship between CD4+ effector and memory T-cells has been under investigation for some time. Some studies suggested the effector to memory transition is a stochastic event. In this setting, all effector cells had equal potential to become memory cells and were randomly chosen to contribute to the memory cell populations (Bousso et al., 1998; Bousso and Kourilsky, 1999; Busch et al., 1998a; Freitas and Rocha, 2000). Our data, along with data from

others, are consistent with a model in which naive T-cells are programmed early during their activation to progress as a separate memory cell lineage, and the strength of TCR stimulation influences this programming event (Kaech and Wherry, 2007; Williams et al., 2008). Interestingly, data from CD8+ cells suggests strong TCR signals lead to terminal differentiation of effector cells with less memory potential than CD8+ effector cells generated as a result of weak TCR signals (Kaech and Wherry, 2007). Here, we propose the opposite for CD4+ T-cells, in that strong TCR signals program memory potential. Why is this the case? It may simply be another disparity between the two lineages, along with their requirements for full activation and expansion (Gett et al., 2003; Iezzi et al., 1998; Kaech and Ahmed, 2001; Mercado et al., 2000; Obst et al., 2005; van Stipdonk et al., 2001). It also leaves open the possibility that graded amounts of TCR signals during activation and expansion may lead to other cellular fates within the CD4+ lineage, aside from long-term memory, as has been suggested (Fazilleau et al., 2009b).

Our studies with antigen-specific responses following acute viral infection showed R effector cells were enriched for CD4+ memory precursors as a result of early and strong TCR-pMHC interactions. Studies investigating transcriptional differences between Ox40-cre/3373 reporter populations generated early during infection may be useful in uncovering unique CD4+ pro-memory genetic programs. We predict such transcriptional programs likely had been initiated by a subpopulation of cells found within the R effector CD4+ T-cells and are worthy of investigation. In these proposed experiments, the use of the Ox40-cre/3373

reporter will be important, as we have shown CD4+ effector populations had various fates not readily apparent by surface marker expression or by effector cytokine secretion.

A significant fraction of T-cells activated *in vitro* expressed OX40 but did not undergo Cre-mediated recombination. We believe this was due to insufficient expression of Cre-recombinase, as a result of lower amounts of TCR signalling, and not due to an intrinsic inability of these T-cells to have recombined the reporter allele. Our reasons for this were twofold. First, R-N cells generated following peptide stimulation consistently contained Cre-recombinase message. The level of *cre* expression was significantly greater than unstimulated transgenic cells but twofold lower than *cre* expression levels seen in R and R-G cells (data not shown). These data showed that R-N cells express *Ox40-cre*, but evidently at an insufficient level to undergo recombination of the reporter allele. R and R-G contained about twice as much *cre* message as R-N cells, showing that reporter allele recombination was sensitive to relatively small differences in Cre expression, and that the threshold for Cre recombination is between the expression levels in R-N and R/R-G cells.

Second, sorted R-N cells having undergone one round of stimulation were capable of recombining the reporter allele following a second stimulation (data not shown). These data, and the observation that low amounts of agonist peptide favored R-N generation while high amounts favored R cell generation (Figure 3-1A), support the notion that R-N cells were created as a result of weak

TCR stimulation (and low Ox40-cre expression) and not as a result of intrinsic deficiencies.

Apoptosis played a major role in regulating the contraction phase following LCMV infection and shaping the memory cell pool (Hildeman et al., 2002; Wang et al., 2004; Wang et al., 2003; Williams et al., 2008). Pre-apoptotic effector lymphocytes, identified by Annexin V binding, did not contribute to the CD8+ memory populations following LCMV infection (Wang et al., 2004; Wang et al., 2003). Programmed cell death following infection had been determined very early during the expansion phase of CD8+ responses (Badovinac et al., 2002). The results presented here suggest that cell death may also be programmed early during CD4+ responses *in vivo*, as a result of relatively weak TCR signal strength.

The preferential accumulation of R effector cells during contraction of the response may be attributed to a greater number of R-N cells undergoing cell death than R cells during the contraction phase, and likely not to differential migration or cell cycling. An increased frequency of pre-apoptotic cells was observed in R-N effector populations following short-term *in vitro* culture compared to R and R-G cells, and in this assay the pre-apoptotic state could not be rescued by the addition of the essential cytokine IL-7 (Kondrack et al., 2003; Li et al., 2003). All reporter populations had equal surface expression of the IL-7-receptor and were capable of signalling following IL-7 stimulation. These data are consistent with TCR signal strength influencing CD4+ effector cell fate by programming survival (with strong signals) or cell death (with weak signals) early

during the response, and supported similar conclusions found in studies that correlated low functional avidity within the effector CD4+ T-cell population with cell death during contraction of an anti-bacterial response (Williams et al., 2008).

Kinetic analyses comparing the MP and antigen-specific CD4+ responses showed the MP CD4+ compartment gradually accumulated R MP CD4+ cells throughout the length of the experiment. The observation that the CD4+ MP compartment underwent constant enrichment for R CD4+ cells during the life of the host was consistent with the notion that R CD4+ effector cells have greater memory potential than R-G and R-N MP CD4+ cells . In general, the CD4+ MP population in mice is a mixture of lymphocytes generated by antigen-dependent and -independent mechanisms as a result of encounter with diverse (unknown) antigens (MacLeod et al., 2009b). Over the life of a host, MP CD4+ T-cells increase in number (Sprent, 1994), and therefore R marked MP CD4+ T-cells (i.e. those generated following strong TCR signals and with greater memory-cell potential than those generated from weak TCR signals) would be predicted to increase in number. We acknowledge, however, that the lack of information regarding antigen-reactivity in the MP compartment necessitates caution with these interpretations that R MP CD4+ accumulated within mice due to greater memory potential than R-N and R-G cells. Chronic antigens and reactivation of MP cells by environmental antigens may drive recombinase expression and reporter recombination leading to similar results. Additional studies mapping the fates of T cells in alternative antigen-specific responses, e.g., to subdominant epitopes, or in other *in vivo* infection model systems, will be insightful.

In some experiments we infected mice that had been engrafted with Ox40-cre/3373 Smarta cells. Because of the Smarta TCR, we generated a population of effector cells with uniform affinity for antigen that should undergo TCR-dependent reporter allele recombination in a common fashion. The fates of the Smarta effector cells were tracked during contraction of the anti-LCMV response. Strikingly, we observed accumulation of R, and to a lesser extent R-G, marked CD4+ effector cells with similar kinetics to those observed in the endogenous responses. These findings showed that TCR-affinity differences within a responding population did not solely control the TCR signal strength differences we observed in Ox40-cre/3373 mice. Other non-affinity based mechanisms contributed to the TCR signal strength and early programming of memory potential in effector cells. Other possible mechanisms include competition for antigen on APCs (Blair and Lefrançois, 2007; Kedl et al., 2000; Whitmire et al., 2008a; Whitmire et al., 2006), timing of interaction with APCs (Catron et al., 2006; Itano et al., 2003), and interactions with suboptimally activated APCs (Zammit et al., 2005), all of which have been shown to be involved in CD4+ memory generation. Additional studies will be necessary to determine which if any of these mechanisms contribute to memory fate determination by modulating TCR signal strength.

In summary, CD4+ effector T-cells indelibly marked following activation on the basis of strength of TCR stimulation have disparate fates following acute infection. Effector CD4+ T-Cells generated as a result of strong TCR stimulation accumulate within the memory T-cell population due to preferential survival

during the contraction of the response. This pro-memory fate is programmed early during the response and independent of effector cell function implying that the manipulation of CD4+ stimulation strength with adjuvants and by other means may have long-lasting effects on the generation of a stable CD4+ memory cell population during immunization.

Chapter 4

Model of CD4+ T-cell Differentiation Based on TCR Signal Strength

Abstract

A simple model of CD4+ T-cell differentiation based upon TCR signal strength is proposed. The significance and implications of our findings are discussed.

Many studies have implicated TCR signal strength in shaping an immune response (Badovinac et al., 2007; Blair and Lefrançois, 2007; Busch and Pamer, 1999a; Foulds and Shen, 2006; Gett et al., 2003; Malherbe et al., 2004; McHeyzer-Williams and Davis, 1995; Mercado et al., 2000; Obst et al., 2005; Savage et al., 1999; Whitmire et al., 2006; Williams et al., 2008; Zehn et al., 2009). Few studies, however, have looked at long-term effects of initial TCR signal strength, in particular within the CD4+ lineage, on subsequent fate during the memory phase.

One study that has investigated these issues correlated effector CD4+ T-cell functional avidity with subsequent fate and found relatively low functional avidity in effector cells expanded by bacteria infection. These low avidity effector cells were destined for apoptosis during the transition to memory. Surprisingly, the same T-cell clones generated following viral infection showed relatively high functional avidity and survived during contraction. Thus, a good correlation existed between effector cell functional avidity and fate, and suggested TCR-pMHC interactions perhaps played a role in CD4+ effector cell fate *in vivo*. These studies lacked evidence to discern whether increased functional avidity within the effector cells led to effector cell fate. Memory T-cell populations may have resulted from earlier (TCR related or non-TCR related) events that determined both memory cell fate and maturation of functional avidity in parallel. Thus, these *in vivo* findings suggested TCR-strength may play a role in CD4+ effector fate during an immune response, but additional approaches were necessary to support this idea.

The work presented in this dissertation provides such supporting evidence and advances our understanding of the role of strength of TCR-pMHC interactions in determining CD4+ T-cell fate *in vivo*. We took the approach of indelibly labeling populations of activated CD4+ T-cells on the basis of the strength of TCR simulation using a novel reporter allele, and then followed these cells as they underwent normal responses to an acute viral infection. We found that the relative strength of stimulation did impact the fate of effector cells. Specifically, those effector CD4+ T-cells generated as a result of strong TCR interactions (and marked by the Red-only reporter state) preferentially survived the contraction phase of the response. Conversely, those effector CD4+ T-cells generated as result of weak TCR interactions preferentially died by apoptosis during this transition to memory. Thus the strength of TCR stimulation experienced by CD4+ T-cells prior to the peak of the response determined subsequent fate during contraction and shaped the resulting memory cell populations. These findings from our work are significant and have important implications.

The ability of Red-only marked effector cells to accumulate within the memory population suggests that Red-only effectors are enriched for CD4+ memory-like precursors. Within the CD8+ lineage, such memory precursors have been phenotypically identified by elevated expression of IL-7 receptor during the peak of the response (Kaech et al., 2003). Similar cells have not been identified within the CD4+ lineage (Lees and Farber, 2010b). Therefore, our findings that the Red-only CD4+ effector cells exhibit greater memory potential

than R-G and R-N cells are significant. As of yet, we have been unable to further discriminate, by surface marker expression or functional assays (Figure 3-4C), those cells with memory-like potential from other cells found at the peak of the response. However Red-only effector cells can readily be isolated and enriched for in number following cell sorting. Enrichment of these cells and in depth analyses of broad gene transcriptional profiles may help in identifying biologically relevant surface markers as well as other transcriptional markers within the predicted rare subset of memory precursors in these reporter marked effectors. Such discoveries have the potential to advance our understanding of CD4+ differentiation and memory cell generation.

The presence of Red-only cells during the effector phase that preferentially give rise to memory cells is consistent with a programmed model of CD4+ differentiation where memory cells are a separate lineage and memory lineage determination occurs early during an immune response. The approach we purposefully took of marking cells following transient TCR activation (as a result of acute infection and Ox40-cre expression) allows us to estimate the timing of events that determine fate. Because reporter states do not change after the marking event (transient Ox40-cre expression), all subsequent effects observed must be due to deterministic events that take place prior to, or at the same time of, Ox40-cre expression. Our approach utilizing transient expression of *Ox40-cr*e during activation on CD4+ T-cells and our findings that OX40 expression and Ox40-cre recombinase activity are very low during and after the peak of the response supports the notion that these fate determining events

occurred prior to the peak of expansion. It remains unclear whether memory and non-memory CD4+ lineages are plastic in nature, and whether early programmed CD4+ T-cells may readily transition between these two lineages. Also at what point is fate secured and what signals allow for this to occur? Is the CD4+ memory lineage a slow and gradual process, as has been observed with the CD8+ lineage (Kaech and Ahmed, 2001)? These questions will need to be answered with additional studies, however, we can say with confidence that TCR signal strength played a role in setting into motion separate fates within the responding CD4+ T-cells prior to the peak of expansion. Studies investigating these early determining signals will be informative with regards to the nature and mechanism of TCR-related fate determination *in vivo*.

We propose the following simple model that is consistent with our data, as well as others, describing the influences of TCR affinity and signal strength on effector and memory cell differentiation. In this model, TCR affinity for pMHC recruits cells into the response (Malherbe et al., 2004). Those cells with relatively low affinity either do not expand or do not expand fully. By mechanisms not entirely clear, and not completely dependent upon TCR affinity, strong TCR interactions lead to programming of the memory cell fate as we have proposed, and this can occur within a clonal population as we have shown. During contraction, those effector cells generated following strong TCR signals preferentially survive, and become memory CD4+ T-cells.

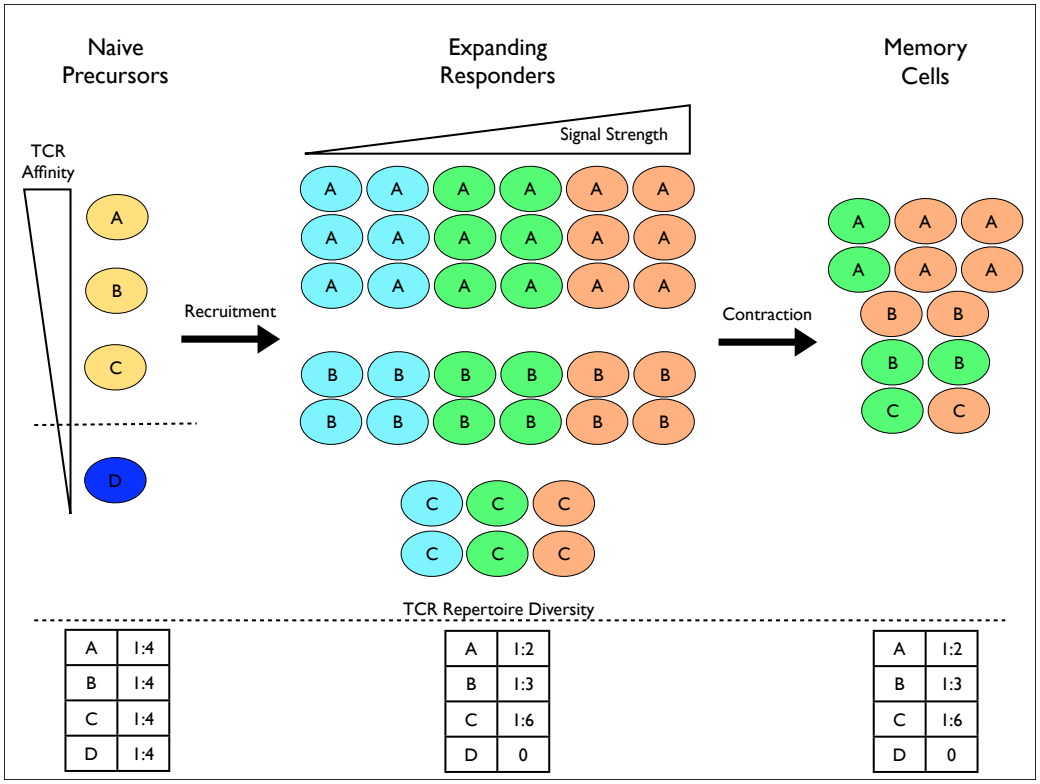

Figure 4-1. Model: TCR Signal Strength Influences CD4+ Fate. Naive precursor cells (A-D) with varying degrees of TCR affinity for antigen are recruited into a response, accordingly. During stimulation with pMHC complexes each clone recruited into the response(A-C) expands in number and receives additional TCR based stimulation. The strength of this stimulation programs memory cell potential during the contraction period. As shown, TCR receptor diversity may be relatively equal between effector and memory phases as a result of TCR signal strength fate determination.

From this model we can make predictions about TCR repertoire diversity. One prediction is that TCR diversity within effector and memory compartments may by quite similar. Although we do not directly look at TCR diversity in our studies, findings consistent with this prediction have been reported (Bousso and Kourilsky, 1999; Malherbe et al., 2004). This model also predicts increasing the precursor frequency of clonal populations may have detrimental effects on memory generation. Data in support of this also have been shown (Blair and Lefrançois, 2007).

The dependence on strong TCR signalling in determining CD4+ memory cell potential, as depicted in this model, is different from observations with CD8+ T-cells. Within the CD8+ lineage it has been proposed that strong signals lead to terminal differentiation of effector cells, whereas weak signals lead to memory cell fate (Kaech and Wherry, 2007). Although studies have suggested CD8+ T-cells can be fully activated and differentiated following short periods of antigen stimulation (Kaech and Ahmed, 2001; Mercado et al., 2000; van Stipdonk et al., 2001), recent studies comparing clonal populations of effector CD8+ T-cells that had been generated following low and high affinity ligands suggest TCR signal strength may impact the developing CD8+ response in other ways. The genetic fate mapping approach we took in our studies may be useful in similar studies of the CD8+ lineage. Unfortunately, *Ox40/Ox40-cre* is not an ideal allele for such an approach because expression of recombinase is low in the CD8+ lineage following certain viral and bacterial infections, and only a small proportion of activated CD8+ T-cells recombine reporter alleles (unpublished data). Other Cre-

expressing alleles responsive to TCR signal strength may be generated with greater potential use in CD8+ T-cells than *Ox40-cre* (see Appendix).

This disparity in signal strength and memory cell determination between the CD4+ and CD8+ lineage may be one of many intrinsic differences, including activation (Gett et al., 2003; van Stipdonk et al., 2001), and expansion (Kaech and Ahmed, 2001; Obst et al., 2005) shown to exist between CD4+ and CD8+ cell lineages. This finding has implications when considering the design of vaccines and immunization strategies targeting both adaptive arms of T-cell immunity. With the goal of generating long-lived immunity in mind, the TCR signals that may be beneficial for CD4+ memory differentiation may inadvertently hinder effective CD8+ memory differentiation, and vice versa.

Appendix

Generation and Characterization of *CD69-cre*/3373 Mice

Abstract

Cd69-cre mice were generated and intercrossed with 3373 3SCS Cre-reporter mice, generated in chapter 2, to determine whether *Cd69/Cd69-cre* expression informed on antigen receptor stimulation strength in lymphocytes *in vivo*. CD4+ and CD8+ single-positive thymocytes, and CD4+ and CD8+ lymph node cells from unmanipulated *Cd69-cre/3373* mice recombined reporter. Double-negative thymocytes were largely negative for reporter expression consistent with CD69-cre expression and recombination occurring during thymocyte selection. Sorted reporter negative lymph node cells from Cd69-cre/3373 mice recombined reporter following in vitro activation, and reporter status correlated well with strength of antigen-receptor stimulation. These results indicate *Cd69-cre* mice may have limited use in marking both CD4+ and CD8+ T-cells *in vivo* on the basis of TCR signal strength as a result of CD69-cre recombinase expression during thymocyte development.

Introduction

The strength of antigen receptor stimulation during lymphocyte development and peripheral activation has been implicated in determining the fate of responding cells in vivo (Cariappa and Pillai, 2002; Haks et al., 2005; Hayes et al., 2005; Hayes and Love, 2006; Jorritsma et al., 2003; Lam and Rajewsky, 1999; Malherbe et al., 2000; Singer et al., 2008; Tao et al., 1997). We have previously shown that *Ox40-cre* mice intercrossed to mice harboring Cre-reporter alleles may be used in lineage mapping approaches to determine the fate of lymphocytes activated following strong selection ligands during thymocyte development (Klinger et al., 2009) and strong versus weak TCR stimulation following infection (Klinger et al., 2010)(Results presented in Chapter 3 of this dissertation).

Ox40-cre mice are of limited use in CD8+ T-cell studies due to low expression of Cre-recombinase in CD8+ T-cells following in vitro and viral stimulation (Klinger et al., 2010)(unpubished data M. Klinger and N. Killeen). Surprisingly, only 15-20% of all CD8+ T-cells specific for the dominant LCMV-derived epitope, gp33, express Ox40-cre at sufficient levels to recombine Cre-reporter alleles, although these effector cell populations underwent massive expansion and differentiation which resulted in long-lived memory cell populations (Murali-Krishna et al., 1998)(M. Klinger and N. Killeen, unpublished data). Other Cre-recombinase expressing alleles that respond to TCR-stimulation transiently and proportionally to strength during T-cell activation in the

CD8+ lineage may be informative in determining the role of TCR signal strength and subsequent fate in vivo.

The *Cd69* locus is one such allele that is transiently expressed in CD4+ and CD8+ T-cells following antigen stimulation (Testi et al., 1994). *Cd69-cre* expressing mice were generated using a modified BAC transgene containing a CFP-hCre fusion protein targeted to the endogenous *Cd69* locus. *Cd69-cre* mice were intercrossed to Cre-reporter expressing mice to analyze Cd69-cre expression in vivo. We found CD4+ and CD8+ peripheral T-cell populations recombined Cre reporter alleles following antigen receptor stimulation, and the reporter state correlated well with the relative amount of antigen receptor stimulation. Thus, CD4+ and CD8+ T-cell populations generated following strong or weak stimulation could be separately identified and resolved using Cd69-cre/3373 reporter expression. However, previous expression of recombinase in *Cd69-cre* mice, likely during selection in the thymus, limits their use in determining the impact of TCR signal strength on lymphocyte cellular fate during an immune response.

Materials and Methods

A DNA fragment containing an IRES-CFP-hCre fragment was inserted into a *Cd69* locus-containing BAC (Wie and Killeen, unpublished data). The BAC containing the newly generated *CD69-cre* locus was used for transgenic expression of Cd69-cre in mice (UCSF Transgenic Core). Founders were intercrossed to Cre-reporter mice to identify Cre-expressing founder lines

(Klinger et al., 2010). One out of three founder lines, designated 965, showed inducible expression of Cre-recombinase following antigen receptor stimulation and PMA/Ionomycin stimulation. The 965 CD69-cre strain was intercrossed to 3373 3SCS reporter mice to generate all experimental mice (CD69-cre/3373).

Results

Single cell suspensions of lymph nodes and thymocytes were analyzed to determine whether cell populations experienced prior Cre-recombinase expression (as a result of CD69-cre expression). CD69-cre expression would result in recombination of the indicator allele generating tdTomato end eGFP expressing cells. Thymocytes were discriminated by CD4 and CD8 expression (Figure A-1A) and subsequently analyzed for reporter recombination. Both CD4+ and CD8+ single positive (SP) thymocyte populations exhibited signs of previous recombinase expression, with 30-45% of all cells recombining reporter resulting in tdTomato expression. Double-positive thymocytes showed less than 1% of these cells had recombined reporter, suggesting that recombinase activity during the late DP stage likely was of sufficient amounts to recombine reporter. This is consistent with the expression of CD69 following selection events in thymocyte development (Testi et al., 1994). Analyses of populations of CD4+ and CD8+ T-cells from lymph nodes showed that a greater portion of cells, compared to CD4-SP and CD8-SP thymocytes, had recombined reporter, with 85% of CD4+, and 60-70% of CD8+ T-cells expressing tdTomato (Figure A-1B).

Previous expression of CD69-cre recombinase in both T-cell lineages (as well as some B-cells and NK cells, data not shown) precluded the use of *Cd69-cre* mice in fate mapping studies of TCR signal strength in lymphocytes during immune responses. We performed one final experiment using sorted Cd69-cre/3373 reporter negative cell populations to ask whether TCR receptor stimulation could be correlated with Cd69-cre/3373 reporter status following receptor stimulation. We found that both CD4+ and CD8+ T-cells recombined reporter, and that the CD4+ and CD8+ lineages generated greater numbers of R cells following relatively high amounts of stimulation than low amounts. Furthermore the ratio of the reporter states all increased with increasing amounts of stimulation indicating the Cd69-cre/3373 reporter has the potential to be used in TCR signal strength studies in some systems.

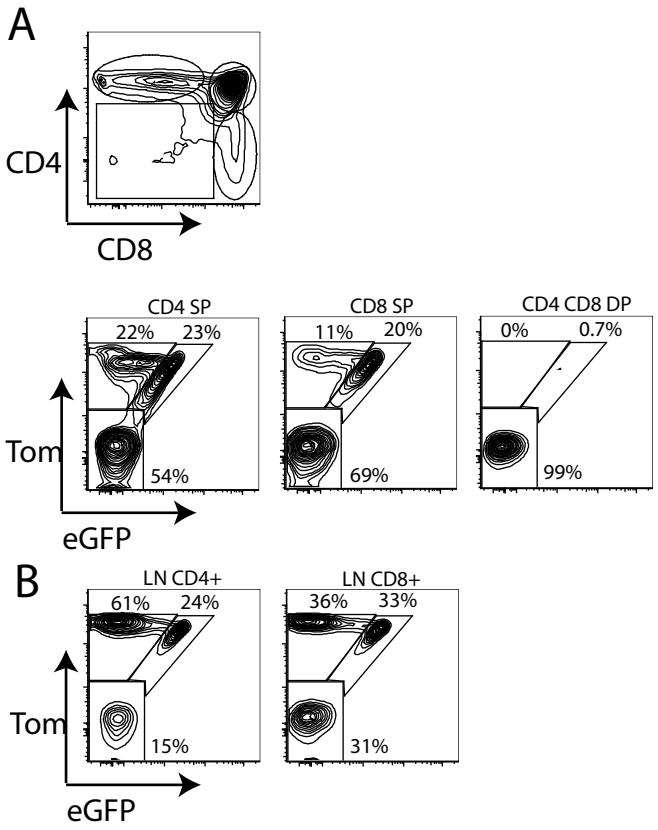

Figure A-1. Thymocytes and T-cells from Cd69-cre/3373 mice show prior expression of CD69-cre. Thymocytes (A) and lymph node cells (B) were analyzed for recombination of the 3373 3SCS reporter.

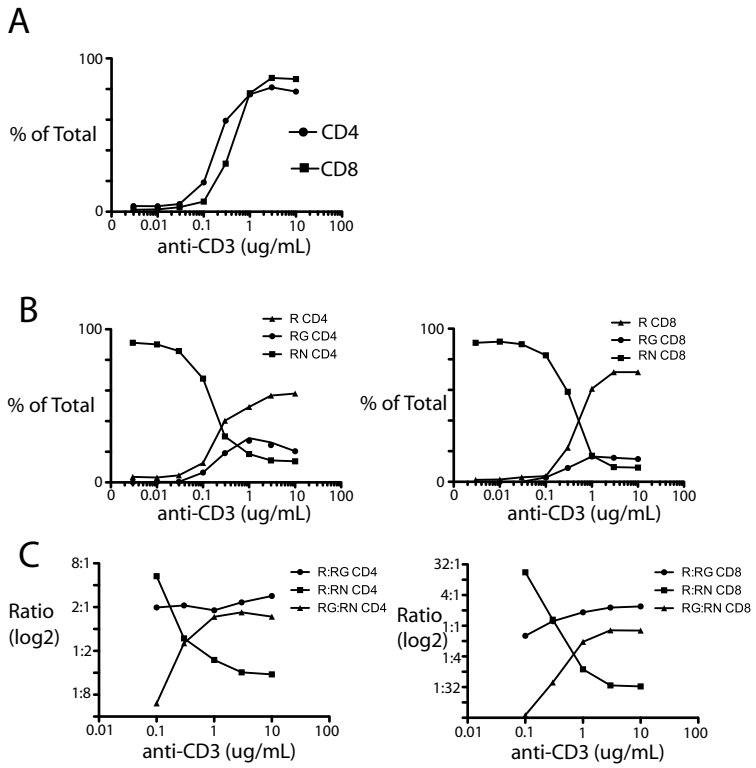

Figure A-2. TCR Signal Strength Correlates with CD69-cre/3373 Reporter Phenotype. Reporter negative cells from Cd69-cre/3373 mice were stimulated with anti-CD3 for three days. (A) Recombination of reporter allele is observed in both CD4+ and CD8+ lineages to a similar degree. (B) Increased stimulation leads to favored generation of Red-only cells. (C) Reporter ratios (log2) are shown.

In summary, expression of recombinase early during T-cell development limits the use of *CD69-cre* mice in fate mapping studies *in vivo*. However, data generated by stimulating the minority of peripheral CD4+ and CD8+ T-cells that had not recombined reporter during development showed that the *Cd69* locus may be useful in studying these cell populations if recombinase expression is limited during development. Strong stimulation favored the generation of Red-only cells and weak stimulation favored the generation of Red-Green and reporter negative cells in both cell lineages. These results indicate that reporter states adopted by stimulated Cd69-cre/3373 T-cells correlate well with the strength of activation. Intercrossing Cd69-cre/3373 mice to weakly selecting transgenic T-cell receptor expressing mice may allow for the generation of naive T-cells with unrecombined Cd69-cre/3373 reporter for *in vivo* studies of TCR signal strength and cellular fate.

References

Alam, S.M., Travers, P.J., Wung, J.L., Nasholds, W., Redpath, S., Jameson, S.C., and Gascoigne, N.R. (1996). T-cell-receptor affinity and thymocyte positive selection. Nature *381*, 616-620.

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

Ansel, K.M., Djuretic, I., Tanasa, B., and Rao, A. (2006). Regulation of Th2 differentiation and Il4 locus accessibility. Annu Rev Immunol *24*, 607-656.

Ansel, K.M., Lee, D.U., and Rao, A. (2003). An epigenetic view of helper T cell differentiation. Nat Immunol *4*, 616-623.

Anthony, R.M., Urban, J.F., Jr., Alem, F., Hamed, H.A., Rozo, C.T., Boucher, J.L., Van Rooijen, N., and Gause, W.C. (2006). Memory T(H)2 cells induce alternatively activated macrophages to mediate protection against nematode parasites. Nat Med *12*, 955-960.

Arstila, T.P., Casrouge, A., Baron, V., Even, J., Kanellopoulos, J., and Kourilsky, P. (1999). A direct estimate of the human alphabeta T cell receptor diversity. Science *286*, 958-961.

Ashe, H.L., and Briscoe, J. (2006). The interpretation of morphogen gradients. Development *133*, 385-394.

Ashton-Rickardt, P.G., Bandeira, A., Delaney, J.R., Van Kaer, L., Pircher, H.P., Zinkernagel, R.M., and Tonegawa, S. (1994). Evidence for a differential avidity model of T cell selection in the thymus. Cell *76*, 651-663.

Badou, A., Savignac, M., Moreau, M., Leclerc, C., Foucras, G., Cassar, G., Paulet, P., Lagrange, D., Druet, P., Guery, J.C., and Pelletier, L. (2001). Weak TCR stimulation induces a calcium signal that triggers IL-4 synthesis, stronger TCR stimulation induces MAP kinases that control IFN-gamma production. Eur J Immunol *31*, 2487-2496.

Badovinac, V.P., Haring, J.S., and Harty, J.T. (2007). Initial T cell receptor transgenic cell precursor frequency dictates critical aspects of the CD8(+) T cell response to infection. Immunity *26*, 827-841.

Badovinac, V.P., Porter, B.B., and Harty, J.T. (2002). Programmed contraction of CD8(+) T cells after infection. Nat Immunol *3*, 619-626.

Bannard, O., Kraman, M., and Fearon, D.T. (2009). Secondary replicative function of CD8+ T cells that had developed an effector phenotype. Science *323*, 505-509.

Bevan, M.J., and Fink, P.J. (2001). The CD8 response on autopilot. Nat Immunol *2*, 381-382.

Blair, D.A., and Lefrançois, L. (2007). Increased competition for antigen during priming negatively impacts the generation of memory CD4 T cells. Proc Natl Acad Sci USA *104*, 15045-15050.

Blattman, J.N., Antia, R., Sourdive, D.J., Wang, X., Kaech, S.M., Murali-Krishna, K., Altman, J.D., and Ahmed, R. (2002). Estimating the precursor frequency of naive antigen-specific CD8 T cells. J Exp Med *195*, 657-664.

Blattman, J.N., Sourdive, D.J., Murali-Krishna, K., Ahmed, R., and Altman, J.D. (2000). Evolution of the T cell repertoire during primary, memory, and recall responses to viral infection. J Immunol *165*, 6081-6090.

Bluestone, J.A., Thomson, A.W., Shevach, E.M., and Weiner, H.L. (2007). What does the future hold for cell-based tolerogenic therapy? Nat Rev Immunol *7*, 650-654.

Bousso, P., Casrouge, A., Altman, J.D., Haury, M., Kanellopoulos, J., Abastado, J.P., and Kourilsky, P. (1998). Individual variations in the murine T cell response to a specific peptide reflect variability in naive repertoires. Immunity *9*, 169-178.

Bousso, P., and Kourilsky, P. (1999). A clonal view of alphabeta T cell responses. Semin Immunol *11*, 423-431.

Brack, C., Hirama, M., Lenhard-Schuller, R., and Tonegawa, S. (1978). A complete immunoglobulin gene is created by somatic recombination. Cell *15*, 1-14.

Branda, C.S., and Dymecki, S.M. (2004). Talking about a revolution: The impact of site-specific recombinases on genetic analyses in mice. Dev Cell *6*, 7-28.

Busch, D.H., and Pamer, E.G. (1999a). T cell affinity maturation by selective expansion during infection. J Exp Med *189*, 701-710.

Busch, D.H., and Pamer, E.G. (1999b). T lymphocyte dynamics during Listeria monocytogenes infection. Immunol Lett *65*, 93-98.

Busch, D.H., Pilip, I., and Pamer, E.G. (1998a). Evolution of a complex T cell receptor repertoire during primary and recall bacterial infection. J Exp Med *188*, 61-70.

Busch, D.H., Pilip, I.M., Vijh, S., and Pamer, E.G. (1998b). Coordinate regulation of complex T cell populations responding to bacterial infection. Immunity *8*, 353-362.

Butz, E.A., and Bevan, M.J. (1998). Massive expansion of antigen-specific CD8+ T cells during an acute virus infection. Immunity *8*, 167-175.

Campbell, C., Risueno, R.M., Salati, S., Guezguez, B., and Bhatia, M. (2008). Signal control of hematopoietic stem cell fate: Wnt, Notch, and Hedgehog as the usual suspects. Curr Opin Hematol *15*, 319-325.

Cariappa, A., and Pillai, S. (2002). Antigen-dependent B-cell development. Curr Opin Immunol *14*, 241-249.

Casanova, J.L., Romero, P., Widmann, C., Kourilsky, P., and Maryanski, J.L. (1991). T cell receptor genes in a series of class I major histocompatibility complex-restricted cytotoxic T lymphocyte clones specific for a Plasmodium berghei nonapeptide: implications for T cell allelic exclusion and antigen-specific repertoire. J Exp Med *174*, 1371-1383.

Catron, D.M., Rusch, L.K., Hataye, J., Itano, A.A., and Jenkins, M.K. (2006). CD4+ T cells that enter the draining lymph nodes after antigen injection participate in the primary response and become central-memory cells. J Exp Med *203*, 1045-1054.

Celli, S., Garcia, Z., and Bousso, P. (2005). CD4 T cells integrate signals delivered during successive DC encounters in vivo. J Exp Med *202*, 1271-1278.

Chang, J.T., Palanivel, V.R., Kinjyo, I., Schambach, F., Intlekofer, A.M., Banerjee, A., Longworth, S.A., Vinup, K.E., Mrass, P., Oliaro, J.*, et al.* (2007). Asymmetric T lymphocyte division in the initiation of adaptive immune responses. Science *315*, 1687-1691.

Croft, M., Bradley, L.M., and Swain, S.L. (1994). Naive versus memory CD4 T cell response to antigen. Memory cells are less dependent on accessory cell costimulation and can respond to many antigen-presenting cell types including resting B cells. J Immunol *152*, 2675-2685.

Crompton, T., Outram, S.V., and Hager-Theodorides, A.L. (2007). Sonic hedgehog signalling in T-cell development and activation. Nat Rev Immunol *7*, 726-735.

Dalton, D.K., Pitts-Meek, S., Keshav, S., Figari, I.S., Bradley, A., and Stewart, T.A. (1993). Multiple defects of immune cell function in mice with disrupted interferon-gamma genes. Science *259*, 1739-1742.

Daniels, M.A., Teixeiro, E., Gill, J., Hausmann, B., Roubaty, D., Holmberg, K., Werlen, G., Hollander, G.A., Gascoigne, N.R., and Palmer, E. (2006). Thymic selection threshold defined by compartmentalization of Ras/MAPK signalling. Nature *444*, 724-729.

Davis, M.M., and Bjorkman, P.J. (1988). T-cell antigen receptor genes and T-cell recognition. Nature *334*, 395-402.

Davis, M.M., Chien, Y.H., Gascoigne, N.R., and Hedrick, S.M. (1984). A murine T cell receptor gene complex: isolation, structure and rearrangement. Immunol Rev *81*, 235-258.

Dooms, H., and Abbas, A.K. (2006). Control of CD4+ T-cell memory by cytokines and costimulators. Immunol Rev *211*, 23-38.

Dooms, H., Kahn, E., Knoechel, B., and Abbas, A.K. (2004). IL-2 induces a competitive survival advantage in T lymphocytes. J Immunol *172*, 5973-5979.

Dooms, H., Wolslegel, K., Lin, P., and Abbas, A.K. (2007). Interleukin-2 enhances CD4+ T cell memory by promoting the generation of IL-7R alpha-expressing cells. J Exp Med *204*, 547-557.

Fazilleau, N., Mark, L., McHeyzer-Williams, L.J., and McHeyzer-Williams, M.G. (2009a). Follicular helper T cells: lineage and location. Immunity *30*, 324-335.

Fazilleau, N., McHeyzer-Williams, L.J., and McHeyzer-Williams, M.G. (2007). Local development of effector and memory T helper cells. Curr Opin Immunol *19*, 259-267.

Fazilleau, N., McHeyzer-Williams, L.J., Rosen, H., and McHeyzer-Williams, M.G. (2009b). The function of follicular helper T cells is regulated by the strength of T cell antigen receptor binding. Nat Immunol *10*, 375-384.

Flynn, J.L., Chan, J., Triebold, K.J., Dalton, D.K., Stewart, T.A., and Bloom, B.R. (1993). An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection. J Exp Med *178*, 2249-2254.

Foulds, K.E., and Shen, H. (2006). Clonal competition inhibits the proliferation and differentiation of adoptively transferred TCR transgenic CD4 T cells in response to infection. J Immunol *176*, 3037-3043.

Foulds, K.E., Zenewicz, L.A., Shedlock, D.J., Jiang, J., Troy, A.E., and Shen, H. (2002). Cutting edge: CD4 and CD8 T cells are intrinsically different in their proliferative responses. J Immunol *168*, 1528-1532.

Freitas, A.A., and Rocha, B. (2000). Population biology of lymphocytes: the flight for survival. Annu Rev Immunol *18*, 83-111.

Friedrich, G., and Soriano, P. (1991). Promoter traps in embryonic stem cells: a genetic screen to identify and mutate developmental genes in mice. Genes Dev *5*, 1513-1523.

Garcia, K.C., Degano, M., Pease, L.R., Huang, M., Peterson, P.A., Teyton, L., and Wilson, I.A. (1998). Structural basis of plasticity in T cell receptor recognition of a self peptide-MHC antigen. Science *279*, 1166-1172.

Gett, A.V., Sallusto, F., Lanzavecchia, A., and Geginat, J. (2003). T cell fitness determined by signal strength. Nat Immunol *4*, 355-360.

Gordon, S. (2003). Alternative activation of macrophages. Nat Rev Immunol *3*, 23-35.

Gramaglia, I., Jember, A., Pippig, S.D., Weinberg, A.D., Killeen, N., and Croft, M. (2000). The OX40 costimulatory receptor determines the development of CD4 memory by regulating primary clonal expansion. J Immunol *165*, 3043-3050.

Gramaglia, I., Weinberg, A.D., Lemon, M., and Croft, M. (1998). Ox-40 ligand: a potent costimulatory molecule for sustaining primary CD4 T cell responses. J Immunol *161*, 6510-6517.

Gu, H., Zou, Y.R., and Rajewsky, K. (1993). Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-loxP-mediated gene targeting. Cell *73*, 1155-1164.

Haks, M.C., Lefebvre, J.M., Lauritsen, J.P., Carleton, M., Rhodes, M., Miyazaki, T., Kappes, D.J., and Wiest, D.L. (2005). Attenuation of gammadeltaTCR signaling efficiently diverts thymocytes to the alphabeta lineage. Immunity *22*, 595-606.

Hammarlund, E., Lewis, M.W., Hansen, S.G., Strelow, L.I., Nelson, J.A., Sexton, G.J., Hanifin, J.M., and Slifka, M.K. (2003). Duration of antiviral immunity after smallpox vaccination. Nat Med *9*, 1131-1137.

Harrington, L.E., Janowski, K.M., Oliver, J.R., Zajac, A.J., and Weaver, C.T. (2008). Memory CD4 T cells emerge from effector T-cell progenitors. Nature *452*, 356-360.

Hayes, S.M., Li, L., and Love, P.E. (2005). TCR signal strength influences alphabeta/gammadelta lineage fate. Immunity *22*, 583-593.

Hayes, S.M., and Love, P.E. (2006). Strength of signal: a fundamental mechanism for cell fate specification. Immunol Rev *209*, 170-175.

He, Y.W., and Bevan, M.J. (1999). High level expression of CD43 inhibits T cell receptor/CD3-mediated apoptosis. J Exp Med *190*, 1903-1908.

Hedrick, S.M., Engel, I., McElligott, D.L., Fink, P.J., Hsu, M.L., Hansburg, D., and Matis, L.A. (1988). Selection of amino acid sequences in the beta chain of the T cell antigen receptor. Science *239*, 1541-1544.

Hildeman, D.A., Zhu, Y., Mitchell, T.C., Bouillet, P., Strasser, A., Kappler, J., and Marrack, P. (2002). Activated T cell death in vivo mediated by proapoptotic bcl-2 family member bim. Immunity *16*, 759-767.

Hoess, R.H., Wierzbicki, A., and Abremski, K. (1986). The role of the loxP spacer region in P1 site-specific recombination. Nucleic Acids Res *14*, 2287-2300.

Hogquist, K.A., Jameson, S.C., and Bevan, M.J. (1994). The ligand for positive selection of T lymphocytes in the thymus. Curr Opin Immunol *6*, 273-278.

Holmes, K., Lantz, L.M., Fowlkes, B.J., Schmid, I., and Giorgi, J.V. (2001). Preparation of cells and reagents for flow cytometry. Curr Protoc Immunol *Chapter 5*, Unit 5 3.

Homann, D., Teyton, L., and Oldstone, M.B. (2001). Differential regulation of antiviral T-cell immunity results in stable CD8+ but declining CD4+ T-cell memory. Nat Med *7*, 913-919.

Iezzi, G., Karjalainen, K., and Lanzavecchia, A. (1998). The duration of antigenic stimulation determines the fate of naive and effector T cells. Immunity *8*, 89-95.

Iezzi, G., Scotet, E., Scheidegger, D., and Lanzavecchia, A. (1999). The interplay between the duration of TCR and cytokine signaling determines T cell polarization. Eur J Immunol *29*, 4092-4101.

Itano, A., Salmon, P., Kioussis, D., Tolaini, M., Corbella, P., and Robey, E. (1996). The cytoplasmic domain of CD4 promotes the development of CD4 lineage T cells. J Exp Med *183*, 731-741.

Itano, A.A., McSorley, S.J., Reinhardt, R.L., Ehst, B.D., Ingulli, E., Rudensky, A.Y., and Jenkins, M.K. (2003). Distinct dendritic cell populations sequentially present antigen to CD4 T cells and stimulate different aspects of cell-mediated immunity. Immunity *19*, 47-57.

Jacob, J., and Baltimore, D. (1999). Modelling T-cell memory by genetic marking of memory T cells in vivo. Nature *399*, 593-597.

Janeway, C. (2005). Immunobiology : the immune system in health and disease, 6th edn (New York: Garland Science).

Janssen, E.M., Droin, N.M., Lemmens, E.E., Pinkoski, M.J., Bensinger, S.J., Ehst, B.D., Griffith, T.S., Green, D.R., and Schoenberger, S.P. (2005). CD4+ T-cell help controls CD8+ T-cell memory via TRAIL-mediated activation-induced cell death. Nature *434*, 88-93.

Janssen, E.M., Lemmens, E.E., Wolfe, T., Christen, U., von Herrath, M.G., and Schoenberger, S.P. (2003). CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. Nature *421*, 852-856.

Jellison, E.R., Kim, S.K., and Welsh, R.M. (2005). Cutting edge: MHC class II-restricted killing in vivo during viral infection. J Immunol *174*, 614-618.

Jenkins, M.K., Khoruts, A., Ingulli, E., Mueller, D.L., McSorley, S.J., Reinhardt, R.L., Itano, A., and Pape, K.A. (2001). In vivo activation of antigen-specific CD4 T cells. Annu Rev Immunol *19*, 23-45.

Jorritsma, P.J., Brogdon, J.L., and Bottomly, K. (2003). Role of TCR-induced extracellular signal-regulated kinase activation in the regulation of early IL-4 expression in naive CD4+ T cells. J Immunol *170*, 2427-2434.

Joshi, N.S., Cui, W., Chandele, A., Lee, H.K., Urso, D.R., Hagman, J., Gapin, L., and Kaech, S.M. (2007). Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor. Immunity *27*, 281-295.

Joyner, A.L., and Zervas, M. (2006). Genetic inducible fate mapping in mouse: establishing genetic lineages and defining genetic neuroanatomy in the nervous system. Dev Dyn *235*, 2376-2385.

Kaech, S.M., and Ahmed, R. (2001). Memory CD8+ T cell differentiation: initial antigen encounter triggers a developmental program in naïve cells. Nat Immunol *2*, 415-422.

Kaech, S.M., Tan, J.T., Wherry, E.J., Konieczny, B.T., Surh, C.D., and Ahmed, R. (2003). Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. Nat Immunol *4*, 1191-1198.

Kaech, S.M., and Wherry, E.J. (2007). Heterogeneity and cell-fate decisions in effector and memory CD8+ T cell differentiation during viral infection. Immunity *27*, 393-405.

Kalia, V., Sarkar, S., Subramaniam, S., Haining, W.N., Smith, K.A., and Ahmed, R. (2010). Prolonged interleukin-2Ralpha expression on virus-specific

CD8+ T cells favors terminal-effector differentiation in vivo. Immunity *32*, 91-103.

Kappler, J., Kubo, R., Haskins, K., White, J., and Marrack, P. (1983). The mouse T cell receptor: comparison of MHC-restricted receptors on two T cell hybridomas. Cell *34*, 727-737.

Kedl, R.M., Rees, W.A., Hildeman, D.A., Schaefer, B., Mitchell, T., Kappler, J., and Marrack, P. (2000). T cells compete for access to antigen-bearing antigen-presenting cells. J Exp Med *192*, 1105-1113.

Khanolkar, A., Badovinac, V.P., and Harty, J.T. (2007). CD8 T cell memory development: CD4 T cell help is appreciated. Immunol Res *39*, 94-104.

Klinger, M., Chmura, S.A., and Killeen, N. (2010). Reporter alleles that inform on differences in Cre recombinase expression. The Journal of Immunology *184*, 6170-6176.

Klinger, M., Kim, J.K., Chmura, S.A., Barczak, A., Erle, D.J., and Killeen, N. (2009). Thymic OX40 expression discriminates cells undergoing strong responses to selection ligands. J Immunol *182*, 4581-4589.

Kondrack, R.M., Harbertson, J., Tan, J.T., McBreen, M.E., Surh, C.D., and Bradley, L.M. (2003). Interleukin 7 regulates the survival and generation of memory CD4 cells. J Exp Med *198*, 1797-1806.

Lakso, M., Sauer, B., Mosinger, B., Jr., Lee, E.J., Manning, R.W., Yu, S.H., Mulder, K.L., and Westphal, H. (1992). Targeted oncogene activation by site-specific recombination in transgenic mice. Proc Natl Acad Sci U S A *89*, 6232-6236.

Lam, K.P., and Rajewsky, K. (1999). B cell antigen receptor specificity and surface density together determine B-1 versus B-2 cell development. J Exp Med *190*, 471-477.

Lanzavecchia, A., and Sallusto, F. (2002). Progressive differentiation and selection of the fittest in the immune response. Nat Rev Immunol *2*, 982-987.

Lee, E.C., Yu, D., Martinez de Velasco, J., Tessarollo, L., Swing, D.A., Court, D.L., Jenkins, N.A., and Copeland, N.G. (2001). A highly efficient Escherichia coli-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. Genomics *73*, 56-65.

Lee, G., and Saito, I. (1998). Role of nucleotide sequences of loxP spacer region in Cre-mediated recombination. Gene *216*, 55-65.

Lees, J.R., and Farber, D.L. (2010a). Generation, persistence and plasticity of CD4 T-cell memories. Immunology *130*, 463-470.

Lees, J.R., and Farber, D.L. (2010b). Generation, persistence and plasticity of CD4 T-cell memories. Immunology *130*, 463-470.

Legue, E., and Joyner, A.L. (2010). Genetic fate mapping using site-specific recombinases. Methods Enzymol *477*, 153-181.

Li, J., Huston, G., and Swain, S.L. (2003). IL-7 promotes the transition of CD4 effectors to persistent memory cells. J Exp Med *198*, 1807-1815.

Liang, S., Mozdzanowska, K., Palladino, G., and Gerhard, W. (1994). Heterosubtypic immunity to influenza type A virus in mice. Effector mechanisms and their longevity. J Immunol *152*, 1653-1661.

Lin, M.Y., and Welsh, R.M. (1998). Stability and diversity of T cell receptor repertoire usage during lymphocytic choriomeningitis virus infection of mice. J Exp Med *188*, 1993-2005.

Lin, Q., Jo, D., Gebre-Amlak, K.D., and Ruley, H.E. (2004). Enhanced cell-permeant Cre protein for site-specific recombination in cultured cells. BMC Biotechnol *4*, 25.

Lobe, C.G., Koop, K.E., Kreppner, W., Lomeli, H., Gertsenstein, M., and Nagy, A. (1999). Z/AP, a double reporter for cre-mediated recombination. Dev Biol *208*, 281-292.

Lyons, D.S., Lieberman, S.A., Hampl, J., Boniface, J.J., Chien, Y., Berg, L.J., and Davis, M.M. (1996). A TCR binds to antagonist ligands with lower affinities and faster dissociation rates than to agonists. Immunity *5*, 53-61.

MacLeod, M.K., Clambey, E.T., Kappler, J.W., and Marrack, P. (2009a). CD4 memory T cells: what are they and what can they do? Semin Immunol *21*, 53-61.

MacLeod, M.K.L., Clambey, E.T., Kappler, J.W., and Marrack, P. (2009b). CD4 memory T cells: what are they and what can they do? Semin Immunol *21*, 53-61.

Macleod, M.K.L., Kappler, J.W., and Marrack, P. (2010). Memory CD4 T cells: generation, reactivation and re-assignment. Immunology *130*, 10-15.

Malherbe, L., Filippi, C., Julia, V., Foucras, G., Moro, M., Appel, H., Wucherpfennig, K., Guery, J.C., and Glaichenhaus, N. (2000). Selective activation and expansion of high-affinity CD4+ T cells in resistant mice upon infection with Leishmania major. Immunity *13*, 771-782.

Malherbe, L., Hausl, C., Teyton, L., and McHeyzer-Williams, M.G. (2004). Clonal selection of helper T cells is determined by an affinity threshold with no further skewing of TCR binding properties. Immunity *21*, 669-679.

Mao, X., Fujiwara, Y., Chapdelaine, A., Yang, H., and Orkin, S.H. (2001). Activation of EGFP expression by Cre-mediated excision in a new ROSA26 reporter mouse strain. Blood *97*, 324-326.

Mao, X., Fujiwara, Y., and Orkin, S.H. (1999). Improved reporter strain for monitoring Cre recombinase-mediated DNA excisions in mice. Proc Natl Acad Sci U S A *96*, 5037-5042.

Masopust, D., Vezys, V., Marzo, A.L., and Lefrançois, L. (2001). Preferential localization of effector memory cells in nonlymphoid tissue. Science *291*, 2413-2417.

Matsui, K., Boniface, J.J., Steffner, P., Reay, P.A., and Davis, M.M. (1994). Kinetics of T-cell receptor binding to peptide/I-Ek complexes: correlation of the dissociation rate with T-cell responsiveness. Proc Natl Acad Sci U S A *91*, 12862-12866.

McHeyzer-Williams, L.J., and McHeyzer-Williams, M.G. (2005). Antigen-specific memory B cell development. Annu Rev Immunol *23*, 487-513.

McHeyzer-Williams, L.J., Pelletier, N., Mark, L., Fazilleau, N., and McHeyzer-Williams, M.G. (2009). Follicular helper T cells as cognate regulators of B cell immunity. Curr Opin Immunol *21*, 266-273.

McHeyzer-Williams, M.G., and Davis, M.M. (1995). Antigen-specific development of primary and memory T cells in vivo. Science *268*, 106-111.

McKinstry, K.K., Golech, S., Lee, W.H., Huston, G., Weng, N.P., and Swain, S.L. (2007). Rapid default transition of CD4 T cell effectors to functional memory cells. J Exp Med *204*, 2199-2211.

McKinstry, K.K., Strutt, T.M., and Swain, S.L. (2008). The effector to memory transition of CD4 T cells. Immunol Res *40*, 114-127.

Mercado, R., Vijh, S., Allen, S.E., Kerksiek, K., Pilip, I.M., and Pamer, E.G. (2000). Early programming of T cell populations responding to bacterial infection. J Immunol *165*, 6833-6839.

Metzger, D., Clifford, J., Chiba, H., and Chambon, P. (1995). Conditional site-specific recombination in mammalian cells using a ligand-dependent chimeric Cre recombinase. Proc Natl Acad Sci U S A *92*, 6991-6995.

Mohrs, K., Wakil, A.E., Killeen, N., Locksley, R.M., and Mohrs, M. (2005). A two-step process for cytokine production revealed by IL-4 dual-reporter mice. Immunity *23*, 419-429.

Moon, J.J., Chu, H.H., Pepper, M., McSorley, S.J., Jameson, S.C., Kedl, R.M., and Jenkins, M.K. (2007). Naive CD4(+) T cell frequency varies for different epitopes and predicts repertoire diversity and response magnitude. Immunity *27*, 203-213.

Murali-Krishna, K., Altman, J.D., Suresh, M., Sourdive, D.J., Zajac, A.J., Miller, J.D., Slansky, J., and Ahmed, R. (1998). Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. Immunity *8*, 177-187.

Nichols, J., Evans, E.P., and Smith, A.G. (1990). Establishment of germ-line-competent embryonic stem (ES) cells using differentiation inhibiting activity. Development *110*, 1341-1348.

Novak, A., Guo, C., Yang, W., Nagy, A., and Lobe, C.G. (2000). Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon Cre-mediated excision. Genesis *28*, 147-155.

Obst, R., van Santen, H.M., Mathis, D., and Benoist, C. (2005). Antigen persistence is required throughout the expansion phase of a CD4(+) T cell response. J Exp Med *201*, 1555-1565.

Oxenius, A., Bachmann, M.F., Zinkernagel, R.M., and Hengartner, H. (1998). Virus-specific MHC-class II-restricted TCR-transgenic mice: effects on humoral and cellular immune responses after viral infection. Eur J Immunol *28*, 390-400.

Peitz, M., Pfannkuche, K., Rajewsky, K., and Edenhofer, F. (2002). Ability of the hydrophobic FGF and basic TAT peptides to promote cellular uptake of recombinant Cre recombinase: a tool for efficient genetic engineering of mammalian genomes. Proc Natl Acad Sci U S A *99*, 4489-4494.

Perez, O.D., Mitchell, D., Campos, R., Gao, G.J., Li, L., and Nolan, G.P. (2005). Multiparameter analysis of intracellular phosphoepitopes in

immunophenotyped cell populations by flow cytometry. Curr Protoc Cytom *Chapter 6*, Unit 6 20.

Pipkin, M.E., Sacks, J.A., Cruz-Guilloty, F., Lichtenheld, M.G., Bevan, M.J., and Rao, A. (2010). Interleukin-2 and inflammation induce distinct transcriptional programs that promote the differentiation of effector cytolytic T cells. Immunity *32*, 79-90.

Poueymirou, W.T., Auerbach, W., Frendewey, D., Hickey, J.F., Escaravage, J.M., Esau, L., Dore, A.T., Stevens, S., Adams, N.C., Dominguez, M.G.*, et al.* (2007). F0 generation mice fully derived from gene-targeted embryonic stem cells allowing immediate phenotypic analyses. Nat Biotechnol *25*, 91-99.

Prlic, M., Williams, M.A., and Bevan, M.J. (2007). Requirements for CD8 T-cell priming, memory generation and maintenance. Curr Opin Immunol *19*, 315-319.

Ramirez-Solis, R., Liu, P., and Bradley, A. (1995). Chromosome engineering in mice. Nature *378*, 720-724.

Ramirezsolis, R., Riveraperez, J., Wallace, J.D., Wims, M., Zheng, H., and Bradley, A. (1992). Genomic DNA Microextraction - a Method to Screen Numerous Samples. Analytical Biochemistry *201*, 331-335.

Rees, W., Bender, J., Teague, T.K., Kedl, R.M., Crawford, F., Marrack, P., and Kappler, J. (1999). An inverse relationship between T cell receptor affinity and antigen dose during CD4(+) T cell responses in vivo and in vitro. Proc Natl Acad Sci U S A *96*, 9781-9786.

Reinhardt, R.L., Khoruts, A., Merica, R., Zell, T., and Jenkins, M.K. (2001). Visualizing the generation of memory CD4 T cells in the whole body. Nature *410*, 101-105.

Rogers, P.R., Song, J., Gramaglia, I., Killeen, N., and Croft, M. (2001). OX40 promotes Bcl-xL and Bcl-2 expression and is essential for long-term survival of CD4 T cells. Immunity *15*, 445-455.

Sallusto, F., Geginat, J., and Lanzavecchia, A. (2004). Central memory and effector memory T cell subsets: function, generation, and maintenance. Annu Rev Immunol *22*, 745-763.

Sallusto, F., Lenig, D., Förster, R., Lipp, M., and Lanzavecchia, A. (1999). Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature *401*, 708-712.

Sant, A., and Yewdell, J. (2003). Antigen processing and recognition. Curr Opin Immunol *15*, 66-68.

Savage, P.A., Boniface, J.J., and Davis, M.M. (1999). A kinetic basis for T cell receptor repertoire selection during an immune response. Immunity *10*, 485-492.

Savage, P.A., and Davis, M.M. (2001). A kinetic window constricts the T cell receptor repertoire in the thymus. Immunity *14*, 243-252.

Schoenberger, S.P., Jonges, L.E., Mooijaart, R.J., Hartgers, F., Toes, R.E., Kast, W.M., Melief, C.J., and Offringa, R. (1998). Efficient direct priming of tumor-specific cytotoxic T lymphocyte in vivo by an engineered APC. Cancer Res *58*, 3094-3100.

Seder, R.A., and Ahmed, R. (2003). Similarities and differences in CD4+ and CD8+ effector and memory T cell generation. Nat Immunol *4*, 835-842.

Shaner, N.C., Campbell, R.E., Steinbach, P.A., Giepmans, B.N.G., Palmer, A.E., and Tsien, R.Y. (2004). Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein. Nat Biotechnol *22*, 1567-1572.

Shedlock, D.J., and Shen, H. (2003). Requirement for CD4 T cell help in generating functional CD8 T cell memory. Science *300*, 337-339.

Singer, A., Adoro, S., and Park, J.-H. (2008). Lineage fate and intense debate: myths, models and mechanisms of CD4- versus CD8-lineage choice. Nat Rev Immunol *8*, 788-801.

Slifka, M.K., and Whitton, J.L. (2001). Functional avidity maturation of CD8(+) T cells without selection of higher affinity TCR. Nat Immunol *2*, 711-717.

Soriano, P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet *21*, 70-71.

Sourdive, D.J., Murali-Krishna, K., Altman, J.D., Zajac, A.J., Whitmire, J.K., Pannetier, C., Kourilsky, P., Evavold, B., Sette, A., and Ahmed, R. (1998). Conserved T cell receptor repertoire in primary and memory CD8 T cell responses to an acute viral infection. J Exp Med *188*, 71-82.

Sprent, J. (1994). T and B memory cells. Cell *76*, 315-322.

Srinivas, S., Watanabe, T., Lin, C.S., William, C.M., Tanabe, Y., Jessell, T.M., and Costantini, F. (2001). Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. BMC Dev Biol *1*, 4.

Sternberg, N., and Hamilton, D. (1981). Bacteriophage P1 site-specific recombination. I. Recombination between loxP sites. J Mol Biol *150*, 467-486.

Steward, M.W., and Steensgaard, J. (1983). Antibody affinity : thermodynamic aspects and biological significance (Boca Raton, Fla.: CRC Press).

Sun, J.C., and Bevan, M.J. (2003). Defective CD8 T cell memory following acute infection without CD4 T cell help. Science *300*, 339-342.

Sun, J.C., Williams, M.A., and Bevan, M.J. (2004). CD4+ T cells are required for the maintenance, not programming, of memory CD8+ T cells after acute infection. Nat Immunol *5*, 927-933.

Surh, C.D., and Sprent, J. (2008). Homeostasis of naive and memory T cells. Immunity *29*, 848-862.

Swain, S.L., Agrewala, J.N., Brown, D.M., Jelley-Gibbs, D.M., Golech, S., Huston, G., Jones, S.C., Kamperschroer, C., Lee, W.H., McKinstry, K.K., *et al.* (2006). CD4+ T-cell memory: generation and multi-faceted roles for CD4+ T cells in protective immunity to influenza. Immunol Rev *211*, 8-22.

Tao, X., Constant, S., Jorritsma, P., and Bottomly, K. (1997). Strength of TCR signal determines the costimulatory requirements for Th1 and Th2 CD4+ T cell differentiation. J Immunol *159*, 5956-5963.

Testi, R., D'Ambrosio, D., De Maria, R., and Santoni, A. (1994). The CD69 receptor: a multipurpose cell-surface trigger for hematopoietic cells. Immunol Today *15*, 479-483.

van Leeuwen, E.M., Sprent, J., and Surh, C.D. (2009). Generation and maintenance of memory CD4(+) T Cells. Curr Opin Immunol *21*, 167-172.

van Stipdonk, M.J., Lemmens, E.E., and Schoenberger, S.P. (2001). Naïve CTLs require a single brief period of antigenic stimulation for clonal expansion and differentiation. Nat Immunol *2*, 423-429.

Vogt, W. (1929). Gestaltungsanalyse am Amphibienkeim mit ortlicher Vitalfarbung. II. Teil Gastrulation und Mesodermbildung bie Urodelen und Anuren. Wilhelm Roux Arch. Entwicklungsmech. Org. *120*, 322.

Wang, X.Z., Brehm, M.A., and Welsh, R.M. (2004). Preapoptotic phenotype of viral epitope-specific CD8 T cells precludes memory development and is an intrinsic property of the epitope. J Immunol *173*, 5138-5147.

Wang, X.Z., Stepp, S.E., Brehm, M.A., Chen, H.D., Selin, L.K., and Welsh, R.M. (2003). Virus-specific CD8 T cells in peripheral tissues are more resistant to apoptosis than those in lymphoid organs. Immunity *18*, 631-642.

Whitmire, J.K., Benning, N., Eam, B., and Whitton, J.L. (2008a). Increasing the CD4+ T cell precursor frequency leads to competition for IFN-gamma thereby degrading memory cell quantity and quality. J Immunol *180*, 6777-6785.

Whitmire, J.K., Benning, N., and Whitton, J.L. (2006). Precursor frequency, nonlinear proliferation, and functional maturation of virus-specific CD4+ T cells. J Immunol *176*, 3028-3036.

Whitmire, J.K., Eam, B., Benning, N., and Whitton, J.L. (2007). Direct interferon-gamma signaling dramatically enhances CD4+ and CD8+ T cell memory. J Immunol *179*, 1190-1197.

Whitmire, J.K., Eam, B., and Whitton, J.L. (2008b). Tentative T cells: memory cells are quick to respond, but slow to divide. PLoS Pathog *4*, e1000041.

Williams, M.A., and Bevan, M.J. (2007). Effector and memory CTL differentiation. Annu Rev Immunol *25*, 171-192.

Williams, M.A., Ravkov, E.V., and Bevan, M.J. (2008). Rapid culling of the CD4+ T cell repertoire in the transition from effector to memory. Immunity *28*, 533-545.

Williams, M.A., Tyznik, A.J., and Bevan, M.J. (2006). Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells. Nature *441*, 890-893.

Wu, C.Y., Kirman, J.R., Rotte, M.J., Davey, D.F., Perfetto, S.P., Rhee, E.G., Freidag, B.L., Hill, B.J., Douek, D.C., and Seder, R.A. (2002). Distinct lineages of T(H)1 cells have differential capacities for memory cell generation in vivo. Nat Immunol *3*, 852-858.

Yang, T.T., Cheng, L.Z., and Kain, S.R. (1996). Optimized codon usage and chromophore mutations provide enhanced sensitivity with the green fluorescent protein. Nucleic Acids Research *24*, 4592-4593.

Zambrowicz, B.P., Imamoto, A., Fiering, S., Herzenberg, L.A., Kerr, W.G., and Soriano, P. (1997a). Disruption of overlapping transcripts in the ROSA beta geo 26 gene trap strain leads to widespread expression of beta-

galactosidase in mouse embryos and hematopoietic cells. Proc Natl Acad Sci USA *94*, 3789-3794.

Zambrowicz, B.P., Imamoto, A., Fiering, S., Herzenberg, L.A., Kerr, W.G., and Soriano, P. (1997b). Disruption of overlapping transcripts in the ROSA beta geo 26 gene trap strain leads to widespread expression of beta-galactosidase in mouse embryos and hematopoietic cells. Proc Natl Acad Sci U S A *94*, 3789-3794.

Zammit, D.J., Cauley, L.S., Pham, Q.-M., and Lefrançois, L. (2005). Dendritic cells maximize the memory CD8 T cell response to infection. Immunity *22*, 561-570.

Zehn, D., Lee, S.Y., and Bevan, M.J. (2009). Complete but curtailed T-cell response to very low-affinity antigen. Nature *458*, 211-214.

Zinyk, D.L., Mercer, E.H., Harris, E., Anderson, D.J., and Joyner, A.L. (1998). Fate mapping of the mouse midbrain-hindbrain constriction using a site-specific recombination system. Curr Biol *8*, 665-668.

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