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MEMORY CELLS – REGULATORY STEPS IN GENERATING PROTECTIVE IMMUNITY

Topic Editors
Gabrielle Belz and Erika Cretney

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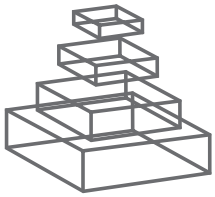

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MEMORY CELLS – REGULATORY STEPS IN GENERATING PROTECTIVE IMMUNITY

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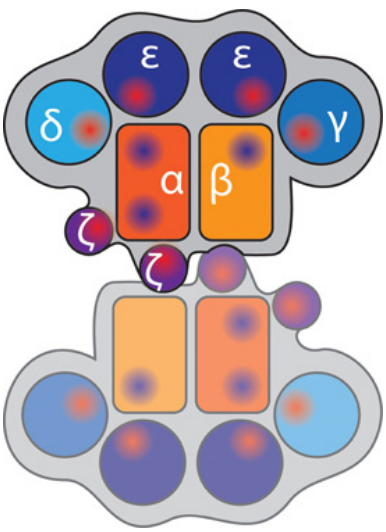

Model of TCR complex dimerization. Heterodimers of TCRα and TCRβ (orange and yellow) are opposed due to the short ectodomains of CD3ζ (purple), which places CD3γ, CD3δ, and the two CD3ε chains (shades of blue) around the outside. The shapes represent immunoglobulin domains of the components; and red and blue shaded regions indicate positive and negatively charged regions, respectively. Figure taken from Rossy J, Williamson DJ, Benzing C and Gaus K (2012) The integration of signaling and the spatial organization of the T cell synapse. *Front. Immun.* 3:352. doi: 10.3389/fimmu.2012.00352 .

The development of protective immunity and memory lymphocytes involves an intricate network of different cell types – each of which is required for the full development of an acute immune response and the formation and maintenance of memory. This series of review articles provides insight into the regulation of a number of these cell types including B cells, CD4+ T cells, CD8+ T cells, regulatory T cells and follicular helper cells. A significant focus of this series is the factors that affect the molecular and transcriptional wiring that is key to the development of these cell types in generating protective memory.

Table of Contents

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CD8 T cell memory: it takes all kinds

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Understanding the mechanisms that regulate the differentiation and maintenance of CD8$^+$ memory T cells is fundamental to the development of effective T cell-based vaccines. Memory cell differentiation is influenced by the cytokines that accompany T cell priming, the history of previous antigen encounters, and the tissue sites into which memory cells migrate. These cues combine to influence the developing CD8$^+$ memory pool, and recent work has revealed the importance of multiple transcription factors, metabolic molecules, and surface receptors in revealing the type of memory cell that is generated. Paired with increasingly meticulous subsetting and sorting of memory populations, we now know the CD8$^+$ memory pool to be phenotypically and functionally heterogeneous in nature. This includes both recirculating and tissue-resident memory populations, and cells with varying degrees of inherent longevity and protective function. These data point to the importance of tailored vaccine design. Here we discuss how the diversity of the memory CD8$^+$ T cell pool challenges the notion that "one size fits all" for pathogen control, and how distinct memory subsets may be suited for distinct aspects of protective immunity.

Keywords: protective immunity, CD8 T cells, immune memory, T cell differentiation, host–pathogen interactions

INTRODUCTION

Memory CD8$^+$ T cells form a unique population, which are able to confer protection against many diverse pathogens. During acute infection, rare, naïve, antigen-specific T cell clones inhabiting secondary lymphoid tissue scan incoming pathogen-derived peptide–MHC I complexes. Once recognition of cognate peptide occurs, in conjunction with appropriate costimulatory and cytokine signals, CD8 T cells undergo massive proliferation and differentiation to form an effector cell population. Effector cells then utilize multiple mechanisms (predominantly cytolysis, IFN-γ, and TNF production) to destroy pathogen-infected cells. After the clearance of infection, a dramatic contraction phase ensues, leaving behind a small, extremely heterogeneous population of long-lived cells that compose the CD8 memory T cell pool (Harty and Badovinac, 2008; Jameson and Masopust, 2009). These cells remain at stable numbers, which are much higher than the starting, naïve population, in the absence of antigen or MHC class I, relying instead on survival cues from homeostatic cytokines IL-7 and IL-15 (Schluns and Lefrancois, 2003; Antia et al., 2005; Surh and Sprent, 2008). In addition to quantitative increases, memory T cells are qualitatively changed from their naïve counterparts, enabling them to respond to reinfection with faster, more robust activity. Recent evidence has revealed the extreme heterogeneity of the memory T cell pool contains, the cues that influence their formation, and the unique challenges which complex pathogens present. Here, we review recent advances, with special emphasis on identification of memory T cells capable of prompt control of acute pathogen infections, and the relevance for vaccine design.

FACTORS INFLUENCING MEMORY T CELL FORMATION

CD8 memory T cell formation is influenced by multiple environmental cues that occur during priming. The combination of these factors regulates the size of the CD8 T cell response and the balance between memory and short-lived effector cell differentiation. Since this has been discussed in several reviews (Ahmed et al., 2009; Jameson and Masopust, 2009; Cui and Kaech, 2010; Rutishauser and Kaech, 2010), we will briefly review key factors that influence generation of CD8 memory cells, highlighting newer findings.

INFLAMMATORY CUES

In addition to encounter with specific peptide/MHC molecules and costimulation (in the form of B7 or other ligands), the inflammatory environment surrounding the cell also has a large impact on the development of memory populations. IL-12 and IFN-α/β are well-defined for providing a "Signal 3," and promote optimal development of both effector and memory cell populations (Mescher et al., 2006; Curtsinger and Mescher, 2010), although the specific cytokine may impact the characteristics of the resulting effector and memory pool. Signal 3 cytokines regulate an impressive number of gene expression changes (including those encoding factors that regulate survival, effector function, and trafficking) and chromatin remodeling may also be an important action of Signal 3 cytokines in CD8 T cells (Agarwal et al., 2009). Stimulatory signals generated during acute bacterial or viral infections can increase the number of effector cells generated during an immune response, but can also delay the onset of memory development (Badovinac et al., 2004, 2005). Cytokines TNF-α, IL-2, and IFN-γ have all been shown to impact the CD8 T cell response as naïve cells differentiate into memory, and limiting early inflammation favors the generation of memory cells (Badovinac et al., 2004, 2005; Harty and Badovinac, 2008). Overall, the data to date show that inflammation and the ensuing cytokine milieu can have a remarkably strong influence on the developing CD8 memory pool.

These effects of inflammation operate, at least in part, though changes in several key transcription factors. Multiple transcription factors (including T-bet, Eomesodermin, Blimp-1, Bcl-6, Id-2, Id-3, TCF, and Stat3) have been shown to modulate the development of short-lived effector versus memory cells, and the expression of several of these factors is influenced by the cytokine milieu (Joshi and Kaech, 2008; D'Cruz et al., 2009; Rutishauser and Kaech, 2010; Ji et al., 2011; Olson and Jameson, 2011; Yang et al., 2011; Xue and Zhao, 2012). These transcriptional regulators often operate in antagonist pairs (perhaps best defined for Blimp-1 and Bcl-6; Nutt et al., 2008; Crotty et al., 2010). A comprehensive picture of how transcription factors and epigenetic changes (Weng et al., 2012) coordinate with each other and additional signals to mold CD8 memory differentiation has yet to emerge – but manipulation of the cytokine environment offers a promising opportunity to regulate the balance between effector and memory CD8 T cell differentiation.

Recent data suggest an interesting twist in the impact of inflammatory cues on effector and memory differentiation. The chemokines CXCL9, CXCL10, and CXCL11 are effectively induced by IFN-γ, and strongly influence migration of CXCR3-expressing immune cells. Hence CXCR3 expression is important for T cell control of various pathogens. However, the ability of CXCR3 signals to retain activated T cells in sites of antigen presentation appears to promote their terminal differentiation toward short-lived effector cells: CXCR3 deficient CD8 T cells form greatly increased numbers of long-lived memory cells, with reduced contraction from the effector phase of the response (Hu et al., 2011; Kohlmeier et al., 2011; Kurachi et al., 2011). Hence, not only the direct response to inflammatory cytokines, but also the response to secondary cues (in this case CXCR3 ligands) may promote effector differentiation, potentially at the expense of memory cell generation.

METABOLIC CONTROL

It is also possible to manipulate the number and type of memory cells formed through metabolic agents. Interestingly, mammalian target of rapamycin (mTOR), a metabolic kinase, has been shown to be a key regulator of CD8 T cells as well as other immune cells (Pearce et al., 2009; Araki et al., 2011). Treatment with rapamycin, an inhibitor of mTOR, has been known for some time to inhibit cellular proliferation and has been used in clinical settings. Recent evidence has shown that inhibiting mTOR will also increase the number of central memory T cells formed, enhancing trafficking to secondary lymphoid organs (Sinclair et al., 2008; Araki et al., 2011). Additionally, either treatment with rapamycin or knocking down components of mTOR during the early phase of the immune response, increases the quantity of memory cells that survive long-term, supporting the concept that mTOR is a component of CD8 memory T cell differentiation (Araki et al., 2009). Although the exact mechanism through which disruption of mTOR signaling enhances lymphoid CD8 T cell memory generation is currently unclear, the concept of capitalizing on differences between the metabolic states of effector versus memory (and naïve) T cells offers an interesting opportunity for therapeutic manipulation of CD8 T cell differentiation (Prlic and Bevan, 2009; van der Windt and Pearce, 2012).

ANTIGEN RESTIMULATION

Repeated acute exposure to foreign antigen has a dramatic effect on the memory $CD8^+$ T cell pool. Characteristics of the memory pool differ between the primary pool (generated by one round of antigen exposure) compared to "secondary" (or tertiary) memory cells induced by boosting. The differentiation of T_{CM} is considerably delayed, and cells bearing effector-like traits (including expression of KLRG1 and granzyme B) are maintained for considerably longer periods (Jabbari and Harty, 2006; Masopust et al., 2006). These differences alter the functional and trafficking characteristics of the memory pool – for example, the relative paucity of T_{CM} in the secondary memory pool limits their ability to traffic through lymph nodes (while the abundance of T_{EM} may enhance survey of peripheral tissues). Depending on the context of reinfection, such changes in localization could be either a benefit or detriment to the host – indeed Nolz and Harty (2011) propose that boosting may impair the ability of CD8 T cells to mount protective responses against certain pathogens (due at least in part to altered trafficking), while control of other pathogens is improved by boosting. Similarly, the number of antigen-specific cells is increased with boosting, and this can allow achievement of a threshold for protective immunity (perhaps most dramatically illustrated for the response to malaria; Schmidt et al., 2008) but this may come at the cost of the boosted memory cells' capacity for proliferation after antigen re-exposure (Masopust et al., 2006; Wirth et al., 2010). Again, depending on the context of the response required, this trait may become a limitation for the immune response. Analysis of boosted memory T cells is important, partly for evaluating optimal vaccination strategies, and partly because pathogen reencounter is likely to occur in natural situations (unlike the artificially controlled exposure used in experimental studies or vaccination), and hence may be a better indication of normal immune function.

Furthermore persistent infections that periodically reactivate from a latent state (such as occur with several herpes viruses) can promote memory CD8 T cell "inflation," producing T cells with the characteristics of boosted memory CD8 T cells (Snyder et al., 2008), and such features have been exploited for induction of protective immunity in models of HIV (Hansen et al., 2011). On the other hand, excessive or sustained antigen exposure (as occur during some chronic infections), can lead to the decline of CD8 T cell function – this has been reviewed extensively by others (Kaech et al., 2002; Virgin et al., 2009; Wherry, 2011), and hence will not be further explored here. However, this raises the important point that considerably more information is needed to understand the conditions that dictate whether multiple antigen encounters leads to enhancement versus impairment of the CD8 T cell response.

HETEROGENEITY AMONG MEMORY CD8 T CELLS

The memory pool contains many distinct subsets of CD8 T cells with differing proliferative, survival, trafficking, and functional qualities (Seder et al., 2008; Jameson and Masopust, 2009). Elegant single cell transfer and bar-coding experiments show that an individual naïve TCR transgenic CD8 T cells is capable of forming diverse effector and memory populations (Stemberger et al., 2007; Gerlach et al., 2010), arguing against the model that distinct memory subsets are occupied by different clones, or cells receiving distinct initial activation cues.

Considerable work has gone into defining cell surface markers to subset the memory pool into functionally distinct populations, in both mice and humans (Seder and Ahmed, 2003; Seder et al., 2008; Masopust and Picker, 2012). Major classifications are discussed below. However, in contrast to the depth of information on the factors regulating effector versus memory differentiation, much less is known about the signals that drive appearance of distinct memory subsets.

EFFECTOR AND CENTRAL MEMORY

The most widely characterized subset division is that of central and effector memory cells (Sallusto et al., 1999; Wherry et al., 2003), which are defined based on the coordinate expression of CCR7 and CD62L. Both molecules interact with components displayed on the high endothelial venules of lymph nodes – CD62L interacting with carbohydrate moieties termed lymph node addressins while CCR7 binds the "homeostatic" chemokines CCL19 and CCL21. Memory cells that express these two molecules are termed central memory (T_{CM}), and efficiently traffic into lymph nodes, but are not predominant in peripheral tissues. In contrast, effector memory (T_{EM}) cells do not express CCR7 or CD62L and are excluded from lymph nodes, but can be found in the spleen (especially in the red pulp; Jung et al., 2010) and are prevalent in non-lymphoid tissues (Masopust et al., 2001). In addition to these trafficking differences, the T_{CM} pool exhibits improved long-term survival and enhanced proliferation upon antigen restimulation, compared to the T_{EM} population, while the T_{EM} subset, especially cells isolated from tissues, show more rapid deployment of effector functions compared to T_{CM} (Kaech and Wherry, 2007; Jameson and Masopust, 2009).

The "T_{EM}" subset is heterogeneous and can be further dissected. First, some $CD62L^{low}$ $CCR7^{high}$ cells have been defined (Unsoeld and Pircher, 2005): given that CCR7 supports T cell migration from some non-lymphoid tissues (Debes et al., 2005), this phenotype may be indicative of a specialized trafficking pattern. Furthermore, the general T_{EM} phenotype includes both a recirculating pool (with special predilection to migration through non-lymphoid tissues) as well as non-recirculating cells, termed resident memory cells, discussed next.

RESIDENT MEMORY

More than a decade ago, seminal studies documented the existence of CD8 memory cells in diverse non-lymphoid tissues, in addition to their counterparts in lymphoid sites (Masopust et al., 2001; Masopust and Lefrancois, 2003). Memory CD8 T cells persist long-term in peripheral tissues, and were noted as having increased granzyme B expression and more potent killing capacity than central memory cells (Masopust et al., 2001; Marzo et al., 2007). Such cells were originally thought to be part of the recirculating T_{EM} pool (with which they share some key phenotypic traits), but more recent studies indicate that there is a distinct non-recirculating population of memory CD8 T cells, termed resident memory (T_{RM}), in many tissues, including the IEL, skin, lung, brain, and salivary gland (Gebhardt et al., 2009; Masopust et al., 2010; Wakim et al., 2010; Jiang et al., 2012; Masopust and Picker, 2012).

T_{RM} cells have been identified at barrier surfaces in mice and non-human primates (Bevan, 2011; Sheridan and Lefrancois, 2011; Masopust and Picker, 2012), with similar cells characterized in human skin (Clark et al., 2012) and this pool is of interest as a critical first line of defense against infection. While there are numerous questions about the pathways involved in establishment and maintenance of T_{RM}, the pool found in the mouse small intestine IEL pool is especially well-characterized. Although phenotypically related to T_{EM}, the SI-IEL pool displays some distinct markers, including upregulated CD103 (the αE integrin chain, which, when paired with the β7 chain, is a receptor for E-cadherin) and CD69 (Sheridan and Lefrancois, 2011; Masopust and Picker, 2012). Surprisingly, despite the common association of CD69 with TCR stimulation, foreign antigen exposure is not required for generation of the SI-IEL pool, which can be induced by homeostatic mechanisms (Casey et al., 2012), arguing against an obligatory role for an antigen depot in sites occupied by T_{RM}. Cytokines, including TGF-β, are important for induction of CD103 on SI-IEL T_{RM} cells, and CD103 itself is important for sustained residency of this population (Casey et al., 2012). It is not yet clear whether these requirements will apply to T_{RM} in all tissues, and whether additional cues are needed to initiate or sustain tissue residency, but these data highlight the sophisticated mechanisms which allow segregation of recirculating from tissue-resident cells.

EFFECTOR-LIKE MEMORY CELLS

Another CD8 memory T cell division scheme was defined by Woodland and colleagues, based on CXCR3, CD27, and a glycosylated form of CD43 (Hikono et al., 2007). These markers further fragment the T_{CM} and T_{EM} pools, offering refinement of functional properties within the memory-stage pool, for example showing that $CD27^{hi}CD43^{lo}$ cells were superior over other subsets in their ability to proliferate after rechallenge (Hikono et al., 2007). $CD27^{lo}CD43^{lo}$ cells on the other hand, showed markers associated with the effector phase, including expression of KLRG-1 and granzyme B, and showed impaired proliferative responses. This "effector-like" population is maintained for many months following the response to respiratory infections (Hikono et al., 2007) as well as system infection with diverse pathogens (Olson et al., unpublished data). Such cells decline over time in the primary immune response: however, cells with this phenotype are maintained long-term and at high frequency following antigen-specific boosting (Olson et al., unpublished data) and, as will be discussed below, show optimal immediate protective control against acute bacterial and viral infections (Olson et al., unpublished data). Notably, this effector-like phenotype (characterized in lymphoid tissues) overlaps with the resident memory pool – for example, cells from the small intestinal IEL are $CD27^{low}$, $CD43^{low}$, granzyme B^{high} – although some other markers are distinct (e.g., SI-IEL cells are KLRG-1^{low} and $CD69^{+}$ while effector-like cells in lymphoid tissues are KLRG-1^{high}, $CD69^{-}$). Hence the potential relationship between effector-like and T_{RM} cells needs to be investigated further.

MEMORY STEM CELLS

Recent evidence has suggested that some memory T cells may have the ability to produce a specialized self-renewing population,

sharing signaling pathways with hematopoietic stem cells. Gattinoni et al. (2009) proposed that induction of Wnt signaling suppresses Eomesodermin, and generates T cells of an unusual phenotype: $CD44^{low}$, but high in expression of Sca-1, CD122, CD62L, and Bcl-2, which bears features of proposed "Memory Stem Cells" (first identified in a transplant setting; Zhang et al., 2005). This change allowed T cells to go through many more cell divisions than normal, as well as proliferate and differentiate in response to antigen. This property was shown to be beneficial in a tumor model, suggesting the possibility that antigen-specific memory stem cells (even in small numbers) may be a useful immunotherapy tool (Gattinoni et al., 2009; Koehn and Schoenberger, 2009). However, other studies, using similar approaches, concluded that activation of the Wnt signaling pathway in mouse CD8 T cells did not promote production of a memory stem cell pool, but rather may attenuate initial naïve response (Driessens et al., 2010; Prlic and Bevan, 2011). Hence, considerable controversy currently surrounds the definition of this memory subset. Nevertheless, the concept of a specialized memory stem cell, paired with the finding that humans possess a similar CD8 memory T cell subset with the ability to survive and reconstitute the T cell pool after depletion events like chemotherapy (Turtle et al., 2009; Gattinoni et al., 2011), will certainly provoke continued research and interest.

WHICH SUBSET(S) OF MEMORY CD8 T CELLS OFFER OPTIMAL PROTECTION AGAINST PATHOGENS?

A fundamental feature of the adaptive immune system (and the primary goal of vaccines) is that immune memory results in improved protection against pathogen reinfection. While this can, in part, be ascribed to the numerical increase in antigen-specific T cells that follows immunization, heterogeneity within the memory T cell pool naturally leads to the question of whether some populations of memory cells are better than others at protective immunity against a given pathogen (**Figure 1**).

Some years ago, the answer seemed relatively clear: T_{CM} had numerous features suggesting these were the critical memory population for long-term protective immunity. First, T_{CM} show very effective long-term maintenance, becoming the predominant memory subset over time following a primary antigen encounter. In addition, T_{CM} exhibit optimal recall proliferative capacity and the ability to quickly differentiate into potent effector cells upon antigen re-encounter. Finally, direct comparisons between T_{EM} and T_{CM} following infection with various pathogens (acute and chronic LCMV, vaccinia virus) suggested the T_{CM} pool was, overall, the superior subset for pathogen elimination (Wherry et al., 2003; Laouar et al., 2008). As discussed earlier, the representation of T_{CM} changes with antigen-specific boosting, which decreases the frequency of T_{CM} and delays their appearance: indeed, with heterologous prime/boost strategies, the frequency of T_{CM} can become quite low, with the antigen-specific memory CD8 T cell population dominated by T_{EM} and effector-like cells (Jabbari and Harty, 2006; Masopust et al., 2006; Wirth et al., 2010). This might lead to the conclusion that boosting the immune response, though clearly of benefit for high affinity B cell responses, could degrade the protective capacity of the CD8 memory pool. In fact, recent studies argued that boosted (or secondary memory cells) are indeed compromised for control of chronic LCMV and MHV infection, although responses to some other pathogens (*Listeria*, vaccinia, and acute LCMV infection) were unchanged or improved by boosting (Nolz and Harty, 2011).

On the other hand, additional studies suggested that cells of T_{EM} phenotype exhibited optimal pathogen control against some of the same pathogens (e.g., vaccinia virus; Bachmann et al., 2005) and against other systemic infections (e.g., *Listeria*; Huster et al., 2006). Furthermore, T_{EM} phenotype cells induced by heterologous prime/boosting were associated with improved protection against mucosal SIV challenge, with important implications for vaccination against HIV infection (Hansen et al., 2009, 2011).

Furthermore, in recent studies we examined the protective capacity of "effector-like" CD8 T cells that persist into the memory phase during primary responses and are the predominant antigen-specific pool following certain prime-boost strategies (Olson et al., unpublished data). These cells, bearing the phenotype of $CD27^{low}$, $CD43^{low}$, KLRG-1^{high}, and $CD127^{int}$ were poor at recall proliferation compared to subsets containing the classic T_{EM} and T_{CM} populations (Hikono et al., 2007; Olson et al., unpublished data): yet these effector-like cells mediated optimal protective immunity against *Listeria* and vaccinia infection at least in part due to preferential utilization of cytotoxic mechanisms (Olson et al., unpublished data). Since this effector-like subset shares some phenotypic traits with typical T_{EM} cells, care must be taken in evaluating data on the protective capacity of the T_{EM} subset. It is interesting to note that the rapid prime-boost strategy described by Harty and colleagues, which leads to highly efficient protection against various viral, bacterial, and parasitic infections (Pham et al., 2010), predominantly induces a $CD27^{lo}$, $CD43^{lo}$, $KLRG1^{hi}$, $CD127^{int}$, effector-like population, which persist long-term (Olson et al., unpublished data). Hence, this population – which might also be termed "long-lived effectors" to contrast with their short-lived counterparts found in the early immune response – represents an appealing goal for vaccination against certain diseases.

However, a limitation on many studies testing the protective capacity of distinct memory subsets is that they typically involves isolation of cells from lymphoid tissues followed by adoptive transfer into the blood. This approach neglects the T_{RM} populations existing in non-lymphoid sites, which (by definition) are not part of the recirculating pool found in lymphoid tissues. Experimentally, this issue is compounded by the finding that T_{RM} are inefficient at homing back to non-lymphoid tissues in the absence of restimulation (Masopust et al., 2010; Masopust and Picker, 2012). However elegant approaches, including parabiosis and selective depletion strategies have been used to test the capacity of T_{RM} to mediate protective immunity in non-lymphoid tissues. For example, Jiang et al. (2012) examined a parabiotic mice mouse model in the context of vaccinia infection in the skin: mice that contained both antigen-specific T_{RM} and recirculating memory cells rapidly cleared the infection, while mice with recirculating memory CD8 T cells alone showed impaired clearance of the virus. Other studies limited the capacity of recirculating memory cells to contribute to pathogen control, and again saw efficient protection mediated through T_{RM} (Hofmann and Pircher, 2011; Mackay et al., 2012). These data highlighted that in the context of

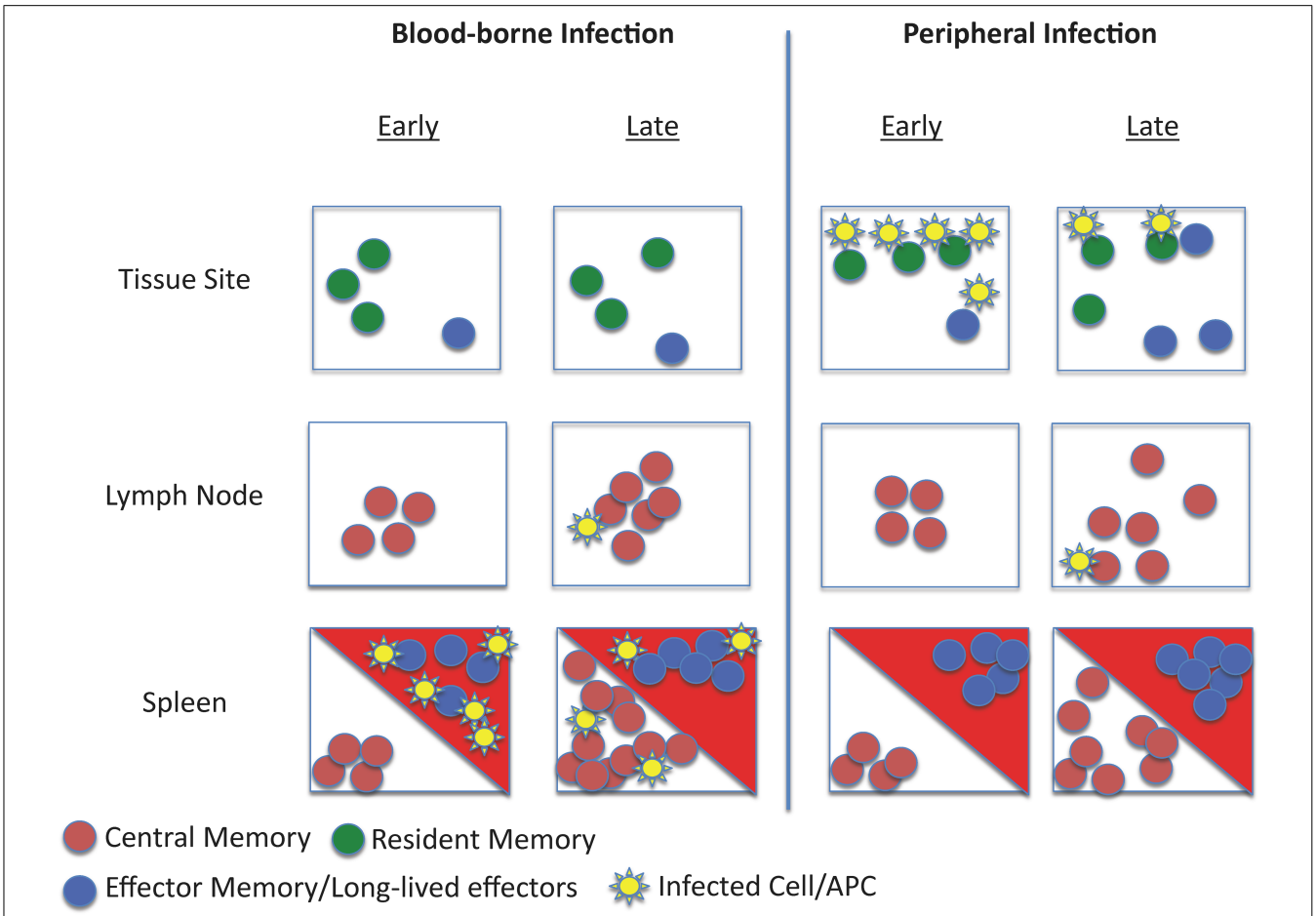

FIGURE 1 | Schematic of memory T cell distribution and reactivation during systemic and local infection. The model depicts the response to infection of animals with pre-existing memory CD8 T cell populations. Different populations of memory cells occupy distinct niches: T_{CM} are restricted to lymphoid tissues, recirculating T_{EM} and long-lived effector-like cells traffic through tissues and certain lymphoid sites (such as the splenic red pulp – red triangles) while T_{RM} are strictly limited to tissue parenchyma. Upon infection via the blood (such as bacterial sepsis), pathogens may initially be controlled by effector-like and T_{EM} populations at entry sites (e.g., the splenic red pulp and marginal zone). Subsequently, activation and clonal expansion of T_{CM} increases the frequency of secondary effector cells. In this scenario, T_{RM} are not engaged in the response. However, during a peripheral infection (for example, viral infection in the skin), initial pathogen control is mediated by the T_{RM} pool. This inflammatory response may recruit circulating T_{EM} (and, potentially T_{CM}) to the site of infection. Subsequent responses initiated in draining lymphoid tissues would control systemic spread.

a pathogen invasion at an epithelial surface, resident memory cells are superior to central memory or naïve CD8 T cells. Likewise, it is likely (although not proven) that the mucosal T_{EM}-like CD8 T cells that offer optimal control of SIV infection (Hansen et al., 2011) are in fact T_{RM} (Masopust and Picker, 2012). Such data suggest that the T_{RM} pool is critical for first-line defense against infection at barrier surfaces, but presumably play a more minor role in responses to blood-borne infections (**Figure 1**).

CONCLUDING REMARKS

The goal of vaccination is to rapidly control infection to prevent or minimize the occurrence of disease. Determining the CD8 memory T cell(s) best able to achieve that goal is critical for future development of effective vaccines as we move to apply bench work to the clinic. Defining a "protective" memory cell is always context dependent. Is the infection acute or chronic? What is the inflammatory environment created? What is the life style of the pathogen and its location in the host? These factors and others impact the developing CD8 T cell response and should be at the forefront of our attempt to create the most useful memory T cell pool by vaccination. Thus, while it is tempting to try to define "The" optimal subset of memory CD8 T cells for protective immunity, the very fact of memory heterogeneity suggests that this diversity is useful for the immune system in different contexts: so, while rapid recall proliferation of a small T_{CM} memory subset may be suitable for control of chronic LCMV infection (Wherry et al., 2003; Nolz and Harty, 2011), very high numbers of T_{EM} and effector-like cells may be important for rapid control of liver-stage malaria infection (Schmidt et al., 2008; Pham et al., 2010) and establishment of a mucosal pool of T_{RM} may be essential for control of SIV (Hansen et al., 2011; Masopust and Picker, 2012). This discussion also raises the question of how quantity versus quality of antigen-specific memory CD8 T cells relates to protective immunity: while ideal immunity may produce a high

frequency of diverse memory subsets, practical limitations force consideration of how many memory cells, of what type and in what locations, are sufficient for protection against a given pathogen. Careful analysis of the protective function mediated by various memory CD8 T cell subsets in distinct locations may provide suggest more streamlined vaccine approaches. Lastly, this review focuses on responses to infectious disease, but there may be quite different criteria for protective immune responses against tumors – indeed there is suggestive evidence that the self-renewing "memory stem cell" pool has key features for sustained responses against the self-antigens often targeted for cancer immunotherapy (Gattinoni et al., 2009; Koehn and Schoenberger, 2009).

Thus we propose two central challenges for optimizing protective CD8 T cell vaccination – first, defining the traits (including function and localization) which characterize protective CD8 T cell subsets (i.e., the correlates of protection); second, developing refined vaccination techniques to optimize production of the relevant subsets. As usual, the natural characteristics of the immune response (for example, the changes in memory populations induced by primary versus boosted immune responses) form a useful guide as to what can be achieved – but the pressing need is for vaccines against pathogens (such as HIV, malaria, tuberculosis) that elicit inefficient protective responses, hence radical strategies may be needed to achieve radical results.

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The smallest unit: effector and memory CD8$^+$ T cell differentiation on the single cell level

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CD8$^+$ T cell immune responses provide immediate protection against primary infection and durable memory capable of rapidly fighting off re-infection. Immediate protection and lasting memory are implemented by phenotypically and functionally distinct T cell subsets. While it is now widely accepted that these diverge from a common source of naïve T cells (T_n), the developmental relation and succession of effector and memory T cell subsets is still under intense debate. Recently, a distinct memory T cell subset has been suggested to possess stem cell-like features, sparking the hope to harness its capacity for self-renewal and diversification for successful therapy of chronic infections or malignant diseases. In this review we highlight current developmental models of memory generation, T cell subset diversification and T cell stemness. We discuss the importance of single cell monitoring techniques for adequately mapping these developmental processes and take a brief look at signaling components active in the putative stem cell-like memory T cell compartment.

Keywords: T memory stem cell, single cell resolution, single cell fate mapping, subset diversification, memory ontogeny

INTRODUCTION

In tissues with high cellular turnover, as for example in the epithelial layers of gut or skin, cells conveying the tissues major functional properties are constantly produced, mature and die (Creamer et al., 1961; Sun and Green, 1976; Celli et al., 2005). A general biological strategy for maintaining tissues subjected to such constant attrition is that of resupplying rapidly cycling short-lived cells from a source of long-lived, locally residing tissue stem cells (Simons and Clevers, 2011). Aside from their longevity, stem cells are characterized by the capacity to self-renew and in parallel generate a diverse offspring of short-lived cells for restocking the tissue's functional layers. Relatively short life spans of differentiated cells can also be observed for many branches of the hematopoietic system. Here, renewal processes can generally be tracked back to pluripotent bone marrow stem cells (Spangrude et al., 1988).

Naïve T cells (T_n) also originate ultimately from hematopoietic stem cells. They are however, over time disconnected from their hematopoietic ancestors by thymic involution, which after puberty hinders further maturation of marrow-borne T cell precursors in the thymus (Steinmann, 1986; Hale et al., 2006). Further on, T cell receptor (TCR) recombination outfits developing T cells with unique epitope-specific receptors. This process of "individualization," followed by some rounds of division, leaves a diverse TCR repertoire (Arstila et al., 1999; Casrouge et al., 2000). This repertoire is maintained, largely independent of hematopoietic precursors, by slow homeostatic turnover of naïve T cells (Jameson, 2002). While this steady state mode of homeostatic tissue maintenance lacks stem cell-like aspects of cell fate diversification, matters appear very different when naïve T cells are strained by infection or vaccination. Under these conditions, clonal T cell populations that recognize their cognate antigen expand vigorously and differentiate into various phenotypically and functionally distinct subsets (Williams and Bevan, 2007). This process can be described by analogy as the rapid outgrowth of an epitope-specific (mono- or oligoclonal) "tissue," whose short-lived layers are quickly lost after resolution of infection, while its long-lived ones serve as a source for quicker and stronger responses to re-infection.

In this review we compile our current knowledge concerning the development and relation of acute and memory CD8$^+$ T cell responses. A special focus is laid on the role of single cell fate mapping for adequately understanding these processes. In the first part we give a short overview on the diverse subsets present in the antigen-experienced T cell compartment and discuss how short-lived effector and long-lived memory T cells could arise from a limited number of epitope-specific naïve precursors. We continue by highlighting the importance of continuous observation and single cell resolution for an unambiguous evaluation of developmental pathways. In the last part we summarize current findings on stem cell-like signaling properties of distinct memory T cells and discuss further experimental routes for the evaluation of stemness in CD8$^+$ memory T cells.

MEMORY AND EFFECTOR SUBSETS

Upon contact with their cognate antigen, naïve epitope-specific $CD8^+$ T cells proliferate vigorously and differentiate to acquire phenotypic and functional properties that are key to resolving acute infection on the one hand and generating long lasting memory on the other hand. Initially it proved difficult to resolve whether these diverse properties are shared by all T cells responding to antigen challenge or are differentially assigned to distinct subsets (Dutton et al., 1998)—possibly even reserved to certain TCR specificities. The development of major histocompatibility complex (MHC) multimer technology (Altman et al., 1996) allowed for the first time to directly visualize endogenous antigen-specific $CD8^+$ T cell responses irrespective of their functional status and revealed that T cell populations harboring TCRs specific to different epitopes of the same pathogen appear to expand, contract and enter the memory phase with similar kinetics (Busch et al., 1998). Recent data show that even very low affinity TCR peptide-MHC complex interactions suffice to generate memory $CD8^+$ T cells, albeit after a weaker initial expansion (Zehn et al., 2009). Thus, largely independent of TCR specificity or affinity $CD8^+$ T cell responses show a conserved pattern of expansion, contraction and memory maintenance.

It further became clear that already during the expansion phase endogenous (polyclonal) and adoptively transferred (monoclonal) TCR-transgenic T cell populations undergo a process of phenotypic und functional diversification that is indicative of their capability to transit into memory (Williams and Bevan, 2007). In general, memory T cells are characterized by their capacity to receive signals for homeostatic maintenance from the common gamma chain cytokines interleukin 7 (IL7) and IL15 (Schluns and Lefrançois, 2003). Like naïve T cells they express the IL7 receptor alpha chain (CD127) (Huster et al., 2004). They however are less dependent on homeostatic signals received from self-MHCI molecules than are naïve $CD8^+$ T cells (Murali-Krishna et al., 1999) but more sensitive to IL15 through their expression of IL15 receptor alpha chain (CD122) (Schluns et al., 2002). While globally receptive to homeostatic cytokines IL7 and IL15, memory T cells can be further subdivided according to their capacity to migrate to secondary lymphoid organs and mount proliferative responses to re-infection (Sallusto et al., 1999). So-called "central memory T cells" (T_{cm}) express lymph node homing molecules L-Selectin (CD62L) and chemokine receptor CCR7 and mount strong proliferation in response to re-infection. "Effector memory T cells" (T_{em}) migrate to epithelial barriers and are capable of rapid effector function but only weak proliferation in response to antigen challenge (Masopust et al., 2001). By the time of peak primary expansion (usually around day 7 upon antigen challenge), the subdivision into $CD62L^+$ $CD127^+$ T_{cm} precursors, $CD62L^-$ $CD127^+$ T_{em} precursors and $CD62L^-$ $CD127^-$ effector T cells (T_{ef}) that die during the contraction phase, is already apparent (Kaech et al., 2003; Huster et al., 2004). Even earlier, still during the expansion phase, the surface molecule killer cell lektin-like receptor G1 can be used to distinguish short- and long-lived T cells (Joshi et al., 2007). An important functional characteristic of memory precursor T cells is their capacity to produce interleukin 2 (IL2) (Sarkar et al., 2008). Initially IL2 signals during priming were described as prerequisite for successful $CD8^+$ T cell recall expansion during memory (Williams et al., 2006). It was unclear however, whether the necessary IL2 was provided autocrine by $CD8^+$ T cells themselves or in a paracrine manner by $CD4^+$ helper T cells. Recently, it could be clarified that autocrine IL2 production during priming is a prerequisite for strong recall expansion of $CD8^+$ T cells (Feau et al., 2011). This finding is well in line with the observation that IL2 production during the primary response serves as an indicator of vaccination success or protectivity in $CD4^+$ (Darrah et al., 2007) and $CD8^+$ T cell responses (Harari et al., 2004; Betts et al., 2006). In this context it is important to emphasize that IL2 producing $CD8^+$ T cells are also capable of producing high amounts of effector cytokines like interferon γ (IFNγ) and tumor necrosis factor α (TNFα), creating a "per cell" pattern of cytokine production generally referred to as "multi-functionality" (Seder et al., 2008). While some aspects of the surface phenotype and cytokine receptivity of memory precursor T cells are clearly reminiscent of naïve T cells, transcriptional profiling (Holmes et al., 2005; Sarkar et al., 2008) and functional studies underline their developmental proximity to T_{ef}. A possible aspect resolving this ambivalence was provided by research showing that master transcription factors T box transcription factor expressed in T cells (Tbet) (Szabo et al., 2000) and Eomesodermin (Eomes) (Pearce et al., 2003), which are essential for the induction of the effector cytokine IFNγ also induce expression of CD122, thus conveying IL15-receptivity to cells that have acquired effector properties (Intlekofer et al., 2005). Further research showed that while both transcription factors act redundantly in inducing effector function they appear to show reciprocal effects on the long-term maintenance of memory T cells. While increased expression of Tbet preferentially induces differentiation into short-lived effector T cells (Joshi et al., 2007), the presence of Eomes appears to support homeostatic memory maintenance (Banerjee et al., 2010; Zhou et al., 2010). Interestingly, during chronic infection with Lymphocytic choriomeningitis virus (LCMV), so called exhausted T cells, lacking the capacity for prolonged proliferation in response to antigen, show increased Eomes, and lower Tbet expression than their proliferation competent predecessors (Paley et al., 2012). This might hint to the fact that successful memory generation and maintenance are not exclusively dependent on one of these transcription factors, but rather on a specific balance of the two. Taken together, the data mentioned in this section point toward T cells having to acquire at least some effector characteristics during their ontogeny to achieve "T cell fitness" (Gett et al., 2003)—that is fitness to receive homeostatic maintenance signals and survive the contraction phase (see **Figure 1**). However, effector differentiation and strong proliferation can also be detrimental for transition to memory (Joshi and Kaech, 2008). Further on, the role of certain transcription factors for memory maintenance appears to differ greatly depending on the specific immunological context of chronic vs. cleared infection (Doering et al., 2012). Overall, the ambivalent positioning of memory $CD8^+$ T cells in between effector and naïve states (Holmes et al., 2005) continues to yield controversy concerning their developmental path. This controversy focuses on two—currently unsolved—key conceptual questions: When during clonal expansion do long-lived memory and short-lived

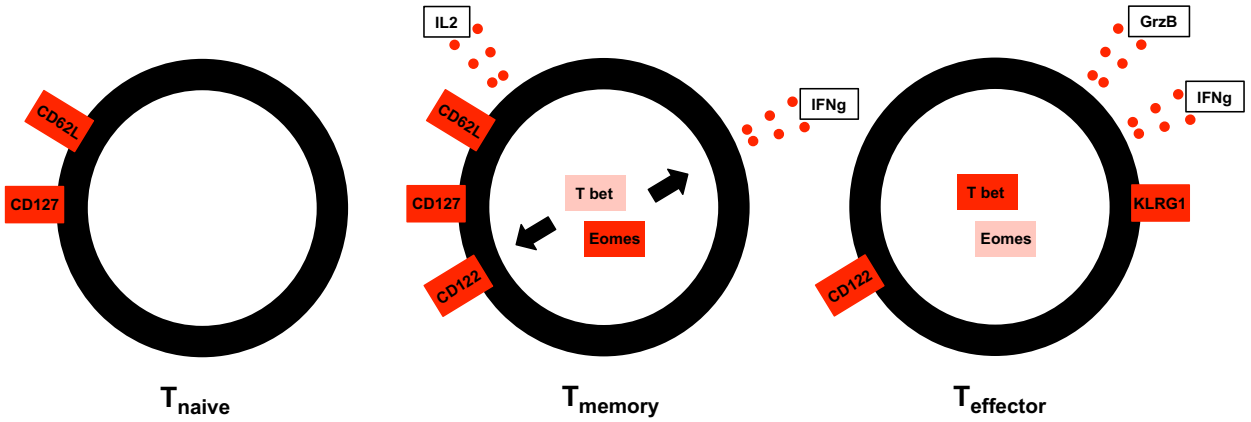

FIGURE 1 | Memory CD8+ T cells share traits of both the naïve and effector compartment. Like T_n, T_{cm} express lymph node homing molecule L-Selectin (CD62L) and IL7 receptor α chain (CD127) conveying recirculation capacity to secondary lymphoid organs and receptivity to homeostatic cytokine IL7. Like T_{ef}, T_{cm} express IL15 receptor β-chain (CD122), and effector cytokine IFNγ. Both molecules are induced by transcription factors Tbet and/or Eomes.

effector fates diverge? And, when are individual cells instructed to follow either one of these fates?

THE ROAD TO MEMORY

In response to recognition of their cognate antigen, naïve $CD8^+$ T cell populations expand and undergo phenotypic and functional diversification. Importantly, both processes occur in parallel, making it a difficult task to distinguish quantitative (cell proliferation or cell death) from qualitative changes (cell differentiation) as driving forces for distinct subset abundance. Thus, the dominance of phenotype A at an earlier time point of the immune response and that of phenotype B at a later one does not necessarily imply that cells of phenotype A have differentiated to B (see **Figure 2**). In order to elucidate the developmental path of a $CD8^+$ T cell, the ultimate goal would be to monitor all the interaction, division and differentiation events that a single naïve T cell and its ancestors have experienced. While current approaches are still far away from achieving this total documentation of T cell history, some crucial insights have been gained using a variety of innovative technologies (Schumacher et al., 2010). Foremost, in order to discriminate global changes in population phenotype from the phenotypic segregation of subsets, technologies providing single cell resolution are warranted (see **Figure 3**). Flow cytometry or fluorescence microscopy both fulfill the prerequisite of single cell resolution and can routinely be used to investigate the expression of 10–15 different molecules per cell (Perfetto et al., 2004). The number of different markers that can simultaneously be detected by staining with fluorochrome-labeled antibodies is however, intrinsically limited due to the spectral overlap of excitation and emission spectra. This restriction has recently been considerably alleviated by a novel approach combining metal-labeled probes and mass spectrometry analysis. Here, heavy metal isotopes are used to label monoclonal antibodies and labeled cells are analyzed (with single cell resolution) for expression of 36 (theoretically up to 100) molecules by a combination of flow cytometry and mass spectrometry (Bendall et al., 2011). This so-called cytometry by time-of-flight approach

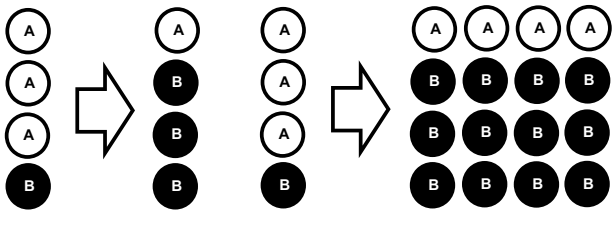

FIGURE 2 | Parallel proliferation hinders delineation of quantitative and qualitative changes in subset abundance. Left panel: In the absence of proliferation or cell death, a change in subset phenotype from A to B can be inferred from the observation. **Right panel:** In the presence of proliferation (and/or cell death) this inference is not valid.

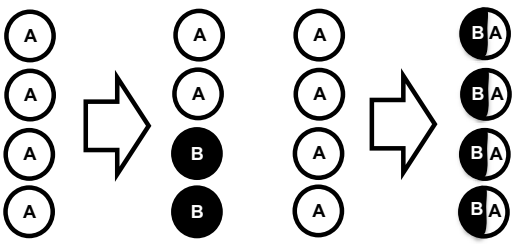

FIGURE 3 | Single cell resolution required to identify subset segregation. Left panel: Global phenotypic composition changes (from 100% A to 50% A and 50% B) due to emergence of a subset expressing marker B instead of A. **Right panel:** Global phenotypic composition changes due to global change in expression of A and B.

provides a wealth of data that emphasizes the heterogeneous phenotypic and functional composition of epitope-specific T cell populations. While confirming previously defined $CD8^+$ T cell subsets (T_n, T_{cm}, T_{em}, and T_{ef}) its major contribution to elucidating $CD8^+$ T cell ontogeny is the definition of transitional states that lie in between major subsets and that connect T_n to T_{cm}, T_{cm} to T_{em}, and T_{em} to T_{ef} (Newell et al., 2012). While

this observation is congruent with a progressive differentiation from naïve to memory to terminally differentiated T_{ef}, it has to be emphasized that these data were gathered in subjects facing chronic and not acute infection. Thus, drawing conclusions concerning the developmental path from naïve to memory CD8+ T cells appears difficult. Further on, identifying transitional states as "missing links" situated on proposed developmental trajectories can only be a supplementary strategy to actually monitoring cells during this transition. Genetic approaches to "follow" CD8+ T cells of a certain phenotype or developmental state throughout their further developmental history, were pioneered by Baltimore and colleagues (Jacob and Baltimore, 1999). Recently, an even more stringently designed transgenic mouse model, linking the transient expression of effector molecule granzyme B (GrzB) to the permanent expression of enhanced yellow fluorescent protein (EYFP), has been developed (Bannard et al., 2009). While GrzB expression was found to be absent from epitope-specific memory CD8+ T cells at 7 weeks post infection with influenza virus, EYFP was readily detectable in a substantial fraction of the same cells. These memory T cells thus had passed through a state of effector functionality before reaching their GrzB− memory state. According to these genetic single cell fate mapping data, memory T cells do not bypass effector differentiation completely. This is consistent with observations showing expression of GrzB by nearly all CD8+ T cells 2.5 days after acute LCMV infection (Sarkar et al., 2008), and with observations mentioned above that show a coupling of IL15 receptivity and effector cytokine IFNγ inducing transcription factors Tbet and Eomes. However, acquisition of effector function cannot be equated with induction of excessive proliferation and loss of longevity. Therefore, a key issue waiting to be resolved is to what end the precursors of memory take part in the massive proliferative expansion characteristic of an acute immune response (see **Figure 4**).

THE LONE TRAVELLER

In order to adhere to the stem cell analogy introduced in the first paragraph of this review, naïve CD8+ T cells are not only required to generate diverse functional subsets and self-renew, but these capabilities should in principal converge within an individual precursor cell capable of generating a complete "epitope specific tissue." For the hematopoietic system this capacity for tissue regeneration was shown in a pioneering study, in which the transfer of a single hematopoietic stem cell sufficed to repopulate diverse hematopoietic lineages after myeloablative treatment (Osawa et al., 1996). In order to investigate the developmental path of an individual T cell and its progeny under physiological conditions in immunocompetent hosts, two general approaches can be envisioned (see **Figure 5**): First, continuous observation and second, individualization by heritable markers (Stemberger et al., 2009; Buchholz et al., 2012). The obvious choice for continuously observing interaction, differentiation and proliferation of single cells and their daughters is intravital microscopy. Two-photon live imaging increased our knowledge concerning the initial events of single T cell development considerably (Henrickson and von Andrian, 2007). However, not every organ can be visualized equally well. Therefore, most insights gained by *in vivo*

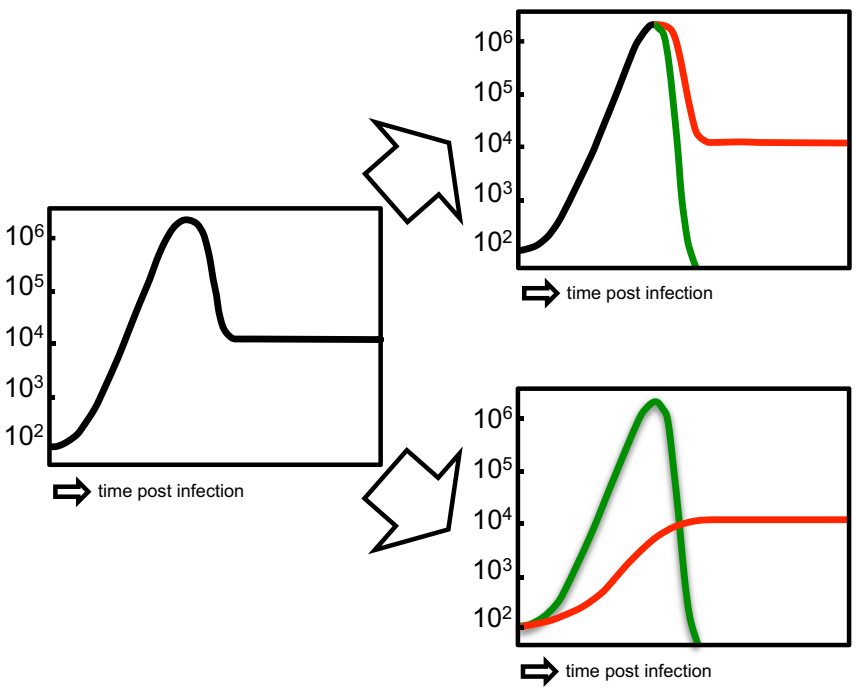

FIGURE 4 | Alternative proliferative histories of memory T cells. Left panel: Overall primary expansion, contraction and memory maintenance of an epitope-specific T cell population. **Upper right panel:** The precursors of short-lived and long-lived subsets both contribute equally to primary expansion ("shared proliferative history"). **Lower right panel:** The precursors of long-lived subsets do not substantially contribute to primary expansion ("distinct proliferative history").

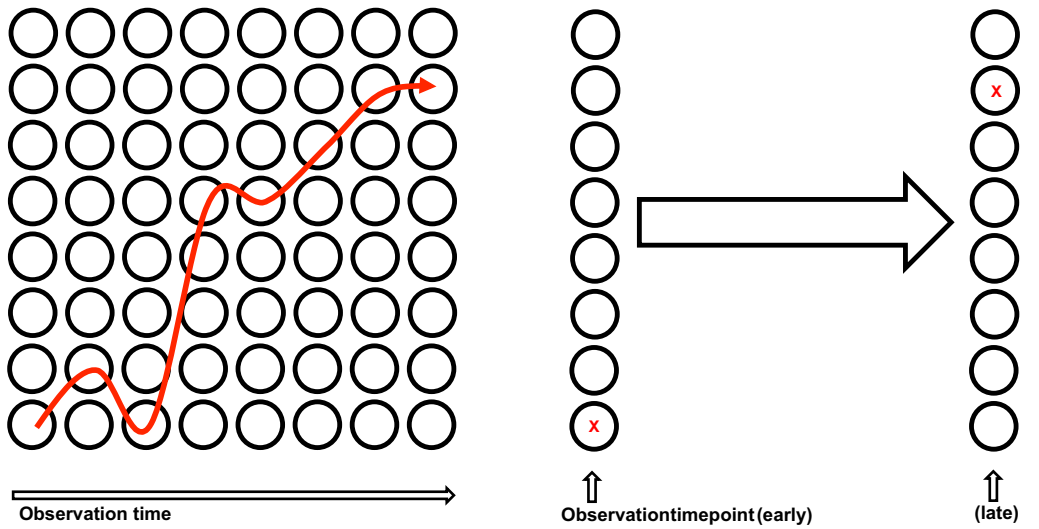

FIGURE 5 | Single cell fate mapping. Left panel: Continuous monitoring of an individual cell (→). **Right panel:** Tagging of an individual cell by a heritable marker (X).

microscopy are currently based on studying immune reactions in lymph nodes draining the site of infection (Stoll et al., 2002). Here, quite representative tissue volumes can be analyzed. Three phases of T cell activation could be defined by this technique. "Phase 1" is characterized by transient contacts of antigen-specific T cells with their cognate peptide presented on MHC-complexes of dendritic cells (DCs). During this phase activation markers like CD44 and CD69 are already up-regulated by responding T cells. "Phase 2" is then marked by stable interactions in between T cells and DCs and coincides with the first production of cytokines. During "phase 3" transient contacts prevail again and T cells begin to divide (Mempel et al., 2004). It could be shown that increased peptide MHC complex density on DCs as well as increased numbers of peptide loaded DCs and higher peptide-TCR affinity shorten "phase 1" considerably and lead to a more rapid establishment of stable contacts (Henrickson et al., 2008). These data together with recent imaging studies implicate that after accumulating a certain amount of signal strength T cells are programmed for a defined developmental fate and then undergo proliferation (Beuneu et al., 2010; Moreau et al., 2012). This mode of signal integration (before proliferation) suggests a homogenous response of the progeny of a single T cell. A study applying multiple waves of antigen-presenting DCs could however show that further signal integration during the process of clonal expansion is possible (Celli et al., 2005). Another stem cell related mechanism of T cell diversification was first described by Reiner and colleagues. Here, the first cell division of *in vivo* activated T cells was imaged (Chang et al., 2007). Strikingly, it became apparent that T cell contacts with antigen presenting cells can lead to an asymmetric distribution of key components of the immunological synapse. After division this uneven distribution is thought to yield two daughter T cells that carry unequal amounts of defined signaling molecules and are fated to generate either short-lived effector (proximal daughter) or long-lived memory T cell progeny (distal daughter). This process has recently also been suggested to occur in memory T cells re-exposed to their cognate antigen (Ciocca et al., 2012) and is thought to be based at least in part on the asymmetric degradation of transcription factors due to the uneven concentration of the protein degradation machinery in one of the daughter cells (Chang et al., 2011). Moreover, asymmetric division was suggested to occur especially in the case of high affinity peptide TCR interaction, while low affinity interactions were biased for symmetric generation of "distal" memory fated daughters (King et al., 2012). These data implicate that a single T cell should be able to generate both effector and memory progeny and that the relative distribution of offspring onto these subsets is determined by the modes of division. However, formal proof for the importance of this partitioning mechanism for subset diversification and stem cell-like capacity of naïve and memory T cells is still lacking. It would require selective means of hindering asymmetric division while leaving other components of the immune response (e.g., peptide density, DC-T cell ratio, or peptide-TCR affinity) unchanged. A possible option to achieve this might be through interference with the orientation and positioning of the division plane as recently explored for the earliest divisions in embryonic development of *Caenorhabditis elegans* (Galli et al., 2011). Following the dynamic differentiation and proliferation process of single T cells via intravital microscopy is intrinsically limited by the volume of tissue monitored and the limited duration of observation. However, comprehensive single cell fate mapping beyond the earliest events of the immune response is possible by individualization. Two pioneering approaches have shed light on the diversification process of progeny originating from individual T cells during the expansion phase. The first approach truly visualizing the diversification potential that is inherent to a single naïve T cell used adoptive transfer of single naïve T cells outfitted with a heritable congenic marker to allow *in vivo* analysis of the

diversification potential of individual T cells (Stemberger et al., 2007). Progeny generated from single precursor cells after infection with *Listeria monocytogenes* was here detected at the peak of clonal expansion by high sensitivity flow cytometry and analyzed for the expression of phenotypic markers (CD62L and CD127) and functional capacity (secretion of IFNγ, TNFα, and IL2). This study not only proved that it is technically feasible to identify single cell-derived progeny in a physiological animal model of acute infection, it also showed that the full diversity of effector and memory fates can originate from a single precursor cell. This obviously is at odds with the notion that T cell fate is determined during or even before the first cell division and rather hints to a continuous process of fate changing events acting upon the expanding progeny of a single naïve T cell. Importantly, a single cell-derived progeny can contain effector and memory subsets in parallel, thus adhering to the notion of stem cell-like capacity for diversification and self-renewal (Stemberger et al., 2009). Another study using the principal of heritably marking individual participants in an immune response is that of Schumacher and colleagues. The authors used an elegant method of integrating unique genetic tags ("barcodes") into the genome of individual T cells and then measuring barcode abundance in progeny that develops during an *in vivo* immune response (Schepers et al., 2008). Drawing on a large library of unique barcodes (around 3000) this approach holds the obvious advantage of tracking hundreds of single cell-derived populations within one host. To date however, this approach is not capable of investigating phenotypic diversification within one single cell-derived progeny. When focusing on evaluating barcode abundance in progeny recovered from different organs at different times after acute infection, it was found that progeny originating from individual cells is capable of acquiring diverse homing patterns. Interestingly, effector and memory phase progenies gathered in the same experimental animals early and late after infection showed a similar barcode composition, which is again supportive of the idea that single naïve T cells have a stem cell-like potential for phenotypic, functional, and migratory diversification and can generate progeny both for the short and long-lived pool of antigen-experienced T cells (Gerlach et al., 2010). In the last part of this review, we will explore the similarities in between certain T cell subsets and stem cells by taking a closer look at shared traits on transcriptional and signaling level.

STEM CELL-LIKE INSIDE?

Compared to other antigen-experienced subsets, T_{cm} show enhanced longevity, self-renewal capacity, and proliferative potential as well as potency to generate T_{ef} and T_{em} cells. However, recent data suggest that only a small subset within the T_{cm} compartment is equipped with truly stem cell-like characteristics.

Several recent studies have been devoted on identifying parallels in between the subcellular organization of memory T cells and stem cells. The overexpression of multidrug efflux proteins of the ATP-binding cassette (ABC) superfamily in hematopoietic stem cells is—besides cell quiescence—one of the mechanisms mediating their resistance to cytotoxic drugs (Chaudhary and Roninson, 1991; Mizutani et al., 2008). An ABCB1-overexpressing $CD161^+$ $IL18R\beta^+$ $cKit^+$ putative memory T cell subset was described in humans. This subset selectively survived chemotherapy and showed enhanced proliferative activity in a lymphopenic environment (Turtle et al., 2009). However, broader phenotypic characterization identified predominantly $V\alpha7.2^+$ IL17-secreting mucosa-associated invariant T cells among the ABCB1 expressing T cells, arguing against the idea of $CD161^+$ $IL18R\beta^+$ $cKit^+$ T cells being a less differentiated, stem cell-like reservoir of adaptive memory T cells (Dusseaux et al., 2011; Turtle et al., 2011; Havenith et al., 2012). Nonetheless, IL17-secreting subsets of $CD4^+$ and $CD8^+$ T cells appear to share some signaling pathways with stem cells: Signal transducer and activator of transcription 3 (STAT3) together with SMAD (human homolog of MAD "mothers against decapentaplegic" and SMA "small body size" protein) signaling mediates the polarization of $CD4^+$ and $CD8^+$ T cells toward IL17-secreting subsets and is also known to be active in stem cells. Interestingly, activation of STAT3 via IL21 suppresses terminal differentiation and exhaustion of T cells, highlighting its importance for sustained immune competence in the face of chronic infections (Leonard and Spolski, 2005; Li et al., 2005; Zeng et al., 2005; Fröhlich et al., 2009; Yi et al., 2009).

Further parallels in between memory T cells and tissue-specific stem cells were identified by a deeper investigation into shared transcriptional programs (Luckey et al., 2006; Fleming et al., 2008; Staal et al., 2008). In this context it was found that several molecular pathways found in stem cells, like Wnt/β-catenin, SMAD, STAT3, and forkhead box O (FOXO) signaling, are also active in T cells. These pathways appear to guide the generation of memory T cells through conserving their longevity, quiescence and self-renewal capacity (Betz and Müller, 1998; Castellino and Germain, 2007; Hand et al., 2010; Rao et al., 2010, 2012; Cui et al., 2011; Ji et al., 2011; Siegel et al., 2011; Yang et al., 2011; Kim et al., 2012; Thaventhiran et al., 2012).

The transcription factors lymphoid enhancer-binding factor 1 (LEF-1) and T cell factor-1 (TCF-1) are downstream targets of the Wnt pathway and are essential for a normal thymic maturation of naive T cells (Verbeek et al., 1995; Staal et al., 2008). The influence of active Wnt signaling on mature T cell differentiation came into focus as LEF-1 and TCF-1 are down-regulated upon T cell activation and are expressed in decreasing order in $T_n \rightarrow T_{cm} \rightarrow T_{em}$ subsets (Willinger et al., 2006; Gattinoni et al., 2009). Taken together proliferative activity, long-term survival and recall potential are affected by downstream targets of the Wnt/β catenin axis (Jeannet et al., 2010; Zhao et al., 2010; Zhou et al., 2010; Muralidharan et al., 2011; Xue and Zhao, 2012).

The enforced generation of less differentiated memory subset by the induction of Wnt signaling during priming and differentiation of naïve T cells (Zhang et al., 2005; Gattinoni et al., 2009) fueled further research into identifying such a stem cell-like undifferentiated subset under physiological conditions *in vivo*. Antigen-experienced cells with a surface phenotype characteristic for the naïve T cell compartment ($CD45RA^+$ $CD62L^+$ $CCR7^+$ $CD27^+$ $CD28^+$ $IL7Ra^+$) that in parallel express molecules associated with effector/memory T cell differentiation (IL2Rb, CXCR3, and CD95) were recently identified in human subjects (Gattinoni et al., 2011). Phenotypic and functional studies suggest enhanced self-renewal capacity and repopulation potential of these so called

"T memory stem cells" (T_{scm}) and propose them as an intermediate differentiation stage in between T_n and T_{cm} (Zhang et al., 2005; Gattinoni et al., 2011, 2009).

In normal homeostasis the pool of T_{scm} cells is believed to comprise a small fraction of 2–3% of all circulating T lymphocytes in human and non-human primates (Lugli et al., 2012). Their phenotype is especially enriched in antigen-specific T cell populations during the acute and chronic phases of immune responses as well as in resting memory (Gattinoni et al., 2011; Cieri et al., 2013). Furthermore, T_{scm} cells specifically accumulate in lymphopenic environments and during *in vitro* culture with a high availability of γ-chain cytokines IL2, IL7, IL15, and IL21 that control the homeostatic turnover of memory T cells (Zhang et al., 2005; Cieri et al., 2013). These observations suggest that instructive and permissive environmental signals provided by growth factors and cytokines, perhaps at site-specific niches, limit the size, and the stem cell-like potential of the memory T cell pool (Jiang et al., 2005; Li et al., 2005; Zeng et al., 2005; Mazzucchelli and Durum, 2007; Morrison and Spradling, 2008).

Possibly these stem cell-like memory T cells could provide a basic principle behind long-term memory maintenance, acting as a tiny reservoir of quiescent, long-lived cells that compensates for the continuous loss of more differentiated effector and memory T cells. However, this principle has never been experimentally proven. Strong T cell activation due to repetitive antigen encounter or chronic inflammation is usually associated with enhanced differentiation, exhaustion and induction of senescence (Wherry et al., 2003; Gattinoni et al., 2005; Appay et al., 2008; Sallusto et al., 2010; Wirth et al., 2010). Thus, a preservation of the regenerative potential within antigen-specific T cell populations by self-renewing, quiescent T_{scm} cells appears exceedingly important for maintaining immune responses against recurrent and chronic infections as well as malignancies. Providing optimally self-renewing T cells by distinct vaccination strategies or selective *in vitro* expansion of T cells out of patient material (Lugli et al., 2012), appears to open new avenues for immunological cell therapy approaches. Especially therapy of malignant diseases could be supported substantially by a self-renewing cellular source providing continuous anti-tumor activity (Gattinoni et al., 2012).

However, until today, only dynamics and properties of populations derived from the designated T_{scm} phenotype have been studied. The behavior and attributes of individual T_{scm} cells remain elusive and no conclusion can be drawn whether self-renewal capacity and multipotency are truly conjoint on the single cell level. A microscopic approach of continuous observation and single cell tracking in long-term cell culture, has proven very successful analyzing lineage development in the hematopoietic system; it could however, be difficult to adapt to the complex nature of *in vivo* T cell responses (Eilken et al., 2009). Adoptive *in vivo* transfer of single cells has already been successfully used to demonstrate that long-term reconstitution of the lymphohematopoietic system can be achieved by single hematopoietic stem cells (Osawa et al., 1996). Similarly, adoptive transfers of single naïve CD8+ T cells proved the multipotent differentiation potential of individual naïve T cells (Stemberger et al., 2007). A similar strategy could be implemented to study self-renewal and multipotency of memory T cells on the single cell level. Challenging the T_{scm} paradigm on the single cell level could contribute substantially to revealing how and to what extent stem cell-like regenerative potential is feeding the pool of memory T cells throughout life.

CONCLUSION

New technologies allowing single cell *in vivo* fate mapping have started to provide novel insights into the diversification process from few naïve antigen-specific T cells into a spectrum of long- and short-living effector and memory T cell subsets. These studies suggest that subset diversity can even be generated from single naïve precursor cells and that this process provides in parallel to highly differentiated effectors a subset of T cells, which seems to resemble the differentiation plasticity of naïve T cells, so-called memory stem cell (T_{scm}). However, physiological T cell responses are believed to be composed by the recruitment of multiple naïve precursor T cells. It is currently unknown whether under these conditions all recruited T cells follow the same diversification pattern or whether they differ on the single cell level. In addition, although the concept of the presence of a unique memory T cell subset with stem cell-like characteristics is intriguing, it still remains elusive to experimentally demonstrate that self-renewal capacity and multipotency can truly be conjoint on the single cell level. Although future studies are still necessary to further prove the novel concept of T cell subset diversification as well as memory T cell generation and maintenance, it is already obvious that these insights will have important implications for the improvement of active (vaccination) and passive (adoptive T cell transfer) immunotherapies for the treatment of infections, defined cancers and autoimmune disease.

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Local immunity by tissue-resident CD8$^+$ memory T cells

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Microbial infection primes a CD8$^+$ cytotoxic T cell response that gives rise to a long-lived population of circulating memory cells able to provide protection against systemic reinfection. Despite this, effective CD8$^+$ T cell surveillance of barrier tissues such as skin and mucosa typically wanes with time, resulting in limited T cell-mediated protection in these peripheral tissues. However, recent evidence suggests that a specialized subset of CD103$^+$ memory T cells can permanently lodge and persist in peripheral tissues, and that these cells can compensate for the loss of peripheral immune surveillance by circulating memory T cells. Here, we review evolving concepts regarding the generation and long-term persistence of these tissue-resident memory T cells (T_{RM}) in epithelial and neuronal tissues. We further discuss the role of T_{RM} cells in local infection control and their contribution to localized immune phenomena, in both mice and humans.

Keywords: T cell, memory, protection, periphery, virus infection

INTRODUCTION

Primary infection results in immunity against reencounter with the same pathogen. This anamnestic "immune memory" builds the conceptual basis for vaccination and relies on different types of adaptive memory cells including B cells and T cells (Welsh et al., 2004). While almost all current vaccines target B cells for the induction of antibody-mediated immunity, this approach has significant limitations for a number of infectious diseases including AIDS, tuberculosis, and malaria (Seder and Hill, 2000). The relative inefficiency of humoral immunity in these cases is related to various evasion strategies employed by the pathogens, including their rapid variation of exposed antigens and their intracellular localization. In addition, serum antibodies have only limited access to some epithelial compartments under non-inflammatory conditions and as a consequence, may fail to provide the heightened level of immediate immune control required to prevent the establishment and persistence of peripheral infections (Zinkernagel, 2002). Thus, protection from certain pathogens relies on potent T cell-dependent cellular immune responses (Seder and Hill, 2000). Understanding the generation and maintenance of such T cell responses, particularly those in barrier locations at body surfaces, is of critical importance for the development of future vaccines.

CIRCULATING CD8$^+$ MEMORY T CELLS

The activation of pathogen-specific T cells in lymphoid tissues draining the site of infection results in the generation of a large pool of effector cells (Jameson and Masopust, 2009). During activation, these cells acquire the expression of certain migration receptors that allow them to infiltrate peripheral tissues where they contribute to pathogen clearance through the elimination of infected cells and the release of proinflammatory and microbicidal mediators (Harty et al., 2000; Nolz et al., 2011). Interestingly, the organ-specific microenvironments in lymphoid tissues can preferentially support the induction of migration receptors required for T cell entry specifically into those tissues that are connected to the respective lymphoid priming sites (Agace, 2006; Nolz et al., 2011). Such organ-specific migration imprinting is best documented for barrier tissues such as skin and gut and involves the induction of ligands for E- and P-selectin as well as the integrin α4β7 and the chemokine receptor CCR9, respectively (Parrott et al., 1976; Guy-Grand et al., 1978; Campbell and Butcher, 2002; Mora et al., 2005; Agace, 2006; Nolz et al., 2011). It should be noted, however, that migration imprinting is by no means absolute and that substantial numbers of effector CD8$^+$ T cells disperse into a broad variety of peripheral tissues even after localized peripheral infections (Marshall et al., 2001; Masopust et al., 2004).

While the vast majority of effector cells disappear from the circulation following pathogen clearance, there remains a heterogeneous population of long-lived memory cells capable of mounting rapid recall responses on antigen reencounter (Jameson and Masopust, 2009). Memory T cells are commonly grouped into two major subsets based on their functional status and expression of certain homing receptors: (1) central memory T cells (T_{CM}) that express the lymph node-targeting molecules CD62L and CCR7, and (2) effector memory T cells (T_{EM}) that largely lack these receptors, but instead express certain migration molecules that target them to peripheral non-lymphoid tissues (Sallusto et al., 1999, 2004). This delineation between the two subsets, as initially defined for human blood cells (Sallusto et al., 1999), is now also widely used to define migratory abilities and the anatomical localization of memory subsets in other species (Seder and Ahmed, 2003).

Early migration studies employing adoptive transfer and lymph canulation experiments in sheep, described memory T cells as constantly recirculating cells that patrol through peripheral tissues including the skin and intestinal mucosa (Gowans and Knight, 1964; Cahill et al., 1977; Mackay et al., 1990, 1992). These seminal studies along with more recent work using parabiotic

systems in mice, have led to the concept that blood-borne memory cells frequently exchange with their counterparts in lymphoid and most extra-lymphoid or peripheral tissues (Butcher and Picker, 1996; Von Andrian and Mackay, 2000), although exceptions for some epithelial and neuronal tissues with limited exchange between blood and tissue cells have been noted (Klonowski et al., 2004). Consistent with the subset-specific expression of migration receptors, peripheral tissues almost exclusively contain CD62L$^-$ cells which, at face value, appear to fit into the T_{EM} category (Masopust et al., 2004). Lymph nodes however, harbor both CD62L$^+$ and CD62L$^-$ memory cells, albeit showing a strong bias toward T_{CM} cells (Sprent and Surh, 2002) that are likely to have entered the lymph nodes from the blood by extravasating through high endothelial venules in a CD62L- and CCR7-dependent fashion (Gallatin et al., 1983; Warnock et al., 1998; Forster et al., 1999). The minor fraction of CD62L$^-$ cells found in lymph nodes instead may be comprised of classical T_{EM} cells that return from peripheral tissues via afferent lymphatics (Mackay et al., 1990, 1992).

Interestingly, while CCR7 is critically involved in T cell homing to lymph nodes via high endothelial venules (Forster et al., 1999), its expression is also required for efficient T cell entry into afferent lymphatic vessels in peripheral tissues (Bromley et al., 2005; Debes et al., 2005). This raises the important question as to whether peripheral T_{EM} cells dynamically regulate CCR7 expression during tissue exit and/or whether there may exist distinct populations of peripheral T cells with varying abilities to return to the circulation depending on their expression levels of CCR7 and their responsiveness to locally produced chemokines (Bromley et al., 2005). Regardless, it appears that the delineation of T cell memory into T_{CM} and T_{EM} subsets based on CD62L and CCR7 expression, may not reflect the full complexity of chemokine receptor usage in T cell recirculation that has now emerged from animal studies. Furthermore, recently described populations of non-migratory memory T cells in peripheral tissues that exist in disequilibrium with the circulating T cell pool, do not easily fit this widely used classification of T cell memory subsets.

IMMUNE PROTECTION BY CIRCULATING CD8$^+$ MEMORY T CELLS

Memory T cells differ from their naïve counterparts by their persistence at elevated frequencies, their broader anatomical distribution and their ability to rapidly acquire effector functions upon reactivation (Kaech et al., 2002). As a consequence, they are regarded as powerful mediators of immune protection from reinfection. In keeping with this, protective immunity by CD8$^+$ memory T cells has been demonstrated in various experimental models of systemic infection, such as *Listeria monocytogenes*, lymphocytic choriomeningitis virus (LCMV), and the malaria parasite *Plasmodium berghei* (Bachmann et al., 1997, 2005; Wherry et al., 2003; Badovinac et al., 2005; Huster et al., 2006; Schmidt et al., 2008). Importantly, T cell immunity in these cases of systemic infection is long-lived and can be conferred to naïve animals by the adoptive transfer of pathogen-specific CD8$^+$ memory T cells (Lau et al., 1994). Thus, it appears that persisting antigen is not required for the maintenance of functional CD8$^+$ T cell memory that is able to control lymphoid replication and systemic dissemination of various pathogens.

MEMORY T CELL MIGRATION AND PERIPHERAL IMMUNE SURVEILLANCE

In contrast to their role in immune protection from systemic infections, the ability of circulating CD8$^+$ memory T cells to deal with localized infections in the periphery is surprisingly limited (Bachmann et al., 1997, 2005; Jiang et al., 2012; Mackay et al., 2012a). This lack of peripheral protection by circulating memory T cells, seen across a variety of models, is best explained by their progressive loss of peripheral migration abilities (Kundig et al., 1996; Bachmann et al., 1997; Woodland and Kohlmeier, 2009). Consistent with this, effector or early memory T cells in the circulation gradually lose expression of homing molecules required to enter peripheral tissues (Masopust et al., 2010; Gebhardt et al., 2011). In addition, the population of circulating T_{EM} cells appears to decline over time, resulting in a progressive conversion of the memory cell pool toward a CD62L$^+$ T_{CM} phenotype (Tripp et al., 1995; Wherry et al., 2003). Collectively, these changes result in a skewing of the overall pattern of memory T cell recirculation from peripheral, non-lymphoid organs early after infection toward secondary lymphoid organs in the absence of continued antigenic stimulation (Gebhardt et al., 2011; Yang et al., 2011). Interestingly, this shift in migratory preference is most pronounced in the CD8$^+$ T cell subset, whereas CD4$^+$ T cells appear to retain their ability to migrate through peripheral tissues including the skin and intestinal mucosa, for prolonged periods of time (Gebhardt et al., 2011; Yang et al., 2011). In line with this, CD4$^+$ memory T cells predominate among the population of peripheral cells returning to the circulation via afferent lymphatics in both sheep and humans (Mackay et al., 1990; Yawalkar et al., 2003). Taken together, it appears that continuous antigenic stimulation is essential for optimal migratory immune surveillance by CD8$^+$ memory T cells, but not for their CD4$^+$ counterparts. This requirement obviously poses significant challenges for the development of future vaccines targeting CD8$^+$ T cell memory. Along these lines, recent studies have shown that ongoing T cell activation by persisting vaccine vectors is necessary for long-lived mucosal immunity against SIV infection in non-human primates (Hansen et al., 2009, 2011), highlighting the requirement for persisting antigenic stimulation to sustain ongoing peripheral CD8$^+$ T cell surveillance.

PERMANENTLY TISSUE-RESIDENT CD8$^+$ MEMORY T (T_{RM}) CELLS

Despite the progressive loss of circulating T_{EM} cells with peripheral migration abilities, extra-lymphoid tissues including the intestinal and vaginal mucosa, skin, brain, salivary glands, and several others can harbor substantial populations of long-lived pathogen-specific CD8$^+$ memory T cells (**Table 1**) (Bevan, 2011; Sheridan and Lefrancois, 2011; Ariotti et al., 2012). Of note, such cells are often localized to epithelial or neuronal tissues, meaning that their access to lymphatic vessels, located in the respective non-epithelial or non-neuronal compartments, is restricted by

Table 1 | Examples of mouse models describing peripheral CD8$^+$ memory T cells that fit the recent definition of CD103$^+$ T_{RM} cells.

Category	Mode of lodgment	Location of T_{RM} cells	References
Virus infection	Influenza virus infection (i.c.)	Brain	Hawke et al., 1998
	Lymphocytic choriomeningitis virus infection	Intestinal and vaginal mucosa, brain, kidney, gastric mucosa, pancreas, and salivary glands	Masopust et al., 2006, 2010; Hofmann and Pircher, 2011; Casey et al., 2012
	Vesicular stomatitis virus infection	Brain, salivary glands, and intestinal mucosa	Masopust et al., 2004; Klonowski et al., 2004; Wakim et al., 2010; Hofmann and Pircher, 2011
	Herpes simplex virus infection	Skin, dorsal root ganglia, and vaginal mucosa	Gebhardt et al., 2009, 2011; Tang and Rosenthal, 2010
	Vaccinia virus infection	Skin and intestinal mucosa	Isakov et al., 2009; Jiang et al., 2012
Non-specific local inflammation	DNFB treatment	Skin	Mackay et al., 2012a
	Nonoxynol-9 treatment	Vaginal mucosa	Mackay et al., 2012a
Local antigen presentation	Intracranial injection of peptide-pulsed DC	Brain	Wakim et al., 2010
Non-specific T cell activation	Lymphopenia-driven T cell activation	Intestinal and vaginal mucosa, brain, kidney, gastric mucosa, pancreas, and salivary glands	Casey et al., 2012

Abbreviations: i.c., intracranial; DNFB, 1-fluoro-2,4-dinitrobenzene; DC, dendritic cells.

structural barriers such as basement membranes and the blood brain barrier, respectively. A common feature of CD8$^+$ memory T cells in epithelial and neuronal tissues is their expression of high levels of CD103, the α-chain of the $\alpha_E\beta_7$ integrin, CD49a, the α-chain of the $\alpha_1\beta_1$ integrin [very late antigen (VLA-1)], and CD69, a surface molecule typically found on recently activated T cells. Interestingly, dendritic epidermal γδ T cells (DETC) in mouse skin, as well as other types of non-conventional T cells in epithelial tissues also express high levels of CD103 and CD69 (Ibraghimov and Lynch, 1994; Lefrancois et al., 1994; Jameson et al., 2004). This suggests that microenvironmental factors in these niches may be responsible for the sustained expression of high levels of these molecules on T cells. While naïve CD8$^+$ T cells express intermediate levels of CD103, effector and resting memory T cells in the circulation lack expression of both CD103 and CD69 (Lefrancois et al., 1994; Masopust et al., 2006; Gebhardt et al., 2009). This noticeable phenotypic difference led to the hypothesis that circulating and peripheral memory T cells may represent distinct populations that show minimal, if any, intermixing with each other (Ibraghimov and Lynch, 1994; Masopust and Lefrancois, 2003). As discussed below, there is now compelling evidence that peripheral CD8$^+$ CD103$^+$ memory T cells indeed represent a non-migratory population of cells that are maintained without replenishment from the circulating memory T cell pool. Emphasizing these unique features, these cells are now widely referred to as tissue-resident memory T (T_{RM}) cells (Bevan, 2011; Di Meglio et al., 2011; Sheridan and Lefrancois, 2011; Ariotti et al., 2012).

ANIMAL MODELS DESCRIBING CD103$^+$ T_{RM} CELLS

Skin infection with herpes simplex virus (HSV)-1 generates a population of CD8$^+$ CD103$^+$ T_{RM} cells in the epidermal layer of the skin that persist at elevated frequencies for more than a year after initial lodgment. Interestingly, these cells are concentrated at sites of previous infection or inflammation, while remote areas of skin harbor only low numbers of these cells (**Figure 1A**) (Gebhardt et al., 2009; Mackay et al., 2012a). Intravital imaging experiments have demonstrated that epidermal T_{RM} cells have an irregular shape with a dendritic morphology (Gebhardt et al., 2011) and thereby resemble other immune cell types present in the same location including DETC and Langerhans cells. Consistent with their site-specific accumulation and persistence, epidermal T_{RM} cells show only very restricted local migration and minimal displacement over time. Notably, this behavior is in stark contrast to the rapid movement seen for CD4$^+$ memory T cells which, in contrast to CD8$^+$ T cells, mainly localize to the dermal layer of the skin (Gebhardt et al., 2011).

Similar populations of persisting CD8$^+$ T_{RM} cells have been described following virus infection of the brain and submandibular glands (Hawke et al., 1998; Wakim et al., 2010; Hofmann and Pircher, 2011), and intracranial labeling experiments have indicated that clusters of neuronal T_{RM} cells are retained in areas of previous infection for at least several weeks (Wakim et al., 2010). Such results are in line with the observation that intestinal infection generates a distinct population of virus-specific epithelial CD8$^+$ memory T cells that differs from their counterparts in the lamina propria or the spleen in terms of T cell receptor rearrangement and usage (Isakov et al., 2009). Interestingly, T_{RM} cells isolated from the brain or gut show impaired survival and defective recall proliferative responses upon transfer into the circulation of recipient mice (Masopust et al., 2006; Isakov et al., 2009; Wakim et al., 2010). Thus, T_{RM} cells may irreversibly adapt to their local environments and therefore, may require a constant supply of microenvironment-specific factors for long-term survival and optimal functionality.

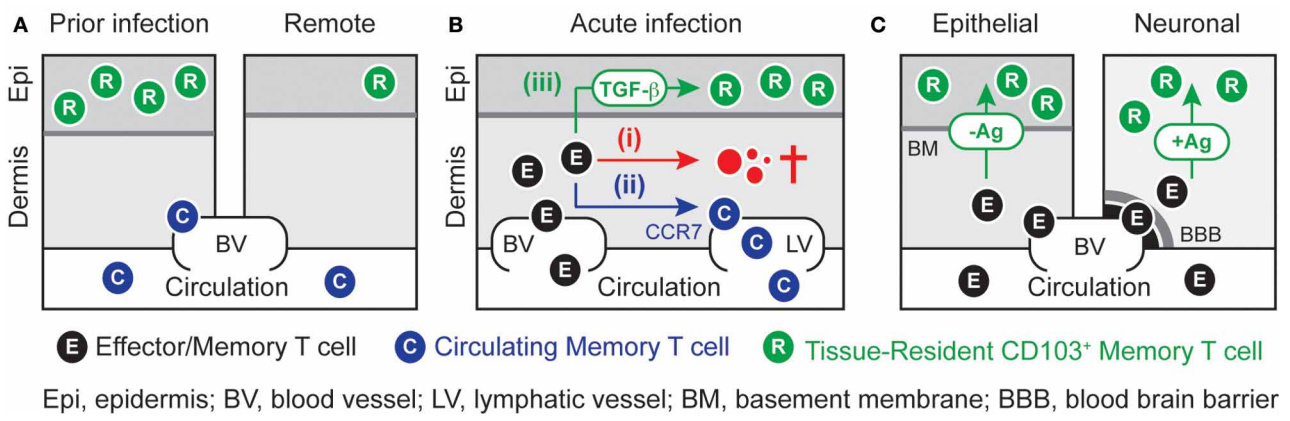

FIGURE 1 | Local differentiation and persistence of T_{RM} cells. (A) Localized infection or inflammation of the skin generates a population of epidermal CD103$^+$ T_{RM} cells that exist in disequilibrium with their counterparts in the circulation. High numbers of T_{RM} cells are concentrated at sites of previous infection or inflammation, whereas remote areas of skin contain only low numbers of these cells. **(B)** Effector CD8$^+$ T cells that have infiltrated infected skin can undergo three principal fates. While the majority of cells are likely to (i) die *in situ*, others may (ii) egress the skin in a CCR7-depdendent fashion and join the evolving pool of circulating memory T cells. A minority of cells may (iii) access the epidermal layer of the skin where they can undergo TGF-β-dependent differentiation into T_{RM} cells during the resolution phase of infection. **(C)** The role of local antigen recognition in T_{RM}-differentiation is dependent on anatomical location. While peripheral tissues such as skin and intestinal mucosa can promote T_{RM}-differentiation in the absence of local antigenic stimulation, the same process in neuronal tissues such as brain and ganglia requires local antigenic T cell restimulation. The basement membrane in the skin and the blood-brain-barrier in the brain sequester T_{RM} cells from the circulation.

The most definitive pieces of evidence for the disequilibrium between T_{RM} and circulating memory T cells stem from experiments employing parabiotic mice (Klonowski et al., 2004; Jiang et al., 2012), tissue transplantation (Gebhardt et al., 2009; Masopust et al., 2010), and pharmacological blockade of T cell recirculation (Masopust et al., 2010; Jiang et al., 2012). In addition, experiments involving the transfer of male T cells into female recipients have demonstrated the long-term survival of epidermal T_{RM} cells even in the face of an anti-male histoincompatibility response that results in the rapid rejection of circulating T cells (Gebhardt et al., 2011). Collectively, these studies demonstrate that circulating memory CD8$^+$ T cells have only very limited access to epithelial and neuronal compartments in the steady state. Instead, these anatomical niches are permanently occupied by an autonomous population of non-migratory T_{RM} cells that are characterized by the expression of a unique pattern of adhesion and migration molecules (**Table 2**).

Non-migratory peripheral cells may also exist amongst the pool of CD4$^+$ memory T cells (Cauley et al., 2002; Clark et al., 2006). For instance, a recent report has described a population of lung-seeking CD69$^+$ CD11a$^+$ memory CD4$^+$ T cells that were retained in the lung parenchyma for at least 3 weeks in parabiotic mice (Teijaro et al., 2011), although others have reported a progressive decline of virus-specific CD4$^+$ memory T cells in the same location (Cauley et al., 2002). A similar population of lung-resident CD4$^+$ memory T cells with a distinct phenotype has also been described in human lungs (Purwar et al., 2011), and given that these cells were enriched for influenza virus-specific cells, it appears likely that these cells play an important role in early local protection from reinfection (Hogan et al., 2001b; Purwar et al., 2011). While in previously HSV-infected mouse skin almost all CD8$^+$ memory T cells have hallmark features of T_{RM} cells, including epidermal localization and expression of high levels of CD103 (Gebhardt et al., 2011), the pool of peripheral CD4$^+$ memory T cells in the skin and other peripheral organs may be considerably more heterogeneous. This is supported by the observation that virus-specific CD4$^+$ memory T cells consist of both CD103$^-$ and CD103^{int} populations in previously infected skin (Gebhardt et al., 2011). Some of these cells may represent truly recirculating cells in transit through the periphery, as indicated by lymph canulation studies (Mackay et al., 1990; Yawalkar et al., 2003), whereas others might be retained in extralymphoid locations for prolonged periods of

Table 2 | Phenotypic differences between CD8$^+$ memory T cell subsets.

Category	Marker	T_{CM}	T_{EM}	T_{RM}
Migration/adhesion	CD62L	++	−	−
	CCR7	++	±	−
	CD44	++	++	+++
	CD69	−	−	++
	CD103	−	−	+++
	E-cadherin	−	−	+
	VLA-1	±	±	++
	ESL[a], PSL[a]	++/−	−	+++
Survival	CD122	++	+	+
	CD127	++	+	+
	Bcl-2	++	++	++
Effector function	Granzyme B	−	+	+

[a] *Shown only for skin T_{RM} cells.*

Abbreviations: ESL, E-selectin ligands; PSL, P-selectin ligands.

time (Teijaro et al., 2011). Further studies will have to clarify whether such cells are indeed permanently resident within peripheral tissues and, if so, whether similar mechanisms are involved in the long-term persistence of both $CD4^+$ and $CD8^+$ T cells subsets.

HUMAN $CD8^+$ $CD103^+$ MEMORY T CELLS WITH CHARACTERISTICS OF T_{RM} CELLS

The expression of CD103 defines a heterogeneous population of intraepithelial T cells in skin and mucosal tissues that exist in many species including rodents and humans (Parker et al., 1992; Agace et al., 2000). Intraepithelial populations of T cells comprise non-conventional T cells expressing CD8α:α homodimers and/or γδ T cells, as well as conventional CD8α:β αβ T cells (Lefrancois et al., 1994; Hayday et al., 2001). While these cells are present in many tissues under normal physiological conditions, a marked accumulation of the $CD8^+$ $CD103^+$αβ T cell subset is often associated with localized immune phenomena in epithelial and neuronal compartments, including graft rejection (Hadley et al., 1999; Wong et al., 2003), chronic inflammation (Ebert et al., 1998), infection (Piet et al., 2011), tumor development (Ling et al., 2007; Masson et al., 2007; Webb et al., 2010), and autoimmune processes (Pauls et al., 2001; Mizukawa et al., 2002; Teraki and Shiohara, 2002). Intriguingly, there is accumulating evidence that such populations of human T cells share major characteristics with the T_{RM} cells described in mouse experiments, including their long-term persistence and expression of T_{RM} signature markers such as CD69 and VLA-1, in addition to CD103.

Psoriasis is a chronic inflammatory skin disease characterized by well-demarcated erythematous plaques that tend to reoccur in identical anatomical locations on cessation of therapy (Nestle et al., 2009). Of note, psoriatic lesions are characterized by the accumulation of epidermal $CD8^+$ $CD103^+$ $CD69^+$ memory T cells (Pauls et al., 2001; Teraki and Shiohara, 2002). Most strikingly, studies using xeno-transplantation of healed skin onto immuno-compromised mice have shown that these cells can locally persist for several weeks after transplantation, and that their activation by as yet unknown antigens results in the VLA-1-dependent local reappearance of psoriatic lesions (Boyman et al., 2004; Conrad et al., 2007). Another example for the contribution of resident T cells to localized skin pathology is the so-called fixed drug eruption, an allergic skin reaction with sharply demarcated lesions that are triggered by the ingestion of certain drugs. Interestingly, lesion development is characterized by the rapid activation of epidermal $CD8^+$ $CD103^+$ memory T cells (Mizukawa et al., 2002). Of note, these lesions strictly reoccur in identical anatomical regions even several years after the last exposure to the disease-causing drug, implicating long-term survival of locally resident memory cells (Mizukawa et al., 2008). Furthermore, predilection sites for lesions are often areas of skin that have previously been exposed to some form of tissue trauma or infection (Mizukawa and Shiohara, 2002), thereby resembling the requirements for optimal T_{RM} cell lodgment described in mouse experiments (Gebhardt et al., 2009; Mackay et al., 2012a). Other examples of T_{RM} cells in humans include influenza virus-specific $CD8^+$ $CD103^+$ T cells that persist in the alveolar epithelium of the lungs (Piet et al., 2011), as well as virus-specific $CD8^+$ memory T cells that are preferentially retained in close proximity to the epidermis and peripheral nerves following HSV-2 infection in vaginal skin (Zhu et al., 2007, 2009). In addition, Epstein-Barr virus-specific $CD8^+$ $CD103^+$ T cells have been detected at high frequencies in the tonsils of persistently infected individuals (Hislop et al., 2005; Woodberry et al., 2005). Collectively, these studies strongly suggest that permanently tissue-resident T cells equivalent to the $CD8^+$ $CD103^+$ T_{RM} cells defined in mice, also exist in humans. Importantly, these cells appear to play a significant role in local infection control and various types of immuno-pathologies.

LOCAL DIFFERENTIATION AND PERSISTENCE OF $CD103^+$ T_{RM} CELLS

Several lines of evidence indicate that the local differentiation of T_{RM} cells occurs early during the resolution phase of infection. For instance, circulating effector cells rapidly lose the ability to infiltrate epithelial tissues and form T_{RM} cells (Masopust et al., 2010; Mackay et al., 2012a). Moreover, substantial populations of male-derived T_{RM} cells can lodge in female recipient mice even in the face of a potent anti-male rejection response that results in the rapid elimination of virtually all effector cells from the circulation (Gebhardt et al., 2011). These results further suggest that cells capable of differentiating into T_{RM} cells must exist amongst the effector cells that infiltrate peripheral tissues at the early stages of infection. Following pathogen clearance, these effector cells may undergo three fundamentally different fates (**Figure 1B**). While the majority of cells are likely to undergo apoptotic cell death *in situ*, others may enter afferent lymphatics in a CCR7-dependent manner and return to the circulation where they can join the evolving pool of circulating memory cells (Brown et al., 2010; Jennrich et al., 2012). A minority of cells however, may access epithelial compartments and further differentiate into long-lived T_{RM} cells. Given that T cell receptor-dependent activation can reduce the responsiveness of effector T cells toward CCR7 ligands (Debes et al., 2004; Schaeuble et al., 2011; Jennrich et al., 2012), it is possible that local antigen recognition, although not strictly required (Casey et al., 2012; Mackay et al., 2012a), supports effector T cell retention and further T_{RM} cell differentiation. While commitment to either of these fates could be a stochastic process, it is tempting to speculate that the ability to differentiate into either circulating memory or T_{RM} cells may be specific traits of distinct committed precursor cells. Future studies will have to establish the nature of T_{RM} precursor cells and will also have to better define the environmental cues that support their differentiation into T_{RM} cells. Such insights may help to determine optimal conditions for the generation of high densities of T_{RM} cells, critical for the development of T cell-based future vaccines, as discussed below.

THE ROLE OF ANTIGEN DEPENDS ON ANATOMICAL LOCATION

The molecular mechanisms that underlie the generation and long-term persistence of T_{RM} cells remain largely unknown. However, recent studies have begun to shed some light on the basic requirements for the establishment of peripheral T_{RM}-cell populations. Although T cells typically require antigenic

activation in order to infiltrate peripheral sites, their subsequent differentiation into T_{RM} cells in extralymphoid tissues does not require ongoing stimulation (**Figure 1C**). Non-specific inflammation alone for instance is sufficient to generate a substantial population of T_{RM} cells that locally persist for more than a year (Mackay et al., 2012a). Remarkably, even lymphopenia-driven activation following transfer of transgenic T cells into lymphocyte-deficient mice results in the generation of T_{RM} populations in a broad variety of peripheral organs (Casey et al., 2012). Exceptions to this, however, are neuronal tissues such as the brain and dorsal root ganglia (DRG) where local recognition of cognate antigen is required for CD103 induction in $CD8^+$ T cells (**Figure 1C**) (Wakim et al., 2010; Casey et al., 2012; Mackay et al., 2012a). Interestingly, even the intracranial injection of peptide-pulsed dendritic cells is sufficient to generate a local population of T_{RM} cells in the brain (Wakim et al., 2010). The additional level of "specificity control" in neuronal tissues may represent a mechanism to restrict the lodgment of potentially autoaggressive T cells, which could have detrimental consequences in the face of the inherently limited regenerative capacity of these organs. This further suggests that autocrine factors produced by the T cells themselves are involved in these specific locations, whereas other tissues such as the epidermis and the intestinal epithelium may promote T_{RM} differentiation through the provision of such factors in a more promiscuous and paracrine manner.

THE ROLE OF TRANSFORMING GROWTH FACTOR-β

Transforming growth factor (TGF)-β induces CD103 expression in activated rodent and human T cells (Kilshaw and Murant, 1990; Cepek et al., 1993). Accordingly, epithelial T cells expressing a dominant negative form of the TGF-β receptor show impaired upregulation of CD103 *in vivo*. This has been demonstrated in several experimental models including intestinal graft-versus-host disease, pulmonary influenza virus infection and lymphopenia-driven induction of intestinal epithelial T cells (El-Asady et al., 2005; Lee et al., 2011; Casey et al., 2012). Thus, TGF-β-mediated signals play a critical role for CD103 induction during T_{RM} differentiation in various body locations (**Figure 1B**). Nevertheless, it remains possible that other local factors are necessary to drive and sustain the remarkably elevated levels of CD103 expression seen particularly for epithelial T cells such as T_{RM} cells and DETC. Several cell types including fibroblasts, mast cells, T cells as well as keratinocytes and enterocytes can be potent sources of TGF-β, particularly during the regenerative wound-healing phase of the immune response (Li et al., 2006). In addition, epithelial injury results in the upregulation of the integrin $\alpha_v\beta_6$ (Breuss et al., 1995) that is involved in the local conversion of latent TGF-β into its biologically active form (Li et al., 2006). For instance, cutaneous wounding induces $\alpha_v\beta_6$ expression selectively in keratinocytes surrounding the wound edges (Breuss et al., 1995), which suggests that these cells may be involved in providing and converting active TGF-β for the induction of CD103 in differentiating T_{RM} cells. The assumption that epithelial accumulation of $CD103^+$ T cells is a consequence of local induction rather than selective recruitment of $CD103^+$ cells is further supported by the observation that effector cells can readily access epithelial and neuronal sites in the absence of CD103 expression and that, in fact, the vast majority of early infiltrating cells lack expression of this integrin subunit (Wakim et al., 2010; Mackay et al., 2012a).

THE ROLE OF THE $\alpha_E\beta_7$ INTEGRIN

CD103, the α-chain of the $\alpha_E\beta_7$ integrin, is one of the signature markers for $CD8^+$ T_{RM} cells and functions as a receptor for E-cadherin, an adhesion molecule specifically expressed by epithelial cells (Cepek et al., 1993, 1994). While some effector $CD8^+$ T cells express killer lectin receptor G1 (KLRG1), which is another E-cadherin binding molecule (Grundemann et al., 2006), the expression of this molecule is usually absent from T_{RM} cells (Masopust et al., 2006; Wakim et al., 2010; Hofmann and Pircher, 2011). Intestinal epithelial T cells and DETC can use CD103 to adhere and interact with enterocytes and keratinocytes, respectively (Cepek et al., 1993, 1994; Schlickum et al., 2008). In line with this, CD103-deficient mice have reduced numbers of these T cells (Schon et al., 1999, 2002; Schlickum et al., 2008), although residual populations are still present even in the absence of CD103. Furthermore, virus-, tumor-, or alloantigen-specific $CD8^+$ T_{RM} cells deficient in CD103 expression show impaired persistence in the brain, lung and the intestinal epithelium (El-Asady et al., 2005; Masson et al., 2007; Wakim et al., 2010; Lee et al., 2011; Casey et al., 2012). These results implicate a functional role for CD103 in the local differentiation and long-term persistence of T_{RM} cells in various epithelial and neuronal tissues, although the requirement of CD103 may not be absolute (Lefrancois et al., 1999). Interestingly, T_{RM} cells themselves also express E-cadherin and this molecule is involved in optimal persistence of virus-specific T_{RM} cells in salivary glands (Hofmann and Pircher, 2011). While binding of CD103 to E-cadherin is likely to promote local retention by tethering T cells to neighboring epithelial cells, other modes of action apart from this adhesive function have also been proposed. These include the regulation of T cell proliferation and cytolytic effector function, but possibly also extend to the functional modulation of epithelial and T cells as a result of E-cadherin ligation by T cell-derived CD103 (Agace et al., 2000). Moreover, $CD103^+$ T_{RM} cells in the brain express higher levels of the anti-apoptotic molecule Bcl-2 in comparison to $CD103^-$ cells isolated from the same location, implicating a role for outside-in signaling through CD103 in promoting T_{RM} cell survival (Wakim et al., 2010). This mechanism may be of particular importance given that T_{RM} cells show only minimal homeostatic turnover when compared to lymphoid $CD8^+$ memory T cells (Masopust et al., 2006; Gebhardt et al., 2009; Wakim et al., 2010), indicating that the maintenance of the T_{RM} pool is mainly regulated by their longevity rather than through constant self-renewal. Consistent with this, T_{RM} cells show only low-level expression of CD122 (Masopust et al., 2006; Gebhardt et al., 2009; Wakim et al., 2010), the receptor for interleukin (IL)-15 known to drive homeostatic turnover in lymphoid $CD8^+$ memory T cells (Surh and Sprent, 2008), and it has been suggested that $CD8^+$ memory T cells can be maintained independently of IL-15 signals following mucosal, as in contrast to systemic infections (Verbist et al., 2011).

THE PUTATIVE ROLE OF OTHER ADHESION AND MIGRATION MOLECULES

Apart from CD103, T_{RM} cells also express a number of other adhesion and migration molecules that are likely to be involved in their local retention. These include the $\alpha_1\beta_1$ integrin (VLA-1), CD44, ligands for E- and P-selectins, and the chemokine receptor CCR9, although expression of some of these molecules may be specific for T_{RM} populations in certain organs (**Table 2**). T_{RM} cells in the epidermis and the salivary glands express high levels of VLA-1 (Gebhardt et al., 2009; Hofmann and Pircher, 2011), a receptor for the extracellular matrix (ECM) proteins collagen type I and IV (Ben-Horin and Bank, 2004), the latter of which is a major structural component of basement membranes. Of note, VLA-1^+ influenza virus-specific $CD8^+$ memory T cells in the lung localize to collagen-rich areas in close association to airways, blood vessels and alveoli, and genetic VLA-1 deficiency or antibody-mediated blockade of VLA-1 function results in strongly reduced numbers of these cells (Ray et al., 2004). It is still under debate as to whether lung-resident $CD8^+$ memory T cells share major characteristics such as longevity and permanent residency with their counterparts in other extralymphoid tissues (Takamura et al., 2010; Lee et al., 2011), and this matter is further complicated by inherent technical difficulties in identifying bona fide tissue-resident as in contrast to intravascular T cells in this particular organ (Anderson et al., 2012). Regardless, these results nevertheless suggest that T_{RM} cells in other organs may also utilize the VLA-1 integrin for their adhesion to basement membranes in epithelial compartments. In a similar fashion, CD44, which is expressed at very high levels on T_{RM} cells (Gebhardt, unpublished results), may also be involved in local retention through its interaction with hyaluronic acid, a major ECM component abundantly expressed in epithelial tissues (Baaten et al., 2012). Furthermore, epidermal T_{RM} cells express remarkably high levels of ligands for E- and P-selectins (Jiang et al., 2012), which represent a heterogeneous group of modified glycoproteins including CD44, CD43, PSGL-1, and possibly others (Woodland and Kohlmeier, 2009). While these molecules play a major role in skin migration of effector and memory T cells through their binding to E- and P-selectin on endothelial cells in the cutaneous microvasculature (Agace, 2006), skin T_{RM} cells may further utilize such receptors to bind to as yet unidentified ligands constitutively expressed in the epidermis. Finally, T_{RM} cells can also express organ-specific chemokine receptors that enable them to respond to gradients of locally produced chemokines. One example of this is the sustained expression of CCR9 by gut-resident T_{RM} cells (Masopust et al., 2010), which is the receptor for the chemokine CCL25, constitutively expressed by enterocytes in the small intestine (Agace, 2006). Interestingly, another adhesion molecule critically involved in gut migration by virus-specific effector $CD8^+$ T cells, the integrin $\alpha_4\beta_7$ that binds to the mucosal addressin MAdCAM-1 on blood vessels (Williams and Butcher, 1997; Lefrancois et al., 1999; Agace, 2006), is rapidly lost in intestinal T cells on epithelial entry (Masopust et al., 2010), and therefore, does not seem to be involved in local persistence of gut T_{RM} cells.

Future studies will have to clarify the precise contribution of the aforementioned adhesion and migration receptors to the local retention and persistence of T_{RM} cells in different anatomical locations. Experimental approaches targeting selected candidate molecules, however, could be complicated by the possibility that many of these molecules may exert redundant or overlapping functions. Furthermore, in addition to their function in tethering T_{RM} cells to their microenvironments, several of these molecules have also been implicated in outside-in signaling in T cells, meaning that their ligation by ECM components may directly impact on cellular functions including long-term survival or the expression of preformed effector molecules such as granzyme B (Agace et al., 2000; Richter and Topham, 2007; Baaten et al., 2012). In line with this, T_{RM} cell express high levels of the activation marker CD69 even in the absence of cognate antigen (Masopust et al., 2006; Gebhardt et al., 2009), possibly reflecting some level of cellular activation maintained by their prolonged interaction with various microenvironmental stimuli.

LOCAL IMMUNITY BY T_{RM} CELLS

While circulating memory T cells provide efficient protection against systemic infections that result in rapid accumulation of pathogens in lymphoid filter organs, their ability to deal with localized infections in the periphery is often limited. This deficiency is partly explained by their progressive loss of peripheral migration as well as by their delayed accumulation at peripheral sites of infection due to the time-consuming nature of T cell recruitment from the circulation. Instead, it has been proposed that memory T cells already residing in peripheral tissues at the time of infection—either permanently as in the case of T_{RM} cells, or temporarily as proposed for $CD4^+$ memory T cells—are key to rapid infection control in barrier tissues such as skin and mucosa (Woodland and Kohlmeier, 2009; Di Meglio et al., 2011; Kupper, 2012; Masopust and Picker, 2012).

Importantly, memory $CD8^+$ T cells isolated from the intestinal mucosa exhibit enhanced effector function such as rapid interferon (IFN)-γ production and target cell lysis following restimulation *ex vivo* (Masopust et al., 2001). Similarly, T_{RM} cells isolated from the brain and skin can rapidly reacquire effector functions and kill peptide-pulsed target cells *in situ* and *ex vivo*, respectively (Hawke et al., 1998; Wakim et al., 2010; Jiang et al., 2012). Our own unpublished results further indicate that virus-specific T_{RM} cells produce IFN-γ within the first 24 h after epidermal or mucosal infection with HSV-1. Such results demonstrate that T_{RM} cells indeed display the full arsenal of effector functions required for immediate local immune control at sites of peripheral infection.

CONTROL OF LOW-LEVEL PERSISTENT AND LATENT VIRUS INFECTIONS

As discussed above, peripheral infections leave behind a population of T_{RM} cells concentrated at sites of previous pathogen encounter. The reason why these cells are formed particularly in epithelial and neuronal tissues sequestered away from the circulation is not entirely clear. It is tempting to speculate, however, that their main evolutionary purpose is to deal with residual, chronic or latent reservoirs of pathogens. Of note, circulating $CD4^+$ and $CD8^+$ memory T cells are largely excluded from epithelial and neuronal tissues under non-inflammatory conditions (Klonowski et al., 2004; Gebhardt et al., 2011). Thus, in the absence of locally

persisting T_{RM} cells, such tissues would be highly vulnerable to pathogens that specifically target these sites to establish locally persisting or latent reservoirs in the absence of overt inflammation (Zinkernagel, 2002), as seen in infections with HSV, varicella zoster virus, and human papilloma virus.

Epithelial HSV infection in the skin or vaginal mucosa results in the establishment of a latent infection that is restricted to sensory ganglia innervating the site of primary infection. This latent infection is characterized by the absence of both infectious viral particles and ongoing inflammation (Stanberry, 1992; Sawtell, 1997) and therefore escapes immuno-surveillance by circulating $CD8^+$ memory T cells (Himmelein et al., 2011). However, T_{RM} cells selectively retained within infected ganglia (Khanna et al., 2003) can recognize latently infected neurons and control viral latency through non-cytolytic mechanisms including the site-directed release of effector molecules such as granzyme B (Van Lint et al., 2005; Knickelbein et al., 2008; Mackay et al., 2012b). Interestingly, these ganglionic T_{RM} cells retain their full functionality even in the face of chronic antigenic stimulation and can readily produce effector cytokines and mount local proliferative recall responses on experimental virus reactivation (Wakim et al., 2008; Mackay et al., 2012b). Importantly, similar populations of HSV-specific $CD8^+$ T cells are also retained in latently infected ganglia in humans (Verjans et al., 2007), and further accumulate at sites of anticipated virus reemergence such as the sensory nerve endings and epidermis of previously HSV-2-infected human vaginal skin (Zhu et al., 2007, 2009).

PROTECTION FROM VIRUS REINFECTION

The local persistence of T_{RM} cells at sites of previous infection or inflammation can be interpreted as a strategy of the immune system to predict or anticipate the site of reencounter with the same or similar pathogens (Bevan, 2011). This is best documented for skin infection, where levels of site-specific immunity in previously infected areas of skin can be compared with control skin not involved in the primary infection. Indeed, following localized skin infection with HSV-1 in B cell-deficient mice, there is strongly enhanced local protection from virus reinfection specifically at the site of previous infection harboring elevated numbers of T_{RM} cells (Gebhardt et al., 2009). Furthermore, following reconstitution of lymphocyte-deficient mice with virus-specific $CD8^+$ T cells, T_{RM} cells can mediate site-specific immunity alone, in the absence of virus-specific antibodies and $CD4^+$ memory T cells (Gebhardt et al., 2009). Importantly, this form of local immunity is long-lived and can be observed for at least 3 months after primary immunization (Gebhardt et al., 2009), which is consistent with the remarkable longevity of T_{RM} cells. Confirming these results, T_{RM} cells generated in skin and salivary glands after vaccinia or LCMV infection, respectively, mediate potent protection from rechallenge infection even when T cell recirculation is pharmacologically inhibited by treatment with the sphingosine-1-phosphate antagonist FTY720 (Liu et al., 2009; Hofmann and Pircher, 2011; Jiang et al., 2012). Similarly, memory T cells in the lung can contribute to local immunity (Hogan et al., 2001a; Ray et al., 2004), although protection from heterosubtypic reinfection in this location appears to wane with time (Liang et al., 1994), which is potentially related to the fact the lung environment is not as conducive to support T_{RM} cell survival as other barrier tissues (Woodland and Kohlmeier, 2009). Collectively, these results strongly support the notion that T_{RM} cells at body surfaces play a major role in protection from localized reinfection, whereas circulating $CD8^+$ memory T cells may be specialized in dealing with pathogens that access lymphoid tissues following systemic infection.

PROTECTION FROM *de novo* INFECTION—T_{RM} CELLS AS VACCINE TARGETS

The induction of long-lived memory T cells able to patrol through peripheral tissues has proven difficult in the absence of continued T cell activation (Kundig et al., 1996; Bachmann et al., 1997; Hansen et al., 2009). However, T_{RM} cells do not rely on persisting antigen (Casey et al., 2012; Mackay et al., 2012a), and as a consequence, targeting these cells with novel vaccination strategies has the potential to overcome obstacles originating from the progressive loss of peripheral immune surveillance by circulating $CD8^+$ memory T cells. Given that T_{RM} cells can mediate rapid immune control at peripheral sites of infection, the generation of high densities of such cells is an appealing goal for future vaccines against pathogens that invade the body via peripheral portals of entry.

Strategically well-positioned at peripheral sites, T_{RM} cells may provide a first line of defense potent enough to control peripheral infection before local pathogen replication results in the establishment of chronic or latent reservoirs as seen in infections such as HSV and human immunodeficiency virus. We have recently shown that the combination of T cell activation and site-specific lodgment by non-specific inflammation generates high densities of long-lived T_{RM} cells in the skin and these that cells are able to (1) control local virus replication, (2) prevent viral skin disease, and (3) significantly reduce the ensuing latent infection in DRG innervating the site of *de novo* challenge infection (Mackay et al., 2012a). While this approach provides regional rather than global protection of the skin, it has recently been suggested that repeated immunization in prime-boost settings can generate high densities of T_{RM} cells even in remote areas of skin not involved in successive immunization steps (Jiang et al., 2012) (**Figure 2**). Furthermore, non-specific inflammation of the vaginal mucosa has been shown to result in the local accumulation of T_{RM} cells capable of controlling a subsequent infection with HSV-1 (Mackay et al., 2012a). Of note, the latter approach represents an example where coverage of a whole organ system, the cervico-vaginal mucosa, is achievable by a single intervention without the need of repeated immunizations. Collectively, these results provide promising proof-of-principle evidence that T_{RM} cells may successfully be exploited in vaccination settings. Future studies are needed to refine the requirements for optimal T_{RM} lodgment and coverage of different organ systems. Finally, complementing such innovative immunization strategies with the simultaneous induction of other adaptive immune elements, including tissue-tropic $CD4^+$ memory T cells and antibody-secreting plasma cells, has the potential to further enhance protection afforded by T_{RM} cells.

CONCLUDING REMARKS

To a large extent, the field of T cell memory has been dominated by the paradigm that memory T cells are constantly recirculating

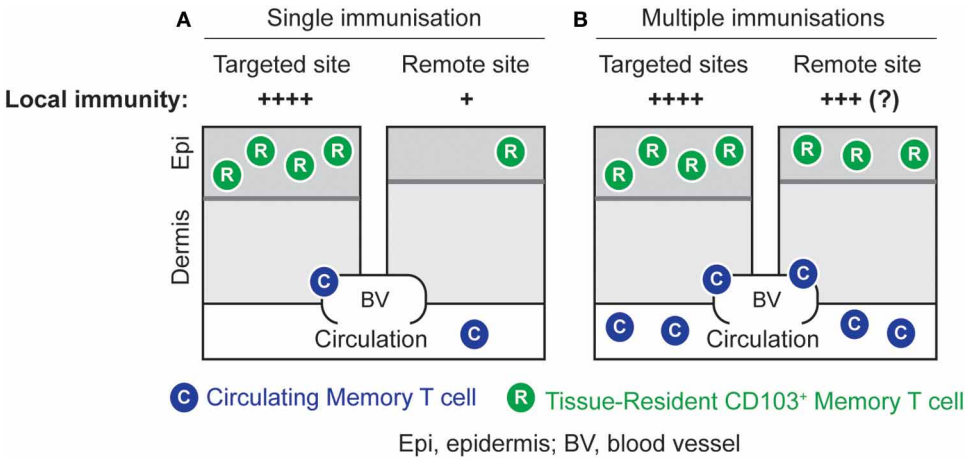

FIGURE 2 | Local immunity by site-specific or global lodgment of T_{RM} cells. (A) High numbers of T_{RM} cells can be generated in the skin by an immunization strategy that combines the induction of effector T cells with the local application of an inflammatory stimulus. This results in potent local immunity at the targeted site. **(B)** Repeated immunizations in different skin sites result in elevated numbers of T_{RM} cells in both targeted and remote areas of skin. This strategy has the potential to generate global T_{RM}-cell-mediated immunity against skin infection, although this remains to be formally proven.

cells that patrol through either lymphoid or extralymphoid tissues as a consequence of subset-specific migratory programs. An important extension to this concept is that these fundamentally different migration patterns correlate with the functional status exhibited by distinct memory subsets, and thereby, predict their participation in different types of immune responses. Recent evidence based on the analysis of antigen-specific memory T cells in various lymphoid and non-lymphoid tissues, however, suggests that a large proportion of peripheral $CD8^+$ memory T cells are permanently sessile and non-migratory and therefore, do not easily fit into the prevailing concept of circulatory immune surveillance. Given that these T_{RM} cells show remarkable similarities in terms of the expression of core signature markers, residency and effector function irrespective of their mode of generation and localization to different peripheral organs, it is tempting to speculate that T_{RM} differentiation represents a default pathway of $CD8^+$ memory T cell generation in non-lymphoid tissues. Their important role in infection control highlights the need to better understand the molecular mechanisms that govern their differentiation and long-term persistence in peripheral sites. Future studies will also have to clarify whether similar populations of non-migratory peripheral cells also exist among the pool of $CD4^+$ memory T cells. Refining the contribution of circulating versus permanently tissue-resident populations of memory T cells to immune protection in barrier tissues such as the skin and mucosa will be critical to the design of future immunization strategies harnessing cellular immunity to protect against pathogens that invade through peripheral portals of entry.

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Use it or lose it: establishment and persistence of T cell memory

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Pre-existing T cell memory provides substantial protection against viral, bacterial, and parasitic infections. The generation of protective T cell memory constitutes a primary goal for cell-mediated vaccines, thus understanding the mechanistic basis of memory development and maintenance are of major importance. The widely accepted idea that T cell memory pools are directly descended from the effector populations has been challenged by recent reports that provide evidence for the early establishment of T cell memory and suggest that the putative memory precursor T cells do not undergo full expansion to effector status. Moreover, it appears that once the memory T cells are established early in life, they can persist for the lifetime of an individual. This is in contrast to the reported waning of naïve T cell immunity with age. Thus, in the elderly, immune memory that was induced at an early age may be more robust than recently induced memory, despite the necessity for long persistence. The present review discusses the mechanisms underlying the early establishment of immunological memory and the subsequent persistence of memory T cell pools in animal models and humans.

Keywords: T cell memory, CD8 T cells, aged, Influenza A virus, CD4$^+$ T lymphocytes

INTRODUCTION

Memory T cells provide protection against re-infection with the same pathogen enabling a more rapid recovery of the host and a milder clinical outcome (Powell et al., 2007). For example, survival from the H2N2 influenza pandemic of 1957 was attributed to pre-existing T cell immunity in the adult population (Epstein, 2006). Furthermore, high levels of pre-existing CD4$^+$ T cell memory correlate with reduced viral loads and clinical scores in a human influenza challenge study, highlighting the importance of T cell memory in viral infection (Wilkinson et al., 2012). Once established, memory T cells can reside in tissues or recirculate between the blood, organs, and lymph nodes. These memory T cells appear to be quite distinct from the recently described tissue resident memory cells (Trm; Gebhardt et al., 2009) displaying a unique set of molecular markers and optimal functions within the tissues (Mackay et al., 2012; Wakim et al., 2012).

For a long time, the traditional view of T cell memory formation involved the expansion of pathogen-specific effector T cells in number in response to infection followed by an extensive contraction in cell numbers (~90–95%), leaving a residual stable memory T cell pool, known as the linear model of memory differentiation. It has been known for decades that CD8$^+$ memory T cells sort into CD62L^{hi} and CD62L^{lo} sets (Tripp et al., 1995), with the former having the capacity to recirculate from blood to lymph nodes via the high endothelial venules, while the latter move readily through other tissue compartments and can return to the lymph node via the afferent lymphatics. Thirteen years ago, a seminal paper by Sallusto et al. (1999) coined the terms "central" (CD62L^{hi}) and "effector" (CD62L^{lo}) to describe these memory T cell subsets. These findings challenged the traditional view of memory formation, suggesting a more complex process, and raised important questions about the generation of T cell memory and which populations actually persist for the life-time of an individual. In fact, the key question has long been whether persistent T cells (CD62L^{hi} or CD62L^{lo}) are survivors from the clonally expanded effector cytotoxic T lymphocyte (CTL) pool or are part of a separate lineage.

More than a decade of research and published studies provide evidence of the early establishment of memory, which can be greatly affected by the inflammatory stimuli and transcription signatures at the acute phase of the infection. Both animal and human studies also show that T cell memory is long-lived and can be detected for up to 75 years in humans (Hammarlund et al., 2003) and for the life-time of a laboratory mouse (Valkenburg et al., 2012). A growing area of research focused on the impact of age on the immune system suggests that age-related changes have potential implications for the generation and maintenance of T cell memory late in life. Interestingly, memory T cell populations directed at acute and long-term persistent infections exhibit different functions and characteristics, as the latter display signs of exhaustion due to the persistence of the antigen. In addition, studies suggest that, in the context of acute infections, the protective capacity of memory CD8$^+$ T cells is pathogen-specific and can be substantially impacted by repeated antigenic stimulation (Nolz and Harty, 2011). In this review, we discuss both the establishment and persistence of primary T cell memory, with a particular focus on CD8$^+$ T cell responses directed at acute readily resolved infections such as respiratory viruses.

ESTABLISHMENT OF T CELL MEMORY

The relatively recent development of the tetramer-magnetic enrichment approach (Moon et al., 2007) has substantially shaped our understanding of the recruitment, expansion, and persistence of endogenous T cell numbers for the naïve, effector, and memory T cell populations. The numbers of naïve T cells per mouse available to respond to a specific antigenic epitope range from tens (e.g., ≈30 naïve precursors for the immunodominant influenza-specific D^bNP_{366} epitope; La Gruta et al., 2010) to hundreds (e.g., ≈600 for the murine cytomegalovirus D^bM45 epitope; Obar and Lefrancois, 2010). After antigenic stimulation, the naïve precursors increase in prevalence by more than 10,000 times (Croom et al., 2011), whilst substantially fewer cells establish stable memory pools. These memory pools increase the numbers of antigen-specific T cells by up to 200–1000 times (Belz et al., 2000; Hogan et al., 2001; Marshall et al., 2001; Croom et al., 2011) compared to the initial naïve precursor pool (La Gruta et al., 2010). Such effects are readily observed in mouse model systems following prime/challenge with influenza A viruses that differ for their surface H and N proteins but share internal components (Kedzierska et al., 2008). It has been reported that the size of antigen-specific memory pools strongly correlates with the level of immune protection (Badovinac et al., 2003; Schmidt et al., 2008). As the establishment of immunological memory is critical for a rational design of any cell-mediated vaccine, understanding the precise mechanisms of memory formation is essential.

EARLY ESTABLISHMENT OF T CELL MEMORY

Recent studies provide important insights into the development of memory T cells. Experiments using *in vitro* stimulated T cells (Kaech and Ahmed, 2001), antibiotic treatment prior to *Listeria monocytogenes* or influenza co-infection (Badovinac et al., 2004), dendritic-cell vaccination (Badovinac et al., 2005), clonal dissection of influenza-specific $CD8^+$ T cells at different stages of infection (Kedzierska et al., 2006, 2008) or transfer studies (Kedzierska et al., 2007) suggest that the full expansion to effector status is not a pre-requisite for the generation of long-term memory T cells.

As mentioned previously, the discovery that memory T cell populations are comprised of distinct T cell subsets (Sallusto et al., 1999) has also had a substantial impact on our understanding of T cell memory. Based on the expression of lymph-node homing markers (CD62L and CCR7), memory T cells have been classified as central memory T cells (T_{CM}; $CD62L^{hi}$, $CCR7^{hi}$), circulating between lymphoid organs, and effector memory (T_{EM}, $CD62L^{lo}$, $CCR7^{lo}$), found principally in the blood, spleen, and peripheral organs (Sallusto et al., 1999; Masopust et al., 2001; Reinhardt et al., 2001). These T_{CM} and T_{EM} memory populations are also considered to differ at the functional level. While human T_{EM} cells display immediate cytotoxic activity *ex vivo*, T_{CM} populations acquire effector function after short-term *in vitro* stimulation (Sallusto et al., 1999; Masopust et al., 2001). Although both subsets can produce anti-viral cytokines IFN-γ and TNF-α, IL-2 production is largely limited to $CD62L^{hi}$ memory cells (Wherry et al., 2003). Significant insights into T cell formation and maintenance have been gained since studies have focused on classifying T_{EM} and T_{CM} cells.

A first important observation from studies of the T_{EM} and T_{CM} subsets is that the proportions of T_{EM} and T_{CM} populations change throughout the course of infection. In contrast to naïve $CD8^+$ T cells expressing the $CD62L^{hi}$ $CD44^{lo}$ phenotype, the majority of $CD8^+$ T cells present at the peak of infection display the surface markers $CD62L^{lo}$ $CD44^{hi}$, although some (around 10%; Kedzierska et al., 2006) remain of the $CD62L^{hi}$ $CD44^{hi}$ phenotype (**Figure 1**). As the virus is cleared and memory is established, the proportion of cells with the $CD62L^{hi}$ phenotype progressively increases. For example, around 50% of the influenza-specific immunodominant $D^bNP_{366}{}^+CD8^+$ and $D^bPA_{224}{}^+CD8^+$ T cell memory populations express $CD62L^{hi}$ at d60 after infection, compared with 90% being $CD62L^{hi}$ by d500 (Kedzierska et al., 2006). Interestingly, the numbers of $CD62L^{hi}$ $CD8^+$ T cells remain relatively stable throughout the infection, while the total numbers of $CD62L^{lo}$ $CD8^+$ T cells rapidly decreases during the memory phase (Schlub et al., 2010; **Figure 1A**). This suggests that, rather than there being a regression from $CD62L^{lo}$ to $CD62L^{hi}$, there is preferential survival of antigen-specific memory $CD62L^{hi}$ $CD8^+$ T cells (T_{CM}) set. This can be also translated into the relative contributions of $CD62L^{lo}$ and $CD62L^{hi}$ $CD8^+$ T cell memory populations to the secondary response to Sendai virus (Roberts and Woodland, 2004; Roberts et al., 2005). Although the recall responses by the $CD62L^{lo}$ $CD8^+$ T cell memory population dominate at the early memory phase of infection (d30), the central memory $CD62L^{hi}$ $CD8^+$ T cells from later (1 year) memory timepoints make a markedly greater contribution to recall responses (Roberts et al., 2005).

Secondly, important insights into the early establishment of T cell memory have been made possible by the differential expression of $CD62L^{lo}$ and $CD62L^{hi}$ throughout the memory phase, which allows the tracking of different subsets starting from the early days after infection. Experiments utilizing transgenic mice suggest that it is possible to transition from the "more activated" $CD62L^{lo}$ to the "less activated" $CD62L^{hi}$ phenotype, indicating that being $CD62L^{lo}$ is not a marker of terminal differentiation and supporting the possibility that there can be a linear model of T_{EM} to T_{CM} memory generation (Wherry et al., 2002). The results of clonotypic analysis of T cell receptor (TCR) signatures in mice (Bouneaud et al., 2005; Kedzierska et al., 2006) and humans (Baron et al., 2003) further expand this model. The indications are that although common TCRs are found in both T_{CM} and T_{EM} populations, the T_{CM} set contains additional TCR clonotypes not found within the T_{EM} set and that TCR diversity within both the T_{CM} and T_{EM} subsets appears to be stable. In mice, such divergent (and consistent) TCR repertoire characteristics are apparent for the T_{CM} and T_{EM} sets from the early acute phase of infection (d8) through to the long-term memory (>d500; **Figure 2**). This indeed suggests the early establishment of clonotypically stable T cell memory pools. In a follow-up study, we found that influenza-specific $CD8^+$ T cells isolated on d3.5 after influenza infection, especially cells isolated from the draining lymph nodes, could survive following the transfer into naïve mice and be recalled after a secondary challenge (Kedzierska et al., 2007). This suggests that stable $CD8^+$ T cell memory is established early in the antigen-driven phase of influenza virus infection. The survival of memory $CD8^+$ T cells isolated early after infection on d3.5 highly resembled

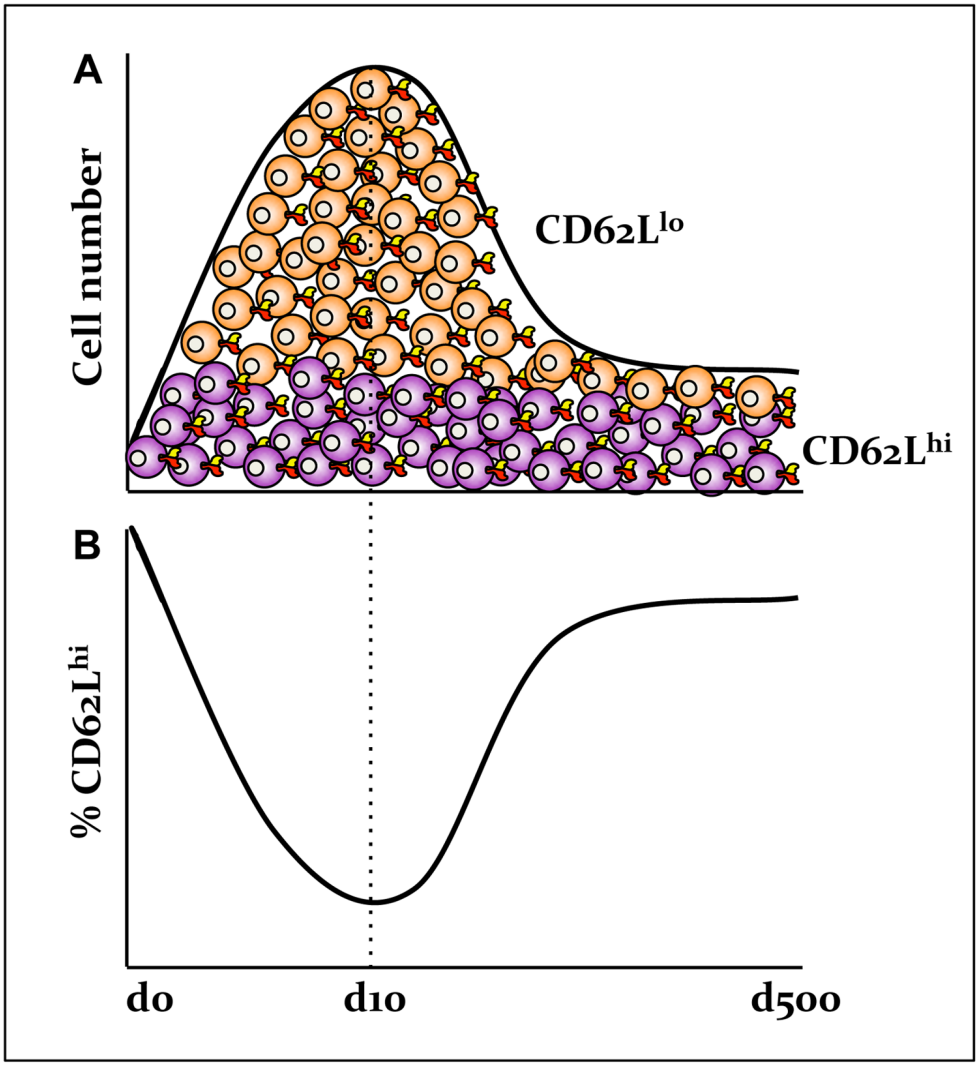

FIGURE 1 | Preferential survival of antigen-specific memory CD62L^{hi} CD8$^+$ T cells following influenza virus infection. (A) The numbers of influenza-specific CD62L^{hi}CD8$^+$ T cells, but not CD62L^{lo} CD8$^+$ T cell sets, are stable following the primary infection. **(B)** This is in contrast to the relative frequencies of CD62L^{lo} and CD62L^{hi} effector and memory T cell populations as based on our published data in Kedzierska et al. (2006).

the survival of CD8$^+$ T cells isolated during the memory phase (d28; **Figure 3**). Conversely, when CD8$^+$ T cells isolated at d8 (i.e., at the peak of the acute phase) were transferred into a naïve animal, the survival potential was lower than those of cells isolated early after the infection. This most likely reflects the high proportion of terminally differentiated short-lived effectors at the peak of the response to influenza viruses (**Figure 3**). Overall, these findings support the idea that antigen-specific CD8$^+$ T cells that have not achieved full effector potential are more likely to be part of the memory pool. For example, CD8$^+$ T cells lacking the cytotoxic capacity thus protected from cell damage during target cell killing. In addition, early d3.5 CD8$^+$ T cells lacking CD25 (the IL-2Rα subunit) displayed enhanced survival into memory (Kedzierska et al., 2007). This was further confirmed, more recently (Belz and Masson, 2010; Kalia et al., 2010; Pipkin et al., 2010) in studies which showed lower CD25 expression, and thus weaker IL-2 signaling, leads to preferential generation of T cells with a memory phenotype capable of long-term survival.

Expression of the IL-7R α-chain (CD127) on CD8$^+$ T cells during the acute phase of the response is also essential, although not sufficient on its own, for identifying the memory precursors at the acute time of infection (Kaech et al., 2003; Huster et al., 2004; Hand et al., 2007; Croom et al., 2011). Evidence for this comes from studies showing that, IL-7Rα-expressing cells at the peak of LCMV infection survive preferentially to give long-lived memory and show increased expression of anti-apoptotic molecules (such as bcl-2; Kaech et al., 2003). Further, FACS separation of LCMV-specific effector CD8$^+$ T cells on the basis of IL-7Rα and KLRG1 expression led to characterization of the IL-7Rα^{hi} KLRG1^{lo} memory precursor effector cells (MPEC), as distinct fromIL-7Rα^{lo} KLRG1^{hi} short-lived effector cells (SLEC) following LCMV infection (Joshi et al., 2007). Acquisition of this memory IL-7Rα^{hi}

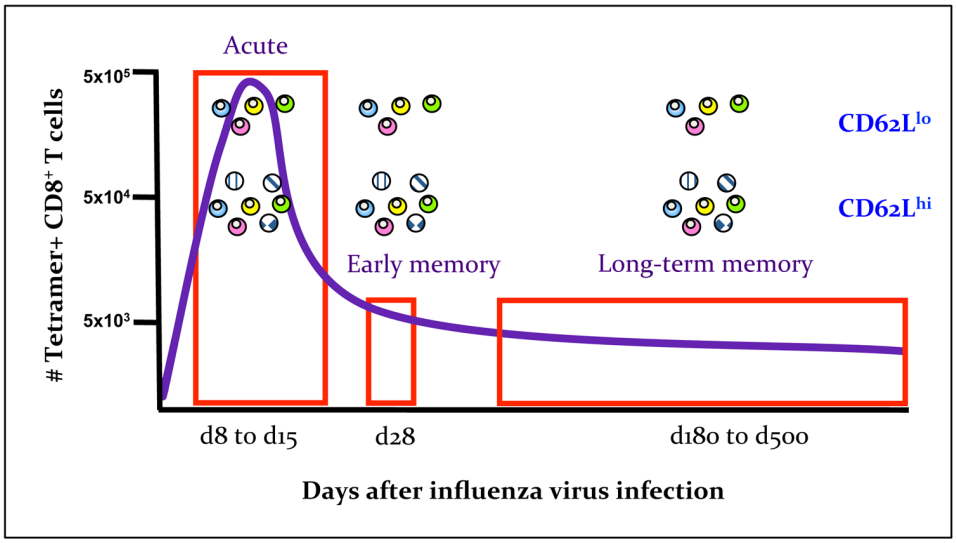

FIGURE 2 | Early establishment of a stable TCR repertoire composition for memory T_{EM} CD62L^{lo} and T_{CM} CD62L^{hi} sets. Schematic summarizing the results of TCR repertoire analysis of the T_{EM} CD62L^{lo} and T_{CM} CD62L^{hi} subsets for influenza-specific $D^bNP_{366}{}^+$ and $D^bPA_{224}{}^+CD8^+$ T cells at the acute phase of infection (d8–d15), early memory (d28) and late memory (d180–d500). Data are based on $n = 3018$ sequences published in Kedzierska et al. (2006).

KLRG1^{lo} phenotype in models of rapid, systemic infection (LCMV and *L. monocytogenes*) depends on the spectrum of inflammatory cytokines present during the acute phase of the disease process (Joshi et al., 2007; Pham et al., 2009).

In influenza infection, a substantial number of antigen-specific $D^bNP_{366}{}^+CD8^+$ and $D^bPA_{224}{}^+CD8^+$ T cells express IL-7Rα at the peak of the acute primary response (70.2 ± 1.5 and 37.5 ± 2.4%, respectively), which does not translate into the relative numbers of T cells in the stable memory pools (Croom et al., 2011). Thus, at least for influenza in a non-TCR-transgenic B6 mouse system, the majority of the antigen-specific IL-7Rα$^+$ CD8$^+$ T cells do not survive into the memory phase in a non-TCR-transgenic B6 mouse system. Further subsetting of influenza-specific CD8$^+$ T cells into the SLEC and MPEC sets showed that, although the IL-7Rα^{hi} KLRG1^{lo} CD8$^+$ population recovered from the spleen and the infected respiratory tract showed evidence of enhanced survival, the number of cells of this phenotype at peak does not define memory numbers. Our data suggested that the most stable MPEC IL-7Rα^{hi} KLRG1^{lo} numbers are observed in the draining mediastinal LN (MLN, draining the lungs), providing further evidence that, following this localized respiratory infection, the draining MLN offers the optimal anatomical niche for memory establishment and maintenance after influenza infection. Furthermore, the IL-7Rα^{hi} KLRG1^{lo} phenotype (but not the ultimate size of the memory pool) can be altered by varying the antigen dose, antigen presentation, extent of T cell division, and CD8$^+$ T cell precursor numbers (Croom et al., 2011). This indicates that cell surface molecule expression on CD8$^+$ T cells predominantly reflects early antigenic experience and to a lesser extent marks the capacity to generate CD8$^+$ T cell memory. And obviously, the potential of the memory cells to survive can be coupled to its replicative capacity (Hikono et al., 2007).

In addition to the important determinants of T cell memory generation discussed above, sufficient CD4$^+$ T cell help is also required for the establishment of stable memory (but not effector) influenza-specific CD8$^+$ T cell populations. This was established by the diminished magnitude of memory and recall CD8$^+$ T cell responses in MHC class II$^-$ ($IA^{b-/-}$) mice primed and then challenged with the influenza viruses (Topham et al., 1996; Doherty and Christensen, 2000; Riberdy et al., 2000; Belz et al., 2002), and mice treated with monoclonal antibody against CD4$^+$ T cells (McKinstry et al., 2012; Swain et al., 2012). Unhelped CD8$^+$ T cells have reduced survival to memory and reduced expansion during recall. The mechanism proposed involves help from CD4$^+$ T cells resulting in down-regulation of TNF-α related apoptosis inducing ligand (TRAIL) on CD8$^+$ T cells (Janssen et al., 2005; Badovinac et al., 2006).

THE ROLE OF INFLAMMATION AND TRANSCRIPTION FACTORS IN THE GENERATION OF STABLE MEMORY CD8$^+$ T CELL POOLS

Infection-induced inflammation can affect T cell immunity at any stage. Evidence suggests that the fate of memory precursors can be determined by the nature of inflammatory stimuli during the antigen driven phase. Elegant studies using antibiotic treatment prior to *L. monocytogenes* infection showed that reduced inflammation (including diminished IFN-γ production) markedly decreased T cell contraction (Badovinac et al., 2004). Conversely, increasing inflammation with CpG treatment restored this effector T cell editing process (Badovinac et al., 2004). These findings suggest that T cell memory pools can be generated independent of whether there is massive contraction in numbers. Furthermore, IL-12 has been shown to affect the progression to MPEC status via decreased T-bet expression (Joshi et al., 2007). While low inflammation (low T-bet expression) promotes MPEC formation, high inflammation (T-bet expression) leads to numerically enhanced SLEC population.

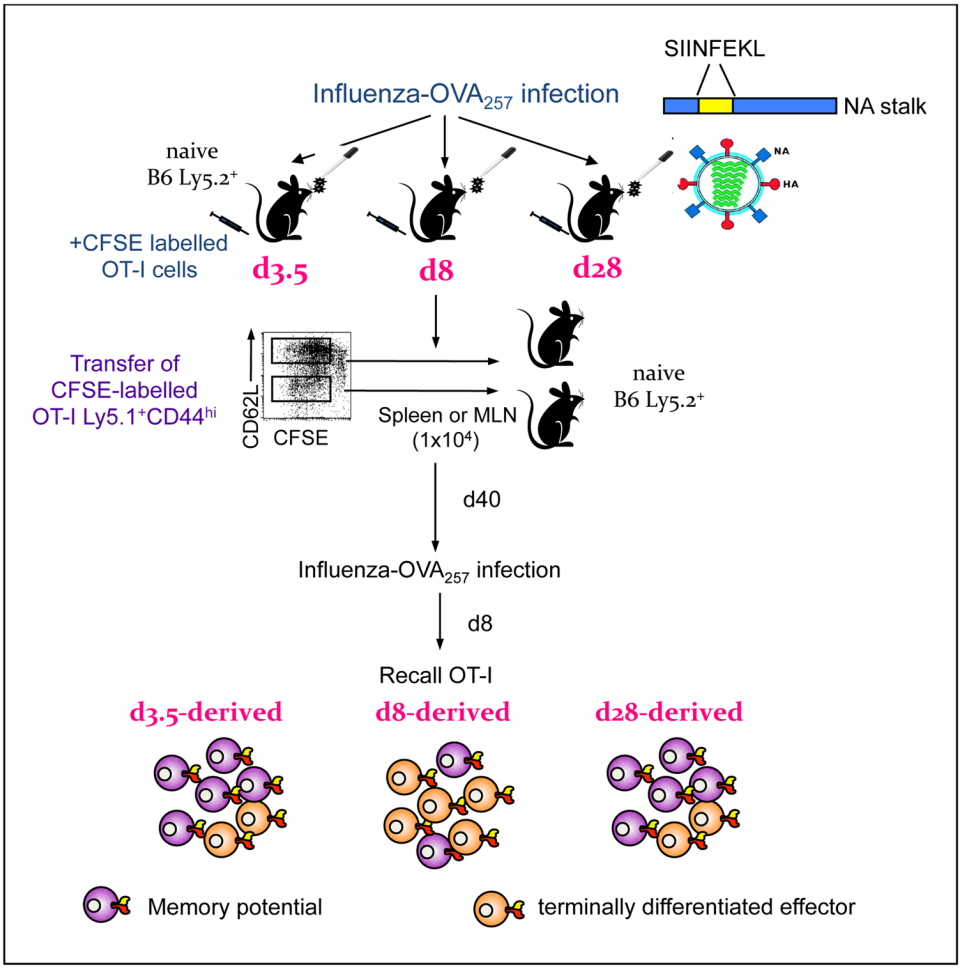

FIGURE 3 | Early establishment and recall of memory precursors in an OT-I transgenic system. Diagrammatic representation of findings published in Kedzierska et al. (2007). OT-I cells were labeled with CFSE, transferred into congenically different B6 mice and infected with an influenza virus expressing the SIINFEKL peptide in the viral NA. On d3.5, d8, or d28 after infection, OT-I $CD44^{hi}$ cells, which have divided, were transferred into naïve B6 mice. After the following 40 days, mice were infected with influenza expressing SIINFEKL to recall any surviving memory cells. OT-I cells from d3.5 after influenza infection could survive and be recalled, findings which reflected the d28 (memory) time-point. The memory potential of d8 effectors was greatly diminished, most likely due to a high proportion of terminally differentiated effectors.

Thus, it appears that the inflammatory milieu, and particularly IFN-γ and IL-12, impact the proportion of cells expressing effector and memory-like markers at peak, and therefore the establishment of T cell memory pools. However, the exact mechanisms by which inflammation affects memory formation are yet to be elucidated.

Studies using peptide-pulsed dendritic cells revealed that antigenic stimulation of T cells without high-level inflammation typically present in infection, led to the earlier establishment of T cell memory, both phenotypically and functionally (Badovinac et al., 2005). Again, this early establishment of memory pools was reversed by the addition of CpG. However, it is important to note that some level of inflammation is needed for T cell memory formation (Shaulov and Murali-Krishna, 2008). There is a substantial body of evidence that the inflammatory stimuli can affect the molecular signatures of the responding T cells. The expression of T-bet, as mentioned above, can clearly direct antigen-specific T cells into either a memory or an effector pathway (Joshi et al., 2007). Similarly, modulation of transcription factors such as Blimp-1 (Kallies et al., 2009; Rutishauser et al., 2009; Shin et al., 2009) and Eomes (Intlekofer et al., 2005) is necessary for the acquisition of effector versus memory lineage commitment. The main cytokines that affect the expression of theses transcription factors for responding T cells are IL-12, IL-2, IL-15, and IL-7, and the type-1 interferons. Dissecting the difference between the "good inflammation" necessary for the establishment of immunological memory versus the "bad inflammation" that leads to damaging immunopathology is imperative for future vaccine design.

LONG-TERM PERSISTENCE OF T CELL MEMORY

INSIGHTS GAINED FROM STUDIES IN AGED ANIMALS

Animal models have provided important insights into the persistence of T cell memory. In particular, experiments in aged animals

allow a controlled analysis of the persistence of memory T cells over the lifetime that may follow an initial exposure to, and recovery from, a pathogenic infection. Such studies also allow us to study the recall of these memory cell populations after a subsequent pathogen challenge. Aged mice and non-human primates, similar to the elderly human population, are particularly susceptible to novel viral and bacterial infections, leading to an increase in the occurrence of severe disease. Thus, the impact of age on T cell responsiveness to infection or vaccination, together with the potential implications for both memory persistence into old age and the *de novo* generation of such memory in the elderly, are priority areas for analysis.

The reduced efficacy of T cell responses to infection and vaccination with age is thought to reflect a decline in both naïve T cell numbers and TCR diversity, together with the functional compromise of the "aged" naïve T cells. The thymic epithelium involutes with age, substantially reducing the generation and export of naïve T cells (Yunis et al., 1973; Simpson et al., 1975). Thus, maintenance of the naïve T cell pool becomes more dependent on homeostatic turnover, evidenced by clonal expansions in the naïve T cell pool (LeMaoult et al., 2000; Cicin-Sain et al., 2007; Ahmed et al., 2009) and the acquisition of a "memory-like" $CD44^{hi}$ phenotype in older mice (Haluszczak et al., 2009; Rudd et al., 2011b; Decman et al., 2012). These time-associated alterations to the naïve T cell pool are accompanied by an age-related increase in the proportion of memory T cells due to pathogen encounter, which is consistent with reported expansions in the memory pool with immunological experience (Vezys et al., 2009). Moreover, large clonal expansions in the memory T cell repertoire associated with acute infection or primary vaccination (Cicin-Sain et al., 2007; Ely et al., 2007) and chronic infections are often observed (Ouyang et al., 2003; Ely et al., 2007; Ahmed et al., 2009; Kohlmeier et al., 2010). These factors collectively contribute to perturbations and a reduction in TCR diversity in the naïve T cell repertoire, which results in the preferential survival of high avidity TCR clonotypes (Cicin-Sain et al., 2007; Ahmed et al., 2009; Rudd et al., 2011b; Decman et al., 2012). As a consequence, aged individuals have impaired capacity to recruit an immune response a diverse array of TCR clonotypes when exposed to some novel infectious process (Yager et al., 2008; Rudd et al., 2011a; Bunztman et al., 2012; Valkenburg et al., 2012), but is instead dominated by clonal expansions or reduced diversity relative to younger counterparts.

Apart from a decline in the number and diversity of naïve T cells, the "aged" naïve T cells are thought to be functionally defective, thus compromising the generation and persistence of T cell memory in aged individuals. Recent reviews on age-related changes for $CD4^+$ (Haynes and Swain, 2012) and $CD8^+$ (Nikolich-Zugich et al., 2012) T cell responses summarize our understanding of such functional defects in antigen-specific T cell responses in the "elderly." These phenotypically obvious defects include reductions in the response magnitude, the capacity to proliferate and differentiate following activation, narrowed T cell polyfunctionality, and the increase in expression in inhibitory receptors associated typically with functional exhaustion. We refer readers to these excellent reviews in order to focus the remainder of this discussion on our recent finding concerning the generation and persistence of influenza-specific $CD8^+$ T cell memory in aged mice.

Previous mouse studies have established that aging is associated with diminished $CD8^+$ T cell efficacy and delayed influenza virus clearance (Effros and Walford, 1983; Bender et al., 1991; Bender et al., 1995; Dong et al., 2000; Po et al., 2002). Recent evidence has further shown that the selective loss of primary, influenza-specific $CD8^+$ T cell responsiveness in older mice is characterized by a narrowing in the spectrum of TCR usage that is seen predominantly for low frequency populations, with this effect being best characterized for the prominent $D^bNP_{366}{}^+$ $CD8^+$ T cell set (Yager et al., 2008; Toapanta and Ross, 2009; Valkenburg et al., 2012). Our work established that primary influenza virus infection of naïve 22-month (versus 12 week) old mice resulted in reduced numbers of immunodominant $NP_{366}{}^+CD8^+$ and $PA_{224}{}^+CD8^+$ T cells in the spleen compared with young adult mice (Valkenburg et al., 2012). In contrast, the difference in the magnitude for epitope-specific $CD8^+$ T cell responses between young and old mice was not observed at the site of infection sampled by bronchoalveolar lavage (Valkenburg and Kedzierska, unpublished). This supports our previous findings that in the event of decreased T cell numbers, virus-specific $CD8^+$ T cells traffic preferentially to the site of infection (Valkenburg et al., 2010). However, despite there being comparable $CD8^+$ T cell counts at the site of infection for young and old, the "aged" "inflammatory" $CD8^+$ T cells displayed impaired cytokine profiles (Valkenburg and Kedzierska, unpublished), similar to those found for the spleen (Valkenburg et al., 2010).

As a consequence, mice primed late (at 22 months) and then challenged even later in life (at 24 months) developed secondary $CD8^+$ T cell responses that were diminished in quantity, quality, and TCR repertoire diversity (Valkenburg et al., 2012). This suggests that the memory $CD8^+$ T cell pools established late in life are defective. Similarly, a recent study (Decman et al., 2010) suggested that infecting mice with LCMV or influenza at an extreme age (18–20 months) leads to defective $CD8^+$ T cell memory and diminished recall responses following virus challenge. Thus, the age at initial priming is a critical determinant of CTL numbers, diversity, and function, with memory $CD8^+$ T cell populations that are generated later in life being generally less efficacious (Eaton et al., 2008).

A key issue that then arises is whether we can recruit and maintain a pool of responsive T cells by priming those cells early in life, especially to diseases such as influenza, which are a particular threat to the elderly. We probed this question for influenza-specific $CD8^+$ T cell memory. Our studies found that the ability to mount a $CD8^+$ T cell response to influenza infection waned with age. However, early vaccination (at 6 weeks of age) prior to the attrition of low frequency anti-influenza $CD8^+$ T cells was important to maintain the numbers, function, and a diverse array of TCRs of the memory pools for the life-time of an animal (Valkenburg et al., 2012). The TCR repertoire in extremely aged mice was "locked in" at an early age following vaccination, which proved advantageous for providing a high avidity and high magnitude response later in life. Consequently, memory T cells generated by influenza priming at a young age had a better protective capacity that was evidenced by accelerated viral clearance and reduced body weight loss in the aged animals as compared to the aged mice responding to primary infection (Valkenburg and Kedzierska, unpublished

data). Hence, it is important to establish influenza-specific $CD8^+$ T cell responses early in life to preserve optimal, T cell responsiveness and protect against the age-related attrition of naïve T cell precursors. These findings also suggest that designing influenza vaccines, which promote as-broad-as-possible spectrum of $CD8^+$ T cell memory in adolescence could be beneficial, even if such benefit emerges long after the subject has first seen the protective immunogen.

LONGEVITY OF HUMAN T CELL MEMORY

Novel insights into the longevity and function of human memory T cell responses have been provided by the elegant studies exploiting early live virus vaccination against yellow fever (YFV-17D) or smallpox (Dryvax; VV; reviewed in Ahmed and Akondy, 2011). Immunization with YFV-17 enabled analysis of YFV-specific T cell responses for individuals residing in countries where they would have had no exposure to wild-type infection. Using this approach, longitudinal analyses showed that a single dose of YFV-17D immunization elicits potent effector $CD8^+$ and $CD4^+$ T cell responses that can then be maintained as long-lasting memory populations (Co et al., 2002, 2009; Akondy et al., 2009; Wrammert et al., 2009). These memory sets had proportionally contracted from larger effector pools, co-incident to expression of CD127 (IL-7Rα; Akondy et al., 2009). Importantly, YFV-specific T cell pools could be detected decades after immunization (Ahmed and Akondy, 2011).

Similarly, exposure to vaccinia virus (VV), to protect against smallpox (or VV recombinants), has enabled the assessment of acute T cell responses and memory in the absence of secondary, "live virus" challenge (Walker and Slifka, 2010). In-depth analysis of memory T cell persistence suggests that the estimated half-life ($t_{1/2}$) for $CD8^+$ T cells is between 8 and 15 years, whereas $t_{1/2}$ for the VV-specific $CD4^+$ set is between 8 and 12 years (Hammarlund et al., 2003; Seder and Ahmed, 2003). At the peak of the acute VV-specific T cell responses, occurring within 2–3 weeks of human vaccination (Terajima et al., 2003; Kennedy et al., 2004; Miller et al., 2008), the numbers of VV-specific effector $CD8^+$ T cells are higher than those of $CD4^+$ T cells within the same individual (Amara et al., 2004; Miller et al., 2008). However, a preferential loss of VV-specific $CD8^+$ T cell memory is experienced by ∼50% of individuals within 20 years post-vaccination (Hammarlund et al., 2003). Interestingly, the $t_{1/2}$ for T cells is much shorter than the $t_{1/2}$ (92 years) for VV-specific antibody responses (Walker and Slifka, 2010). However, though the T cell $t_{1/2}$ appears to be <16 years, VV-specific T cells can be detected in some individuals for up to 75 years by IFN-γ/TNF-α staining (Hammarlund et al., 2003). In comparison, measles virus (MV)-specific memory T cells induced after vaccination can be found for at least 34 years, with $CD8^+$ T cell memory being more stable than $CD4^+$ T cell memory (Naniche et al., 2004). Taken together, the above studies with YFV, VV and MV suggest a long-term persistence of human T cell memory that is in every sense comparable to the profiles analyzed experimentally in mice.

The persistence of human $CD8^+$ T cell memory after influenza virus infection is also of a particular interest, given the commonplace recurrence and the observed susceptibility in elderly populations. In contrast to strain-specific antibodies, T cells elicit broader immunity to a spectrum of influenza strains (seasonal and pandemic) as they generally recognize more conserved, internal components of the virus. Both $CD4^+$ and $CD8^+$ T cell sets play an important role in influenza-specific T cell-mediated immunity (McMichael et al., 1983b; Wilkinson et al., 2012). The effector $CD8^+$ T cells function to recognize and destroy influenza virus-infected cells. Pre-existing $CD8^+$ T cell memory promotes more rapid recovery via the production of pro-inflammatory cytokines and direct killing of virus-producing cells (Doherty et al., 1996). Both experimental and epidemiological evidence indicates that memory $CD8^+$ T cells primed by prior exposure to a seasonal influenza infection provide protection against subsequent challenge with novel, HA- and NA-distinct strains. Some virus replication still occurs, but the disease is less severe and the clinical outcome is improved. Evidence from animal models (Christensen et al., 2000) and humans (McMichael et al., 1983b; Epstein, 2006; Kreijtz et al., 2008; Lee et al., 2008) supports this protective role for $CD8^+$ T-cells following heterologous priming and challenge between H1N1, H7N7, H3N2, and H5N1 influenza viruses. However, persistence of influenza-specific memory T cells in humans is understudied, although early study by McMichael et al. (1983a) used ^{51}Cr-killing activity assay in PBMCs from individuals in 1977–1982 to infer contraction of T cell memory. It is, however, possible that a decrease in ^{51}Cr lysis could be partially related to the loss of the killing capacity (Stambas et al., 2007) or cytolytic molecule expression (gzm A, gzm B) in the long-term memory $CD8^+$ T cells (Jenkins et al., 2007) as shown previously by our studies in a mouse model of influenza infection. Similar observations have been made for human $CD8^+$ T cells, with both granzyme A and granzyme B expression decreasing from 60% 1 month after infection to 33% within 1 year after infection (Rock et al., 2005). Further studies epitope-specific $CD8^+$ T cells (across different HLAs) using the more recent tetramer technology is needed to further explore this issue of memory $CD8^+$ T cell persistence, and acute T cell responses, after human influenza virus infection.

Our recent antigenic analysis of the 2009-H1N1 virus, showed that this newly emerged pandemic virus shared immunogenic peptides with the catastrophic 1918-H1N1 strain (Gras et al., 2010), and shared antigenic similarity with viruses present prior to 1945. This resulted in the detection of cross-reactive $CD8^+$ T cell immunity between the H1N1-2009-infected donors and 1918 derived peptides for the NP_{418} epitope restricted by the large HLA-$B7^+$ family. Cross-reactive immunity between the pandemic 2009-H1N1 strain and the 1918-H1N1 strain (as well as other H1N1 viruses from the beginning of the century) at both T cell and antibody levels may have resulted in lower susceptibility to the H1N1-2009 in individuals >65 years of age, with the majority of severe cases occurring in young adults (the mean patient age was 24 years; Cao et al., 2009; Agrati et al., 2010). This was in contrast to the usual scenario of the elderly population being more susceptible to annual, seasonal epidemics caused by influenza A viruses (Couch et al., 1986; Webster, 2000). Therefore, the lack of T cell immunity in younger adults and children, and the persistence of cross-reactive memory T cells in older individuals may partially account for the demographics of infection. The question thus still remains whether memory $CD8^+$ T cell populations to influenza strains encountered early in life persist for a life-time.

A previous report on $CD8^+$ T cell responses in elderly individuals showed no difference in the frequency of influenza-specific CTLs between the 18 and 70 years-old cohort, however there was lower lytic capacity in the 68–70 years versus the 18–20-year-old donors (Boon et al., 2002). Then, while there were no significant differences in IFN-γ^+ $CD3^+$ $CD8^+$ or $CD4^+$ T cell numbers with age, the proportion of IL-4^+ $CD3^+$ $CD8^+$ and IFN-γ^+ $CD3^+$ $CD8^+$ (detected following PBMC stimulation with PMA/inomycin) increased in the elderly. Furthermore, the T cell proliferative response was significantly higher in the 18–20 years-old cohort, and was not antigen-dose dependent. That is, increasing antigen dose did not compensate for the reduced PBMC proliferation in 68–70 years-old individuals.

Interestingly, CD45RO (memory) and CD45RA (naïve) expression varied between different age groups. In the 18- to 20-year-old cohort, the prevalence of CD45RO versus CD45RA expression was similar for $CD3^+CD4^+$ T cells, while a CD45RA > CD45RO distribution was found for the $CD3^+CD8^+$ set. By contrast, the elderly (Ouyang et al., 2003; Yager et al., 2008; Kohlmeier et al., 2010) group showed a CD45RO > CD45RA profile for both $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells, together with reduced CD28 expression (for both $CD4^+$ and $CD8^+$ T cells) when compared with the youngsters. Furthermore, there was a trend for lower lytic capacity in the age 68–70 set following *in vitro* PBMC stimulation (Boon et al., 2002). This could be related to lower levels of perforin/granzyme expression. Overall, the analysis to date suggests that influenza-specific $CD8^+$ T cells persist, though their cytolytic- and cytokine-producing potential may decline with increasing years. But the findings so far are limited in both scope and sophistication. There is clearly a need for much more detailed analysis of aging, influenza-specific human T cells, from the aspects of numbers, function, repertoire diversity, and capacity for effective recall.

SUMMARY

The future design of T cell-based vaccination strategies that can provide effective and optimal protection across the lifespan of an individual crucially depends upon an in-depth understanding of the development and maintenance of T cell memory and the factors that impact the protective capacity of memory T cell populations. Studies in recent years have made substantial progress in dissecting the complexities of T cell memory populations, resulting in the identification of major factors that influence the composition and stability of these T cell memory populations. While these advances have moved us closer to elucidating the mechanisms contributing to optimal T cell memory generation, there remain many aspects of T cell memory to be investigated. Studies continue to reveal increased phenotypic, functional, and anatomical heterogeneity of T cell memory populations. The temporal changes to the composition and protective abilities of these T cell memory subsets require additional study in order to determine what constitutes optimal T cell memory. An important consideration in future investigations of T cell memory is the increasing evidence that the protective abilities of memory T cells are dependent on the pathogen and the nature of the infection. One potential complicating factor in the maintenance of life-long immunity is the changes at the cellular, environmental, and population level of T cells that occur in later life that have been associated with impaired immune responsiveness in the elderly. However, studies to date indicate that these age-related defects in T cell responses primarily affect the generation of T cell memory in old age. This suggests that to address the increased immune susceptibility in the elderly will require either the development of vaccines to be administered earlier in life, to generate T cell memory that will provide protection the against those pathogens likely to be encountered in later life, or the development of strategies to prevent or treat age-related defects in T cell immunity. Either approach provides substantial challenges for immunological research in terms of improving our understanding of temporal changes to T cell immunity.

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Relevance of long-lived CD8$^+$ T effector memory cells for protective immunity elicited by heterologous prime-boost vaccination

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Owing to the importance of major histocompatibility complex class Ia-restricted CD8$^+$ T cells for host survival following viral, bacterial, fungal, or parasitic infection, it has become largely accepted that these cells should be considered in the design of a new generation of vaccines. For the past 20 years, solid evidence has been provided that the heterologous prime-boost regimen achieves the best results in terms of induction of long-lived protective CD8$^+$ T cells against a variety of experimental infections. Although this regimen has often been used experimentally, as is the case for many vaccines, the mechanism behind the efficacy of this vaccination regimen is still largely unknown. The main purpose of this review is to examine the characteristics of the protective CD8$^+$ T cells generated by this vaccination regimen. Part of its efficacy certainly relies on the generation and maintenance of large numbers of specific lymphocytes. Other specific characteristics may also be important, and studies on this direction have only recently been initiated. So far, the characterization of these protective, long-lived T cell populations suggests that there is a high frequency of polyfunctional T cells; these cells cover a large breadth and display a T effector memory (TEM) phenotype. These TEM cells are capable of proliferating after an infectious challenge and are highly refractory to apoptosis due to a control of the expression of pro-apoptotic receptors such as CD95. Also, they do not undergo significant long-term immunological erosion. Understanding the mechanisms that control the generation and maintenance of the protective activity of these long-lived TEM cells will certainly provide important insights into the physiology of CD8$^+$ T cells and pave the way for the design of new or improved vaccines.

Keywords: memory, vaccines, CD8, adenovirus

GENETIC VACCINATION USING THE HETEROLOGOUS PRIME-BOOST REGIMEN

Genetic vaccination using naked DNA or recombinant viruses is being pursued as an alternative to traditional vaccines. This strategy could be particularly important in the case of intracellular pathogens and neoplastic cells, where the effectiveness relies heavily on the capacity of the vaccine to elicit specific immune responses mediated by cytotoxic CD8$^+$ T cells (reviewed in Rice et al., 2008; Lasaro and Ertl, 2009 Bassett et al., 2011; Gómez et al., 2011; Mudd and Watkins, 2011; Saade and Petrovsky, 2012).

Whereas the use of single-vector delivery for priming and boosting is usually the initial option, one of the most prolific areas of genetic vaccine development is the strategy known as the heterologous prime-boost regimen. This strategy uses two different vectors, each carrying a gene that encodes the same antigenic protein for priming and boosting immunizations. The utility and importance of this strategy was first proposed in the early 1990s using a combination of recombinant viruses (influenza and vaccinia) to induce protective immunity against malaria (Li et al., 1993; Rodrigues et al., 1994). Subsequently, this approach was extended and simplified by incorporating naked DNA for priming followed by a booster injection of a recombinant poxviral vector (i.e., modified vaccinia Ankara, MVA); this was also used to generate sterile protective immunity against rodent malaria (Schneider et al., 1998; Sedegah et al., 1998). Collectively, these studies demonstrated that the heterologous prime-boost regimen generated significantly higher immune responses and proved more effective than the use of any of these genetic vectors individually. The initial use of rodent malaria parasites as a model system may have delayed the development of the field, because malaria is not a conventional model system for developing antiviral or antibacterial vaccines. Nevertheless, this fortuitous fact established the important concept that the difficulty in generating immunity to malaria, and perhaps to other intracellular parasites, in many ways recapitulates the struggles encountered to elicit protective immunity against viruses and bacteria that case chronic infections such as HIV and tuberculosis (TB).

In subsequent years, the heterologous prime-boost vaccination regimen was adopted worldwide as a powerful means to elicit strong type 1 effector $CD8^+$ T cell-mediated immune responses (Tc1) against viral, parasitic, and neoplastic antigens in rodents and non-human primates (NHP; Irvine et al., 1997; Amara et al., 2001; McShane et al., 2001; Zavala et al., 2001; Moore and Hill, 2004; Ellenberger et al., 2006; Gilbert et al., 2006; Aidoo et al., 2007; Nigam et al., 2007; Martinon et al., 2008; Sadagopal et al., 2008). Based on pre-clinical studies, a number of human clinical trials have also been initiated. However, to our knowledge, heterologous prime-boost regimens using plasmid DNA and recombinant poxviruses have not yet provided meaningful protective immunity in humans (McConkey et al., 2003; Moorthy et al., 2004; Keating et al., 2005; Vuola et al., 2005; Cebere et al., 2006; Dunachie et al., 2006; Goonetilleke et al., 2006). The precise reason for such failures is not yet clear. It may be due to the target antigens chosen or to the possibility that the combination of vectors may elicit a type of effector $CD8^+$ T cells in humans that are not functionally and/or phenotypically related to mice, as discussed below.

A number of possible vector combinations that significantly improved cell-mediated immunity, particularly the generation of specific $CD8^+$ T cells, have been described in parallel. Among them, heterologous prime-boost vaccination using naked plasmid DNA for priming followed by a booster injection of recombinant replication-deficient human adenovirus 5 (AdHu5) has recently received significant attention. This strategy has proved successful in some relevant experimental models such as simian immunodeficiency virus (SIV), malaria, Marburg, and Ebola virus infection, and Chagas disease (American trypanosomiasis), providing a considerable degree of protective immunity (Gilbert et al., 2002; Casimiro et al., 2003, 2005; Santra et al., 2005; Acierno et al., 2006; Letvin et al., 2006; Mattapallil et al., 2006; Sun et al., 2006; Wilson et al., 2006; de Alencar et al., 2009; Geisbert et al., 2010; Hensley et al., 2010; Martins et al., 2010; Dominguez et al., 2011; Rigato et al., 2011). These relative successes obtained in pre-clinical experimental models fueled human phase I trials (Freel et al., 2010; Jaoko et al., 2010; Koup et al., 2010; Schooley et al., 2010; Churchyard et al., 2011; De Rosa et al., 2011).

Very recently, improved vector combinations have yielded results (measured in terms of protective immunity) that are slightly better than the results obtained by using plasmid DNA followed by replication-deficient AdHu5 viruses. These new strategies include (i) prime with plasmid DNA in the presence of cytokine genes such as IL-12 or GM-CSF (Lai et al., 2011; Winstone et al., 2011), (ii) genes encoding multimeric proteins (Lakhashe et al., 2011), (iii) boost of adenovirus-immunized animals with an optimized plasmid DNA (Hutnick et al., 2012), (iv) prime with rhesus cytomegalovirus (Hansen et al., 2011), and (v) prime with a different heterologous strain of adenovirus (Barouch et al., 2012).

The precise reason for the superior performance of the heterologous prime-boost vaccination compared to the sequential use of the same vector is still a matter of controversy. Some evidence indicates the possibility that the intense immunity to epitopes present on the priming vector prevents the boosting effect. For example, a recent study in humans shows that a second dose of a recombinant AdHu5 does not provide significant boosting. In parallel, recombinant AdHu5 boosting of DNA-primed individuals resulted in significantly higher immune responses (De Rosa et al., 2011). The anti-vector immunity can be either antibody mediated or independent (Cockburn et al., 2008; Schirmbeck et al., 2008; Frahm et al., 2012). Haut et al. (2011) found that in B cell-deficient mice transgene-specific $CD8^+$ T cell responses were significantly higher in systemic compartments. In contrast, recent studies in humans showed that neutralizing antibodies titers to AdHu5 did not correlate with the magnitude of specific $CD8^+$ T cell of priming after immunization with a recombinant AdHu5. In these experiments, the frequency of specific $CD4^+$ T cells negatively correlated with the intensity of specific $CD8^+$ T cells priming (Frahm et al., 2012).

In spite of the clear evidences that pre-existing immunity may interfere with the use of viral vectors, still, the heterologous prime-boost regimen of immunization is described as a possible solution to this problem. This can be achieved by strong priming with cytokine genes (for example, see Barouch et al., 2003).

Independent of the reasons why the heterologous prime-boost vaccination regimen is superior to the sequential use of the same vector, the main purpose of this review is to examine the characteristics of the protective $CD8^+$ T cells generated by this vaccination regimen.

CHARACTERISTICS OF PROTECTIVE $CD8^+$ T CELLS ELICITED AFTER HETEROLOGOUS PRIME-BOOST VACCINATION

HIGH FREQUENCIES OF SPECIFIC $CD8^+$ T CELLS

One hallmark of the heterologous prime-boost regimen is the elicitation of a higher frequency of epitope-specific $CD8^+$ T cells across multiple models. This high number of effector T cells was initially estimated by the presence of epitope-specific IFN-γ-producing cells using the *ex vivo* ELISPOT assay (Murata et al., 1996; Schneider et al., 1998; Sedegah et al., 1998; Bruña-Romero et al., 2001). Subsequently, the hypothesis was further validated by intracellular staining for IFN-γ (Pinto et al., 2003) and tetramer staining of epitope-specific $CD8^+$ T cells (Tao et al., 2005). More recently, intracellular staining for TNF, IL-2, MIP1-β, T cell surface mobilization of CD107a, and *in vivo* cytotoxicity provided extended evidence (for examples, see Masopust et al., 2006; Mattapallil et al., 2006; Cox et al., 2008; de Alencar et al., 2009; Freel et al., 2010; Reyes-Sandoval et al., 2010; Rigato et al., 2011).

Because most studies are performed with T cells collected from the spleen or peripheral blood lymphocytes (PBL) of mice or NHP, respectively, it is not clear whether these results reflect an overall increase in every tissue. The presence of a large number of epitope-specific T cells in several tissues has been documented in the case of mouse lung, liver, intraepithelial lymphocytes, and PBL (Masopust et al., 2006; Reyes-Sandoval et al., 2011); however, because parallel comparison was not performed with animals that were immunized with a single vector, it is not clear whether these levels were particularly higher than the other vaccination protocols. Conversely, the frequency of epitope-specific $CD8^+$ T cells seems to decrease in mouse lymph nodes (Masopust et al., 2006). This may be due to the lack of CD62L expression on the surface of these activated T cells, as discussed below. In addition, the pattern of circulation and recirculation of these lymphocytes has

been poorly explored. A single study, however, demonstrated that, after a recombinant plasmid DNA prime-AdHu5 boost, $CD8^+$ T cells need to recirculate in order to exert protective immunity against an infectious challenge with the protozoan parasite *Trypanosoma cruzi*. In these vaccinated mice, treatment with the drug FTY720 significantly reduced the efficacy of the protective immunity by interfering specifically with signaling through sphingosine-1-phosphate receptor-1, thereby inhibiting the egress of T cells from the lymph nodes (Dominguez et al., 2012). This observation is important because immunity to certain pathogens is not dependent on the recirculation of T lymphocytes (Pinschewer et al., 2000; Kursar et al., 2008; Jiang et al., 2012).

The importance of recirculation is likely dependent on factors such as (i) the host, (ii) the vectors used for prime and/or boost, and (iii) the route of administration of each vector (for examples, see Mattapallil et al., 2006; Kaufman et al., 2010). These are important characteristics that will certainly influence the protective immunity, as pathogens can cause either tissue-specific or systemic infections.

The T cell protective immunity elicited by heterologous prime-boost regimen is not only significantly higher than the immunity elicited by the traditional vaccine regimen but is also longer-lived. Several experimental models have shown that the immunity lasts for significant periods of time (Amara et al., 2001; Bruña-Romero et al., 2001; de Alencar et al., 2009; Reyes-Sandoval et al., 2010; Rigato et al., 2011).

POLYFUNCTIONALITY OF SPECIFIC $CD8^+$ T CELLS

Based on the assays described above, it became clear that the specific $CD8^+$ T cells elicited by the heterologous prime-boost regimen could exert different immunological functions, as measured by *ex vivo* or *in vivo* assays. Confirmation of the polyfunctionality of these cells was made possible through FACS analyses coupling intracellular cytokine staining and cell surface mobilization of the degranulation marker CD107a.

Accordingly, a number of studies confirmed that distinct heterologous prime-boost regimens elicited polyfunctional $CD8^+$ T cells as defined by the cells' capability to exert two or more functions at the same time. The most frequent example of this across different models is specific $CD8^+$ T cells producing IFN-γ and TNF simultaneously. High frequencies of polyfunctional specific $CD8^+$ T cells were described in (i) mice (Masopust et al., 2006; Duke et al., 2007; de Alencar et al., 2009; Elvang et al., 2009; Reyes-Sandoval et al., 2010; Rigato et al., 2011; Rodríguez et al., 2012; Vijayan et al., 2012), (ii) NHP (Mattapallil et al., 2006; Sun et al., 2006; Cox et al., 2008; Liu et al., 2008; Magalhaes et al., 2008; Cayabyab et al., 2009; Wilks et al., 2010; Hutnick et al., 2012), and (iii) humans (Beveridge et al., 2007; Harari et al., 2008; Winstone et al., 2009; Freel et al., 2010; Jaoko et al., 2010; Koup et al., 2010; Schooley et al., 2010; Churchyard et al., 2011; De Rosa et al., 2011).

It is noteworthy that although these T cells have a high frequency of polyfunctionality, their ability to mediate multiple immunological functions has never been clearly linked to their protective capacity. It is possible that this characteristic aids in but is not critical for their effector functions, depending on the mechanism necessary for pathogen elimination. In a single study performed using genetically deficient mice, in the absence of either IFN-γ or perforin, heterologous prime-boost vaccination failed to mediate protective immunity against infection with the intracellular parasite *T. cruzi*. In the case of perforin-deficient mice, the lack of protective immunity was associated mainly with a significant decrease in the induction of polyfunctional T cells (de Alencar et al., 2009). A second study correlated the presence of higher frequencies of polyfunctional T cells to protective immunity against liver stages of malaria parasite (Reyes-Sandoval et al., 2010). Although these studies may suggest a role for polyfunctional T cells during protective immunity, it is still too early to conclude that they are critical for the protective immunity exerted by $CD8^+$ T cells elicited following the heterologous prime-boost vaccination regimen.

BREADTH OF SPECIFIC $CD8^+$ T CELLS

T cell immune responses are often restricted to a few immunodominant epitopes, a phenomenon termed immunodominance (Akram and Inman, 2012). The precise reason for such a restriction is not clear; however, it may have evolved to maximize the immune response, while at the same time reducing the risk of autoimmunity. For the purpose of vaccine development, having only a narrow number of recognized epitopes may be dangerous, as the pathogens will rapidly select for escape mutants to avoid effector immune responses (reviewed in Streeck and Nixon, 2010; Chopera et al., 2011).

Although it has been possible to increase the frequency of T cells specific for immunodominant epitopes, it is still a challenge to broaden the vaccine-induced $CD8^+$ T cell response to a number of subdominant T cell epitopes. There is evidence that heterologous rAdHu5 boosting improved not only the magnitude but also the breadth of specific $CD8^+$ cells (Liu et al., 2008). However, the impact of this response on protective immunity is not clear. Two recent studies indicated that immunity to subdominant epitopes might participate in vaccine-induced protective immunity following a DNA prime-AdHu5 boost vaccination regimen. The first study provided a correlation between the breadth of the immune response and the protective immunity observed in individual rhesus monkeys vaccinated with SIV genes (Martins et al., 2010). A second study formally demonstrated that heterologous prime-boost vaccination with plasmid DNA followed by recombinant AdHu5 elicited strong immune response to two subdominant epitopes that were not recognized during infection (Dominguez et al., 2011). Based on these observations, mutant genes were generated in which the dominant epitope was removed. Heterologous prime-boost vaccination with these mutant genes-induced $CD8^+$ T cell immune responses only to the subdominant epitopes. Most importantly, strong $CD8^+$ T cell-mediated immunity was still observed (Dominguez et al., 2011). These results unequivocally demonstrate the importance of immunity to the subdominant epitopes and the ability of the heterologous prime-boost to elicit them. Nevertheless, other groups still have difficulty improving the immune response to subdominant epitopes following heterologous prime-boost vaccination of NHP (Vojnov et al., 2011). Therefore, new strategies to improve the breadth of the immune response might be developed in order to potentiate vaccine formulation.

TEM PHENOTYPE OF SPECIFIC LONG-LIVED CD8$^+$ T CELLS

The current immunological paradigm divides antigen-experienced CD8$^+$ T cells into three main types: (i) T effector (TE), (ii) T effector memory (TEM), and (iii) T central memory (TCM). These populations of T cells can be distinguished by the presence of activation markers, as well as by differences observed in their localization and recirculation patterns and their ability to proliferate and present certain effector functions/molecules (Angelosanto and Wherry, 2010; Cui and Kaech, 2010; Ahmed and Akondy, 2011; Sheridan and Lefrançois, 2011). More recently, other subpopulations of CD8$^+$ T cells have also been described (Wakim et al., 2010; Sheridan and Lefrançois, 2011; Jiang et al., 2012). The concept of TE/TEM/TCM was primarily established by experimental infections with self-curing pathogens. In these cases, TE are short-lived effector cells (CD44^{High} CD11a^{High} CD62L^{Low} CD127^{Low} KLRG1^{High}); TCM are long-lived memory cells (CD44^{High} CD11a^{High} CD62L^{High} CD127^{High} KLRG1^{High}); and TEM are transitional cells that exist for a short period of time and have distinct surface marker expression (CD44^{High} CD11a^{High} CD62L^{Low} CD127^{High} KLRG1^{High}). However, even by using models of self-curing infections, the existence of other memory T cell subpopulations that might be more relevant for long-term T cell immunity have been proposed (Hikono et al., 2007).

In addition, very recently, considerable interest has been placed on tissue-resident memory T (TRM) cells. These cells are potent long-lived effector cells present in a variety of peripheral tissues including the skin and sensory ganglia, gut, brain, lung, etc. (Wakim et al., 2008; Gebhardt et al., 2009; Masopust et al., 2010; Purwar et al., 2011). They mediate protective immunity against brain infection with vesicular stomatitis virus or skin infection with vaccinia or herpes simplex virus (Wakim et al., 2010; Jiang et al., 2012; Mackay et al., 2012). Their phenotype is CD44^{High}, CD62L^{Low}, CD103^{High}, CD69^{High}, CD127^{Low}, and PD-1^{Low}. The relationship of TRM cells with TEM or TCM is yet to be determined. Some authors propose that these cells constitute an independent lineage primed within the tissue (Wakim et al., 2010, 2012).

Very recently, the development of a methodology to perform transcriptional profiling at the single cell level has detected further differentiation profiles among TEM and TCM. The analysis of specific splenic CD8$^+$ T cells from mice immunized with distinct vaccination protocols yields significant differences among these subpopulations (Flatz et al., 2011). For example, while 74% of the specific TEM elicited by the heterologous DNA prime-recombinant AdHu5 boost vaccination were Klrk1$^-$ Klrg1$^-$ Ccr5$^-$, only 20% of the cells generated by HuAd5-prime-recombinant LCMV boost had a similar phenotype (Flatz et al., 2011). This type of analysis further highlights the heterogeneity still being uncovered within these memory cells.

It has been observed on multiple occasions that after heterologous prime-boost vaccination, the boosting immunization drives not only an expansion of the T cells but also a different phenotype in the long-lived memory T cells. Different doses of the vaccine also caused a significant increase in the frequency of specific CD8$^+$ T cells with a TEM phenotype (CD44^{High} CD11a^{High} CD62L^{Low} CD127^{High} KLRG1^{High}; Masopust et al., 2006). TEM cells were described initially as highly protective against certain viral and bacterial infections (Bachmann et al., 2005a,b; Huster et al., 2006). Likewise, protective immunity afforded by the different heterologous prime-boost vaccination protocols has been associated with the presence of this type of T cell (Hansen et al., 2011; Reyes-Sandoval et al., 2011; Rigato et al., 2011; Xiao et al., 2011; Barouch et al., 2012; Yamamoto et al., 2012).

Based on the relatively poor knowledge of the surface activation markers present on long-lived specific TEM CD8$^+$ T cells, we performed a detailed analysis of the different T cells markers following intramuscular DNA prime-adenovirus boost immunization. We identified transgenic epitope-specific T cells in the spleen of immunized mice 14 or 98 days after the boost vaccination. **Figure 1** summarizes the surface marker phenotype of these epitope-specific T cells compared to the phenotype of the naïve cells.

PROLIFERATIVE CAPACITY OF SPECIFIC CD8$^+$ T CELLS

The proliferative capacity of specific T cells elicited by heterologous prime-boost vaccination has not been thoroughly studied to date. In general, after resolution of experimental self-curing infections, the frequency of total CD8$^+$ T cells declines to less than 10% of the maximal number of specific T cells observed during the peak of the immune response; this is known as the contraction phase. The decrease in the number of specific T cells occurs mainly among the short-lived effector cells (Angelosanto and Wherry, 2010; Cui and Kaech, 2010; Ahmed and Akondy, 2011; Sheridan and Lefrançois,

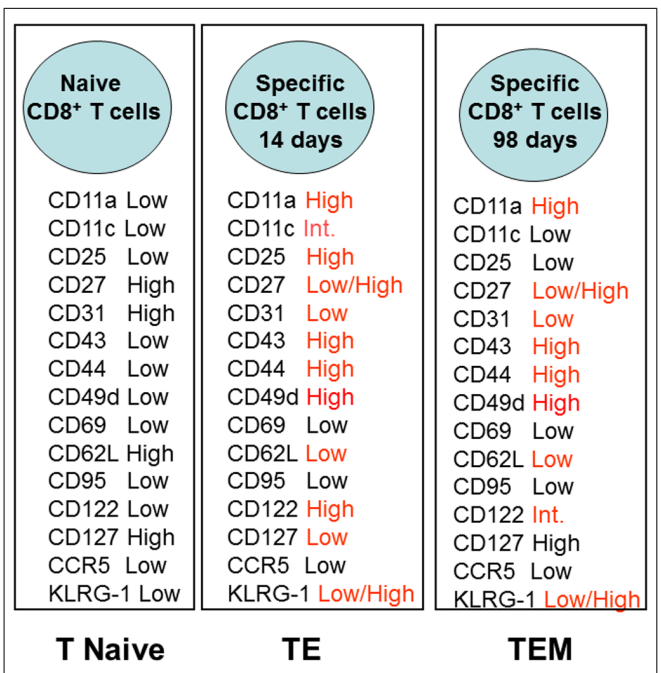

FIGURE 1 | Phenotype of specific CD8$^+$ T cells elicited by heterologous prime-boost vaccination using recombinant plasmid DNA and AdHu5. Prime-boost regimen was performed as detailed described by Rigato et al. (2011). Mice were primed i.m. with plasmid DNA (100 μg) and boosted 21 days later with AdHu5 (2×10^8) both expressing the gene encoding the amastigote surface protein-2 of *T. cruzi*. Expression of distinct adhesion/activation receptors on the surface of splenic CD8$^+$ specific T cells is shown at day 14 or day 98 after the boost immunization.

2011). However, this does not seem to be the case for the TE cells elicited by the heterologous prime-boost vaccination regimen (Jameson and Masopust, 2009). In this case, the cells expand and are maintained at a high frequency in the spleen for a long period of time (Masopust et al., 2006; Vezys et al., 2009; Rigato et al., 2011). This is a somewhat unexpected finding and might be of great relevance for the success of this vaccination protocol. Because this expansion and maintenance contrasts with the retraction observed after self-curing infections, it opens a number of questions that should be studied in depth: (i) How can cells expand out of control to reach frequencies higher than the primary immune response? (ii) How do T cells avoid the apoptotic process observed for the short-lived effector cells during the contraction phase? (iii) How are T cells maintained for these long periods? These questions are keys to understanding the physiology of $CD8^+$ T cells and developing better T cell vaccines.

After an infectious challenge, specific T cells proliferate. However, it is still unknown whether this proliferative response occurs among the antigen-primed TE(M) cells, rare TCM or naïve T cells. To address this question, it is now possible to use the gzm-BCreERT2/ROSA26EYFP transgenic mouse line (Bannard et al., 2009). Specific TE $CD8^+$ T lymphocytes can be indelibly labeled with enhanced yellow fluorescent protein (EYFP). Following an infectious challenge, it will be possible to follow the expansion of EYFP-labeled TE cells. This result will confirm or not whether the cells that expand after the infectious challenge are indeed mainly antigen-experienced TE. Also, by the adoptive transference of green-fluorescent naïve specific T cells prior to the infectious challenge it will be possible to determine whether these cells proliferate or not together with the specific TE cells. Whether this is true for all different protocols of heterologous prime-boost vaccination strategies is still unknown and might be relevant for the development of vaccines to be used in individuals primed with conventional vaccines such as Bacillus Calmette–Guérin (BCG; Hoft et al., 2012).

REFRACTORINESS TO APOPTOSIS OF SPECIFIC $CD8^+$ T CELLS

As mentioned above, after an intense immune response and pathogen elimination, the number of specific $CD8^+$ T cell is drastically reduced during a period called the contraction phase. In that period, TE or short-lived effector cells are eliminated by mechanisms that involve apoptosis mediated by BCL-2-interacting mediator of cell death (Bim) and CD95 (also known as FAS; Green, 2008; Hughes et al., 2008; Weant et al., 2008; Bouillet and O'Reilly, 2009). We observed that following prime-boost immunization with recombinant plasmid DNA and AdHu5, TEM cells do not upregulate surface CD95 expression and are refractory to anti-CD95-induced apoptosis *in vitro* (Rigato et al., 2011). Because CD95 is an important initiator of the intrinsic pathway of apoptosis in T lymphocytes, low levels of expression may protect T cells from apoptotic death.

Decreased levels of expression or resistance to activation of other receptors that control T cell death, such as TNF receptor and TRAIL, might also play a role during the survival of specific TEM cells. In contrast, increased expression of other receptors may act to protect these specific T cells. Recently, expression of PDL-1 (B7-H1) on antigen-activated $CD8^+$ T cells made these cells more resistant to Ca^{2+}-dependent and Fas ligand-dependent killing by cytotoxic T lymphocytes (Pulko et al., 2011). However, more details are necessary to understand the expression levels of many of these molecules controlling T cell survival.

It is plausible that Bim is poorly activated, or not activated at all, on these cells. Because Bim is activated in response to the lack of certain external stimuli, these activation signals might be maintained for long-term. One such mechanism is antigen presentation by dendritic cells. Using a replication-deficient adenoviral vector, Tatsis et al. (2007) demonstrated that the transgene product remains available for antigen presentation for as long as 16 weeks after a single immunizing dose. The importance of continuous transgene expression in the maintenance of the specific $CD8^+$ T cells after AdHu5 vaccination was also demonstrated by a causal relationship between both using an inducible system to turn off the transgene expression (Finn et al., 2009). Bassett et al. (2011) showed that both hematopoietic and non-hematopoietic antigen-presenting cells are necessary for the maintenance of $CD8^+$ T cells following immunization with recombinant adenovirus. However, it is not clear whether antigen persistence is also observed with other vectors.

We tested whether the IFN-γ or IL12/IL23 signaling pathways might be important for maintaining these long-lived $CD8^+$ TEM cells. The absence of either pathway individually made little difference in the generation of specific $CD8^+$ T cells. The absence of the IL12/IL23 pathway, but not the IFN-γ pathway, was important for the long-term survival of these cells (Rigato et al., 2011). Further details regarding the relevance of these signaling pathways in maintaining a high frequency of $CD8^+$ T cells are unknown. In addition, little is known about the impact of the lack of the IL12/IL23 pathway in the maintenance of specific $CD8^+$ T cells after other heterologous prime-boost vaccination regimens. We consider this area of critical importance in understanding how T cells can be maintained at high frequencies for long periods of time. A possible explanation that remains to be tested is whether the lack of contraction could be due to the fact that many specific long-lived $CD8^+$ T cells express low levels of the chemokine receptors CXCR3 and CCR5 (Flatz et al., 2011). This possibility is supported by recent observations that genetically deficient $CD8^+$ T cells that do not express both these receptors were refractory to contraction and accumulated in higher numbers in the spleen (Kohlmeier et al., 2011).

Recently, we described a new and potentially very important aspect of $CD8^+$ TE cells. After recombinant AdHu5 vaccination, $CD8^+$ TE cells do not undergo apoptosis, as well as prevent the development of a pro-apoptotic phenotype that occurs during experimental infection with the protozoan parasite *T. cruzi*. This phenomenon was observed when the administration of the recombinant AdHu5 vaccine was provided before (as part of a prime-boost regimen or alone) or at the time of the infectious challenge. AdHu5 treatment modulated specifically the $CD8^+$ TE cells to express lower levels of CD95 (FAS) and become resistant to CD95-induced apoptosis. The determination of the distinct adhesion/activation receptors on the surface of the $CD8^+$-specific T cells elicited by either infection or recombinant AdHu5 immunization showed very limited differences that were almost exclusively confined to the apoptotic receptor CD95 (**Figure 2**).

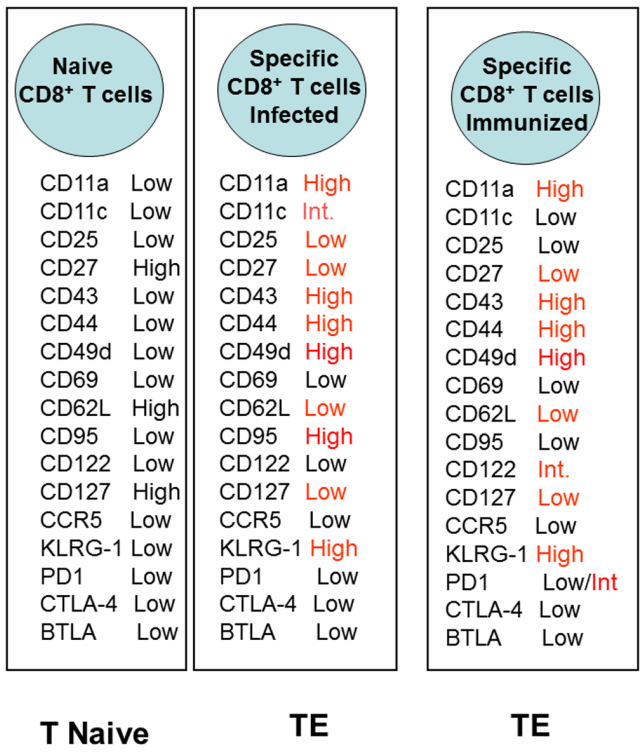

FIGURE 2 | Expression of distinct adhesion/activation receptors on the surface of specific CD8+ T cells elicited by either *T. cruzi* infection or recombinant AdHu5 immunization (Vasconcelos et al., 2012). Mice were infected s.c. with *T. cruzi* (150 blood stream trypomastigotes) or immunized i.m. with AdHu5 (2×10^8 pfu) expressing the gene encoding the amastigote surface protein-2 of *T. cruzi* 19 days earlier.

Despite the above results, we have not ruled out that other pro-apoptotic signaling pathways might also be altered. In these immunized and challenged mice, the CD8+ T cell population expanded largely and protected mice against an otherwise lethal infection (Vasconcelos et al., 2012). An important mechanism that controls the expression of CD95 on the surface of specific CD8+ T cells has just been described during development of immune responses to viral infections. TCR activation led to an increase of the RIG-I-like receptor LGP2 expression which down-regulated CD95 expression. In KO mice, the absence of this molecule significantly increased the apoptosis reducing the effectiveness of the immune response and resistance to the viral infection (Suthar et al., 2012). We consider this a promising area of study because it suggests that perhaps the control of T cell survival might be the key element for the development of new or improved vaccines. The proposed pathway of specific CD8+ T cell activation following prime-boost vaccination and infection is shown in **Figure 3** (based on Vasconcelos et al., 2012).

REDUCED IMMUNOLOGICAL EROSION OF SPECIFIC CD8+ T CELLS

Memory CD8+ T cells are not a stable pool; subsequent infections may cause attrition and erosion of the memory T cell pool (Selin et al., 1996, 1999; Dudani et al., 2008; Huster et al., 2009; Schmidt and Harty, 2011). However, different heterologous prime-boost regimens generated a pool of CD8+ T cells that was not eroded by subsequent viral infections (Vezys et al., 2009; Rigato et al., 2011). After a vaccination regimen consisting of recombinant plasmid DNA prime-AdHu5 boost, we observed that viral infections had limited impact on the number or quality of the TEM cells as measured by different functional immunological assays. Most importantly, protective immunity mediated by these CD8+ TEM cells was not altered in these mice (Rigato et al., 2011).

In contrast, using a different prime-boost vaccination protocol consisting of dendritic cells coated with circumsporozoite protein peptide and a booster immunization with recombinant *actA–inlB*-deficient *Listeria monocytogenes* expressing the same epitope elicited an immune response that could be eroded by multiple subsequent infections (Schmidt and Harty, 2011). The discrepant results highlight the importance of determining the characteristic of each TEM elicited by the distinct regimen of vaccination, as suggested earlier (Flatz et al., 2011).

The resistance to immunological erosion is one more interesting characteristic of these long-lived TEM cells that has been poorly explored and may have an important impact in the development of efficient vaccines. As mentioned above, this high-resistance immunological erosion can be linked to the differential expression of surface molecules (death receptors such as CD95/FAS) or apoptosis mediators (such as Bim).

UNKNOWN FATE OF THE SPECIFIC CD8+ TEM CELLS

Although some studies described that theses TEM can be long-lived in some mouse models, it is not clear what will be the fate of these cells on extended periods of time. Will they become TCM by upregulating CD62L? Will they simple die? Can they be boosted? Perhaps these questions can be addressed by using the gzmBCreERT2/ROSA26EYFP transgenic mouse line (Bannard et al., 2009). Labeling specific TE CD8+ T lymphocytes will allow us to follow theses cells for longer periods of time.

Also, their long-term fate in NHP or humans is even more important for the purpose of development of practical vaccines. Due to the obvious constrains, few studies so far have addressed this issue.

CONCLUDING REMARKS AND PERSPECTIVES

Genetic vaccination using heterologous prime-boost regimen protocols has the potential to serve as the basis for the development of new vaccines against many pathogens. In spite of the progress over the past 20 years, much work is required to improve the relevance of vaccine research, considering that human immune response is still at least one order of magnitude lower than that observed in mouse or even NHP models.

Several areas can be pursued for this matter. So far, protective immunity is clearly associated with the presence of a high number of long-lived specific polyfunctional TEM cells. Nevertheless, several points should be considered. First, TEM may vary from one protocol to the other. It is also important to improve the number of specific TCM cells, for example, by using pharmacological modulators (Li et al., 2012; Takai et al., 2012). However, it will be a challenge to increase the number of TCM cells without reducing the number of TEM cells. Fine tuning the TEM

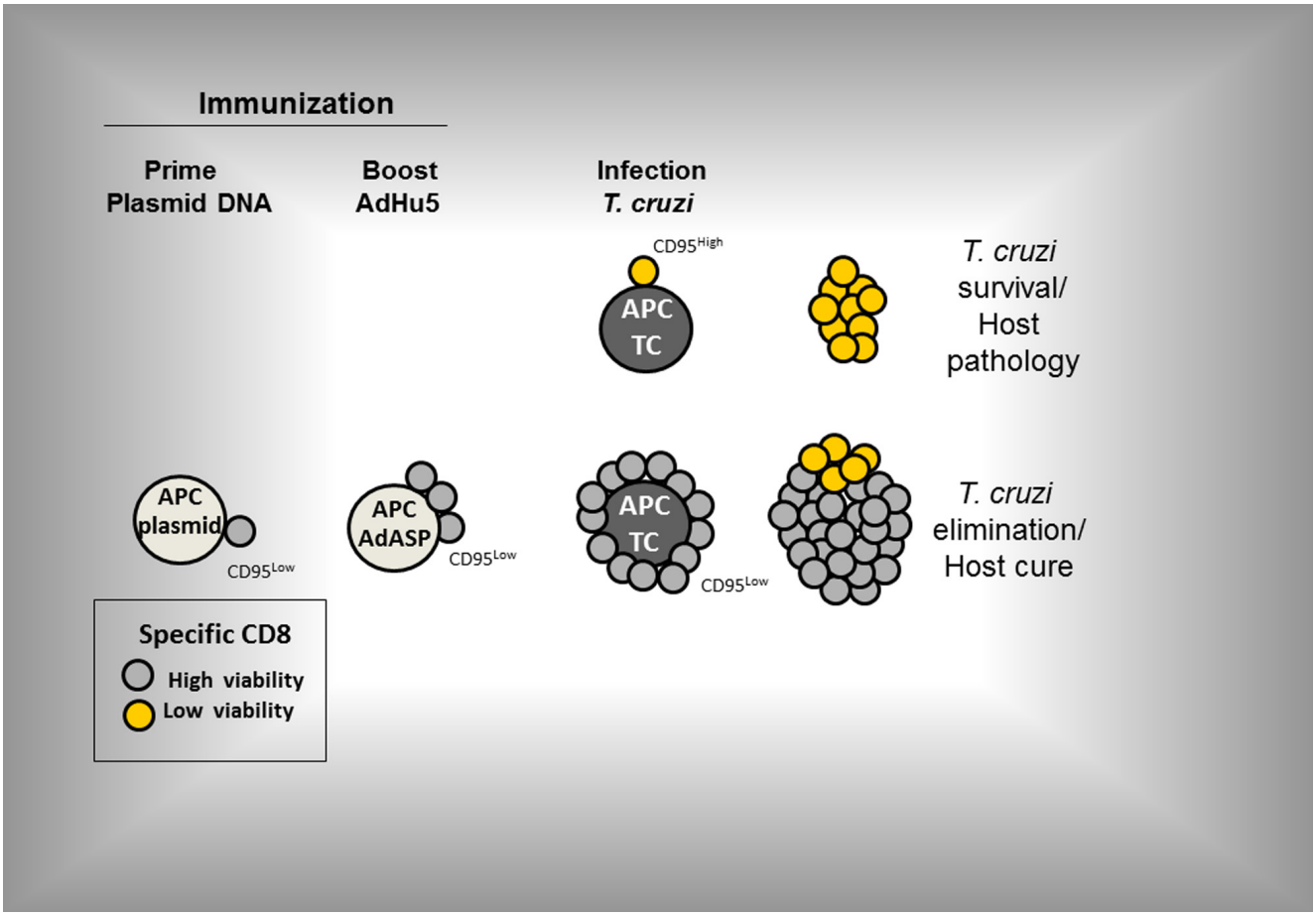

FIGURE 3 | The proposed pathway of activation of specific CD8$^+$ T cells following prime-boost vaccination and infection (based on Vasconcelos et al., 2012). Prime-boost regimen was performed as detailed described by Rigato et al. (2011). Mice were primed i.m. with plasmid DNA (100 μg) and boosted 21 days later with AdHu5 (2×10^8) both expressing the gene encoding the amastigote surface protein-2 of *T. cruzi*. Mice were infected s.c. with *T. cruzi* (150 blood stream trypomastigotes).

subpopulations and increasing the number of TCM cells could further improve and prolong protective immunity beyond the levels currently achieved. Furthermore, new strategies to broaden the epitopes recognized by protective T cells would also improve both the quantity and the quality of the immune response. Finally, the circulation of these cells should be further studied for the purpose of personalizing the vaccination regimen for different types of infections, considering that the site of entry of each pathogen will vary, as will the localization of the T cells at the time of the infection.

In summary, the study of the control of memory T cell generation, maintenance, quality, and recirculation after distinct heterologous prime-boost vaccination regimens will provide important clues regarding the physiology of lymphocytes and the immune system, with potential applications in public health.

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Protecting and rescuing the effectors: roles of differentiation and survival in the control of memory T cell development

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Vaccines, arguably the single most important intervention in improving human health, have exploited the phenomenon of immunological memory. The elicitation of memory T cells is often an essential part of successful long-lived protective immunity. Our understanding of T cell memory has been greatly aided by the development of TCR Tg mice and MHC tetrameric staining reagents that have allowed the precise tracking of antigen-specific T cell responses. Indeed, following acute infection or immunization, naïve T cells undergo a massive expansion culminating in the generation of a robust effector T cell population. This peak effector response is relatively short-lived and, while most effector T cells die by apoptosis, some remain and develop into memory cells. Although the molecular mechanisms underlying this cell fate decision remain incompletely defined, substantial progress has been made, particularly with regards to $CD8^+$ T cells. For example, the effector $CD8^+$ T cells generated during a response are heterogeneous, consisting of cells with more or less potential to develop into full-fledged memory cells. Development of $CD8^+$ T cell memory is regulated by the transcriptional programs that control the differentiation and survival of effector T cells. While the type of antigenic stimulation and level of inflammation control effector $CD8^+$ T cell differentiation, availability of cytokines and their ability to control expression and function of Bcl-2 family members governs their survival. These distinct differentiation and survival programs may allow for finer therapeutic intervention to control both the quality and quantity of $CD8^+$ T cell memory. Effector to memory transition of $CD4^+$ T cells is less well characterized than $CD8^+$ T cells, emerging details will be discussed. This review will focus on the recent progress made in our understanding of the mechanisms underlying the development of T cell memory with an emphasis on factors controlling survival of effector T cells.

Keywords: $CD8^+$ T cells, memory cells, $KLRG1^{hi}CD127^{lo}$, Bim, Bcl-2

IMMUNOLOGICAL MEMORY

The concept of immunological memory has dated back to as early as the fifth century B.C. as the Athenian author Thucydides mentioned in his scripts that people who survived plague would not be attacked a second time (Thucydides and Marchant, 1899). In seventh century, people drank snake venoms to get toxoid-like immunity (Plotkin et al., 2008). In ancient China, people blew powdered scabs of smallpox pustules into their nose to be protected from smallpox, a process called variolation (Plotkin et al., 2008). The process of variolation transferred to westward to the Middle East along shipping routes when Lady Mary Wortley Montagu witnessed this process and popularized variolation in England in the 1700s. By the time Edward Jenner immunized a child with cowpox and challenged him with smallpox, the concept of immune "memory" or "immunity" existed. Nearly 100 years elapsed before purposeful development of vaccines was attempted against cholera toxin and the rabies virus by Pasteur (Plotkin et al., 2008). Thus, the concept that prior exposure to a disease-causing microorganism (or a close relative) could provide long-lasted protection against subsequent infection has been around for a very long time. The subsequent large-scale development of effective vaccines against yellow fever, smallpox, rabies, influenza, polio, measles, mumps, diphtheria, Bordetella, hepatitis B, and, more recently, rotavirus have saved countless lives and are one of the greatest improvements to human health. Over the last few decades with the advent of cellular and molecular approaches we are started to unravel the mechanisms underlying immunological memory.

Immunological memory has been defined simply as the heightened immune response against a previously encountered pathogen that is due to the increased numbers of antigen-specific cells and their increased capacity to respond to secondary stimulation (Murphy et al., 2011). Both arms of adaptive immunity; antibody responses and T cell responses are quantitatively and qualitatively better than the primary responses. Immunological memory has been utilized successfully for generating protective immunity against many pathogens (Rappuoli, 2007). While it is clear that B cell production of antibody is critical for the protective features of many vaccines; long-lived T cell immunity is also critical component induced by vaccines. This review will focus on recent advances made in our understanding of mechanisms underlying the development of memory T cell responses.

TRACKING T CELL RESPONSES

One of the substantial developments in T cell biology over the past few decades has been the ability to monitor T cells responses at the single-cell level. Early work examining T cell function was restricted to population based assays such as proliferation (^{3}H incorporation) for CD4^{+} T cells and CTL assays (^{51}Cr release) for CD8^{+} T cells. The development of TCR Tg mice and adoptive transfer approaches for the first time allowed tracking antigen-specific (albeit monoclonal) T cell responses to nominal antigens like ovalbumin (Kearney et al., 1994), to autoantigens (Katz et al., 1993), or to viral antigens (Pircher et al., 1990). It was not until the development of intracellular cytokine analysis by flow cytometry, that endogenous, polyclonal, antigen-specific T cell responses could be tracked at the single-cell level (Jung et al., 1993). While this was a critical development, it also required a brief stimulation of T cells either *in vitro* (Jung et al., 1993) or *in vivo* (Liu and Whitton, 2005), which could change the gene expression and phenotype of the cells. In addition, it only allowed for examination of cells whose cytokines are being measured, not necessarily all of the T cells responding to the antigen/infection. In contrast, the development of MHC tetramers was an absolutely critical tool for the tracking and analysis of endogenous T cell responses without the need for secondary stimulation (Altman et al., 1996). The development of these tools for tracking endogenous T cell responses has taught us a lot about T cell expansion, differentiation, and localization.

KINETICS OF T CELL RESPONSES

The initial reports tracking endogenous T cell responses characterized a massive expansion phase, in which responding T cells undergo 15–20 rounds of division, a "contraction" phase in which 80–90% of the responding T cells undergo apoptosis, and a "maintenance" phase in which the remaining effector cells persist as memory T cells and are maintained for the life of the animal (Butz and Bevan, 1998; Murali-Krishna et al., 1998; Williams and Bevan, 2007). For acute infections, the decline of T cell responses occurs just after the infection is cleared (**Figure 1**). Further, the expansion and contraction of CD8^{+} T cell responses are of a significantly greater magnitude compared with CD4^{+} T cell responses (**Figure 1**). While CD8^{+} T cell memory appears relatively stable over time, the CD4^{+} memory T cell population undergoes a gradual attrition (**Figure 1**). Nonetheless, a central question regarding the development of T cell memory is how some T cells avoid death and develop into memory T cells. Over the last decade, significant progress has been made regarding our understanding of the molecular mechanisms that contribute to the death of most effector T cells and to the transcriptional network that controls development of cells that are destined to become memory T cells. Herein, we will describe the current understanding of how T cells transit from potent effectors to lifelong protectors.

HETEROGENEITY OF EFFECTOR T CELLS

Effector CD8^{+} T cells are a heterogeneous population as defined by differential expression of surface markers. As only a small fraction of effector T cells develop into memory cells, there has been a quest to identify memory precursors early after infection.

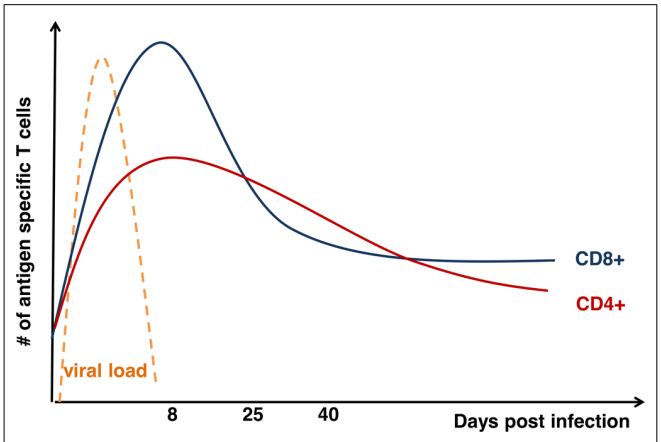

FIGURE 1 | Kinetics of T cell response after acute viral infection. Graph shows total numbers (*y*-axis) of antigen-specific CD8^{+} (blue) and CD4^{+} (red) T cells days (*x*-axis) after acute infection as modified from Hinds et al. (2007). Kinetics of viral load (orange) is also displayed on the graph.

Initially, it was unclear if memory cells went through an effector stage or whether they were a distinct lineage without effector characteristics. By using IFN-γ reporter mice or granzyme B promoters, it has been shown that memory CD8^{+} T cells were derived from IFN-γ producing and granzyme expressing effector cells, respectively (Harrington et al., 2008; Bannard et al., 2009). Importantly, adoptive transfer of a single naïve TCR transgenic T cell into congenic mice generated heterogeneous subsets of effector and memory CD8^{+} T cells in response to *L. monocytogenes* (Stemberger et al., 2007). Although these studies showed that memory cells are derived from effector cells, not every effector cell maintains the same potential to become memory cell over the course of infection. Many markers including cytokine receptors, chemokine receptors, and stimulatory/inhibitory receptors (described in more detail below) have been found to be differentially expressed among effector cells at the peak of the response (days 8–10 after infection). Among these markers, IL-7Rα (CD127) which is down-regulated on most of the effector cells early after infection (Schluns et al., 2000), but the proportion of cells expressing CD127 increases as the response contracts (Kaech et al., 2003).

Further characterization of these effector CD8^{+} T cells has revealed inverse expression of another marker, killer cell lectin-like receptor subfamily G, member 1 (KLRG1) relative to expression of CD127 (Joshi et al., 2007). At the peak of the response, two major CD8^{+} effector T cell populations emerge, one being KLRG1^{hi}CD127^{lo} and another being KLRG1^{lo}CD127^{hi} (Joshi et al., 2007). Although the two subsets had similar cytotoxicity and IFN-γ production, KLRG1^{lo}CD127^{hi} cells had better production of IL-2 (Sarkar et al., 2008). Adoptive transfer experiments using TCR Tg cells have revealed that KLRG1^{hi}CD127^{lo} cells slowly declined over time after transfer while KLRG1^{lo}CD127^{hi} cells were maintained at a greater level and persisted as long-lived memory T cells (Joshi et al., 2007; Sarkar et al., 2008). Because of these results, KLRG1^{hi}CD127^{lo} have been referred to as "short-lived effector cells or SLECs" and KLRG1^{lo}CD127^{hi} have been

referred to as "memory precursor effector cells or MPECs" (Joshi et al., 2007). While these markers have been helpful in identifying certain populations of cells that have enrichments of cells with more or less potential to develop into memory, further work is necessary to more precisely define cells with memory potential. For example, while many SLECs die during contraction of the response, not all do, and after contraction of the response is largely complete, roughly half of the $CD8^+$ T cells have an SLEC phenotype (Joshi et al., 2007; Kaech and Wherry, 2007). Likewise, when assessing the numbers temporally, roughly 30–40% of MPECs die during contraction of the response (Sarkar et al., 2008; Kurtulus et al., 2011).

Other markers in addition to KLRG1 and CD127 are also used to determine the memory potential of effector T cells. For instance, $CD127^{hi}$ cells also express high levels of CD27, which is a member of tumor necrosis factor receptor (TNF-R) family and the chemokine receptor, CXCR3, but these cells are found to have low expression of CD43 after infection with the Sendai virus (Kaech et al., 2003; Hikono et al., 2007). Thus, while these markers have helped identify cells with more or less ability to form long-lived memory cells, further work is necessary to more precisely define the cells within these subsets.

As primary infections have been difficult to assess in humans, it is unclear whether or not these precise effector subsets exist amongst human T cells. However, recent studies have shed light on effector cells in humans after vaccination with yellow fever virus and the smallpox vaccine (Miller et al., 2008; Akondy et al., 2009). Using MHC–peptide tetramers, the authors characterized the antigen-specific T cell response across the effector response and into memory from the peripheral blood. The phenotype of activated $CD8^+$ T cells were characterized by high expression of HLA-DR and CD38 along with high expression of the proliferation marker Ki-67 and low expression of anti-apoptotic protein Bcl-2 and CD127 (Miller et al., 2008). As antigen-specific human T cells progressed into memory, they upregulated expression of CD127, CCR7, CD45RA, CD28, and Bcl-2 (Miller et al., 2008; Akondy et al., 2009). These memory cells were poly-functional and maintained after 2 years (Akondy et al., 2009). Although KLRG1 expression was not assessed in these studies, they showed that CD127 expression was similar in human and mouse T cells after infection. Thus, these studies showed that human and mouse effector $CD8^+$ T cells share similar expression of several markers.

A more recent study performed a comprehensive analysis of 17 cell surface markers and 9 functional qualities of human $CD8^+$ T cell subsets using single-cell spectrometric analysis (cytometry by time-of-flight or CyTOF; Newell et al., 2012). Functional qualities including expression of six different cytokines and cytotoxic granule components granzyme B and perforin were examined together with surface markers including CD62L, CD45RA, CD45RO, CD27, CD43, and KLRG1. This study found that naïve cells ($CD45RA^+$ $CD27^+$ $CD62L^+$ $CCR7^+$), central memory $CD8^+$ T cells (T_{CM}; $CD45RA^-$ $CD27^+$ $CD62L^+$ $CCR7^+$), effector memory $CD8^+$ T cells (T_{EM}; $CD45RA^-$ $CD27^-$ $CD62L^-$ $CCR7^-$) cells and terminal effector cells ($CD45RA^+$ $CD27^-$ $CD62L^-$ $CD28^-$ $KLRG1^+$ $CD57^+$) represented quite distinct subsets as previously described (Sallusto et al., 1999). However, this study also found a range of cells with combinatorial diversity of phenotypic and functional markers in between these subsets suggesting a continuum of T cell phenotypes (Newell et al., 2012). Unfortunately, this did not longitudinally assess the response to infection as samples were obtained from chronically infected individuals. Thus, more work is needed to temporally examine the effector T cell subsets in humans during both acute and chronic infections in greater detail.

GENERATION OF EFFECTOR $CD8^+$ T CELL SUBSETS

Recent work from a few labs has examined the potential *in vivo* plasticity of these subsets and has tracked their emergence from their naïve precursors. Interestingly, at the earliest times after the response when the cells can be reliably detected, a population appears that is $KLRG1^{lo}CD127^{lo}$, which have been termed "early effector cells or EECs" (Obar et al., 2010). When EECs were adoptively transferred into timed-infected recipient mice, they were able to generate both $KLRG1^{lo}CD127^{hi}$ and $KLRG1^{hi}CD127^{lo}$ cells; transferred $KLRG1^{lo}CD127^{hi}$ cells were able to give rise to some EECs early after transfer but predominantly remained as $KLRG1^{lo}CD127^{hi}$; while transferred $KLRG1^{hi}CD127^{lo}$ cells were largely unable to generate $KLRG1^{lo}CD127^{hi}$ cells (Obar et al., 2011). Thus, shortly after the response, naïve T cells lose expression of CD127, some cells stably reacquire CD127 expression, while others upregulate KLRG1 and largely fail to upregulate CD127 (Joshi et al., 2007; Sarkar et al., 2008). At a molecular level this regulation of CD127 appears to be due to the competing effects of Gfi-1 and GABP-α at the CD127 locus (Chandele et al., 2008). However, the mechanism(s) that control expression of Gfi-1 and GABP-α remain unclear.

As differential expression of KLRG1 and CD127 has allowed some demarcation of cells with more or less memory potential, much work has been focused on mechanisms underlying their generation. For example, one critical question is whether CD127 or KLRG1 are involved in the fate of effector T cells or whether they are simply markers. One initial idea was that expression of CD127 allowed effector $CD8^+$ T cells to compete for IL-7 and, in doing so, was instructive for their survival and/or development into memory cells. However, while exogenous IL-7 could protect effector $CD4^+$ and $CD8^+$ T cells from contraction of the response (Tripathi et al., 2007; Nanjappa et al., 2008), transgenic expression of CD127 failed to prevent contraction of the response (Hand et al., 2007; Haring et al., 2008). Similarly, neutralization or inhibition of IL-7 after infection failed to substantially exacerbate contraction of the effector $CD4^+$ or $CD8^+$ T cell responses (Klonowski et al., 2006; Tripathi et al., 2007, 2010). A recent study has shown that KLRG1-deficient mice have no defects in memory T cell development (Grundemann et al., 2010), demonstrating that KLRG1 is not necessary for effector/memory T cell differentiation. However, given that there are multiple KLRG family members and the fact that KLRG1 possesses an immunoreceptor tyrosine-based inhibition motif (ITIM)-domain, makes it possible that KLRG1 contributes redundantly with other KLRG family members to limit signaling events within $KLRG1^{hi}CD127^{lo}$ cells. On the other hand, if neither KLRG1 nor CD127 are instructive, what are the mechanisms that control generation of these two subsets?

INFLAMMATION DIRECTS EXPANSION OF KLRG1^{hi}CD127^{lo} CELLS

Recent work has revealed an intriguing and complex interrelationship between transcriptional programs that balance input from surrounding inflammatory stimuli to promote a self-renewal program that maintains lifelong immunity. The transcription factor t-bet, initially described as a master regulator of Th1 fate, favors the generation of KLRG1^{hi}CD127^{lo} CD8^{+} T cells (Joshi et al., 2007). Loss of tbx21 (gene encoding t-bet) reduced the formation of KLRG1^{hi}CD127^{lo} effector CD8^{+} T cells, while graded increases in t-bet expression, whether retrovirally overexpressed or induced by varying amounts of inflammatory stimuli (e.g., TLR stimuli, IL-12, etc.) gradually increased the generation of KLRG1^{hi}CD127^{lo} CD8^{+} T cells (Badovinac and Harty, 2007; Joshi et al., 2007). Importantly, the overall numbers of KLRG1^{lo}CD127^{hi} CD8^{+} T cells in these t-bet titration experiments did not change, suggesting a critical role of t-bet in the formation of cells with a KLRG1^{hi}CD127^{lo} phenotype, but not an MPEC phenotype.

Importantly, KLRG1^{hi}CD127^{lo} cells express more t-bet compared to KLRG1^{lo} CD127^{hi} cells, and the reverse is true for eomesodermin (eomes; Joshi et al., 2007, 2011). While neither subset is truly negative for either molecule, both are required for expression of CD122 and a lower t-bet:eomes ratio correlates with KLRG1^{lo}CD127^{hi} cells and long-lived memory (Intlekofer et al., 2005, 2007; Banerjee et al., 2010). Thus, control of the t-bet:eomes ratio, as dictated by the level of inflammation is likely critical in controlling CD8^{+} T cell memory generation.

Understanding the regulation of this t-bet:eomes balance is the focus of several recent papers, which have outlined a complex interplay between t-bet and the mTORC1/AKT/FOXO signaling network (**Figure 2**). Overexpression of a constitutively active (ca) AKT transgene led to significantly increased expression of t-bet and a concomitant decrease in eomes expression (Kim et al., 2012). Conversely, caFOXO overexpression decreases expression of t-bet and increases expression of eomes (Rao et al., 2012). Inflammation, via IL-12 (and possibly other inflammatory mediators) has been shown to increase mTORC1/AKT, which in turn decrease FOXO activity and enhance t-bet expression (Rao et al., 2010, 2012). However, a complicating factor in many of these studies is that caAKT appears to also decrease expression of CD127 (Hand et al., 2010), likely through inactivation of FOXO1 (Kerdiles et al., 2009), making it difficult to clearly distinguish the effector CD8^{+} subsets. Thus, while the current data suggest that mTORC1/AKT/FOXO signaling is differentially balanced between the subsets, it is also formally possible that a proper balance of mTORC1/AKT/FOXO signaling is necessary to emerge from the

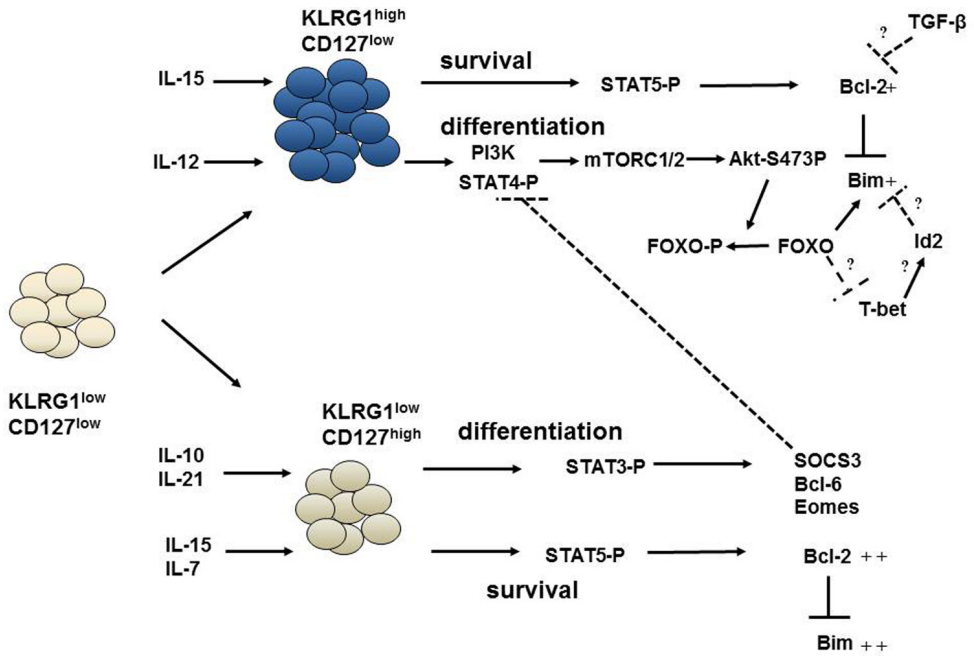

FIGURE 2 | Pathways governing the survival and differentiation of effector CD8^{+} T cells. The differentiation of early effector cells (KLRG1^{lo}CD127^{lo}) into KRLG1^{hi}CD127^{lo} or KLRG1^{lo}CD127^{hi} cells is regulated by inflammatory cytokines and by IL-10 and IL-21. IL-12 can activate STAT4 and PI-3K signaling which modulates mTOR kinases and subsequent Akt phosphorylation at s473. Phosphorylated Akt can phosphorylate and inactivate FOXO proteins. This favors an increased t-bet:eomes ratio and differentiation into KLRG1^{hi}CD127^{lo} cells. On the other hand, Stat3 phosphorylation by IL-10 and IL-21 increases eomes and other transcription factors required for differentiation of KLRG1^{lo}CD127^{hi} cells. SOCS3 induced by Stat3 can, then, inhibit IL-12 signaling, effectively shielding KLRG1^{lo} CD127^{hi} cells from inflammation. Interestingly, survival of effector subsets are regulated by γc cytokines IL-15 and IL-7 via signals driven through STAT5 which appear largely independent of differentiation. While KLRG1^{hi}CD127^{lo} cells can only receive IL-15 signals; IL-15 and IL-7 can both activate Stat5 signaling in KLRG1^{lo}CD127^{hi} cells. This results in Bcl-2 upregulation and inhibition of Bim-mediated apoptosis. IL-15 becomes limiting for KLRG1^{hi} CD127^{lo} cells reducing their ability to sustain Bcl-2 levels in the face of TGF-β signaling. Id2 may also control Bim levels in KLRG1^{hi}CD127^{lo} cells. FOXO proteins may be at the intersection of survival and differentiation pathways as it can regulate both Bim expression and influence the t-bet:eomes ratio.

EEC compartment. More work will be necessary to cleanly dissect the factors that control mTORC1/AKT/FOXO signaling between the effector subsets.

THE ROLE OF ANTIGEN PRESENTATION IN THE GENERATION OF EFFECTOR AND MEMORY CD8$^+$ T CELLS

Obviously, antigen initially drives the metamorphoses of naïve to effector T cell. Previous studies showed that limiting antigen exposure to the first 24 h was sufficient to drive expansion and differentiation into full-fledged effector (van Stipdonk et al., 2001) and memory (Kaech and Ahmed, 2001) cells *in vitro*. However, stimulation longer than 40 h in the presence of IL-12 resulted in a substantial increase in CD8$^+$ clonal expansion compared to shorter stimulation, indicating the role of inflammatory cytokines in the magnitude of the response (Curtsinger et al., 2003). These studies suggested at least two interactions of T cells with antigen-presenting cells (APCs) promoted optimal effector and memory responses. Interestingly, limiting antigen display during *Listeria* infection by antibiotic treatment 24 h after infection resulted in a decreased magnitude of the response but a similar contraction (Badovinac et al., 2002). Secondary challenge of the antibiotic treated mice revealed an enhanced secondary response, despite the decreased magnitude of the primary response (Badovinac et al., 2002). Subsequently, it was found that antibiotic treatment prior to infection in this same model resulted in significantly enhanced generation of cells with a memory phenotype (CD127^{hi}; Badovinac et al., 2004). However, in this study, it was shown that antibiotic treatment significantly decreased inflammation, and it was this attribute, rather than effects on antigen display that likely contributed to the increased memory cells (Badovinac et al., 2004). Similarly, adoptive transfer of naïve TCR Tg cells into mice with an ongoing immune response (as inflammation is waning), results in accelerated development of memory cells (D'Souza and Hedrick, 2006; Sarkar et al., 2007). Furthermore, attempts to restrict antigen display by elimination of dendritic cells (DCs) using CD11c-DTR mice also resulted in a decreased magnitude of the CD8$^+$ T cell response, but accelerated development of cells with memory characteristics (Prlic et al., 2006). However, as DCs are also the same cells that secrete pro-inflammatory mediators, the degree to which their role as antigen presenters versus producers of inflammation is difficult to separate. Thus, while it is likely that limiting antigen display may contribute to memory cell development, further work is necessary to cleanly separate inflammatory stimuli from antigen-presentation.

MIGRATION AND LOCALIZATION OF EFFECTOR AND MEMORY CD8$^+$ T CELL SUBSETS

Being at the right place at the right time may also be important for memory cell development. Indeed, recent work has shown that, in the spleen KLRG1^{lo}CD127^{hi} CD8$^+$ T cells are mostly localized to the T cell zones in the white pulp, while KLRG1^{hi}CD127^{lo} CD8$^+$ T cells are localized to the red pulp (Jung et al., 2010). CXCR3 signals may also be critical in attracting KLRG1^{hi}CD127^{lo} cells to the marginal zone areas, where they may be exposed to more inflammatory stimuli (Kurachi et al., 2011). Also, T cells in CXCR3/CCR5-deficient mice had similar problems with localization, actually failed to undergo contraction in the spleen, and had an emergence of KLRG1^{lo}CD127^{hi} cells (Kohlmeier et al., 2011). In these studies, it was also clear that the absence of CXCR3/CCR5 restricted the accumulation of effector T cells to sites of infection/inflammation as plenty of cells were recruited to the lung, but not to areas of viral replication within the infected lung. While overall tissue localization may direct CD8$^+$ T cells to general areas of inflammation, the finer tuning of their migration within these organs is likely mediated by signals through CXCR3/CCR5. Intriguingly, CXCR3 signals through AKT/FOXO transcription factors raising the intriguing possibility that, in addition to promoting appropriate localization, differentiation signals driven by these chemokine receptors may also contribute to effector T cell heterogeneity. Conversely, high expression of CCR7 on KLRG1^{lo}CD127^{hi} cells likely fosters their migration to/retention within the T cell zones where the ligands CCL19 and CCL21 are highly expressed (Jung et al., 2010). This differential expression of CCR7 may be part of the effector T cell transcriptional program as t-bet and B lymphocyte-induced maturation protein-1 (Blimp-1) can suppress CCR7 expression. Further, in the lymph nodes, fibroblastic reticular cells in T cell zones produce CCL19 and IL-7 (Link et al., 2007), thereby linking localization to T cell zones by CCR7 to IL-7 signals supporting survival of effector cells. It will be of great interest to determine whether it is simply the localization driven by chemokines that is critical for effector CD8$^+$ T cell differentiation, or whether signaling by these chemokine receptors also contributes to effector cell heterogeneity and memory development.

TRANSCRIPTIONAL PROGRAMING OF KLRG1^{hi}CD127^{lo} EFFECTOR T CELLS

In addition to t-bet, other transcription factors have been shown to contribute to the formation of KLRG1^{hi}CD127^{lo} cells including the inhibitor of differentiation 2 (Id2) and Blimp-1 (Kallies et al., 2009; Yang et al., 2011). Id family proteins act as transcriptional repressors and often combat e-box proteins (Murre, 2005). Of the four members of Id family, both Id2 and Id3 are reciprocally expressed in effector CD8$^+$ T cell subsets. Id2 is more expressed in KLRG1^{hi}CD127^{lo} cells, while Id3 is more expressed in KLRG1^{lo}CD127^{hi} cells (Yang et al., 2011). Id2-deficient mice generated a substantially reduced effector CD8$^+$ T cell response to *L. monocytogenes* (Cannarile et al., 2006). This was further characterized in a follow-up study, where Id2 was found to be required for formation of KLRG1^{hi}CD127^{lo} cells; and Id3 was required for formation of KLRG1^{lo}CD127^{hi} effector CD8$^+$ T cells (Yang et al., 2011). Further studies showed that E proteins, E2A and HEB were required for generation of memory precursor KLRG1^{lo}CD127^{hi} effector CD8$^+$ T cells (D'Cruz et al., 2012). The limitation of E2A/HEB activity by Id proteins appears to set the balance between these two important effector T cell subsets.

Blimp1 is a transcription repressor in the PRDI-BF1 and RIZ homology domain containing (PRDM) family and also appears to contribute to formation of KLRG1^{hi}CD127^{lo} cells (Kallies et al., 2009; Rutishauser et al., 2009). Similar to Id proteins and E-box proteins, Blimp1 and another transcription repressor in the BTB/PZ family, Bcl-6 act as antagonists of each other (Tunyaplin et al., 2004; Cimmino et al., 2008). Blimp1 expression is higher in KLRG1^{hi}CD127^{lo} cells and the absence of Blimp-1

impairs development of these cells (Rutishauser et al., 2009). Multiple mechanisms may contribute to Blimp-1's role in promoting KLRG1^{hi}CD127^{lo} cells, including antagonization of Bcl-6 (Martins et al., 2006; Kallies et al., 2009), repression of IL-2 production (Martins et al., 2008). A recent report suggests that Blimp-1 may repress expression of Id3 in KLRG1^{hi}CD127^{lo} cells and that lack of this repression (i.e., in Blimp-1-deficient mice) allows for their persistence into the memory compartment and for expression of E2A-driven genes important for genomic stability (Ji et al., 2011). Thus, the current data suggest a model in which inflammation drives expression of t-bet and an AKT/mTOR/FOXO signaling network that may contribute directly (by inducing Id2/Id3) or potentially in parallel with a Bcl-6/Id2/Id3 repressive network.

TRANSCRIPTIONAL PROGRAMING OF MEMORY PRECURSOR EFFECTOR T CELLS

Memory precursor effector cells are the Yin to the SLEC Yang and as such are often intertwined, experimental interpretations notwithstanding. Nonetheless, several factors have been reported to control the development of this effector cell population, including Bcl-6, TCF-1, and Stat3 (**Figure 2**). Deficiency in Tcf-1, an effector of the Wnt signaling pathway, impairs proliferative responses against *Listeria* infection and generation of KLRG1^{lo}CD127^{hi} effector CD8^{+} T cells after *Listeria* (Zhou et al., 2010) and lymphocytic choriomeningitis virus (LCMV) infections (Jeannet et al., 2010). Zhou et al. (2010) also showed that Tcf-1 is essential for optimal eomes and IL-2Rβ expression and forced overexpression of eomes partially prevented the decline of effector cells, although it did not appear to affect their surface marker phenotype. However, a role for β-catenin/wnt signaling on memory generation is controversial as two recent studies found that loss of β-catenin did not impair generation of effector responses (Driessens et al., 2010; Prlic and Bevan, 2011). In these studies, T cell-specific loss of β-catenin did not impair effector or secondary responses (as assessed by the frequency of tetramer^{+} T cells up to day 30 after infection); however, the expression of KLRG1/CD127 markers were not assessed in this study (Prlic and Bevan, 2011). Although it is possible that a β-catenin-independent function of Tcf-1 could contribute to formation of KLRG1^{lo}CD127^{hi} cells, at least one study suggests that the effects of Tcf-1 on memory T cell development require its ability to interact with β-catenin (Jeannet et al., 2010). Thus, more work is required to determine the role of the wnt/β-catenin/Tcf-1 pathway on KLRG1^{lo}CD127^{hi} cell formation and memory development.

Another recent study implicated STAT3, downstream of IL-10 and IL-21 signaling as a critical regulator of development of memory precursor cells (Cui et al., 2011). Interestingly, this study showed that T cell-specific loss of STAT3 or neutralization of IL-10 in an IL-21-deficient background lead to decreased percentage and number of KLRG1^{lo}CD127^{hi} cells but an increased number of KLRG1^{hi}CD127^{lo} cells (Cui et al., 2011). Thus, while the overall numbers of effector cells did not change, their phenotype did, an important distinction and potential separation of the effects of differentiation from effects on survival at a time when responses are crashing. In this study, Stat3-deficient effector T cells had normal expression of eomes, Blimp-1, and Bcl-6 at the peak of the response their levels decreased over time (Cui et al., 2011). However, it was not apparent if this was a selective decrease in KLRG1^{lo}CD127^{hi} cells or the decrease was reflective of a shift in the effector subpopulations (Cui et al., 2011). SOCS-3, a known STAT-3 target gene was increased in wild type (WT) KLRG1^{lo}CD127^{hi} cells at the peak of the response, and these levels were decreased in STAT-3-deficient cells, but again, subset-specific expression was not clear. Nonetheless, SOCS-3 overexpression in effector T cells reduced their ability to activate STAT4, whilst SOCS-3 knockdown promoted emergence of KLRG1^{hi}CD127^{lo} cells. However, it remains unclear as to how these target genes may be selectively activated in KLRG1^{lo}CD127^{hi} cells because stimulation of effector CD8^{+} T cells with IL-10 and IL-21 lead to homogenous STAT3 activation (Cui et al., 2011). Together, the data suggest an intriguing model whereby KLRG1^{lo}CD127^{hi} cells are shielded from the differentiating effects of inflammation by STAT3-driven induction of SOCS-3.

PARALLELS BETWEEN EFFECTOR AND MEMORY SUBSETS

First described in humans, T_{CM} express lymph node homing receptors CD62L and CCR7 and are mostly found in the lymph nodes and spleen as opposed to the T_{EM} that lack CD62L and CCR7 expression and instead express a variety of chemokine receptors and tissue-specific homing receptors (Sallusto et al., 1999; Masopust et al., 2001). These two subsets also differ in their functional properties. T_{CM} cells are capable of IL-2 production, self-renewal and they are multi-potent cells that can rapidly proliferate upon activation and generate effector cells (Wherry et al., 2003). Numbers of T_{CM} cells gradually increase over time and outnumber T_{EM} cells. While some studies suggest that the T_{EM} subset converts to T_{CM} over time (Wherry et al., 2003), others suggest that these lineages branch out early during memory differentiation and they are not convertible (Marzo et al., 2005). Adoptively transferred T_{EM} cells were able to convert to CD62L^{hi}, CCR7^{hi} CD27^{hi} cells that could produce IL-2 (Wherry et al., 2003). However, responses of non-physiologically high numbers of P14 TCR transgenic T cells were shown to be different qualitatively compared to endogenous effector cells (Marzo et al., 2005). Nevertheless, both endogenous T_{EM} cells and transfers of low number of P14 cells were shown to convert to T_{CM} subset upon transfer (Sarkar et al., 2007). Although the conversion contributes to the increase in T_{CM} numbers, CD62L^{hi} effector T cells can be detected early after the infection and they are enriched within the KLRG1^{lo}CD127^{hi} subset in the lymph nodes (Obar et al., 2011). On the other hand, KLRG1^{hi}CD127^{lo} cells are low for the expression of CD62L (Sarkar et al., 2008). Thus, in addition to conversion, higher proliferation and better survival of T_{CM} cells also contributes to the outgrowth of T_{CM} cells later, after the infection has cleared. Reacquisition of T_{CM} phenotype can be much slower after prime-boost immunizations (Jabbari and Harty, 2006; Masopust et al., 2006). Also, a greater fraction of secondary memory cells are KLRG1^{hi}CD127^{hi}CXCR3^{lo}CD27^{lo} phenotype (Masopust et al., 2006; Joshi et al., 2011). Although the recall responses of the adoptively transferred secondary memory cells were found to be even more potent than the responses of primary memory cells (Jabbari and Harty, 2006; Masopust et al., 2006), third generation memory cells had lower recall

responses upon adoptive transfers as a result of further differentiation into KLRG1^{hi} phenotype (Masopust et al., 2006). However, if the prime-boost immunizations are done in the same host; increased numbers of pre-existing memory cells prevent further differentiation into KLRG1^{hi}CXCR3^{lo}CD62L^{lo}CD27^{lo} phenotype (Joshi et al., 2011). Thus, the numbers of memory cells generated and the context of secondary priming conditions may affect the phenotype of secondary memory cells and these differences could play a role in the efficacy of prime-boost immunizations.

There are different models to explain the differentiation of memory cells from effector cells:

The early fate determination model predicts that memory cell heterogeneity, CD62L^{hi} – CD62L^{lo} or CD127^{hi} – CD127^{lo} are fixed (pre-determined) at early times after infection. Indeed, effector cells with CD62L expression (Obar and Lefrancois, 2010) or CD127 expression (Kaech et al., 2003) can be detected before the peak of immune response. Similarly, Chang et al. (2007) visualized the TCR transgenic cells after priming and just before the first division and found that certain cell surface molecules or TCR signaling components segregated asymmetrically during division. They showed that certain receptors segregated to the putative distal pole relative to the microtubule organizing center (MTOC) which is formed close to the immunological synapse. This resulted in asymmetric cell division and the daughter cell containing the distal pole as to the synapse had more characteristics of memory T cells such as CD62L. This study, although incomplete, provided a mechanism as to how heterogeneity can be generated from a single CD8^{+} T cell during the first division (Chang et al., 2007). However, CD62L^{lo} effector cells can also convert to CD62L^{hi} cells (Wherry et al., 2003; Sarkar et al., 2007) which suggest that there is some flexibility during memory differentiation.

The decreasing potential model suggests that every effector cell has the potential to develop into a memory cell, but exposure to inflammation and antigen for longer periods of time can further differentiate effector cells into terminal effector cells (KLRG1^{hi}CD127^{lo}) and decrease their potential to become memory cells (Ahmed and Gray, 1996; Badovinac et al., 2005; D'Souza and Hedrick, 2006).

A modified model proposed by Kaech and Wherry (2007), fate commitment with progressive differentiation suggests that there are memory precursors within the KLRG1^{lo}CD127^{hi} generated early in the immune response, but these cells are not fully mature memory cells and they require further differentiation. Although these cells have the potential for memory differentiation, they can develop into terminal effector cells (KLRG1^{hi}CD127^{lo}) if they encounter inflammatory signals. Importantly, this model appears to be consistent for the host response to several diverse infections (D'Souza and Hedrick, 2006; Badovinac and Harty, 2007; Joshi et al., 2007). As mentioned previously, IL-10 and IL-21 act to "shield" KLRG1^{lo}CD127^{hi} cells from the effects of inflammation, by increasing expression of SOCS3, which limits STAT-driven signals from inflammatory receptors (Cui et al., 2011). Thus, while differential expression of KLRG1 and CD127 can crudely mark cells with more or less memory potential, they likely require additional maturation signals and shielding from pro-inflammatory cytokines as they develop into full-fledged memory T cells.

ENDOGENOUS MEMORY CELLS – IRRELEVANT BYSTANDER OR ACTIVE PARTICIPANT?

In addition to infection-induced memory cells, it is well known that mice harbor populations of pre-existing memory T cells that bear markers of memory (CD44, Ly6c, etc.). Notably, these cells arise in mice that have not been purposefully challenged with infection. Admittedly, some of these cells might be specific for infections existing in some mouse colonies, for environmental antigens, or for gut flora. However, endogenous memory cells exist in gnotobiotic mice and recent data suggest that a fair number of these cells arise during thymic development (Dobber et al., 1992; Weinreich et al., 2010). A complex cellular and cytokine network, involving NKT cells and IL-4 appears to contribute to the development of pre-existing memory T cells, at least in Balb/c mice (Weinreich et al., 2010). Interestingly, in the process of quantifying the pre-existing naïve T cell compartment in unchallenged animals using peptide–MHC tetramers, Kedl's group found a significant frequency of T cells isolated from unchallenged mice bore memory markers (Haluszczak et al., 2009). They showed that, after purification these endogenous memory cells responded more robustly to stimulation, raising the intriguing possibility that this heterogeneity in the naïve compartment might contribute to effector T cell heterogeneity. On the other hand, in other experiments, transfer of a single TCR Tg T cell shows that effector and memory populations can arise from a single cell, a demonstration that differentiation from a common precursor is sufficient for effector and memory development. Whether or not there is a significant contribution of endogenous memory T cells to effector heterogeneity or whether these pre-existing cells contribute to epitope dominance at the population level (or both) remains to be determined.

APOPTOSIS AND THE DEVELOPMENT OF T CELL MEMORY

The molecular mechanisms responsible for apoptotic cell death have been investigated intensely over the last few decades. Mammalian cells have two major pathways to execute apoptosis: the extrinsic pathway (activated by death receptors of the TNF-R superfamily); and the intrinsic pathway (mostly controlled by members of the Bcl-2 gene family; Strasser, 2005). A considerable amount of experimental effort has been put into understanding T cell apoptosis. Initially, based on the discovery that defects in Fas signaling led to the accumulation of T cells in autoimmune lymphoproliferative syndrome (ALPS) patients and lpr/gld mice, and the requirement for Fas in an *in vitro* model of activated T cell death it was assumed that Fas signaling was required for the contraction of T cell responses (Watanabe-Fukunaga et al., 1992; Takahashi et al., 1994; Brunner et al., 1995; Dhein et al., 1995; Fisher et al., 1995; Rieux-Laucat et al., 1995). However, while *in vitro* experiments readily showed a role for Fas/FasL signaling in activated T cell death, several experiments showed that contraction of T cell responses occurred readily *in vivo* in the absence of Fas signaling (Desbarats et al., 1998; Hildeman et al., 2002; Pellegrini et al., 2003). Thus, although disruptions of either pathway can affect T cell homeostasis, recent research has suggested a critical role for Bcl-2 family members and the intrinsic pathway in controlling contraction of T cell responses (Hildeman et al., 2002; Pellegrini et al., 2003; Wojciechowski et al., 2006).

The Bcl-2 family can be classified into three subfamilies that have either pro- or anti-apoptotic function. Group 1 consists of anti-apoptotic Bcl-2-like molecules that contain most or all of the four Bcl-2 homology (BH) domains. Group 2 consists of Bax-like molecules that are pro-apoptotic and contain BH domains 1–3. Group 3 consists of BH3-only molecules that are pro-apoptotic and whose only homology to Bcl-2 lies in a short 9–10 amino acid stretch termed the BH3 domain. Group 3 has the most members, which appear to be expressed in a relatively tissue-specific fashion (Youle and Strasser, 2008). BH3-only molecules appear to transmit apoptotic signals to group two Bax-like molecules. In the absence of the two predominant Bax-like molecules, Bax and Bak, BH3-only proteins fail to induce apoptosis (Zong et al., 2001). The mechanism(s) by which BH3-only molecules transmit signals to Bax-like molecules remains the subject of some controversy. One model proposes direct interactions between certain BH3-only molecules and Bax-like molecules (Letai et al., 2002; Kuwana et al., 2005; Kim et al., 2006), while another proposes that BH3-only molecules sequester anti-apoptotic molecules from Bax-like molecules and there is no direct interaction between BH3-only and Bax-like molecules (Willis et al., 2007). Despite the controversy both of these models highlight the importance of physical interactions between Bcl-2 family members in cell death/survival decisions. Thus, a major control point for cell death lies in the regulation of the balance between the levels of pro- and anti-apoptotic molecules.

The first experiment implicating Bcl-2 family members in activated T cell death showed that overexpression of Bcl-2 was sufficient to prevent T cell deletion in response to staphylococcal enterotoxin B (SEB; Strasser et al., 1991). We repeated this experiment and found that, in contrast to loss of Fas and TNF-R signaling, Bcl-2 overexpression gave a substantial protection from SEB-induced deletion (Hildeman et al., 2002). Likewise, loss of Bim provided a similar protection from deletion (Hildeman et al., 2002). Although previous reports showed that transgenic Bcl-2 overexpression failed to prevent contraction of viral-specific T cell responses, the level of the transgene across the response was never examined (Petschner et al., 1998). Notably, by mechanisms that remain unclear, the expression of endogenous Bcl-2 in the human Bcl-2 transgenic mice that were used is substantially decreased, if not all together absent (Jorgensen et al., 2007). Further, following T cell activation, the levels of the Bcl-2 transgene also decline, making it less potent. Subsequently, our and other groups have observed that the loss of the Bcl-2 antagonist, Bim, prevents contraction of antigen-specific $CD4^+$ and $CD8^+$ T cell responses to viral, bacterial, and parasitic infection (Pellegrini et al., 2003; Wojciechowski et al., 2006; Prlic and Bevan, 2008; Reckling et al., 2008). A major question is how T cells normally avoid Bim-driven death on their way to becoming memory T cells.

REGULATION OF Bcl-2 BY γc CYTOKINES

Recent work from our and others groups have begun to address that question. In T cells, major controllers of Bcl-2 expression are the common gamma chain cytokines (Nakajima et al., 1997; Schluns et al., 2000; Berard et al., 2003; Wojciechowski et al., 2007). It has been known for some time that addition of IL-2, IL-4, IL-7, and IL-15 to activated or resting T cells promotes the expression of Bcl-2 (Vella et al., 1997, 1998) and Bcl-2 is largely required for *in vitro* T cell survival in response to these cytokines (Wojciechowski et al., 2007). The decreased expression of CD127 on the surface of $KLRG1^{hi}CD127^{lo}$ $CD8^+$ T cells renders them less sensitive to IL-7 and largely dependent upon IL-15 (Joshi et al., 2007; Rubinstein et al., 2008; Tripathi et al., 2010). $CD127^{hi}$ effector cells, on the other hand, require IL-7 and IL-15 for their optimal survival, although neutralization of IL-7 in an IL-15-deficient background only led to the loss of roughly half of this population (Tripathi et al., 2010). Thus, other γc cytokines probably also play a role in effector T cell survival because the loss of STAT5 signaling during the response led to a dramatic loss of both effector $CD8^+$ T cell subsets (Tripathi et al., 2010). Mechanistically, STAT5 is critical for the ability of IL-7 and IL-15 to promote Bcl-2 expression (Tripathi et al., 2010). Thus, a common g cytokine/STAT5/Bcl-2 network is critical for maintaining effector $CD8^+$ T cell responses (**Figure 2**).

While cytokine signaling through STAT5 promotes expression of Bcl-2 it has been reported that TGF-β signaling can antagonize Bcl-2 expression in $KLRG1^{hi}CD127^{lo}$ cells (Sanjabi et al., 2009). Adoptively transferred T cells expressing a dnTGF-βR transgene had substantially increased expansion of effector cells, which was accompanied by increased expression of Bcl-2 (Sanjabi et al., 2009). Further, there appeared to be an intersection with IL-15 in this model, as transfer of dnTGF-βR Tg T cells into IL-15 deficient mice led to a partial restoration of Bcl-2 levels compared to WT controls (Sanjabi et al., 2009). However, the increases in Bcl-2 were transient in dnTGF-βR Tg T cells and although there were increased T cells at the peak of the response, the contraction of the response was equal if not greater than the control, non-Tg animals (Sanjabi et al., 2009). Also, following T cell activation, levels of endogenous TGF-βR decline dramatically making it unclear if this pathway is operative during the normal response or whether it is magnified by transgenic dnTGF-βR overexpression.

Bim/BcL-2 BALANCE IN EFFECTOR $CD8^+$ T CELL SUBSETS

Initial work describing $KLRG1^{lo}CD127^{hi}$ and $KLRG1^{hi}CD127^{lo}$ cells, showed that Bcl-2 expression was higher in $KLRG1^{lo}CD127^{hi}$ cells and this was attributed to their prolonged survival (Joshi et al., 2007; Sarkar et al., 2008), however this was not formally tested. Using a combination and genetic and pharmacologic approaches, we tested the role of Bcl-2 in effector T cell survival, and its role in combating Bim within the effector subsets. Interestingly, we found that while Bcl-2 levels were higher in $KLRG1^{lo}CD127^{hi}$ cells than $KLRG1^{hi}CD127^{lo}$ cells, that Bim levels were also higher (Kurtulus et al., 2011). Genetic loss or inhibition of Bcl-2 led to a massive, Bim-dependent loss of $KLRG1^{lo}CD127^{hi}$ cells, and a less profound, but still significant loss of $KLRG1^{hi}CD127^{lo}$ cells (Kurtulus et al., 2011). Notably, the cells that survived in the absence of Bcl-2 had significantly decreased levels of Bim (Kurtulus et al., 2011). This phenomenon may also explain the "Bcl-2 independence" of memory T cell survival reported in mice with a mutant IL-7Rα transgene that is incapable of activating STAT5 and maintaining significant levels of Bcl-2 (Osborne et al., 2007). Thus, it is likely that Bcl-2 is an obligate defender of Bim to maintain survival of the memory precursor population, although the additional loss of Bim did not completely restore precursor cell numbers, suggesting that, in addition to restraining Bim, Bcl-2 may antagonize

other pro-apoptotic molecules. Nonetheless, these data showed that Bcl-2 levels determined the levels of Bim that effector T cells can tolerate and survive (**Figure 2**).

While decreased expression of Bcl-2 certainly contributes to the demise of effector $CD8^+$ T cells, changes in Bim expression are difficult to detect because once past a certain Bcl-2 level, cells having higher expression of Bim would be lost by apoptosis. We circumvented this issue by using mice that were deficient in Bak, but had a T cell-specific loss of Bax, making them insensitive to death driven by BH3-only Bcl-2 family members (Zong et al., 2001; Kurtulus et al., 2011). Loss of Bax and Bak led to accrual of T cells with significantly increased levels of Bim, suggesting that there is indeed a rather significant transcriptional induction of Bim during the response (Kurtulus et al., 2011). Importantly, the levels of Bcl-2 were also decreased significantly in these "undead" cells effectively uncoupling concordant Bim and Bcl-2 expression. Because of the inherent toxicities associated with altered expression of Bim, it has been difficult to determine the factors that control Bim expression in T cells. However, recent work has suggested that FOXO3a and Id2 may be regulators of Bim within effector T cells, as loss of FOXO3a led to decreased Bim protein (Sullivan et al., 2012), while loss of Id2 led to increased Bim mRNA (Cannarile et al., 2006). It is possible that there are intersections between FOXO3a and Id2 proteins, as deficiencies in either molecule led to major effects on expansion but rather minor effects on contraction of the response (**Figure 2**). Thus, more work will be necessary to clearly examine the complex transcriptional network underlying effector T cell contraction.

DEVELOPMENT OF CD4 MEMORY – CONSIDERABLY DIFFERENT FROM DEVELOPMENT OF CD8 MEMORY

Although much more work has been done to define effector T cell subsets and control of $CD8^+$ T cell memory, clues are emerging to define effector CD4 responses and the development of memory $CD4^+$ T cells. Interestingly, it appears that the markers expressed on effector $CD8^+$ T cells and those expressed on effector $CD4^+$ T cells are quite distinct. For example, expression of CD127 on effector $CD4^+$ T cells is more dynamic; $CD127^{lo}$ effector $CD4^+$ T cells readily re-express CD127. Several recent studies have examined heterogeneity within effector $CD4^+$ T cells. One study found that subsets of effector $CD4^+$ T cells could be defined based expression of P-selectin ligand-1 (PSGL-1) and Ly6C into three distinct, PSGL-1^{low}Ly6C^{low}, PSGL-1^{hi}Ly6C^{low}, and PSGL-1^{hi}Ly6C^{hi} (Marshall et al., 2011). Over time after infection, there was a slight enrichment for PSGL-1^{hi}Ly6C^{low} cells, but this enrichment was not nearly as dramatic as the enrichment for KLRG1^{lo}CD127^{hi} cells within the effector CD8 compartment. However, similar to the KLRG1^{hi}CD127^{lo} $CD8^+$ T cells PSGL-1^{hi}Ly6C^{hi} $CD4^+$ population required t-bet expression (Marshall et al., 2011). Thus, while effector PSGL-1^{hi}Ly6C^{low} cells appeared to be more capable of expanding in response to a secondary challenge, and that they share a similar transcriptional profile with memory $CD4^+$ T cells (Marshall et al., 2011), suggests that this subset most likely contains memory precursors.

On the other hand, another study defined effector $CD4^+$ subsets via expression of CXCR5 and PD-1 (Pepper et al., 2011). Effector $CD4^+$ T cells were again divided into three major subsets, cells expressing CCR7 along with intermediate levels of CXCR5 and lacking PD-1 (termed Tcm), those expressing t-bet, but not CCR7, CXCR5 nor PD-1 (Th1), and those expressing CXCR5 and PD-1 (Tfh). While Tfh cells waned dramatically over time, Th1 cells contracted more vigorously and Tcm cells contracted less vigorously (Pepper et al., 2011). In secondary responses, Tcm cells gave rise to all three subsets, while Th1 cells gave rise to only Th1 cells, suggesting that the Th1 cells, when they exist as memory cells are less able to give rise to the other subsets, while subset differentiation ability is maintained in the Tcm population. This study also showed that the Th1 cells largely required CD25 expression, while Bcl-6 was critical for Tcm cells as was inducible costimulator (ICOS) stimulation from B cells (Pepper et al., 2011). Importantly, while Tcm and Tfh both required Bcl-6, it is notable that they are discrete populations due to their differential localization after adoptive transfer and the fact that Tcm cells are inefficient at producing Tfh cells in secondary responses (Pepper et al., 2011). The overlap and relationship between the effector $CD4^+$ T cell subsets identified by these two studies remains unclear and awaits further investigation.

The expansion and contraction of the $CD4^+$ T cell response also shares both similarities and differences contraction of the $CD8^+$ T cell response. It has been known for some time that expansion of the CD4 response is less robust than the CD8 response (Homann et al., 2001). Further, the decline of the antigen-specific effector $CD4^+$ T cells after the peak of the response is less steep (90–95% of effector CD8s are lost; compared to 75–80% of effector CD4s) within the 2–3 weeks after the peak of the response (**Figure 1**). After that early contraction, memory $CD8^+$ T cells are maintained at a constant level while $CD4^+$ T cells decline slowly over time (Homann et al., 2001; Pepper et al., 2011; **Figure 1**). However, the pro-apoptotic molecule Bim is critical to the demise of both populations; the absence of Bim spares roughly 80% of the effector $CD8^+$ T cells and >90% of the effector $CD4^+$ T cells (Wojciechowski et al., 2006). Interestingly, there are some basic differences in how effector $CD4^+$ versus $CD8^+$ T cells combat Bim in order to enter the memory compartment. We and others recently showed that IL-7 and IL-15 contribute to survival of effector $CD8^+$ T cells by promoting expression of Bcl-2 through STAT5 (Schluns et al., 2000, 2002; Rubinstein et al., 2008; Tripathi et al., 2010). However, we found that neutralization of IL-7 in IL-15-deficient mice did not result in significantly increased contraction of the $CD4^+$ T cell response (Tripathi et al., 2010). Further, we found that, in contrast to $CD8^+$ T cells, effector $CD4^+$ T cells were much more able to tolerate the loss of STAT5 and persisted for some time as $STAT5^{low}$ effector T cells (Tripathi et al., 2010). Similarly, neutralization of Bcl-2 does not exacerbate contraction of the $CD4^+$ T cell response (Tripathi et al., 2007), suggesting that, in effector $CD4^+$ T cells, something other than Bcl-2 combats Bim. Thus, while there are some similarities with effector to memory transition between $CD4^+$ and $CD8^+$ T cells more work is necessary to untangle the mechanisms that control this transition.

FUTURE DIRECTIONS

Recent progress has greatly improved our understanding of how memory T cells emerge from the effector pool. Death and differentiation work together to shape the effector T cell response.

Most effector T cells that are generated die shortly after the peak of the response. This death process is largely mediated by the pro-apoptotic Bcl-2 family member, Bim. Bim function is negatively controlled by the levels of Bcl-2, which are regulated by the availability of common gamma chain cytokines. Death and differentiation could be manipulated to enhance the death of autoreactive T cells. On the other hand, manipulation of death and differentiation processes could be exploited to improve vaccine responses. For example, recent work from us and other have suggested that IL-7 may be an excellent vaccine adjuvant, promoting strong effector T cell responses to help B cells make antibody as well as promoting strong effector $CD4^+$ and $CD8^+$ T cell responses (Tripathi et al., 2007; Nanjappa et al., 2008; Nam et al., 2010; Pellegrini et al., 2011). However, the effects of IL-7 are somewhat short-lived as they wane with the withdrawal of the cytokine. Thus, factors that restrict cellular differentiation (i.e., IL-10, IL-21) may be combined with IL-7 therapy to boost long-lived central memory T cells. This may be particularly advantageous for vaccines that require boosting to achieve immunity, such as the hepatitis B vaccine. Conversely, other vaccines may benefit from effector memory T cells, which are maintained in the tissues and provide substantial protection from tissue borne infections (Bachmann et al., 2005). For example, adenoviral vaccines appear to promote strong effector T cells that appear to persist as effector memory cells (Reyes-Sandoval et al., 2011). Thus, more research is necessary to define successful immunization strategies that maximize protective immunity. Exploitation of combinatorial strategies aimed at controlling the type of inflammation with enhancing effector T cell survival may provide approaches that could be tailored to the particular infectious disease.

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The integration of signaling and the spatial organization of the T cell synapse

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Engagement of the T cell antigen receptor (TCR) triggers signaling pathways that lead to T cell selection, differentiation and clonal expansion. Superimposed onto the biochemical network is a spatial organization that describes individual receptor molecules, dimers, oligomers and higher order structures. Here we discuss recent findings and new concepts that may regulate TCR organization in naïve and memory T cells. A key question that has emerged is how antigen-TCR interactions encode spatial information to direct T cell activation and differentiation. Single molecule super-resolution microscopy may become an important tool in decoding receptor organization at the molecular level.

Keywords: T cell receptor, membrane organization, receptor oligomerization, signaling assembly, T cell activation

TCR SIGNALING

Activation of T cells is a key element in adaptive immunity and requires the coordination of highly complex signal transduction networks (**Figure 1A**). The process begins when the T cell receptor (TCR) binds to peptide-loaded major histocompatibility complexes (pMHC) (Huppa and Davis, 2003; van der Merwe and Dushek, 2011). While the TCR's peptide-recognizing αβ heterodimer has no intrinsic catalytic activity, it forms a multimolecular complex with the dimers CD3εγ, CD3εδ, and CD3ζζ, which have long cytoplasmic domains containing immunoreceptor tyrosine-based activation motifs (ITAMs) (Call et al., 2004). For signaling to proceed, it is necessary that at least two ITAMs are phosphorylated by the Src family kinase lymphocyte-specific protein tyrosine kinase (Lck) that is anchored to the inner leaflet of the plasma membrane (Palacios and Weiss, 2004). Curiously, a proportion of Lck is already activated in resting cells and there is no evidence for TCR or co-receptor induction of Lck activity (Paster et al., 2009; Nika et al., 2010) so it is currently not clear how Lck distinguishes between non-engaged and engaged TCR (Zhang et al., 2011). One possibility is the spatial segregation of TCR and Lck from phosphatases such as CD45 (Davis and Van Der Merwe, 2006; Rossy et al., 2012). Phosphorylated ITAMs serve as recruitment and activation sites for zeta chain-associated protein kinase of 70 kDa (ZAP-70), whose activity is essential in conventional T cells but not in regulatory T cells (Au-Yeung et al., 2010).

The next step in the signaling cascade is the recruitment and phosphorylation of linker for activation of T cells (LAT) that is essential for TCR signaling (Finco et al., 1998), T cell activation (Zhang et al., 1999a) and development (Zhang et al., 1999b). LAT serves as a platform for several adapter and effector molecules (**Figure 1A**) including phospholipase C gamma (PLCγ) (Zhang et al., 2000), phosphatidylinositol 3-kinase (PI3K) (Cruz-Orcutt and Houtman, 2009) Src-homology-2-domain-containing leukocyte protein of 76 kDa (SLP76) (Wu and Koretzky, 2004), growth factor receptor-bound protein 2 (Grb2) (Zhang et al., 2000) and the Grb2-homologous adapter (GADS) (Zhang et al., 2000; Liu et al., 2001). The mechanism of LAT recruitment is controversial, as discussed below, and it has been suggested that there are LAT-dependent and -independent signaling pathways (Malissen and Marguet, 2011).

Ultimately, TCR activation-induced signaling cascades result in actin cytoskeleton restructuring and induction of gene expression and cytokine secretion. Whether the signals from various pathways are integrated downstream so that the different arms of the signaling process function as independent controls or whether hierarchies exist where specific signaling signatures dominate others is currently not known. Here we focus on signaling molecules up to and including LAT, as each of these steps in early TCR signaling are essential.

TCR MICROCLUSTERS AND THE IMMUNOLOGICAL SYNAPSE

The interface between a T cell and an antigen-presenting cell (APC) is referred to as the immunological synapse (Huppa and Davis, 2003; van der Merwe and Dushek, 2011). In the original model, the synapse is organized into supramolecular activation clusters (SMACs) comprising three distinct concentric regions (**Figure 1B**): a central region, designated as the cSMAC, contains a high number of TCRs. This layer is surrounded by the peripheral region, the pSMAC, which is enriched in adhesion proteins such as leukocyte function-associated antigen 1 (LFA1) and talin. The third and outermost region is the distal SMAC (dSMAC), which contains actin and CD45 (Monks et al., 1998; Grakoui et al., 1999). This classic bull's-eye pattern of the immunological synapse emerges from the dynamic interactions of the TCR with its signaling partners. After initial contact, TCR engagement triggers the formation of TCR microclusters of the proteins

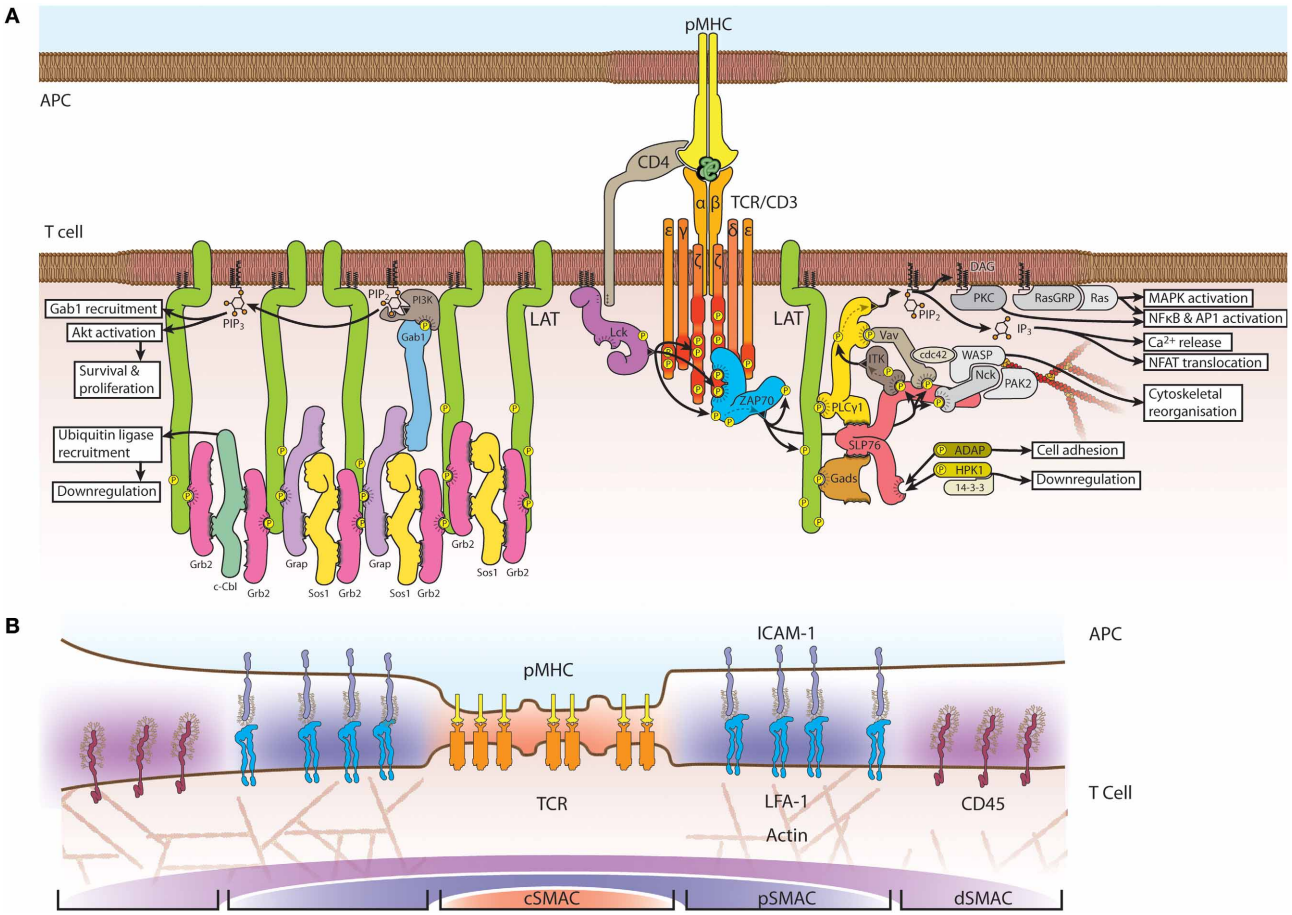

FIGURE 1 | Schematic structure of the TCR signaling assembly and immunological synapse. (A) Engagement of TCR ligation and co-receptors, such as CD4 and CD28, recruits Lck, which phosphorylates CD3 at immunoreceptor tyrosine-based activation motifs (ITAMs). ZAP70 binds double-phosphorylated ITAM residues whereupon it is phosphorylated and activated by Lck. LAT is recruited to the activated TCR complex and is phosphorylated by ZAP70 at several tyrosine residues on its C-terminal tail. LAT phosphorylation induces the assembly of signaling adapters and effectors; multi-point binding options between LAT, Grb2, Sos1 and Grap facilitate the formation of an extended protein network. LAT-nucleated signaling also occurs through Gads and SLP76. **(B)** At larger scales, the immunological synapse forms into distinct supramolecular activation clusters (SMACs), with the central SMAC (cSMAC, orange) rich in TCRs. Surrounding this layer is the peripheral SMAC (pSMAC, blue) enriched in signaling cofactors and adhesion complexes. A further region, the distal SMAC (dSMAC, purple) comprises glycoproteins such as CD45 and CD44.

Lck, ZAP70, LAT, and SLP76 (Bunnell et al., 2002, 2006). In a mature synapse with a fully formed cSMAC and pSMAC, TCR microclusters continuously form in the pSMAC (Campi et al., 2005; Varma et al., 2006) and are transported to the cSMAC in an actin- and myosin-dependent manner (Kaizuka et al., 2007; Ilani et al., 2009); the cSMAC is also where signaling is thought to be terminated and the receptor internalized (Liu et al., 2000; Coombs et al., 2002). The dynamic spatial organization of the synapse, as observed mainly on supported lipid bilayers, influences signaling activity and *vice versa*. The formation of TCR microclusters and initiation of signaling precede the formation of the cSMAC and initially occur throughout the entire T cell-APC contact area (Lee et al., 2002; Yokosuka et al., 2005). Additionally, the phosphatase CD45 is excluded from TCR microclusters in immature synapses (Varma et al., 2006) and from the cSMAC where phosphorylated TCRs accumulates in fully established synapses (Leupin et al., 2000), suggesting that segregation of the receptor from CD45 is mechanistically linked to receptor phosphorylation.

The bull's eye pattern of mature synapses was originally observed in helper T cells but has since been described for cytotoxic T cells (Anikeeva et al., 2005), regulatory T cells (Zanin-Zhorov et al., 2010), B cells (Depoil et al., 2008; Randall et al., 2009) and natural killer (NK) cells (McCarthy et al., 2007). Surprisingly, a SMAC organization is not required for T cell signaling. T cell interfaces with dendritic cells (Brossard et al., 2005) and Th2 cells (Thauland et al., 2008) result in multiple focal structures lacking the SMAC architecture. Naïve T cells in the lymph node form TCR clusters independent of antigen presentation while the dominant feature observed in the presence of antigen was TCR internalization, which was also not contingent on cSMAC formation (Friedman et al., 2010). In a detailed study, Schubert et al. recently examined the patterns of immunological synapses in self-reactive T cells clonally derived from patients

with multiple sclerosis and type 1 diabetes (Schubert et al., 2012) and found that despite strong TCR phosphorylation and signaling activity, essentially no cSMAC was formed in self-reactive T cells.

While cSMACs may not be required for signal initiation (Lee et al., 2002), they appear to function in signal modulation and termination. TCR microclusters are actively transported toward the cSMAC where they co-localize with markers of protein degradation such as LBPA (Varma et al., 2006) and components of the ubiquitin pathway (Vardhana et al., 2010). Internalization of TCR bound to pMHCs at the cSMAC could be mediated by phagocytic processes (Alarcón et al., 2011). Although TCR signaling of strong agonist is terminated at the cSMAC, weak activation results in TCR signaling within the cSMAC (Cemerski et al., 2008), thus acting as a signal modulator. A further role for the SMACs may be in focused secretion of lysosomes from cytotoxic T cells to virally infect and tumor cells or of cytokines to antigen presenting cells (Griffiths et al., 2010).

The underlying mechanisms of synapse patterning and TCR microcluster formation are still not fully understood. Both, inhibition of actin flow and myosin-II activity impair TCR microcluster and synapse formation (Campi et al., 2005; Kaizuka et al., 2007; Ilani et al., 2009). Recently, it has been suggested that under subtle perturbation of actomyosin dynamics (rather than complete inhibition of the network), actin retrograde flow is the main driver for TCR microcluster accumulation in the cSMAC (Babich et al., 2012). Interestingly, an intact actin cytoskeleton is required for initial TCR microclusters formation but, once established, TCR microclusters are sufficiently stable without a functional actin network. Hence actin and acto-myosin contraction are only required at the early stages of synapse formation. *In vivo*, the T cell-APC contact zone is fluid due to the T cells motility (Mempel et al., 2004; Miller et al., 2004) and immunological synapses are not as stable as in cells activated on bilayers. The duration of these transient T cell-APC interactions may determine the signaling switch between tolerance and activation (Katzman et al., 2010). Further, in motile synapses, the movement of TCR microclusters is aligned with the cell migration and not oriented toward the cSMAC. Both TCRs and cSMAC are consistently relocated to actin-poor regions that required local actin depolymerisation (Beemiller et al., 2012). Whether cortical actin is simply a means to compartmentalize the T cell membrane, as proposed in the "picket-fence" membrane model (Kusumi et al., 2011), or plays a more active role in TCR cluster formation remains to be seen.

An association of signaling proteins with protein networks (Douglass and Vale, 2005) and membrane domains (Viola et al., 1999; Janes et al., 2000) has been proposed as an underlying mechanism for the lateral organization of the plasma membrane. Indeed the membrane environment at T cell activation sites is considerably more ordered than in resting cells (Gaus et al., 2005; Owen et al., 2010) and biochemically resembles lipid rafts due to cholesterol and sphingomyelin enrichment (Zech et al., 2009). In addition, preventing membrane condensation resulted in fewer TCR microclusters at the cell surface and impaired signaling and activation responses (Rentero et al., 2008). However, whether the protein affinity for this membrane environment is sufficient to drive protein sorting and clustering is still unknown. The lipid anchor of Lck, for example, does not control Lck distribution and diffusion (Douglass and Vale, 2005), lipid raft reporters are not associated with TCR microclusters (Hashimoto-Tane et al., 2010) and do not cluster upon TCR activation (Glebov and Nichols, 2004). Furthermore, the two palmitoylation groups on LAT are mainly responsible for delivery of the protein to the plasma membrane (Tanimura et al., 2006; Hundt et al., 2009) rather than imposing an association to lipid raft domains (Zhang et al., 1998; Lin et al., 1999). Although the contribution of lipid rafts to TCR signaling remains controversial, lipids clearly play a role in T cell activation (Geyeregger et al., 2005; Galli and Calder, 2009). In addition, a specific membrane environment may stabilize TCR microclusters (Choudhuri and Dustin, 2010) and control the interaction of basic residue-rich stretches in the ITAM domains with the plasma membrane (Zhang et al., 2011).

NEW MODELS FOR LAT SIGNALING

Insights into the spatial organization of immunological synapses have been made possible by total internal reflection fluorescence (TIRF) microscopy and the use of supported planar lipid bilayers within which adhesion and MHC molecules are laterally mobile. More recently the exquisite signal-to-noise ratio of TIRF microscopy has been exploited for super-resolution techniques, namely photoactivated localization microscopy (PALM) (Betzig et al., 2006; Hess et al., 2006) and stochastic optical reconstruction microscopy (STORM) (Rust et al., 2006) that can localize individual proteins molecule in intact cells with nanometre precision (**Table 1**). PALM and STORM [and its derivative direct STORM or dSTORM (Heilemann et al., 2008)] achieve high imaging resolution by employing switchable fluorescent signals (**Figure 2**). By controlling the fluorescence of labeled molecules from dark to bright states, individual molecules are temporally separated, and thus identified, from their unswitched neighbors within a small, diffraction-limited, spatial area. The practical execution of these techniques requires fluorescent proteins (in the case of PALM) or organic dyes (for STORM and dSTORM) which are able to transition from dark to bright states (photoactivation) or from one emission spectra to another (photoswitching) when irradiated with a specific switching or activation laser, usually operating at a sufficiently low power to ensure only a few molecules in the population are driven into the switched fluorescent state. Once a sparse set of molecules are switched, they can be excited into fluorescence by a much higher power imaging laser. In the case of PALM, the high intensity of the excitation laser is usually sufficient to destroy the protein (or at least its chromophore) through photobleaching, thus removing it from the total pool of labeled molecules. For STORM and dSTORM, the high intensity activation laser drives the dye into a dark state, from which it can be recovered by the activation laser for multiple fluorescence cycles before photobleaching. This cycle of photoactivation, fluorescence emission, and photobleaching is repeated until all the labeled molecules have been registered. The fluorescence intensity profile, known as the point-spread function (PSF) of each individual molecule is analyzed to determine the localization coordinates for each molecule. The fitting process also returns the

Table 1 | Advantages and limitations of TIRFM, PALM, and STORM.

Acronym	Name	Description
TIRFM	Total Internal Reflection Fluorescence Microscopy	Principle of operation: Directing an excitation source at a critical angle to the glass coverslip, such that the beam is totally internally reflected, generates an evanescent wave penetrating approximately 100 nm into the sample. Fluorophores within this range are excited whereas material deeper in the sample will remain dark, effectively eliminating out-of-focus fluorescence, including autofluorescence. Advantages: • Minimal background signal; increased signal-to-noise. • Tight field depth, corresponding to the evanescent field penetration. Limitations: • Subject to the diffraction limit. • Only samples which are adjacent to the glass-water interface can be accessed.
PALM	Photo-Activatable Localization Microscopy	Individual photoswitchable or photoactivatable proteins are converted, at very low frequency, into the imaging channel. These sparse, switched molecules are then excited, their spatial positions localized, and bleached. Thousands of successive rounds of switching/activation, excitation, and bleaching are performed to generate a map of all the molecule positions. Advantages: • High resolution, single molecule localization to 20–50 nm in XY. • Excellent labeling specificity conferred by fusion proteins. • Compatible with live cell imaging. • Easily adapted into 3D with additional optics. Limitations: • Long acquisition and processing times. • Poor photon yield from fluorescent proteins decreases molecule localization precision. • Care must be taken to avoid transfection and over-expression artefacts. • Endogenous proteins cannot be studied.
STORM	STochastic Optical Reconstruction Microscopy	The same principle as for PALM with conventional dyes conjugated to antibodies as fluorophores. Advantages: • High resolution, single molecule localization to 20–50 nm in XY. • Conventional immunofluorescence dyes can be used. • Endogenous proteins can be studied, including modified (e.g. phosphorylated) proteins. • Easily adapted into 3D with additional optics. Limitations: • Long acquisition and processing times. • Less compatible with live-cell imaging. • Care must be taken to avoid fixation and staining artefacts.

localization precision and number of photons emitted from each molecule.

Mark Davis and his team used PALM and electron microscopy to put forward the model that the TCR and LAT are segregated in sub-micrometer "protein islands" (**Figure 3**) that coalesce, but do not mix, upon receptor activation (Lillemeier et al., 2010). The notion of such islands comes from their previous work showing that protein-rich domains are surrounded by a protein-poor "lipid sea" on the plasma membrane (Lillemeier et al., 2006). The implication of this model is that an insulating layer exists around the receptor and LAT islands, which needs to be overcome in order for signaling to be initiated (Dustin and Depoil, 2011). We also used PALM to quantify LAT clustering but came to a very different conclusion (Williamson et al., 2011). Unexpectedly, we found a 2.7-fold increase in the number of LAT molecules at the TCR activation site, which were not laterally recruited from non-activated areas of the plasma membrane. When surface-expressed LAT was bound to streptavidin-coated beads outside the activation zone, LAT recruitment and phosphorylation was normal, clearly indicating that an intracellular pool of LAT is sufficient to drive signaling under these conditions. The existence of LAT sub-synaptic vesicles (**Figure 3**) was previously demonstrated but whether LAT vesicles are phosphorylated in *trans* and act as signaling endosomes or whether LAT vesicles stay tethered to, or even fuse with, the plasma membrane is currently not known. The two models of pre-existing clusters or islands of LAT and LAT vesicles are not mutually exclusive (**Figure 3**). In addition to the linear signaling pathway of TCR→Lck→Zap70→LAT→SLP76 that is viewed to take place at the plasma membrane, an alternative pathway may exist in which LAT vesicles dock to the plasma membrane at sites of SLP76-GADS complexes (Purbhoo et al., 2010). Support for this second pathway comes from genetic studies in which LAT was deleted in CD4+ T cells after thymic selection (Mingueneau et al., 2009). Not only did these LAT-deficient CD4+ T cells respond to TCR engagement with Lck and ZAP70 phosphorylation of their targets

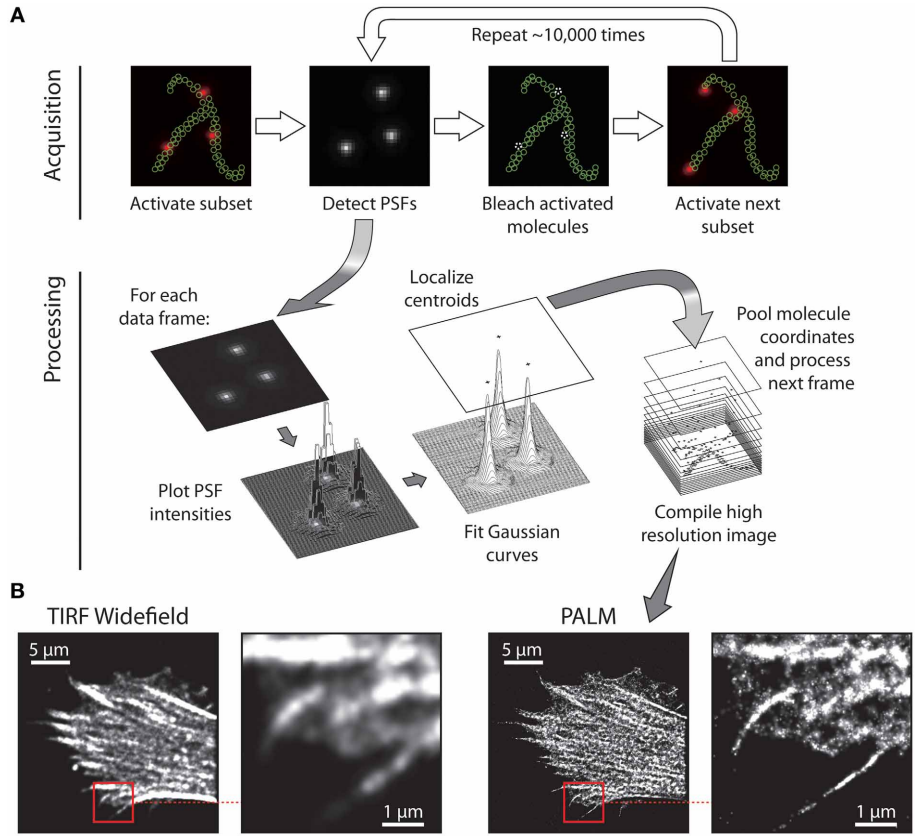

FIGURE 2 | Principle of single molecule localization microscopy. (A) Imaging sequence for data acquisition in which a spare subset of molecules is activated, imaged, and bleached. Following acquisition of the raw PSF data, the image sequence is processed to localize each molecule's point-spread function (PSF) with nanometre precision by fitting Gaussian distributions to the intensity profile of each molecule. By repeating photo-activation imaging and fitting for typically 15,000–20,000 frames, a coordinate map of all detected molecules is generated. **(B)** Example data: HeLa cell expressing Lifeact (an F-actin binding protein) fused to the photoswitchable protein tdEos, observed with TIRF, widefield microscopy and then with TIRF PALM. The substantial increase in resolution is evident by comparing the zoomed TIRF and PALM regions, indicated by the red boxes.

including SLP76, they fully recapitulated the lymphoproliferative disorders associated with constitutive LAT mutation. Hence SLP76 can participate in T cell signaling independently from LAT (Malissen and Marguet, 2011). If in wild-type T cells the two pathways coexist, it will be interesting to see whether the manner of TCR activation (number of engaged TCR, peptide affinity, off/on rates etc.) selects one pathway over the other; if there is synergy or redundancy between the two pathways, and whether this leads to differential signaling outcomes. In this context it is interesting to note that phosphorylation of LAT occurs within 4 s of TCR-pMHC engagement and calcium fluxes after 6–7 s but while diacylglycerol production is strongly desensitized shortly after TCR activation, LAT phosphorylation is not (Huse et al., 2007). It is also possible that the pre-existing compartmentalization of LAT into membrane domains and vesicles determines TCR signal strength, signal maintenance, and/or contributes to T cell specialization.

SPATIAL ORGANIZATION OF TCRs

The report of TCR islands (Lillemeier et al., 2010) has been unexpected since previous studies described a different spatial organization of the TCR. Using a fluorescence technique called dynamic single-molecule colocalization (Dunne et al., 2009), a form of single-molecule spectroscopy, David Klenerman, Simon Davis and colleagues showed that the αβ heterodimer of the TCR/CD3 complex is essentially monomeric (James et al., 2011). These measurements were taken at the apical surface of T cells that is not in contact with the glass coverslip or supported lipid bilayer, and activation of TCR occurred through soluble agonists. Whether the experimental conditions account for the differences in TCR organization remains to be seen. Alternatively, individual TCRs may diffuse freely within TCR islands that are positionally stable (Lillemeier et al., 2010). Indeed, James et al. commented that single αβ heterodimers exhibit non-random confinement (James et al., 2007). By what mechanism such confinement occurs will be important to know because it not only determines the compartmentalization of the receptor but also which interactions are available following receptor engagements. Given the mobility of the receptor, it is unlikely that high affinity protein-protein interactions are responsible for confinement, as previously proposed of LAT and Lck (Douglass and Vale, 2005). Alternative mechanisms of receptor confinement are

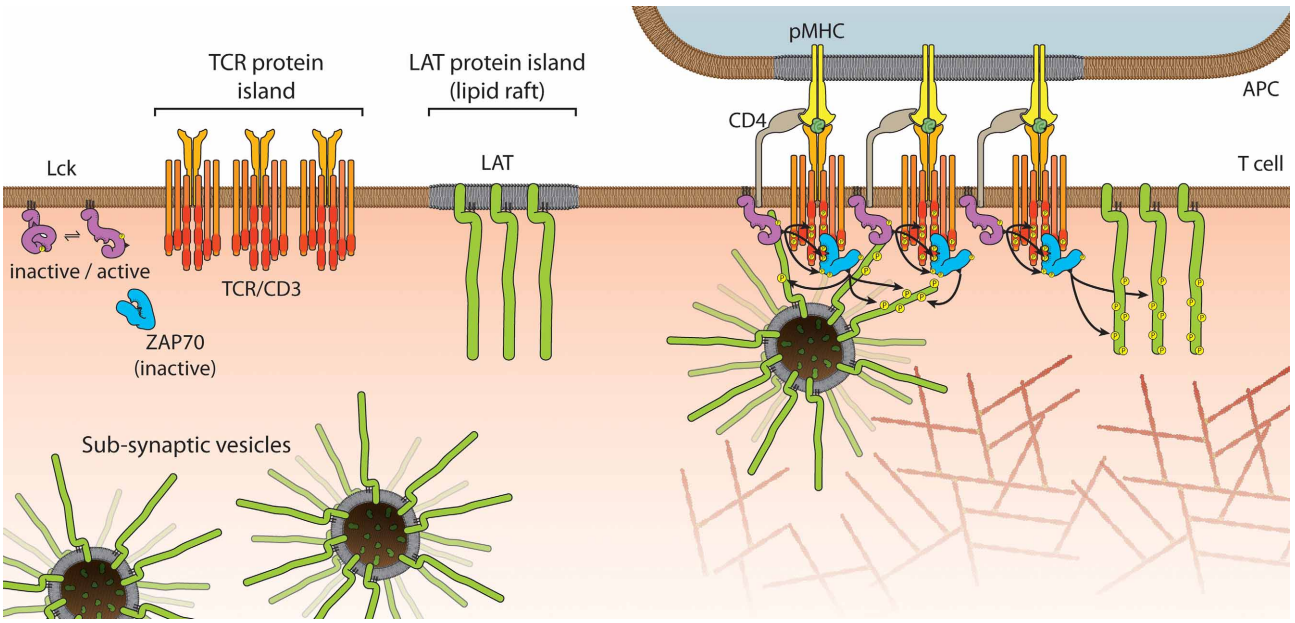

FIGURE 3 | Models of LAT recruitment. Protein islands in the T cell membrane can aggregate or coalesce during TCR activation to facilitate phosphorylation and signal transduction. An alternative model invokes sub-synaptic vesicles, which could be signaling endosomes, which translocate to sites of activated TCR during signal transduction. The two models are not mutually exclusive.

similar to those explored in the context of TCR microclusters, i.e., membrane domains and restrictions imposed by the underlying, membrane-proximal cytoskeleton.

To which extent does the spatial organization of the TCR contribute to T cell signaling and function? This intriguing question has been asked by many researchers but is experimentally difficult to examine. In a comprehensive analysis of four different class II- and I-restricted TCR transgenic mouse models, Prutic et al. found receptor clustering was only important in one specific low affinity/avidity T cell system where TCR accumulation at the cSMAC facilitated integration with costimulatory signals (Purtic et al., 2005). When cSMAC/pSMAC patterns are disturbed by placing T cells on lipid bilayers that are themselves compartmentalized by metal lines or grids, T cell signaling can be prolonged because TCRs cannot accumulate in the cSMAC or be down-regulated as co-receptors are segregated from the TCR (Manz and Groves, 2010). An alternative approach is to target the expression of downstream signaling proteins that do not directly impact on early TCR signaling events. The loss of the tyrosine kinase interleukin-2 (IL2)-inducible T cell kinase (Itk) resulted in unusual spatial organization of the immunological synapse with a mislocalization of the Rho GTPase CDC42 and a concomitant loss of actin accumulation at the synapse (Singleton et al., 2011). These findings illustrate that a downstream signaling molecule can influence the synapse organization of upstream signaling proteins, suggesting that the non-linearity of signaling pathways is interlinked with protein trafficking and membrane compartmentalization at multiple stages.

In 2011, two very different papers were published that we believe will become landmark publications. Firstly, Manz et al. used supported lipid bilayers on metal grids to control the number of peptides that can cluster together without altering the total number of peptides engaged by the T cell (Manz et al., 2011). It was therefore possible to determine how TCR clustering supports the astonishing sensitivity of T cells, which can respond to even a few agonists peptide molecules (Sykulev et al., 1996; Irvine et al., 2002). Limiting TCR clustering at a fixed total pMHC density indeed reduced T cell sensitivity and the probability of intracellular calcium fluxes (Manz et al., 2011). Such stochastic analysis revealed an activation threshold for the number of activating ligands per individual TCR cluster and not per cell, with a minimum of four pMHC in a signal cluster required for calcium signaling. Similar to CD4 blocking which increases the number of peptides required to initiate a T cell responds several fold (Irvine et al., 2002; Krogsgaard et al., 2005), this study suggests that costimulation of CD28 could lower the ligands-per-cluster threshold. In the second study that we would like to highlight, Kumar et al. established a link between the ability of TCR to form oligomers (Schamel et al., 2005; Lillemeier et al., 2010) and the T cell response to antigen stimulation (Kumar et al., 2011). Previously stimulated T cells displayed larger TCR oligomers at their surface than naïve cells and the increased sensitivity of experienced and memory T cells correlated with a higher level of TCR oligomerization. Importantly, a point mutation in the transmembrane domain of CD3ζ involved in tetramer formation (Torres et al., 2002) resulted in a diminution in TCR oligomers and a concomitant decrease in the TCR response to stimulation (Kumar et al., 2011). Hence TCR clustering could be responsible for setting the TCR activation threshold and a key discriminating factor between naïve and memory T cells.

DOES TCR AFFINITY AND QUATERNARY STRUCTURE CONTRIBUTE TO TCR TRIGGERING?

One of the key features of the T cell system is that ligand-receptor interactions occur on the 2-dimensional surfaces of cell membrane. Comparing 2D affinities of TCR-pMHC binding kinetics with 3D affinities in solution revealed unexpected results. By assuming that the TCR and its ligand fully access the whole intermembrane space, Huppa et al. showed that the association rate measured in 2D was 100 fold faster than the one measured in 3D (Huppa et al., 2010). Using a micropipette and a biomembrane force probe to quantify the deformation of a red blood cell or the thermal fluctuation of a bead that were both functionalized with pMHC, Huang et al. observed that 2D affinities had a broad range over a panel of pMHCs that matched T cell proliferation responses (Huang et al., 2010). Association and dissociation rates were both significantly faster in these 2D assays compared to 3D solution measurements. In solution, dissociation rates were the best predictor for T cell responses suggesting that slow pMHC dissociation induces T cell activation. Conversely, in the 2D scenario, it was the extremely fast association rates that drove TCR-pMHC responses. This opens the possibility that rapid antigen sampling and possibly serial engagement, where a few pMHC are repeatedly engaged by the same TCRs or TCRs within the same cluster (Aleksic et al., 2010) are mechanisms by which the high concentration of self-pMHC background is overcome *in vivo*. The efficiency of serial TCR-pMHC engagements would be enhanced by a non-random distribution of TCRs and the relative immobility to TCR clusters or islands (Lillemeier et al., 2010), simply because dissociated pMHC can be recaptured by neighboring TCRs. This model was supported by a single-molecule fluorescence resonance energy transfer (smFRET) study where the duration of TCR-pMHC interactions was driven by the high on-rate (Huppa et al., 2010). Interestingly, the authors showed in the same study that blocking CD4 engagement with antibodies did not alter TCR-pMHC binding. In self-reactive T cells that failed to form synapses and did not accumulate TCR in the cSMAC, the off-rates of TCR-pMHC binding were normal while the on-rates were significantly slower compared to TCRs binding corresponding peptides of viral-specific T cells (Schubert et al., 2012), further lending weight to the TCR-pMHC serial engagement model. However, recently it was shown that serial engagement of TCRs is not necessary for activation when pMHC monomers are cross-linked to TCRs (Xie et al., 2012).

Are there any clues in the TCR structure and pMHC binding topography that tell us about the arrangements of αβ heterodimer with the CD3 dimers (i.e., quaternary structure) or whether a TCR forms dimers and higher-order oligomers? Like immunoglobulin (Ig) Fab fragments, the αβ TCR heterodimer has subunits consisting of one variable (*V*) and one constant (*C*) Ig domain in the extracellular segment. However, unlike antibodies, there is an elongation connecting the F and G β-strands in the Cβ domain, called the Cβ FG loop (Wang et al., 1998). This structural feature is conserved in all mammalian αβ TCRs studied to date and probably co-evolved with the development of distinct CD3δ and CD3γ genes (Kim et al., 2010). It has been proposed that the Cβ FG loop enforces a high level of rigidity to the αβ heterodimer, and consequently the structure of the TCR complex does not undergo major conformational changes upon pMHC binding (Rudolph et al., 2006). Although the Cβ FG loop is not involved in antigen binding, its deletion impairs cytokine production and T cell proliferation upon receptor stimulation. CD3ε, CD3γ, and CD3δ have each a single extracellular Ig domain while CD3ζ has essentially no extracellular domain. All CD3 units have negatively charged residues in the transmembrane domain that drives paring of the dimers—CD3εδ, CD3εγ, and CD3ζζ—with TCRα and TCRβ because the paired acidic domains can interact with positive charges at the same depth in the transmembrane regions of the TCR (Call et al., 2010). The CD3εγ and CD3εδ dimers have a highly conserved hydrophobic interface and adopt a side-by-side configuration (Sun et al., 2004). A model of the entire TCR complex (**Figure 4**) has evolved from the topography of the heavily glycosylated ectodomains of αβ, CD3εγ, and CD3εδ and recapitulates the known chain association with CD3ε-CD3γ-TCRα-CD3ζ-CD3ζ as one cluster and CD3ε-CD3γ-TCRβ as a second cluster (Sun et al., 2004). Mark Davis and colleagues engineered a dimerization reporter system—based on the erythropoietin receptor—that only signals and drives cell proliferation when signaling domains are juxtaposed (Kuhns et al., 2010). They showed that the CD3 heterodimers are assembled in tandem on one side of the αβTCR, and leave the other side free to interact with other αβTCR units. Kai Wucherpfenning and his colleagues placed the CD3ζζ on the other side of the αβ TCR (Call et al., 2002, 2006), and as CD3ζ lacks an extracellular domain, this arrangement leaves one side of the αβ TCR open for dimerization (**Figure 4**). Importantly, the Cβ FG is in close proximity with one of the CD3ε units and therefore does not prevent dimerization.

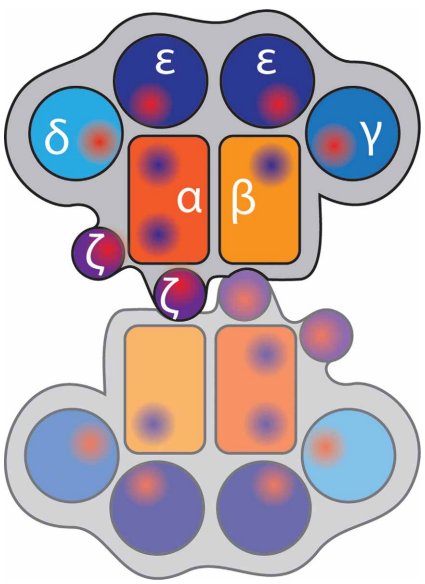

FIGURE 4 | Model of TCR complex dimerization. Heterodimers of TCRα and TCRβ (orange and yellow) are opposed due to the short ectodomains of CD3ζ (purple), which places CD3γ, CD3δ, and the two CD3ε chains (shades of blue) around the outside. The shapes represent immunoglobulin domains of the components; and red and blue shaded regions indicate positive and negatively charged regions, respectively.

Mutations at the αβ TCR "dimerization interface" in the AB loop, C and F strand in Cα of TCRα-transmembrane domain (TM) and TCRβ-TM chimeras slightly impaired calcium fluxes and severely impacted on TCR accumulation in the cSMAC (Kuhns et al., 2010) indicating that TCR dimerization is important for synapse organization. These findings are interesting because TCR dimerization has been previously proposed as a mechanism to initiate signaling (Krogsgaard et al., 2005). Here, soluble pMHC heterodimers—where one peptide was an agonist while the other was an endogenous self-peptide—could stimulate T cell activation and synapse formation as long as CD4 could be engaged on the agonist side of the pMHC dimer. Hence TCR dimerization may go beyond signal initiation but currently it is not clear how TCR dimerization relates to the formation of higher-order oligomers that have been described by Manz et al. (2011) and Kumar et al. (2011).

The requirement of TCR dimerization is an attractive model to explain why certain peptide binding models are not "allowed" despite distinct 3D and 2D affinities. Adams et al. compared the crystal structure of an alloreactive TCR αβ in complex with four different, but not naturally occurring, peptides all bound to the same MHC (Adams et al., 2011). While three peptides utilized germline-preserved TCR-MHC interactions and induced signaling, the fourth had a vastly different docking mode and failed to initiate signaling. The non-stimulatory peptide had a more parallel binding orientation along the α1/α2-helix of the MHC than the other stimulatory peptides. However, this cannot be the full story since an even more parallel orientation was reported for the CD1d-lipid antigen (αGalCer) of a NK TCR (Borg et al., 2007). Likewise, the 2D biophysical parameters of the non-stimulatory peptides were within the range of other agonistic pMHCs. Hence other explanations, like the formation of dimers and higher-order oligomers are needed to explain why a specific docking topology does not initiate signaling.

Ellis Reinherz proposed that selective TCR signaling may require dynamic interactions between the TCR αβ and the CD3 dimers, rather than a static on/off-switch, resulting in dynamic quaternary changes upon TCR ligation and triggering (Kim et al., 2012). In this model, the dynamic interaction between ectodomains rearranges the positioning of the CD3 dimers thereby affecting access to the cytoplasmic ITAM domains. A related dynamic model was put forward as the "safety" model of TCR triggering (Kuhns and Davis, 2008), in which electrostatic interactions sequester basic residue-rich stretches of the ITAM domains into negatively charged lipids in the inner leaflet of the plasma membrane (Aivazian and Stern, 2000; Xu et al., 2008). It was postulated that this lipid association of the cytosolic tails would prevent ITAM phosphorylation by restricting Lck access. However, ITAM phosphorylation triggered the release of these domains from the membrane (Zhang et al., 2011). This recent study also showed that mutations in the basic residue-rich stretch of CD3ζ impair TCR signaling and affect TCR localization in respect to Lck (Zhang et al., 2011). Other possible mechanisms that may dislodge ITAM motifs from the membrane are mechanical forces and changes in local membrane environment, or even a combination of the two. A change in lipid environment (which has been observed microscopically (Gaus et al., 2005) and biochemically (Zech et al., 2009) after the assembly of TCR signaling complexes) would require an initiation signal that is independent of ITAM phosphorylation. This brings us back to the question of what drives membrane restructuring and the recruitment of vesicles, such as the LAT-containing vesicles described above, and whether these processes bypasses TCR triggering. A possibility is that cell adhesion and/or the restructuring of the actin cytoskeleton trigger vesicle recruitment and fusion with the plasma membrane but how this fits into the timeline of TCR signaling and the onset of calcium fluxes within seconds of TCR triggering (Huse et al., 2007) is not clear.

TCR AS MECHANOSENSOR

Several groups have recently provided evidence that physical forces applied to the TCR or TCR subunits activate T cells, meaning that the TCR is a mechanosensor (Kim et al., 2009; Li et al., 2010; Husson et al., 2011; Judokusumo et al., 2012; Ma et al., 2012). These observations were made when beads coated with pMHC or monoclonal antibodies against CD3ε were manipulated with optical tweezers. *In vivo*, such forces could be exerted when migrating T cells attach to pMHC on APCs prior to a stop signal, or during sequential and repetitive contacts between T cells and APCs (Gunzer et al., 2000). This would mean that the affinity of pMHC-TCR interaction is translated into mechanical force, which in turn could affect the quaternary structure of the TCR/CD3 complex. Ellis Reinherz proposed that a pulling force from the pMHC causes the Cβ FG loop to push on the ectodomain of CD3ε. He speculates that multimeric crosslinking (and possibly soluble antibodies) applies a torque on the TCR to achieve the same outcome as monomeric interactions under applied mechanical force (Kim et al., 2012). If this is correct, the need for TCR dimerization could be the application of torque and the subsequent quaternary restructuring, rather than dimerization *per se*. Hence, rupture force (Husson et al., 2011) and bond lifetime under load can potentially determine the potency of pMHC stimulation. Furthermore, force on an individual TCR αβ heterodimer will be greater if fewer cognitive TCR-pMHC per cell are formed during T cell-APC contact. Hence the mechanosensing properties of the TCR could integrate sensitivity and specificity. In adhesion biology, so-called catch bonds have been described (Marshall et al., 2003) that reinforce binding under tensile forces that expose cryptic binding sites. The characteristic of catch bond engagement is that the lifetime of bonding is no longer linear. In this context, certain pMHC-TCR interactions would be stabilized while others are not. To generate sufficient torque to expose potential catch bonds, it is likely that the TCR-pMHC docking topography is critical (Kim et al., 2009). Whether catch bonds exist in the TCR-pMHC interaction and modulate on- and off-rates and how TCR docking orientations fit in to this scenario is yet to be explored.

CONCLUSION

There is much to be learned about this pivotal immune receptor and how antigen binding initiates the assembly of multimolecular complexes for signal initiation. What is now needed is the integration of information from TCR docking topography obtained by crystallography (Borg et al., 2007; Adams et al., 2011),

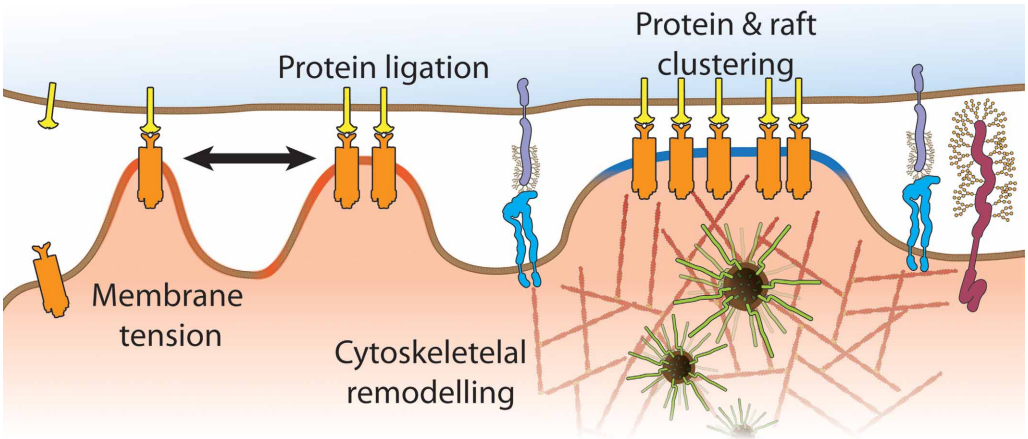

FIGURE 5 | Mechanisms that contribute to the spatial organization of TCR signaling processes include mechanical forces (black arrow), membrane tension due to convoluted membrane topography (red regions), variable inter-membrane distances across the immunological synapse, membrane compartmentalization by cortical actin and positioning and abundance of lipid domains (blue regions).

to measurements and manipulations (Li et al., 2010) of mechanical forces with optical tweezers (Kim et al., 2012), biomembrane force probes (Huang et al., 2010; Husson et al., 2011) and single molecule imaging approaches (Lillemeier et al., 2010; Sherman et al., 2011; Williamson et al., 2011) that take us beyond the plasma membrane. Importantly, recent papers have given us the motivation for such cross-disciplinary work as they highlight that this uniquely complex receptor system holds the key for T cell activation (Kumar et al., 2011; Manz et al., 2011). These discoveries have given us a glimpse that the distinction between naïve and memory T cells could also lay in the spatial organization of the TCR itself. Distributions of TCR dimers, clusters and islands relative to other signaling proteins may explain why we see enhanced basal phosphorylation of LAT and ZAP70 (Kersh et al., 2003) and diminished Lck dependency (Tewari et al., 2006) in memory T cells as well as the differential activation of MAP kinases in experienced and naïve T cells (Adachi and Davis, 2011). The TCR spatial organization itself could be influence by membrane domains (Kersh et al., 2003; Tani-ichi et al., 2005), expression of adaptor proteins (Singleton et al., 2011), membrane topography (James and Vale, 2012) and applied forces (**Figure 5**). This will make for exciting times ahead.

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Defining the molecular blueprint that drives CD8$^+$ T cell differentiation in response to infection

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A cardinal feature of adaptive, cytotoxic T lymphocyte (CTL)-mediated immunity is the ability of naïve CTLs to undergo a program of differentiation and proliferation upon activation resulting in the acquisition of lineage-specific T cell functions and eventual establishment of immunological memory. In this review, we examine the molecular factors that shape both the acquisition and maintenance of lineage-specific effector function in virus-specific CTL during both the effector and memory phases of immunity.

Keywords: cytotoxic T cells, memory T cells, transcription factors, epigenetics, histone modifications

INTRODUCTION

A cardinal feature of adaptive, cytotoxic T lymphocyte (CTL)-mediated immunity is the ability of naïve CTLs to undergo a program of differentiation and proliferation upon activation resulting in the acquisition of lineage-specific T cell functions and eventual establishment of immunological memory (Kaech et al., 2002a; van Stipdonk et al., 2003). CTLs contribute to the control and eventual elimination of a myriad of pathogen (intracellular bacteria and viruses) and tumor challenges via the coordinated interplay of varied effector mechanisms that include; (1) the production of pro-inflammatory cytokines such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α (La Gruta et al., 2004); and (2) the expression of cytolytic effector molecules including perforin (Pfp; Kagi et al., 1994) and the granule enzymes (granzymes, Gzm) A, B, and K (Jenkins et al., 2007; Peixoto et al., 2007; Moffat et al., 2009). Once infection is cleared, the expanded effector T cell population contracts with establishment of a pool of long-lived, pathogen-specific memory T cells (Marshall et al., 2001; Kaech et al., 2002b; La Gruta et al., 2004). Although quiescent, memory CTLs demonstrate rapid effector function without the need for further differentiation (Lalvani et al., 1997; Oehen and Brduscha-Riem, 1998; Cho et al., 1999; Veiga-Fernandes et al., 2000). The combination of a high frequency and rapid effector function enables memory CTLs to respond more rapidly upon secondary infection, enabling earlier control and clearance of infection. Importantly, our understanding of the factors that not only shape the cell fate decisions to be a memory versus effector T cells are unclear, but the molecular mechanisms that enable stable maintenance of rapid effector function in the long-term are also not well understood. This review will examine new advances in our understanding of the molecular mechanisms that control effector and memory CD8$^+$ T cell differentiation.

INITIATION OF T CELL RESPONSES

Initiation of naïve CTL activation requires recognition of pMHC complexes on a specialized subset of antigen-presenting cells, termed dendritic cells (DCs). DCs exist as two general populations – tissue-resident and lymph node-resident DCs (Heath and Carbone, 2001). Importantly, in the context of peripheral infections such as respiratory influenza A virus infection and herpes simplex virus infection of the skin, both tissue-resident and lymph node-resident DCs appear to play roles in the induction of T cell immunity (Allan et al., 2003; Belz et al., 2004; Heath and Carbone, 2009). A primary role for tissue-resident DCs is continual surveillance of their environment for the presence of invading pathogens. Upon infection, tissue-resident DCs are activated via pathogen pattern receptors, such as Toll-like receptors or intracellular sensors such as RIG-I, MDA-5, and members of the inflammasome complex, resulting in activation and trafficking of these DCs from the tissues to the draining lymph node. The activation of DCs also results in the concomitant up-regulation of co-stimulatory molecules, such as CD80/CD86. Thus, these migratory DCs not only carry antigen from the infected tissue to the draining lymph node, but are now capable of providing the necessary secondary signals to promote activation of naïve and memory antigen-specific T cells (Heath and Carbone, 2001; Belz et al., 2007). A third signal, provided via signaling induced by pro-inflammatory cytokines such as type I IFNs and interleukin (IL)-12, is also required for full priming of mature effector T cell responses (Curtsinger et al., 2003a,b). Thus the integration of multiple signals received via the T cell receptor, co-stimulation and inflammatory cytokine receptors is required to drive differentiation of naïve T cells to effector and memory cells.

THE EFFECTOR PHASE: ACQUISITION OF CTL EFFECTOR FUNCTION

As little as 2 h of *in vitro* peptide stimulation is sufficient to initiate an autonomous program of T cell proliferation and differentiation (Kaech and Ahmed, 2001; van Stipdonk et al., 2003). These initial observations were supported by *in vivo* studies demonstrating that early termination of antigen-presentation did not overly impact effector and memory CTL differentiation after infection (Wong and Pamer, 2001, 2003; Prlic et al., 2006). Thus, it would appear that naïve $CD8^+$ T cells are pre-programed for differentiation prior to any antigen exposure. This concept is aligned with the recent data demonstrating that dynamic changes in genomic and transcriptional programing occurring during T cell development are key for establishing a genetic blueprint that likely underpins the fate of naïve T cells after activation (Zhang et al., 2012).

Progressive differentiation is a key factor that shapes both the phenotypic and functional heterogeneity of pathogen-specific CTL responses (Marzo et al., 2005; Badovinac et al., 2007). The acquisition of IFN-γ (Lawrence and Braciale, 2004), Pfp (Jenkins et al., 2008), and granzyme expression (Oehen and Brduscha-Riem, 1998; Jenkins et al., 2008; Moffat et al., 2009) is clearly linked to ongoing lymphocyte proliferation (Badovinac et al., 2007; Jenkins et al., 2008). In addition, functional profiling of effector and memory CTL induced after primary influenza A virus infection of C57BL/6J mice demonstrated that profiles of intracellular cytokine expression (both mRNA and induced protein) followed a strict hierarchy and most likely reflected sequential acquisition of multiple effector functions due to progressive differentiation following activation (La Gruta et al., 2004).

In terms of cytokine production, recent observations suggest that polyfunctional potential ($TNF\text{-}\alpha^+IFN\text{-}\gamma^+$) is acquired within three to four divisions with acquisition of IFN-γ production (Denton et al., 2011). However, extended cycling leads to the loss of TNF-α production for a substantial set of activated CTLs leading to a progressive diminution in polyfunctional capacity. This is supported by the observation that activation of TCR transgenic T cells with "low affinity" ligands leads to an inability to sustain extended proliferation with these less differentiation CTL exhibiting co-expression of IFN-γ and TNF-α (Zehn et al., 2009). This contrasts with acquisition of cytolytic gene expression (Pfp and the granzymes) where continued cell division leads to a broader spectrum of effector gene expression (Jenkins et al., 2007, 2008; Peixoto et al., 2007). Recent data also suggests that there is a hierarchy of expression with granzyme B acquired early after activation with extended proliferation required for both GzmA and GzmK expression (Jenkins et al., 2008; Moffat et al., 2009; Zehn et al., 2009). Differences in the temporal expression of regulatory factors required for Gzm and cytokine gene loci likely explains these differences.

THE MEMORY PHASE

Memory T cells can be broadly divided into "central" and "effector" memory subsets, with the two differing in both phenotypic and functional characteristics that reflect the different roles they play in response to secondary infection (Sallusto et al., 1999, 2004). Effector memory T cells (or T_{EM}) typically express tissue-specific homing markers such as CCR5, CXCR3, and integrins and while they can be found in the circulation, significant numbers are found in the non-lymphoid tissues (Masopust et al., 2001). Moreover, T_{EM} are associated with decreased proliferative capacity and immediate effector function, such as cytotoxicity in the case of CTL (Masopust et al., 2001). While T_{EM} are capable of entering non-lymphoid tissues from the circulation in the steady state (Wakim et al., 2008; Kohlmeier et al., 2011), recent reports have identified tissue-resident T_{EM} that persist in the long-term at the original site of infection (Gebhardt et al., 2009, 2011; Mackay et al., 2012).

Central memory T cells (T_{CM}) typically express the lymph node homing markers, CD62L (L-selectin) and CCR7, and exhibit greater proliferative capacity when compared to T_{EM} (Masopust et al., 2001). The fact that T_{CM} localize to lymph nodes in greater numbers and are capable of proliferation in response to secondary infection ensures greater numbers of effector CTL are generated earlier. Thus, T_{CM} provide a more rapid response and provide a second wave of effector CTL capable of clearing any remaining active infection that T_{EM} have failed to control (Wherry et al., 2003).

Just when after infection memory T cells are generated is the basis of some conjecture. However, there is strong evidence that T cell memory can be established very early after infection, especially when inflammation is limiting. For example, the prophylactic use of antibiotics prior to *Listeria monocytogenes* infection, or vaccination with peptide pulsed DCs, demonstrated that functional memory T cells can be generated as soon as 4–6 days after priming (Badovinac et al., 2005). This is supported by a study where it was shown that T cells isolated from IAV infected mice as early as 3–4 days after infection could form memory when adoptively transferred into a second host (Kedzierska et al., 2006, 2007).

Recent studies have suggested that effector or memory T cell fate can be predicted during the primary effector phase based on the cell surface expression of both the killer cell lectin-like receptor G1 (KLRG1) and IL-7 receptor subunit-α (IL-7Rα; Kaech et al., 2003; Joshi et al., 2007). Activated T cells that express high levels of KLRG1 and low levels of IL-7Rα ($KLRG1^{hi}IL\text{-}7R\alpha^{lo}$) are largely destined to be terminally differentiated effector cells and are termed short-lived effector cells (SLECs). In contrast, the small population of activated T cells that are $KLRG1^{lo}IL\text{-}7R\alpha^{hi}$ demonstrate memory potential and are termed memory precursor effector cells (MPECs). While the models of effector versus memory T cell fate are still an area of debate (reviewed by Kaech and Cui, 2012), recent advances have started to provide insights into the molecular factors that shape the outcomes of T cell activation.

MOLECULAR FACTORS THAT SHAPE ACQUISITION OF EFFECTOR FUNCTION

Specific transcription factors determine T cell effector and memory function and fate (Intlekofer et al., 2005; Joshi et al., 2007; Cruz-Guilloty et al., 2009; Kallies et al., 2009; Pipkin et al., 2010; Zhang et al., 2010; **Figure 1**). In the case of CTL effector function three transcription factors appear to play "pioneering" roles in determining effector T cell differentiation. Two T-box transcription factors, Tbx21 (T-bet) and Eomesodermin (Eomes; Intlekofer et al., 2005) play essential roles in effector CTL differentiation. While T-bet is normally associated with $CD4^+$ T_H1 lineage

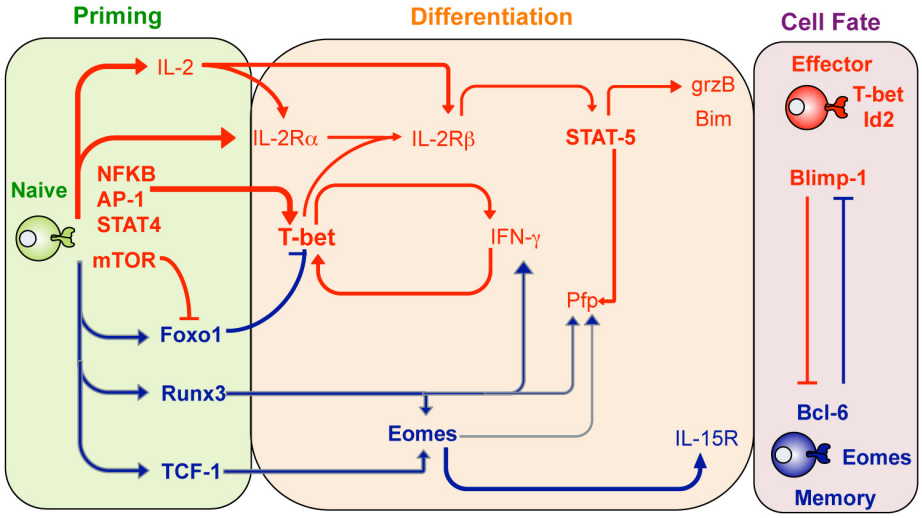

FIGURE 1 | Transcription factor regulation of effector and memory T cell differentiation. Shown are transcription factors that are expressed and act to promote either effector CTL (red lines) or memory CTL (blue lines) differentiation at the different stages after infection including the priming phase (green), differentiation stage (orange) or once the cell fate has been determined (purple shading). Adapted from Pipkin and Rao, Cell, Snapshots Archive, Effector and memory T cells.

commitment, in part, by promoting expression of IFN-γ (Szabo et al., 2000), it is also rapidly up-regulated in activated CTL, contributing to rapid acquisition of IFN-γ production and helping promote GzmB expression (Cruz-Guilloty et al., 2009). Eomes, a homolog of T-bet, was originally implicated in the regulation of $CD8^+$ T cell GzmB expression (Pearce et al., 2003), however recent studies suggest that Eomes is expressed later during CTL differentiation and contributes more to acquisition of Pfp expression and maintenance of the capacity to express IFN-γ (Cruz-Guilloty et al., 2009). The fact that Eomes over-expression does not rescue diminished GzmB expression in T-bet-deficient CTL suggests that the contribution of Eomes in determining specific T cell function is highly dependent on the timing and extent of expression (Cruz-Guilloty et al., 2009).

It is becoming clear that various transcription factors work cooperatively to reinforce the commitment of naïve T cells to become fully differentiated effector T cells. For example, the runt-related transcription factor 3 (Runx3), expressed by naïve CTLs, promotes expression of a variety of signature CTL effector molecules such as IFN-γ, GzmB, and Pfp (Cruz-Guilloty et al., 2009). Runx3 appears to have both a direct affect via binding to promoter and regulatory regions within the *Ifn-γ*, *gzmB*, and *pfp* gene loci. Moreover, Runx3 also promotes Eomes transcription further promoting CTL differentiation and acquisition of effector function (Cruz-Guilloty et al., 2009). Given that Runx3 is constitutively expressed in naïve T cells, just what regulates Runx3 activity is not clear. However, upon T cell activation Runx3 can bind to IL-2 responsive regulatory elements within the *pfp* gene locus (Cruz-Guilloty et al., 2009). Thus it is likely that IL-2 signals received upon CTL activation are required for Runx3 activity on a subset of effector gene loci.

Interleukin-2 is a key cytokine required for inducing proliferation and survival of activated T cells (Miyazaki et al., 1995). Importantly, high levels of IL-2 signaling at the time of CTL activation also contribute to signature effector gene expression, including expression of GzmB and Pfp (Janas et al., 2005; Pipkin et al., 2010). IL-2-dependent regulation of effector CTL differentiation is primarily via IL-2Rβ signaling and subsequent activation of STAT5. This results in binding of activated STAT5 to the GzmB promoter, thus helping promote gene transcription. In contrast, up-regulation of Pfp expression first requires STAT5 to bind to regulatory elements within the Eomes promoter with subsequent Eomes expression able to promote Pfp expression (Cruz-Guilloty et al., 2009). This IL-2-STAT5 pathway also likely explains the IL-2 dependency of Runx3 promotion of effector gene expression (Cruz-Guilloty et al., 2009).

Another key transcription factor in CTL effector differentiation is the B lymphocyte-induce maturation protein-1 (Blimp-1) encoded by *Prdm1*. A role for Blimp-1 in lymphocyte differentiation was first observed in activated B cells where it is required for the terminal differentiation and subsequent maintenance of long-lived antibody-secreting cells (Shapiro-Shelef et al., 2003). Recent studies have demonstrated that Blimp-1-deficient T cells are unable to fully differentiate into effector CTL in response to virus infection. Rather, Blimp-1-deficient CTL preferentially differentiate into effector CTL that have "memory like" characteristics such as high levels of IL-7α and Bcl6 expression (see below) and low levels of typical effector markers such as KLRG1 and GzmB (Kallies et al., 2009; Rutishauser et al., 2009). Thus, Blimp-1 appears to ensure that activated lymphocytes, including CTL, become terminally differentiated effectors. Importantly, the same high IL-2 activating conditions that contribute to CTL effector gene expression, also contribute to up-regulation of Blimp-1 and repression of the transcription factor Bcl6 and IL-7Rα expression (Pipkin et al., 2010). This transcriptional profile is a hallmark of terminal CTL differentiation (Kaech et al., 2002b) and hence IL-2 not only

contributes to acquisition of effector function but also to effector CTL differentiation.

So what signals dictate the decision for activated CTL to differentiate toward either an effector or memory T cell fate? A major driver appears to the response of activated CTL to pro-inflammatory mediators produced upon infection. For example, T-bet up-regulation in activated CTLs is clearly induced via TCR ligation and inflammatory mediators such as IL-12 and IFN-γ (Mullen et al., 2001; Szabo et al., 2002; Sullivan et al., 2003; Takemoto et al., 2006), and results in differentiation of effector T cells. Moreover, IL-12 signaling serves to both simultaneously promote and suppresses T-bet and Eomes expression, respectively (Takemoto et al., 2006). Thus, the degree of inflammatory stimulation serves to establish higher levels of T-bet and tips the balance toward effector CTL differentiation (Joshi et al., 2007).

REGULATING THE T-bet/Eomes NEXUS AND MEMORY T CELL FATE

Current evidences suggest that the programing of T cell memory occurs early during the priming phase (Feau et al., 2011). Thus, what precisely are the factors that translate signals received during priming into this memory capacity? While initially considered a key driver of signature CTL effector gene expression, and hence effector differentiation, it has recently emerged that Eomes may play a more prominent role in memory T cell formation and persistence (Intlekofer et al., 2005; Banerjee et al., 2010). This in part likely due to Eomes-dependent up-regulation of the IL-2β receptor (CD122), enabling responsiveness to IL-15, a cytokine needed for maintenance of memory T cells (Intlekofer et al., 2005).

As described earlier, induction of high levels of T-bet expression in response to pro-inflammatory cytokines serves to promote effector T cell differentiation. Thus, it is interesting that T-bet deficiency within virus-specific CTL results in fewer T_{CM} (Intlekofer et al., 2007). Thus, T-bet appears to not only promote effector T cell differentiation, but to actively suppress memory T cell formation. Just how T-bet does this is not clear but is tempting to speculate that it may act as a transcriptional repressor inhibiting expression of gene loci required for memory T cell programing. This will be a key area of research in the future.

The importance of regulating the T-bet/Eomes nexus in determining effector versus memory T cell fate is highlighted by the fact that a number of extrinsic signaling pathways serve to regulate the balance of T-bet and Eomes levels in activated T cells. Expression of the transcription factor Foxo1 actively represses effector differentiation by blocking T-bet expression, while at the same time promoting Eomes expression and maintenance of memory T cell generation (Rao et al., 2012). Activation of the mammalian target of rapamycin (mTOR) kinase inactivates Foxo1 function thereby releasing T-bet from Foxo1 inhibition and thus, promoting effector CTL differentiation (Rao et al., 2012). Therefore, the use of rapamycin, or some other inhibitor of mTOR activity may be a useful intervention that promotes memory T cell generation.

Another member of the fork head family of transcription factors, Foxo3a, has also been implicated in the regulation of effector versus memory T cell fate (Riou et al., 2007; van Grevenynghe et al., 2008). Comparison of transcriptional signatures between polyclonal human $CD4^+$ T_{CM} and T_{EM} demonstrated differential expression of genes regulated by Foxo3a (Riou et al., 2007), including Bim. Phosphorylation results in the exclusion of Foxo3a from the nucleus and subsequent transcriptional inactivation (Brunet et al., 1999), thus it was of interest that T_{CM} had higher levels of phosphorylated Foxo3a compared to T_{EM}. Importantly, it was determined that both TCR and signals via the common γ-chain cytokine receptor induce Foxo3a phosphorylation and subsequent protection from Bim-mediated apoptosis. Hence, this provides a molecular mechanism for how homeostatic signals regulate memory T cell persistence (Riou et al., 2007). Moreover, inhibition of Foxo3a expression has been shown to prolong human immunodeficiency virus-specific memory T cell survival, indicating that Foxo3a is a potential target for therapeutic intervention that could promote memory T cell establishment (Riou et al., 2007; van Grevenynghe et al., 2008).

In another example of extrinsic signals promoting memory T cell differentiation, activation of the Wnt-β-catenin signaling pathway has been shown to promote expression of the transcription factor T cell factor-1 (TCF-1), with subsequent up-regulation of Eomes (Zhou et al., 2010). As observed in Eomes-deficient mice (Banerjee et al., 2010), TCF-1-deficient mice fail maintain a $CD62L^{hi}$ T_{CM} population after challenge, supporting the notion that programing of memory CTL requires expression of TCF-1 that in-turn promotes Eomes expression (Zhou et al., 2010).

THE ROLE OF OTHER TRANSCRIPTIONAL REGULATORS IN MEMORY T CELL DIFFERENTIATION

Given that inflammation is a key driver of effector T cell differentiation, a question that arises is how does memory arise in the face of a robust infection? Recent evidence suggests that environmental cues may serve to limit the impact of inflammatory-driven effector T cell differentiation allowing for memory T cell formation early after infection. Both IL-10 and IL-21 have been reported to promote memory T cell differentiation (Yi et al., 2010). Further, it has been recently demonstrated that after LCMV infection, signaling via STAT3, a transcription factor necessary for transmitting IL-10R and IL-21R signals, is necessary for memory CTL formation (Cui et al., 2011). Significantly, STAT3-dependent signals promote the expression of the transcription factor BCL6, known to be up-regulated within memory CTL while at the same time, repressing Blimp-1 expression. Moreover, STAT3-deficient CTL had lower levels of suppressor of cytokine signaling-3 (SOCS-3) expression and were more responsive to IL-12-dependent differentiation (Cui et al., 2011). Thus, signaling via immune regulatory cytokines, such as IL-10 and/or IL-21, can promote memory T cell differentiation by both up-regulating key memory T cell fate governing genes, while at the same time actively limiting the impact of inflammatory signals and subsequent effector CTL differentiation.

Finally, the inhibitor of DNA binding (Id)-2, and Id3 proteins have opposing roles in determining effector versus memory CTL generation. While pathogen-specific CTL within Id2-deficient mice could respond and differentiate into effectors, they exhibited a diminished response magnitude after infection and delayed

pathogen clearance (Cannarile et al., 2006; Yang et al., 2011). Importantly, there was a failure to establish a T_{EM} population upon clearance of infection. These data suggest that Id2 up-regulation is required for sustained effector differentiation and establishment of the T_{EM} repertoire. Moreover, STAT5 and STAT4 binding sites have been identified within the Id2 promoter suggesting that the inflammatory signals known to drive effector differentiation, such as IL-2 and IL-12, can act on Id2 to promote this fate (Yang et al., 2011).

In contrast to Id2, Id3 is down-regulated upon CTL activation but is then re-expressed by memory T cells (Yang et al., 2011). Interestingly, while effector differentiation is normal in Id3-deficient mice, there is a failure to maintain a long-lived memory T cell population upon clearance of infection (Ji et al., 2011). Thus, Id3 expression is a key checkpoint that contributes to establishment of a robust memory T cell population. In fact, up-regulation of Blimp-1 has been shown to inhibit Id3 expression, representing a key switch in effector versus memory T cell fate determination (Ji et al., 2011; Yang et al., 2011).

In summary, a complex transcriptional network interprets and integrates the various extrinsic signals received by an activated T cell soon after infection. While there is significant understanding about the precise role these transcriptional networks have in T cell differentiation, little is known about regulation of the genomic template they bind and how changes in the biochemical and structural composition of the genome regulates this activity. The next section of this review will examine the role of epigenetics, or modifications of chromatin, in regulating effector and memory T cell differentiation.

EPIGENETIC REGULATION: THE ROLE OF HISTONE MODIFICATIONS

Within eukaryotic cells, genomic DNA is wrapped around a complex of histone proteins that organize to form a structure termed chromatin (**Figure 2**). The basic unit of chromatin is the nucleosome, where DNA is wrapped around an octameric histone complex, typically containing two each of the core histones H2A, H2B, H3, and H4. Importantly, the composition of chromatin structure and biochemical modifications of histone proteins cannot only regulate short-term gene expression patterns within a cell, but can be propagated as a proliferates ensuring stable inheritance of a cellular phenotype, a process termed epigenetics.

Histone modifications in particular are thought to modulate gene expression by either changing chromatin structure and/or by providing a platform that promotes binding of transcriptional regulators (Kouzarides, 2007). Histone proteins can be modified by a vast array of covalent modifications, particularly on the solvent-exposed N-terminal tail with the combination of histone PTMs and their genomic location a predictor of transcriptional activity (**Table 1**; Zhang and Reinberg, 2001; Wang et al., 2008). For example, acetylation of the histone H3 at lysine 9 (H3K9Ac) that is associated with gene promoters is a positive correlate of transcriptional activation (Wang et al., 2008). Broadly speaking, histone acetylation is thought to promote a more open chromatin structure by masking the overall positive charge of histones (Aoyagi et al., 2002). This relaxes chromatin structure,

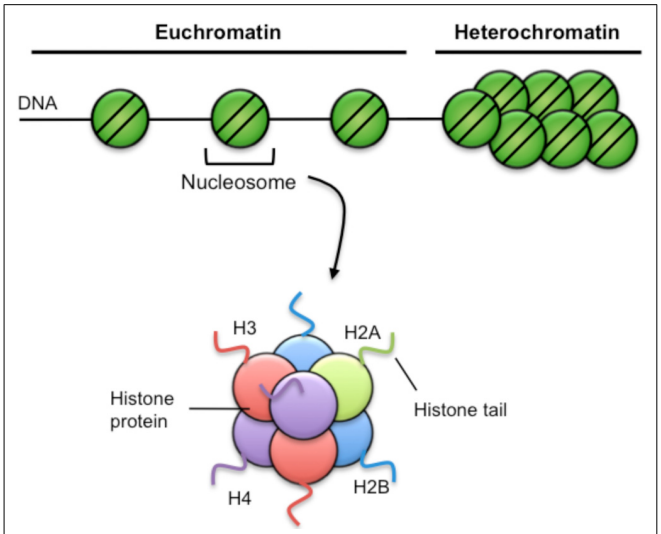

FIGURE 2 | Chromatin structure. Chromatin has two broad structures. The first is euchromatin, characterized by sparse nucleosome density and is generally associated with active gene transcriptional activity. Heterochromatin is characterized by high nucleosome density, is very compacted and is generally associated with repression of gene transcription. Nucleosomes consist of 147 bp of DNA wound 1.65 turns around a complex of histone proteins, comprising two each of the H2A, H2B, H3, and H4 histone variants. Each histone has a soluble amino terminal tail that can be covalently modified by specific epigenetic marks such as acetylation, methylation, and phosphorylation.

and potentiates transcription factor binding and the recruitment of the core transcription machinery. Conversely, a lack of histone acetylation restricts chromatin accessibility and can render the DNA inaccessible to the transcriptional machinery (Ura et al., 1997).

Methylation of histone proteins is somewhat more complicated. For example, tri-methylation of histone 3 at lysine 4 (H3K4me3) is associated with almost all actively transcribed genes, and has a strong correlation with histone acetylation and recruitment of RNA polymerase II, indicative of a transcriptionally permissive gene (Bernstein et al., 2002; Santos-Rosa et al., 2002; Heintzman et al., 2007; Muse et al., 2007). Conversely, H3K4 dimethylation can be associated with either active or repressed genes (Bernstein et al., 2005), suggesting dual roles for this mark. In contrast to transcriptional activation, deposition of tri-methylation of H3K27 (H3K27me3) within promoter regions is strongly linked to transcriptional repression (Wang et al., 2008). Another repressive mark, H3K9me3, is implicated in heterochromatin formation through its interaction with heterochromatin protein 1 (HP1; Jacobs and Khorasanizadeh, 2002).

It is becoming increasingly evident that it is the combination and degree of enrichment of histone modifications that is key for fine-tuning gene transcription or silencing (Barski et al., 2007; Wang et al., 2008). The combination of different active and repressive histone modifications at specific loci defines the transcriptional state of individual genes (Strahl and Allis, 2000), thus defining cell fate. This is most evident when considering patterns of H3K4me3 and H3K27me3 within the same promoter regions. Genome-wide mapping of these two

Table 1 | Histone modifications and their association with gene expression[a].

Histone	Site	Modification	Activity[b]
H2A	S1	Phosphorylation	–
	K5	Acetylation	+
	K119	Ubiquitylation	+/–
H2B	K5	Acetylation	+
	K12	Acetylation	+
	K15	Acetylation	+
	K20	Acetylation	+
	K120	Ubiquitylation	+/–
H3	R2	Methylation	–
	K4	Methylation	+
		Acetylation	+
	K9	Methylation	–
		Acetylation	+
	S10	Phosphorylation	+
	K14	Acetylation	+
	R17	Methylation	+
	K18	Acetylation	+
	K23	Acetylation	+
	K27	Methylation	–
		Acetylation	+
	K36	Methylation	+/–
	K79	Methylation	+
H4	R3	Methylation	+
	K5	Acetylation	+
	K8	Acetylation	+
	K12	Acetylation	+
	K16	Acetylation	+
	K20	Methylation	–

[a] *Adapted from Yi et al. (2010).*
[b] *Associated with activation (+) or repression (–) of genes.*

modifications in embryonic stem cells (ESCs) has demonstrated that regions important for maintaining ESC pluripotency are enriched for both H3K4me3 and H3K27me3, termed "bivalent" loci (Bernstein et al., 2006; Cui et al., 2009; Hawkins et al., 2010). Importantly, upon differentiation, the vast majority of bivalent loci within stem cells resolve to H3K27me3 ensuring that inappropriate gene expression within specific cell lineages does not occur (Bernstein et al., 2006). These data suggest that bivalency is a switch mechanism by which genes can be rapidly activated or repressed depending on the differentiation pathway initiated.

APPROACHES FOR THE ANALYSIS OF HISTONE MODIFICATIONS WITHIN THE GENOME

A standard approach for examining histone modification within the genome is chromatin immunoprecipitation. Initially, DNA–protein complexes within nuclei are cross-linked by formaldehyde fixation, followed by fragmentation of the DNA by enzymatic digestion or mechanical disruption (i.e., sonication). The DNA–protein complexes are then immunoprecipitated using antibodies specific for either DNA binding proteins (such as transcription factors) or specific histone covalent modifications. The purified complexes are then treated to reverse the cross-links, and the DNA isolated and used as a real-time PCR template to interrogate specific genomic regions of interest. The focused nature of this approach means that only small genomic regions are probed in any one reaction (typically 100–300 bp) with extensive analysis of a particular gene locus requiring a laborious and systematic approach.

The advent of next-generation sequencing technology has revolutionized the study of epigenetic modifications by enabling genome-wide profiling of chromatin modifications, an approach termed ChIP-seq (Kharchenko et al., 2008; Park, 2009). ChIP-seq involves "deep-sequencing" the immunoprecipitated DNA with the subsequent short sequences (or reads) being mapped back onto a reference genome. This approach has yielded an unprecedented level of resolution identifying not just the genomic location of specific modifications, but the specific patterns of enrichment, as well as their association with particular genomic features such as promoter and enhancers. In combining such data with large-scale transcriptional profiling (i.e., by microarray), our understanding of how epigenetic modifications underpin key cellular processes is undergoing a renaissance.

EPIGENETIC REGULATION OF CD8$^+$ T CELL EFFECTOR FUNCTION: ACQUISITION AND MAINTENANCE

A defining characteristic of T cell immunity is the acquisition of lineage-specific effector function that is readily maintained into memory. There is a large body of work has determined that specific epigenetic mechanisms underpin CD4$^+$ effector T cell lineage commitment from a naïve state into different effector subsets (reviewed by Ansel et al., 2003; Kanno et al., 2012). Similarly, there is a growing body of work that has examined epigenetic regulation of CD8$^+$ effector T cell differentiation.

Granzyme B expression by activated CD8$^+$, but not CD4$^+$ effector cells, generally reflects differences in the lineage-specific functions observed for the CD4$^+$ and CD8$^+$ T cell subsets. Recently we demonstrated that differences in GzmB expression by *in vitro* activated CD4$^+$ and CD8$^+$ T cells correlates with difference in epigenetic modifications within the *gzmB* locus (Juelich et al., 2009). While, CD8$^+$ T cell expression of GzmB was coupled to a significant increase in chromatin accessibility, H3K9ac and H3K4me3 deposition, and docking of RNA polymerase II at the *gzmB* promoter region, few of these changes occurred within activated CD4$^+$ T cells. Strikingly, this study suggests that differential programing of CD4$^+$ and CD8$^+$ T cell subsets during T cell development dictates the what lineage-specific effector function will be acquired upon activation. In the case of mature, naïve CD8$^+$ T cells, it is most likely the combination of transcription factors including Eomes and Runx3 that direct acquisition of GzmB expression within activated CD8$^+$, but not CD4$^+$ T cells. It still remains to be determined whether these lineage-specific transcription factors play a role in directing the observed epigenetic

changes during differentiation, or drive effector gene expression after chromatin remodeling has occurred.

In terms of acquisition and maintenance of CTL-specific functions recent analysis has demonstrated that dynamic changes in specific histone modifications can underpin observed phenotypic and functional changes during $CD8^+$ T cell differentiation. It is under appreciated that naïve T cells can exhibit rapid effector function upon TCR ligation whereby they rapidly produce TNF-α, but not IFN-γ prior to initiation of division (Brehm et al., 2005; Priyadharshini et al., 2010; Denton et al., 2011). Co-expression of TNF-α^+ and IFN-γ^+ is observed within three to four divisions, but extended proliferation leads to the loss of TNF-α production for a substantial proportion of activated $CD8^+$ T cells leading to a progressive diminution in polyfunctional capacity (Denton et al., 2011). Importantly, the capacity of naïve, effector and memory T cells to make IFN-γ and TNF-α is directly linked to the presence of defined epigenetic signatures within the proximal promoters (Denton et al., 2011). For example, the *tnfA* proximal promoter of naïve OT-I cells has an overall permissive epigenetic landscape with increased chromatin accessibility, enrichment for H3K4me3/H3K9ac and lack of H3K27me3. This is compared to the generally repressive epigenetic signature for the *ifnG* promoter with a closed chromatin structure, lack of H3K4me3/H3K9ac and enrichment of H3K27me3 (Northrop et al., 2006; Denton et al., 2011; Zediak et al., 2011).

The *ifn-γ* promoter region undergoes significant remodeling in effector $CD8^+$ T cells, becoming more accessible and acquiring a permissive epigenetic signature (Northrop et al., 2006; Denton et al., 2011; Zediak et al., 2011). The fact that such events are required for the acquisition of effector gene expression and take time to eventuate probably explains the link between continued cell division and the acquisition of lineage-specific functional capacity within recently activated T cells (Lawrence and Braciale, 2004; Jenkins et al., 2008; Denton et al., 2011). While extended effector T cell differentiation leads to permissive epigenetic marks being deposited within the *ifn-γ* promoter to allow IFN-γ expression (Northrop et al., 2006; Denton et al., 2011; Zediak et al., 2011), there was progression to a more repressive *tnfA* epigenetic signature (decreased chromatin accessibility and increased H3K27me3 deposition; Denton et al., 2011). Importantly, the fact that both activating and repressing chromatin remodeling events were apparent at different loci, but within the same effector CTL effector population, suggests that opposing regulatory mechanisms can act simultaneously at distinct gene loci.

EPIGENETIC AND THE MAINTENANCE OF EFFECTOR FUNCTION WITHIN MEMORY T CELLS

A cardinal feature of memory T cells is their ability to elicit rapid effector function upon antigen recognition without the need for further differentiation. A number of studies have demonstrated the ability to maintain this functional capacity in the resting state is likely underpinned by maintenance of permissive epigenetic signatures at key gene loci. For example, virus-specific memory $CD8^+$ T cells exhibit an open chromatin structure with enrichment of H3K4me3, H3K9ac and loss of H3K27me3, at the *ifnG*, *tnfA*, *gzmB*, and *pfp* effector gene loci (Northrop et al., 2006, 2008; Araki et al., 2008, 2009; Denton et al., 2011; Zediak et al., 2011).

Importantly, maintenance of a permissive epigenetic signature at the IFN-γ and TNF-α gene loci within virus-specific memory T cells coincided with docking of RNA polymerase II, a core component of the transcriptional machinery, at the proximal promoter (Denton et al., 2011; Zediak et al., 2011). This "stalling" of RNA pol II at the transcriptional start site of proximal promoters is associated with genes that are "poised" or "at the ready" for transcriptional activity (Margaritis and Holstege, 2008). Thus, epigenetic re-programing that results in maintenance of permissive epigenetic signatures at key effector gene loci enables memory T cells to keep RNA pol II "on-hold" at the TSS. Subsequent to TCR ligation, the RNA polymerase is released, allowing rapid gene expression and ensuring rapid memory T cell effector function (**Figure 3**). Interestingly, not all effector gene loci appear to be "poised" in memory $CD8^+$ T cells with RNA poll II docking not observed at the *gzmB*, *pfp* (Zediak et al., 2011) or *gzmA* (L. Hatton, B. E. Russ, and S. J. Turner, unpublished data) proximal promoters, despite the presence of a permissive epigenetic signature. While gzmB and gzmA expression is up-regulated in reactivated memory $CD8^+$ T cells, it is delayed when compared to cytokine production (Lawrence and Braciale, 2004; Jenkins et al., 2007, 2008; Moffat et al., 2009; Denton et al., 2011; Zediak et al., 2011). Thus, it appears that different molecular mechanisms work

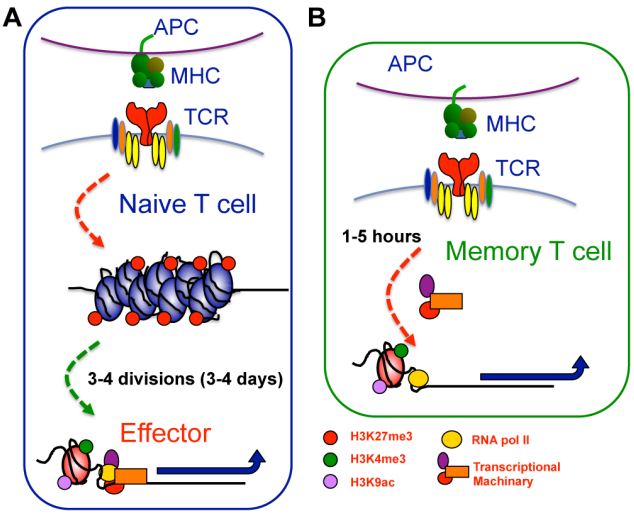

FIGURE 3 | Epigenetic reprogramming within key loci of memory T cells enables rapid effector function upon restimulation. (A) Within naive CTL, key effector gene loci, such as IFN-γ or gzmB, exhibit repressive epigenetic signatures characterized by a heterochromatin structure, deposition of H3K27me3, and lack of H3K4me3 and H3K9ac. Upon T cell activation, these gene loci undergo significant chromatin remodeling becoming more accessible and acquiring a permissive epigenetic signature characterized by loss of H3K27me3, and deposition of H3K4me3 and H3K9ac. This permissive signature acts as a platform allowing recruitment of gene specific TFs and the transcriptional machinery needed to drive transcription. **(B)** In memory cells, the permissive epigenetic signature at effector gene loci is maintained in the long term. The maintenance of permissive epigenetic signatures at key effector gene loci within memory T cells serves to keep RNA pol II "on-hold" at the TSS. Subsequent to TCR ligation, the RNA polymerase is released, allowing rapid gene expression and thus, rapid memory T cell effector function.

to impart different kinetics of cytokine versus cytolytic effector gene expression within reactivated memory T cells.

A more recent study has utilized high-throughput sequencing in combination with ChIP to map genome-wide H3K4me3 and H3K27me3 deposition within polyclonal naïve and memory human CD8$^+$ T cell populations (Araki et al., 2009). In general agreement with previous studies, a direct positive or negative correlation between gene expression and respective H3K4me3 or H3K27me3 deposition was observed (Araki et al., 2009). Interestingly, this genome-wide analysis also identified other correlates of gene transcriptional activity. For example, some loci within memory T cell populations exhibited H3K4me3 deposition, but only active transcription upon anti-CD3 stimulation (termed poised loci), a pattern not dissimilar to poised effector loci identified in previous analyses of virus-specific memory CTL (Denton et al., 2011; Zediak et al., 2011). Thus, epigenetic re-programing is a likely mechanism that underpins both the acquisition of lineage-specific T cell effector function and the rapid responsiveness that exhibited by memory T cells. The Araki study also suggests that different epigenetic signatures may be key in regulating different types of transcriptional responses during T cell differentiation. This is difficult to fully ascertain due to the polyclonal nature of the T cell populations analyzed. It will be of interest to examine T cell populations that have a linked differentiation history (i.e., are known to be responding to the same differentiation signals). Moreover, the T cell subsets examined in the Araki study were static populations and thus, there is no insight into the dynamics of epigenetic re-programing upon T cell activation. For example, do such changes occur quickly upon activation and do they all require cellular division?

SIGNALS THE DRIVE THE DYNAMIC CHANGES IN EPIGENETIC SIGNATURES WITHIN ACTIVATED T CELLS

Interestingly, there are few studies that have identified the specific signals that shape the epigenetic re-programing of T cell differentiation in response to activation. It is appreciated that the provision of both co-stimulatory and cytokine signals at the time of CTL activation promotes up-regulation of specific transcriptional programs associated with full maturation of effector and memory CD8$^+$ T cell responses (Agarwal et al., 2009). In particular, the combination of IL-12 and IFN-α signaling promotes up-regulation of effector genes such as gzmB, IFN-γ, and the TFs, T-bet, and Eomes (Agarwal et al., 2009). Importantly, up-regulation of signature CD8$^+$ T cell effector genes in response to these third signals is associated with chromatin remodeling and an increase in histone acetylation within these effector and TF gene loci (Agarwal et al., 2009). Thus, IL-12 and type I IFN signals induced the appropriate chromatin remodeling events required to promote increased transcriptional activity at those gene loci key for both effector and memory T cell differentiation.

As mentioned earlier, provision of CD4$^+$ T cell help is essential for the establishment and maintenance of CD8$^+$ T cell memory. Given the dynamic epigenetic remodeling that occurs with memory T cell differentiation, CD4 help likely plays a key role in establishing the appropriate permissive epigenetic signatures within effector gene loci of memory T cells. This is supported by the observation that memory virus-specific CD8$^+$ T cells, generated in the absence of CD4$^+$ T cell help, had diminished histone acetylation at the *ifnG* gene, with this correlating with decreased IFN-γ expression (Northrop et al., 2006, 2008). It will be of particular interest to determine the extent to which a lack of CD4$^+$ T cell help contributes to inappropriate epigenetic re-programing during virus-specific memory T cell differentiation. Does such help only result in remodeling of a limited number of gene loci, or are there broader consequences? We are currently utilizing ChIP-seq approaches to examine genome-wide changes in the epigenome between "helped" and "unhelped" memory T cells to help pinpoint both the precise mechanisms, and gene loci within virus-specific T cells that undergo CD4-dependent epigenetic re-programing upon activation.

Questions remain regarding what the precise enzymes and transcription factors are that come to together to rewrite the epigenetic signature during T cell differentiation. The answers to these questions will be important if such processes are ever to be targeted for the optimization (in the case of vaccine strategies for example) or the attenuation (in the case of autoimmune disease) of T cell immunity. One thing that is clear is that with the recent advances in systems biology approaches, our appreciation of just these molecular mechanisms fine tune our T cell immune responses can only grow and will provide new opportunities to think about how to best harness T cell immunity to fight infection, treat cancer and ameliorate autoimmune disease.

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Role of PI3K/Akt signaling in memory CD8 T cell differentiation

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The clonal expansion, differentiation into effectors and establishing an immunological memory are crucial components of the adaptive immune response. Following the initial encounter with a pathogen, clonal CD8 T cell expansion yields at least two distinct populations of effector cells, short-lived effector cells (SLECs) and memory precursor effector cells (MPECs). SLECs are the terminally differentiated cells, which play an active role in pathogen clearance and undergo apoptosis once the pathogen is eliminated. In contrast, MPECs persist and give rise to self-renewing memory cells. These memory CD8 T cells maintain a state of heightened alertness and are poised to rapidly respond and swiftly clear the pathogen upon antigen re-encounter. As one of the goals of vaccination is to induce the development of these memory CD8 T cells, understanding the cellular and molecular basis of memory cell differentiation is critical to rational vaccine design. It is clear that memory differentiation is complex and involves multiple interrelated signaling pathways. It is influenced by factors such as the strength and duration of antigen receptor signaling and concurrent exposure to cytokines. Several signaling pathways that influence T cell fate have been recently described, and many culminate in the differential expression of specific transcription factors. Unfortunately, the mechanisms underlying the coordination and confluence of these signaling pathways remain largely unknown. In this review, we will discuss the role of the phosphatidylinositol 3-kinase signaling pathway as a central signaling node, and the function of Akt as a rheostat in orchestrating the differentiation of memory CD8 T cells.

Keywords: memory T cell, PI3K/Akt, mTOR, FOXO, differentiation, metabolism

INTRODUCTION

CD8 T cells are highly specialized lymphocytes with a remarkable capacity to selectively target and kill tumor cells and cells infected with intracellular pathogens. As such, they play an important immunologic role in defending against tumors and infection by pathogenic organisms, particularly viruses and intracellular bacteria and protozoa. The development and maturation of antigen-specific CD8 T cells is a complex process involving numerous interrelated signaling pathways. This response has been most extensively characterized in animal models of acute viral infection, and this discussion is largely based on the findings from these models.

Antigen receptor engagement in the presence of appropriate co-stimulatory signals and exposure to cytokines such as type I interferons, activate CD8 T cells to undergo clonal expansion and differentiation into effector cells. At the peak of the T cell response, the expanded population of CD8 T cells is comprised of at least two distinct populations of effector cells, the short-lived effector cells (SLECs) and memory precursor effector cells (MPECs). SLECs are the terminally differentiated cytotoxic cells active in pathogen clearance and represent the majority of effector cells. CD8 T cell-mediated cytotoxicity depends upon recognition of specific viral antigens presented by class I major histocompatibility complex (MHC) molecules on the surfaces of infected cells. Antigen recognition induces the effector CD8 T cells to release molecules including perforin and granzymes, and cytokines such as interferon-gamma (IFNγ) and tumor necrosis factor-alpha (TNFα). Upon successful clearance of the pathogen, approximately 90% of effector cells including SLECs are eliminated by apoptosis.

The remaining 10% of effector cells represent the MPECs, which will differentiate into a self-renewing population of memory CD8 T cells. These memory CD8 T cells do not maintain a strong cytotoxic capacity, however, they persist for years in a state of heightened preparedness that enables them to rapidly proliferate and/or develop effector functions upon re-encounter of pathogens. This secondary response capacity of memory cells is significantly more rapid than the initial clonal expansion and leads to swift and expeditious control of the recurrent pathogen (Sallusto et al., 2010; Zhang and Bevan, 2011). Thus, the differentiation and maintenance of a functional memory CD8 T cell population provides effective, long lasting immunity.

The goal of vaccination is to prevent disease by pre-establishment of immunological memory similar to that induced by natural infection. Clearly, a detailed understanding of the cellular and molecular basis of memory cell differentiation is critical to rational vaccine design. Fortunately several important molecules involved in memory T cell differentiation have been identified and some have been well-characterized. However, the relationships between the individual molecules and the mechanisms by

which their signaling is coordinated to ultimately make cell-fate decisions have been incompletely described or remain unknown. In this review, we will focus on the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway and how it may integrate multiple extracellular cues and function as an immunologic rheostat that is able to spearhead a coordinated complex cellular response to govern this crucial differentiation of memory CD8 T cells.

PI3K/Akt SIGNALING

Phosphatidylinositol 3-kinase/Akt signaling pathways exist in all mammalian cells and exert profound effects on multiple diverse processes including cell proliferation, survival, differentiation, migration, and metabolism. The importance of PI3K and its position as a central node in cell signaling pathways has been further demonstrated by studies which show that aberrant regulation of PI3K/Akt signaling is pathologic and results in diseases such as cancer and autoimmunity (Oak and Fruman, 2007; Jiang et al., 2009).

Phosphatidylinositol 3-kinase are divided into classes I, II, and III, based on structural and functional differences. Class I PI3Ks are further classified into class IA PI3Ks (PI3Kα, PI3Kβ, and PI3Kδ) and class IB PI3K (PI3Kγ), and they are well-characterized, while the significance and role of the other PI3K classes remains largely undetermined (Vanhaesebroeck et al., 2010). The class I PI3Ks are heterodimeric enzymes comprised of a regulatory subunit (p85) and a catalytic subunit (p110). Class IA PI3K's place in the signaling chain is typically downstream of signals originating from receptor activation. Extracellular signals such as growth factors and cytokines bind to their receptors and stimulate receptor tyrosine kinases (RTKs). RTKs activate PI3K, which phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to generate phosphatidylinositol-3,4,5-trisphosphate (PIP3). PIP3 interacts with pleckstrin homology (PH) domain-containing target proteins such as Akt and phosphoinositide-dependent protein kinase (PDK1) on the inner leaflet of the plasma membrane.

Akt, also known as protein kinase B (PKB), has three isoforms – Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ. Akt1 is ubiquitously expressed in various tissues including lymphocytes, whereas Akt2 is abundantly expressed and controls insulin-mediated glucose metabolism in muscle and adipocytes. Akt3 expression appears to be restricted to brain and testes (Hers et al., 2011). The kinase domains of all three isoforms have strong homology within kinase domains to the members of the protein kinase A, G and C families (AGC) kinase family (Manning and Cantley, 2007). At the plasma membrane, the interaction between PH domain of Akt and PIP3 results in important conformational changes in Akt, which enable subsequent modifications of Akt by PDK1. To achieve full activation, Akt has to be phosphorylated at T308 and S473 by PDK1 and mammalian target of rapamycin (mTOR) complex 2 (mTORC2), respectively (Alessi et al., 1997; Sarbassov et al., 2005; **Figure 1**).

Regulation of PI3K/Akt signaling interaction can occur via multiple mechanisms. Phosphatases such as phosphatase and tensin homolog (PTEN) and SH2 domain containing inositol 5′-phosphatase (SHIP) work as negative regulators of PI3K signaling by dephosphorylating PIP3 (Sly et al., 2003). Deletion of these molecules results in the elevated activation of PI3K signaling (Aman et al., 1998; Stambolic et al., 1998). Moreover, Akt activity is down-regulated by dephosphorylation at T308 and S473 by protein phosphatase 2 (PP2) and by the PH domain and leucine rich repeat protein phosphatases (PHLPP), respectively (Andjelkovic et al., 1996; Gao et al., 2005).

When fully activated, Akt becomes a powerful signaling molecule, which translocates from the cell membrane to the cytosol and nucleus where it can alter a large number of important signaling pathways. Akt modulation of these pathways is accomplished by serine and/or threonine phosphorylation of the targeted signaling molecules. Several examples common to most cells illustrate the potential impact of Akt activation. Akt phosphorylation of two negative regulators, tuberous sclerosis complex 2 (TSC2) and proline rich Akt substrate of 40 kDa (PRAS40), leads to mTORC1 activation. mTORC1 activation in turn controls protein synthesis, cell growth and metabolism (Laplante and Sabatini, 2012). Glycogen synthase kinase 3 (GSK3) is another direct substrate of Akt; by inhibitory phosphorylation of GSK3, Akt increases cellular glycogen synthesis. In addition, nucleic GSK3 regulates cell survival by phosphorylating cyclins and the transcription factors c-jun and c-myc (Hers et al., 2011). Apart from modulating the activities of mTORC1 and GSK3, Akt also phosphorylates and inactivates forkhead box O (FOXO) transcription factors in the nucleus thereby dampening the expression of FOXO target genes involved in proliferation, apoptosis, motility, and metabolism (Li et al., 2007; Hedrick et al., 2012; **Figure 1**). The PI3K/Akt pathway can indirectly control cellular functions by interfacing with other signaling pathways such as the canonical Wnt/β-catenin pathway, the nuclear factor κB (NF-κB) pathway, and the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway (Okkenhaug and Vanhaesebroeck, 2003; Manning and Cantley, 2007; Delgoffe et al., 2011).

The preceding examples illustrate mechanisms by which PI3K/Akt signaling generally promotes cell growth and survival, while inhibition of PI3K/Akt signaling can growth and decrease cell survival. Although it is this association that has made the PI3K/Akt pathway an attractive target for anti-cancer therapies, and the proper regulation of many of these signaling pathways is also important for the generation of T cell responses. Because the PI3K/Akt signaling pathway is strategically positioned to influence so many aspects of the T cell response, the elucidation of its role in CD8 T cells is critical not only to understanding the immune response, but also to advancing rational vaccine design and development.

In T cells, the PI3Kδ and PI3Kγ isoforms are known to play critical roles during development. Deletion or inactivation of both isoforms during thymopoiesis results in a block at the CD4 CD8 double negative (DN) stage of T cell development. By contrast, PI3Kδ appears to be the isoform that is important for PI3K signaling in mature T cells (Okkenhaug and Vanhaesebroeck, 2003; Finlay, 2012). Although the biochemical mechanisms underlying the activation of PI3K in T cells are not fully elucidated (Chi, 2012), the PI3K/Akt signaling has important roles in the activation process as well as cytokine signaling in peripheral CD8 T cells (Kane and Weiss, 2003). In CD8 T cells, class IA PI3Ks are primarily activated by tyrosine kinase-associated receptors such as the T cell receptor (TCR), co-stimulatory and cytokine receptors. Signaling triggered by exposure to IL-12 and common gamma chain (γc)

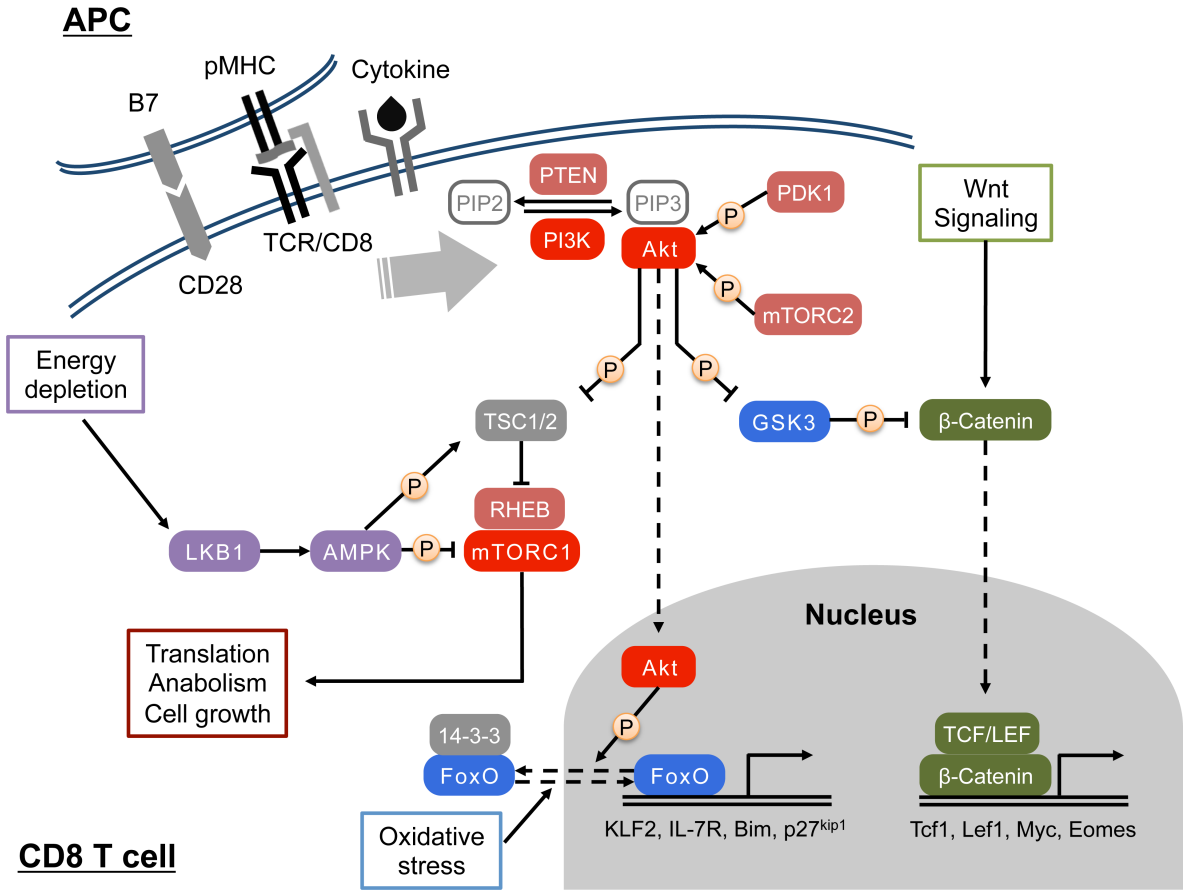

FIGURE 1 | Activation and function of PI3K/Akt signaling in CD8 T cell. Upon engagement of the TCR by peptide/MHC I complex, PI3 kinase (PI3K) is activated in CD8 T cells. Signals from costimulation, cytokines, and chemokines can also activate the PI3K. At the plasma membrane, activated PI3K phosphorylates PIP2 to generate PIP3, which recruit PH domain-containing proteins such as Akt and PDK1. Full activation of the Ser/Thr kinase Akt requires phosphorylations by PDK1 and mTORC2. In the cytosol, Akt phosphorylates and inhibits TSC1/2, a negative regulatory complex of mTORC1, which in turn promotes mTORC1-mediated protein synthesis and cell growth through modulating S6K and 4E-BP. Cytosolic Akt also inhibits GSK3, and regulates glucose metabolism and the canonical Wnt/β-catenin pathway. In addition, Akt translocates to the nucleus, and triggers the nuclear exclusion of FOXO transcription factors that are important for cell quiescence and apoptosis. AMPK senses cellular energy status by interacting with ADP/AMP and LKB1 and regulates cellular metabolism by antagonizing mTORC1-mediated glycolysis.

receptor-related cytokines such as IL-2, IL-7, IL-15, and IL-21 stimulates PI3K/Akt signaling pathway in CD8 T cells (**Figure 1**). Among γc cytokines, IL-2 generates high and sustained levels of PIP3, whereas stimulation of PI3K by IL-15 is relatively weak and results in low levels of PIP3 (Cornish et al., 2006). In contrast, class IB PI3K is activated by G protein coupled receptors (GPCRs) such as chemokine receptors. Additionally, alterations in the cellular microenvironment that can regulate PI3K/Akt signaling pathway include the abundance of growth factors and immunomodulatory factors, and metabolic cues primarily derived from nutrients. Upon activation, T cells augment their metabolism to meet the high-energy needs of cellular processes such as proliferation, cytokine synthesis and secretion and cell-mediated cytotoxicity. While it is accepted that PI3K promotes the uptake of glucose and amino acids and enhance protein synthesis in activated T cells, the role of Akt in T cell metabolism during expansion has been questioned by a recent study (Macintyre et al., 2011). This study suggested that PI3K might regulate T cell metabolism by Akt-independent mechanisms, however, alterations in CD8 T cell trafficking and development of effector functions all require Akt activity. In contrast to Ag- and co-stimulation-mediated PI3K/Akt activation that strongly prime the initial proliferation and differentiation program of CD8 T cells, cytokines such as IL-2, IL-12, IL-15, and IL-21 might shape further differentiation after activation since each cytokine signaling is likely to work at distinct differentiation states (Schluns and Lefrancois, 2003; Cox et al., 2011).

DIFFERENTIATION OF EFFECTOR AND MEMORY CD8 T CELLS

Understanding the differentiation of effector and memory CD8 T cells is an area of intense investigation in immunology. As discussed in the introduction, at the peak of clonal expansion there are two populations of effector T cells, the SLECs and MPECs. These two effector subsets can be identified by the differential expression of the cell surface molecules CD127 (the IL-7 receptor) and killer cell lectin-like receptor G1 (KLRG1) (senescence marker). The

characteristic SLECs phenotype is $CD127^{LO}KLRG1^{HI}$ while the MPEC phenotype is $CD127^{HI}KLRG1^{LO}$. The SLECs are destined for deletion upon resolution of the infection and are highly prone to apoptosis. Because they also have diminished proliferative potential they are considered to be in a terminally differentiated state (Joshi et al., 2007; Sarkar et al., 2008). In contrast, MPECs persist beyond the resolution of the infection and ultimately differentiate into long-lived, self-renewing CD8 memory T cells. Tremendous progress has been made in deciphering the mechanisms underlying the disparate fates of these cells: apoptosis versus differentiation into memory CD8 T cells.

Differentiation of MPECs and SLECs is regulated by multiple mechanisms including asymmetric cell division, exposure to cytokines such as IL-2 and IL-12 and the strength/duration of TCR signaling (Joshi et al., 2007; Sarkar et al., 2007; Jameson and Masopust, 2009; Kalia et al., 2010). It has been reported that exposure of CD8 T cells to IL-2 or IL-12 promotes terminal differentiation into SLECs at the expense of MPECs (Joshi et al., 2007; Kalia et al., 2010). Additionally, it was reported that the duration and intensity of antigenic stimulation is a key factor that controls the magnitude of CD8 T cell response and the differentiation of memory CD8 T cells (Sarkar et al., 2007; Teixeiro et al., 2009; Zehn et al., 2009). In recent years, seminal studies from several laboratories have identified key transcription factors that regulate disparate fate of SLECs and MPECs. Notably, high levels of T-bet, Blimp-1, ID2, and XBP-1 promote differentiation of SLECs. By contrast, high levels of Eomes, Bcl-6, ID3, Mbd2, and Bmi-1 favor differentiation of MPECs (Rutishauser and Kaech, 2010). According to the current paradigm, the relative levels of the opposing transcription factors (e.g., T-bet and Eomes) and/or their mutually antagonistic activities (e.g., Blimp-1 and Bcl-6) might control the differentiation of SLECs and MPECs (Finlay and Cantrell, 2011; Zhang and Bevan, 2011). From the signaling point of view, apart from antigen receptor signaling, IL-12 produced by dendritic cells increases T-bet expression, which promotes terminal differentiation of effector CD8 T cells (Joshi et al., 2007). Moreover, sustained IL-2 signaling favors the differentiation of SLECs in association with elevated expression of T-bet and Blimp1 (Kalia et al., 2010). Eomes is required for sustaining CD8 T cell effector function, but promotes memory differentiation by antagonizing the effects of T-bet and increasing the expression of IL-15R (Intlekofer et al., 2005; Zhou et al., 2010).

These findings are consistent with the hypothesis that it is the collective signaling of the TCR, the IL-2 receptor, and the IL-12 receptor that alters expression levels of the cell-fate-determining transcription factors, which in turn govern the differentiation of memory CD8 T cells. It is important to note, however, that the complex circuitry underlying this fateful pathway remains poorly defined, even though its characterization appears to be fundamental to our understanding of CD8 T cell differentiation. It is clear that this circuitry must facilitate the integration of signals emanating from diverse receptors and signaling pathways. The TCR, IL-2 receptor and IL-12 receptor signaling have all been demonstrated to stimulate the PI3K/Akt signal transduction pathway. Therefore, PI3K/Akt is a logical target for investigation into the complex circuitry underlying CD8 T cell differentiation. Nevertheless, a strong case can be made that the cumulative strength of Akt activation in effector cells, controlled by signaling emanating from multiple receptors including TCR, IL-2 receptor and IL-12 receptors control the balance between terminal differentiation and generation of CD8 T cell memory.

ROLE OF PI3K/Akt SIGNALING PATHWAY IN CD8 T CELL DIFFERENTIATION

Akt appears to be situated in a position to coordinate the convergence of the CD8 T cell-fate-determining pathways, and it has been clearly demonstrated to regulate diverse cellular processes impacting CD8 T cell fate. This has generated considerable interest in investigating its roles as well as those of its downstream effectors, mTOR, FOXOs, and GSK3 in CD8 T cell homeostasis (Araki et al., 2009; Kerdiles et al., 2009; Ouyang et al., 2009; Rao et al., 2010, 2012; Sullivan et al., 2012). Macintyre et al. (2011) examined the role of Akt in controlling the metabolism and development of effector functions of CD8 T cells *in vitro*. These studies provided important insights into how the strength and duration of Akt activation might regulate the trafficking and differentiation of effector CD8 T cells by controlling the cellular transcriptome. First, they demonstrated that high levels of Akt activation down-regulate the expression of adhesion molecules, CD62L, CCR7, and sphingosine-1-phosphate receptor (SIP), thereby redirecting the trafficking of effector CD8 T cells away from the secondary lymphoid tissues into the sites of inflammation. Conversely, low levels of Akt activation did not down-regulate the expression of these adhesion molecules and CD8 T cells continued to traffic into the lymph nodes, and express a transcriptome that resembles the one present in memory CD8 T cells. Second, it was demonstrated that proliferation can occur in the apparent absence of Akt, but Akt activation appears to be essential for development effector functions in activated CD8 T cells (Macintyre et al., 2011). Kim et al. (2012) also showed that terminal differentiation of CD8 T cells induced by sustained exposure to IL-2 was associated with higher Akt activation *in vivo*. They demonstrated that sustained Akt activation *in vivo* invoked a transcriptional program that favored terminal differentiation of CD8 T cells at the expense of CD8 T cell memory, consequent to excessive activation of mTOR, loss of FOXO activity and down-regulation of the Wnt/β-catenin pathway (Kim et al., 2012). It is unclear how constitutive Akt activation leads to down-regulation of Wnt pathway effectors Tcf1, Lef1, and Myc *in vivo*. Additionally, the effects of sustained Akt activation on the metabolic state of effector CD8 T cells warrant further investigation.

Exposure to cytokines such as IL-7 and IL-15 also stimulate the PI3K/Akt signaling pathway (Barata et al., 2004; Hand et al., 2010). Therefore, an interesting topic of discussion is the role of homeostatic cytokines such as IL-7 and IL-15 on the differentiation of CD8 T cells. One possible explanation is that the magnitude of PI3K/Akt signaling triggered by TCR signaling is much higher compared to stimulation with IL-7 and IL-15. Additionally, signaling triggered by IL-7 or IL-15 might activate the PI3K/Akt signaling, but the downstream activation of mTORC1 might be limited. Second, the phosphorylation sites on Akt will likely differ depending upon the nature of the stimuli, and therefore leads to drastically different outcomes. Third, the spectrum of signaling pathways triggered by antigen versus

IL-7/IL-15 are likely to be different and the interplay between various pathways might dictate the cellular response. It is also worth noting that IL-7R is rapidly down-regulated by TCR ligation, and gets selectively re-expressed in memory precursors (Kaech et al., 2003). Although IL-15Rβ (CD122) expression is enhanced by activation, IL-15 signaling may not be strong early in the response because this subunit also functions as a co-receptor for IL-2 (Kalia et al., 2010). Further, exogenous administration of IL-7 or IL-15 fails to elicit dramatic effects pertaining to formation of memory CD8 T cells (Melchionda et al., 2005; Nanjappa et al., 2008). However, *in vitro* exposure of naïve or memory human CD8 T cells to IL-15 can induce proliferation and effector functions, in the absence of TCR signaling (Liu et al., 2002; Alves et al., 2003). It is worth emphasizing that these studies were performed *in vitro*, where naïve/memory T cells were exposed to presumably high and non-physiological concentrations of IL-15 (≥10 ng/ml). Although these studies clearly show that IL-15 at concentrations of ≥10 ng/ml can exert effects comparable to that of TCR signaling, it is unknown whether T cells are exposed to such concentrations *in vivo*, due to the limited availability of these cytokines. However, it should also be noted that specialized T cells do display immediate effector functions at mucosal sites such as the intestines, where IL-15 is available at higher concentrations (Fehniger and Caligiuri, 2001). Therefore, we hypothesize that signaling triggered by IL-15 in naïve or memory CD8 T cells can mimic the effects similar to those exerted by TCR signaling depending upon the concentrations of IL-15 in the immunological milieu. And we further propose that low levels of IL-7 or IL-15 may not exert pronounced effects on the differentiation program of CD8 T cells. Rather, they may promote the survival and proliferation of memory precursors.

REGULATION OF CD8 T CELL MEMORY BY mTOR

One of the important downstream effectors for the PI3K/Akt signaling is mTOR, a serine–threonine kinase that has substantial sequence homology with the members of the PI3K family. Traditionally, mTOR is known as a nutrient sensor that regulates cell growth and protein synthesis, and is selectively inhibited by the immunosuppressive drug, rapamycin. Cellular mTOR is present as two distinct complexes: mTORC1 and mTORC2. The mTORC1 complex is composed of the proteins mTOR, Raptor, mLST8, PRAS40, and Deptor, and promotes protein translation through 4E-BP and S6K. The mTORC2 complex is composed of mTOR, Rictor, mLST8, and mSIN1, and mTORC2 is less sensitive to rapamycin than mTORC1 (Sarbassov et al., 2005). A heterodimeric complex consisting of TSC1 and TSC2 has been identified as a negative regulator of mTORC1 activity in T cells. TSC1/TSC2 complex maintains quiescence of naïve T cells by regulating cell size, cell cycle entry, and cell survival (Yang et al., 2011). Initiation of the PI3K/Akt signaling pathway inactivates TSC1/TSC2 and stimulates the small Ras-related GTPase Rheb, which in turn directly triggers mTORC1 activity (Laplante and Sabatini, 2009, 2012).

There is evidence that mTORC1 might limit the differentiation of memory CD8 T cells. Studies by Araki et al. (2009) showed that mTORC1 negatively regulates the differentiation of MPECs and their subsequent differentiation into memory CD8 T cells. Following an acute lymphocytic choriomeningitis virus (LCMV) infection, treatment with rapamycin during the expansion phase promoted MPEC formation and consequently, enhanced the number of memory CD8 T cells. Alternatively, when rapamycin treatment was restricted to the contraction phase, the phase of effector to memory transition was accelerated and the differentiation of central memory CD8 T cells was substantially increased. This effect appears to be CD8 T cell intrinsic since silencing Raptor expression in CD8 T cells largely recapitulated the effects of rapamycin treatment on memory formation. Rao et al. (2010) also reported that inhibition of mTORC1 activity by rapamycin *in vitro* enhanced the development of MPECs. Furthermore, terminal differentiation of effector cells induced by sustained Akt activation is at least in part due to hyper-activation of mTOR (Kim et al., 2012). In summary, mTORC1 activity promotes terminal differentiation of effector cells at the expense of memory precursors but the underlying mechanism remains to be determined. It is proposed that mTOR might promote terminal differentiation of effector cells by increasing the T-bet:Eomes ratio because, mTORC1 activation promotes the expression of the transcription factor T-bet and also suppresses the expression of Eomes (Rao et al., 2010; Li et al., 2011). How T-bet drives terminal differentiation of effector CD8 T cells and how mTOR modulates expression of T-bet and Eomes remain to be determined. As compared to mTORC1, relatively little is known about the role of mTORC2. mTORC2 regulates Akt activation by phosphorylation at S473 (Sarbassov et al., 2005) and enhances cell survival without activating mTORC1 (Chen et al., 2010). Whether mTORC2 has significant roles in orchestrating memory CD8 T cell differentiation awaits further investigation. Notably, mTOR is well known as an integrative metabolic sensor that is also regulated by 5′ AMP-activated protein kinase (AMPK; Powell and Delgoffe, 2010). The role of mTOR in T cell metabolism will be discussed later.

REGULATION OF CD8 T CELL MEMORY BY FOXOs

Members of the FOXO family transcription factors are direct substrates of Akt. There are four FOXO members namely FOXO1, FOXO3, FOXO4, and FOXO6. While FOXO1, FOXO3, and FOXO4 are widely expressed, the expression of FOXO6 is restricted to the nervous system (Hedrick et al., 2012). Because FOXOs oppose cell cycle entry and promote apoptosis, they are considered as tumor suppressors (Paik et al., 2007). Additionally, FOXOs might promote organismal longevity by detoxifying reactive oxygen species and supporting DNA repair (Salih and Brunet, 2008). Peripheral T cells express FOXO1 and FOXO3, and it is becoming increasingly clear that these proteins play crucial roles in the maintenance of peripheral T cell homeostasis (Hedrick et al., 2012). In their active unphosphorylated form, FOXOs localize to the nucleus where they promote the expression of target genes that suppress cell cycle entry or promote apoptosis. Activated Akt phosphorylates FOXOs resulting in their nuclear exclusion and translocation to cytoplasm through interaction with the nuclear shuttle, 14-3-3 (Hedrick, 2009; Hedrick et al., 2012). However, exposure of cells to oxidative stress or nutrient deprivation can induce nuclear retention of FOXOs, thereby promoting the transcription of FOXO target genes. In

addition to Akt, AMPK, c-jun N-terminal kinase (JNK), and MST1 are known to cause posttranslational modification of FOXOs (Ouyang and Li, 2011).

The role of FOXO1 and FOXO3 in regulating T cell homeostasis has been examined by ablating FOXO1 and/or FOXO3 in mice. In one study, global loss of FOXO3 led to lymphoproliferative disease and multi-organ inflammation, however, further studies have failed to reproduce these results (Lin et al., 2004; Dejean et al., 2009). Studies of LCMV infection in global and T cell-specific conditional FOXO3 null mice showed that FOXO3 might constrain T cell responses by both T cell-intrinsic and extrinsic mechanisms (Dejean et al., 2009; Sullivan et al., 2012). In studies by Dejean et al. (2009) increased accumulation of CD8 T cells in FOXO3 null mice during an acute LCMV infection was linked to overproduction of IL-6 from FOXO3-deficient dendritic cells. However, studies by Sullivan et al. (2012) suggested that FOXO3 might also limit the accumulation of LCMV-specific CD8 T cells by T cell-intrinsic mechanisms that include BIM-dependent apoptosis. By virtue of increased accumulation of CD8 T cells during the primary response, FOXO3 deficiency augmented the magnitude of CD8 T cell memory without affecting their phenotype or function (Sullivan et al., 2012).

While the functions of FOXO3 in T cells are largely consistent with its growth inhibitory properties in other cells, the role of FOXO1 in mature T cells is quite unique. FOXO1 controls multiple facets of T cells including trafficking, tolerance, and survival. First, unlike FOXO3, which promotes apoptosis of T cells (Sullivan et al., 2012), FOXO1 supports the survival of T cells by inducing the expression of the IL-7Rα chain, which promotes IL-7-induced Bcl-2 expression. Additionally, FOXO1 controls T cell trafficking by promoting the expression of the transcription factor KLF2, which in turn induces the transcription of molecules involved in trafficking, CD62L, CCR7, and S1P1 (Kerdiles et al., 2009; Ouyang et al., 2009). Unlike the seemingly opposing effects on T cell survival, FOXO1 and FOXO3 co-operatively protect against autoimmunity. Loss of FOXO1 and FOXO3 in T cells results in uncontrolled T cell activation and autoimmunity, which is at least in part linked to defects in the generation of regulatory T cells (Ouyang and Li, 2011). In addition, disruption of T cell homeostasis in the absence of FOXOs could result from dysregulated expression of $p15^{Ink4b}$, $p21^{Cip1}$, and $p27^{Kip1}$ by itself and/or in association with TGF-β/Smad signaling pathway (Ouyang et al., 2010; Hedrick et al., 2012). More recently, *in vitro* studies of T cells by Rao et al. (2012) showed that FOXO1 might directly induce Eomes expression, indirectly repress T-bet expression, and promote memory CD8 T cell differentiation. Rao et al. (2012) also reported that *in vitro*-activated FOXO1-deficient CD8 T cells have diminished ability to survive after adoptive transfer into syngeneic mice. However, neither do we know how FOXO1 regulates T-bet expression nor it is clear how FOXO1 might support survival of memory CD8 T cells. It is worth investigating whether loss of IL-7R expression, consequent to loss of FOXO1 leads to demise of FOXO1-deficient memory CD8 T cells. Interestingly the cyclin-dependent kinase inhibitor $p27^{Kip1}$, a major target gene for FOXOs curtails the primary expansion of CD8 T cells and limits the number of highly functional memory CD8 T cells during an acute LCMV infection (Singh et al., 2010). This phenotype has not been recapitulated either in FOXO3 or FOXO1 null mice (unpublished observations; Tejera and Suresh).

CONTROL OF T CELL METABOLISM BY PI3K/Akt SIGNALING

During the phase of antigen-driven clonal expansion, CD8 T cells proliferate intensively with an estimated doubling time of 4–6 h (Murali-Krishna et al., 1998; Badovinac et al., 2007). In order to support such rapid proliferation and effector functions including cell-mediated cytotoxicity and cytokine production, activated CD8 T cells increase uptake of glucose, amino acids, and iron (Fox et al., 2005), and switch glucose metabolism from fatty acid oxidation (catabolism) to aerobic glycolysis and glutaminolysis (anabolism) by mechanisms orchestrated by transcription factor c-myc consequent to Akt/Erk1/2 activation (Wang et al., 2011). PDK1 but not Akt appears to be required for metabolic programming of activated CD8 T cells to aerobic glycolysis. While aerobic glycolysis may be required for clonal expansion and effector functions, effector CD8 T cells do switch back to catabolism during effector to memory transition (Prlic and Bevan, 2009). The metabolic switch to catabolism might be a necessary event for generation of CD8 T cell memory because defects in the fatty-acid oxidation pathway induced by TRAF6 deficiency can dramatically decrease memory T cell generation (Pearce et al., 2009). Interestingly, TRAF6-deficient CD8 T cells exhibit hyper-activation of PI3K/Akt signaling, which suggests a role for this signaling pathway in regulating fatty acid metabolism and generation of CD8 T cell memory (King et al., 2006). Pharmacological augmentation of AMPK activation (by metformin treatment) and suppression of mTORC1 (by rapamycin treatment) improve memory formation from TRAF6-deficient CD8 T cells (Pearce et al., 2009). This study confirmed another report, which showed that rapamycin treatment during contraction phase accelerated the differentiation of central memory cells, implicating PI3K/Akt/mTOR pathway in controlling CD8 T cell metabolism and differentiation of memory CD8 T cells (Araki et al., 2009). In a recent report, van der Windt et al. (2012) showed that IL-15 promotes the generation of memory CD8 T cells by supporting fatty acid oxidation and enhancing the mitochondrial respiratory capacity of CD8 T cells. While collective evidence support the idea that PI3K/Akt signaling pathway might regulate cellular metabolism and differentiation of memory CD8 T cells, further studies are clearly needed to fully decipher the underlying mechanisms.

CROSS TALK BETWEEN PI3K/Akt AND OTHER SIGNALING PATHWAYS

Wnt/β-CATENIN SIGNALING PATHWAY

Accumulating data supports the Wnt/β-catenin signaling pathway might be important for generation and maintenance of CD8 T cell memory. The expression of the Wnt target genes is dynamically regulated during a T cell response. Expression of *tcf7* (encodes Tcf1), *lef1*, and *myc* is highest in naïve and central memory CD8 T cells, but substantially down-regulated in SLECs (Kim et al., 2012; Xue and Zhao, 2012). Thus, terminal differentiation into SLECs is associated with the loss of Wnt target gene expression and high-level expression of these genes correlates with survival or quiescence (Driessens et al., 2011). Studies that involved constitutive expression of β-catenin or loss of function

mutants indicated that clonal expansion of CD8 T cells might require down-regulation of Wnt/β-catenin signaling but survival and maintenance of memory CD8 T cells are Wnt/β-catenin-dependent, especially Tcf1 (Jeannet et al., 2010; Zhao et al., 2010; Zhou et al., 2010). Mechanistically, Tcf1 could support CD8 T cell memory formation by directly inducing the expression of transcription factor Eomes, which is critical for sustained expression of the IL-2 receptor β chain (CD122; Zhou et al., 2010). Whether continued action of Tcf1 is required for maintenance of memory CD8 T cells remains unknown. Studies from Restifo's group suggested that augmented Wnt signaling consequent to GSK3β inhibition reduced terminal differentiation of effector cells and promoted development of memory CD8 T cells with stem cell-like properties (Gattinoni et al., 2009b). It is GSK3β that provides a conduit for crosstalk between Wnt signaling and the PI3K signaling pathway. GSK3β is one of the central regulators of canonical Wnt signaling pathway and it is a direct substrate for Akt. Akt phosphorylates and inactivates GSK3β resulting in stabilization and nuclear localization of cytosolic β-catenin. Surprisingly, however, instead of potent activation, constitutively active Akt resulted in the strong inhibition of the downstream effectors of the canonical Wnt signaling in effector CD8 T cells (Kim et al., 2012). The mechanisms underlying the suppression of Wnt/β-catenin signaling by constitutively active Akt in CD8 T cells are yet to be determined, but it was recently reported that FOXO1 binds to the intergenic region of Tcf1 gene and induces its expression in regulatory T cells, with the implication that the loss of FOXO activity might impair the expression of Tcf1 in CD8 T cells (Ouyang et al., 2012).

NF-κB SIGNALING PATHWAY

The nuclear factor κB signaling pathway regulates immune cell survival and various facets of innate and adaptive immunity (Vallabhapurapu and Karin, 2009). In an un-stimulated state, NF-κB family of transcription factors remain in the cytosol as homo- or hetero-dimers in complexes with the inhibitor of κB (IκB) proteins. Upon exposure to ligands for toll-like receptors (TLRs) or cytokines such as TNF and type I IFNs, IκB is phosphorylated and degraded by IκB kinases (IKKs). Consequent to the degradation of IκB, NF-κB re-localizes to the nucleus and alters transcriptional activity (Vallabhapurapu and Karin, 2009). There is precedent for regulation of CD8 T cell memory by the NF-κB signaling pathway. Constrained NF-κB signaling not only diminished clonal expansion of CD8 T cells, but also resulted in defective CD8 T cell memory (Hettmann et al., 2003). Further, defective NF-κB signaling triggered by a mutant TCR lead to a substantive reduction in the formation of memory CD8 T cells (Teixeiro et al., 2009). It is unclear how NF-κB signaling regulates the generation of CD8 T cell memory. Although debatable, there is evidence that PI3K/Akt signaling might interact with the NF-κB pathway at multiple levels (Salminen and Kaarniranta, 2010). For example, Akt potentiates transactivation activity of NF-κB through IKKβ and p38 mitogen-activated protein kinase (MAPK). And, PDK1, a downstream kinase of PI3K, directly phosphorylates IKKβ and activates NF-κB signaling. More recently, it was reported that suppression of Akt during T cell activation reduced NF-κB binding to its target gene promoters and diminished the expression of TNF and IL-6 (Cheng et al., 2011). Therefore, it is possible that PI3K/Akt signaling further tunes the differentiation of CD8 T cells through NF-κB pathway.

JAK/STAT SIGNALING PATHWAY

The Janus kinase/signal transducers and activators of transcription signaling pathway is the principal signaling mechanism conveying biochemical signals from many growth factors and cytokines (Schindler et al., 2007; O'Shea and Plenge, 2012). Stimulation of this pathway induces dimerization and translocation of STAT to the nucleus. In the nucleus, STAT functions as a trans-activator of numerous target genes involved in cell proliferation, differentiation, survival, and migration (Schindler et al., 2007; O'Shea and Plenge, 2012). There is evidence that cytokines such as IL-7 and IL-15 trigger the JAK/STAT signaling pathway and modulate the differentiation and homeostasis of memory CD8 T cells (Schluns and Lefrancois, 2003; Hand et al., 2010; Tripathi et al., 2010). There is scant direct evidence that PI3K/Akt signaling can influence JAK/STAT signaling, but a recent study has suggested that either mTORC1 or mTORC2 could differentially affect JAK/STAT signaling through regulating the expression of suppressor of cytokine signaling (SOCS) in mouse primary T cells (Delgoffe et al., 2011). Significant to memory CD8 T cell differentiation, loss of memory CD8 T cells induced by constitutively active Akt in CD8 T cells was associated with impaired STAT5 signaling in response to cytokines such as IL-2, IL-7, and IL-15 possibly due to hyperactive Akt-mediated inhibition of IL-7R and IL-2R β chain expression. Conversely, constitutively active STAT5 enhanced the generation and/or survival of memory CD8 T cells (Hand et al., 2010). Thus, it is possible that a balance between STAT and Akt signaling could determine the survival of memory CD8 T cells.

THERAPEUTIC MODULATION OF THE PI3K/Akt PATHWAY TO ENHANCE CD8 T CELL MEMORY

It is becoming increasingly clear that vaccines against diseases caused by complex pathogens such as AIDS, tuberculosis, and malaria need to elicit potent humoral and cell-mediated immunity. CD8 T cell-dependent protective immunity depends upon the quantity, quality, and anatomical localization of memory CD8 T cells. Conventional approaches to enhance memory responses by vaccines include the use of different forms and/or doses of antigen, adjuvant, and boosting strategies (Sallusto et al., 2010). Despite decades of research, very few adjuvants are licensed for use in humans. In the US and Europe, only aluminum salts (alum), AS04 (aluminum hydroxide in combination with TLR 4 ligand monophosphoryl lipid A [MPL]), and oil-in-water emulsions (MF59, AS03, and AF03) have been approved for human use (Coffman et al., 2010; Nordly et al., 2011; Pulendran and Ahmed, 2011; Foged et al., 2012). But, none of these adjuvants are known to induce potent CD8 T cell memory. With an in-depth understanding of the signaling pathways that regulate CD8 T cell memory, it is conceivable that targeted immunotherapies could be developed to enhance the quantity and quality of CD8 T cell memory (Gattinoni et al., 2009a). Studies by the Ahmed and Pearce groups have already demonstrated the feasibility of utilizing pharmaceutical agents to augment CD8 T cell memory

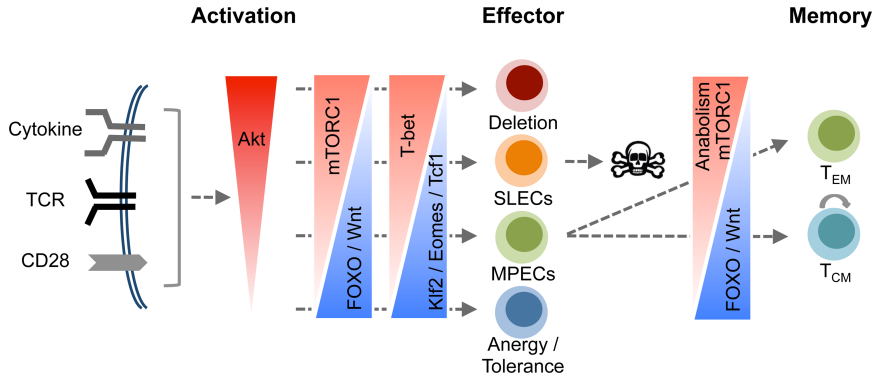

FIGURE 2 | A model for orchestration of CD8 T cell differentiation by the PI3K/Akt pathway. Signals resulting from engagement of cell surface receptors including TCR, co-stimulatory molecules, and cytokine receptors converge to activate Akt, and the magnitude of Akt activation is a function of the cumulative signal strength from these receptors. Increase in the magnitude of Akt activation progressively drives cytotoxic T lymphocytes (CTLs) toward terminal differentiation. We propose a model where balanced Akt activation fosters development of effector functions without impeding the differentiation of MPECs and their descendent memory CD8 T cells. However, activation of Akt above a certain threshold drives differentiation of CD8 T cells into terminal effectors at the expense of MPECs by paralyzing a multitude of cell survival mechanisms including incapacitation of FOXO and the Wnt/β-catenin pathways, and stimulation of the mTOR pathway. Thus, Akt functions as a cellular fulcrum controlling distinct facets of the program that governs differentiation of antigen-activated CD8 T cells into terminal effector cells or memory CD8 T cells.

in vivo (Araki et al., 2009; Pearce et al., 2009). In studies by Araki et al. (2009) inhibition of mTORC1 activity by rapamycin treatment during expansion phase or contraction phase significantly improved the generation of memory CD8 T cells in terms of quantity and/or quality. Likewise, Pearce et al. (2009) showed that treatment of mice with rapamycin and AMP kinase activator metformin enhanced the differentiation of memory CD8 T cells by metabolically reprogramming effector CD8 T cells. Because the amplitude of Akt activation correlated with terminal differentiation of effector CD8 T cells, Kim et al. explored the possibility of Akt blockade as a therapeutic strategy to enhance CD8 T cell memory. Treatment of mice with the pan-Akt inhibitor A-443654 during the expansion phase reduced mTOR activation and significantly enhanced the number of memory CD8 T cells. There have been considerable efforts to develop selective Akt inhibitors as treatment options for cancer. It is challenging to develop selective Akt inhibitors because, not only does Akt kinase has three isoforms, but these isoforms are highly homologous to AGC kinases (e.g., PKA, PKC, and S6K). However, Merck & Co., Inc. introduced MK2206, an allosteric inhibitor of Akt. MK2206 possesses low nanomolar potency against all three Akt isoforms, and has recently entered a Phase I clinical trial in patients with solid tumors. It would be interesting to assess whether MK2206 can augment CD8 T cell memory to vaccinations. The use of pharmaceutics to enhance CD8 T cell memory may be more enticing for the field of adoptive tumor immunotherapy. For example, tumor-infiltrating lymphocytes can be reprogrammed by pharmaceutics during *in vitro* expansion prior to adoptive transfer into patients (Restifo et al., 2012). Since transfer of central memory T cells provided superior anti-tumor effect compared to effector memory or effector T cells, (Gattinoni et al., 2005; Klebanoff et al., 2005), pharmacological modulation to promote the differentiation of central memory CD8 T cells during *in vitro* expansion would greatly improve the efficacy of immunotherapy.

CONCLUDING REMARKS

During an immune response, CD8 T cells are exposed to multiple extracellular signals, temporally and spatially, and the confluence of these signals not only determines the fate of antigen-activated CD8 T cells, they shape the quantity and quality of memory CD8 T cells. In this review, we have discussed how the PI3K signaling pathway might integrate multiple signals and control distinct facets of effector and memory differentiation by modulating specific downstream substrates of Akt (**Figure 2**). The emerging consensus from published work is that strong Akt signaling is required for effective development of effector functions and guiding the effector cells away from the secondary lymphoid organs. By contrast, less intense Akt signaling might favor the differentiation of memory CD8 T cells. This forms the basis for the signal strength model for effector and memory differentiation (**Figure 2**). However, this model leads to an unresolved question how and why only some activated CD8 T cells receive appropriate strength of signals and differentiate into memory cells? First, the duration and intensity of antigen receptor signaling depends on the: (1) nature and duration of infection; (2) expression of chemokine receptors (CXCR3 and CCR5) that regulate T cell/antigen-presenting cell (APC) interactions and the anatomical localization of the responding cells (Hu et al., 2011; Kohlmeier et al., 2011; Kurachi et al., 2011); (3) the stage of infection at which naïve T cells are recruited to the response (early responders versus latecomer cells; Badovinac et al., 2004; D'Souza and Hedrick, 2006). Second, the factors described above regulate the exposure of T cells to IL-2 and IL-12, which in turn promotes heterogeneity in the differentiation states. Third, diversification of effector CD8 T cells may be programmed at the first cell division, which occurs in an asymmetric manner. It is believed that the daughter cells that are in close proximity to the APC receives stronger TCR and co-stimulatory signals due to asymmetric receptor and cellular components and therefore differentiate into terminal effectors (Chang et al., 2007, 2011). Interestingly, the development of

effector functions is closely associated with terminal differentiation, and it is currently unclear how these two processes are linked. Perhaps, transcription factors like T-bet promotes effector functions and at the same time controls genes that drive terminal differentiation. The molecular mechanisms underlying the T-bet-driven terminal differentiation of effector CD8 T cells including the identification of target genes for T-bet warrants further investigation. By the same token, while mTORC1 is known to drive terminal differentiation of effector CD8 T cells, the underlying mechanisms are still elusive. Furthermore, the role of FOXO1 in regulating CD8 T cell memory is unclear. Elucidating the specific roles of key players of the PI3K/Akt signaling pathway might lead to the development of pharmaceutics that can modulate diverse aspects of CD8 T cell memory.

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Memory CD8 T cells specific for *Plasmodia* liver-stage antigens maintain protracted protection against malaria

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Immunologic memory induced by pathogenic agents or vaccinations is inextricably linked to long-lasting protection. Adequately maintained memory T and B cell pools assure a fast, effective, and specific response against re-infections. Studies of immune responses amongst residents of malaria endemic areas suggest that memory responses to *Plasmodia* antigens appear to be neither adequately developed nor maintained, because persons who survive episodes of childhood malaria remain vulnerable to persistent or intermittent malaria infections. By contrast, multiple exposures of humans and laboratory rodents to radiation-attenuated *Plasmodia* sporozoites (γ-spz) induces sterile and long-lasting protection against experimental sporozoite challenge. Protection is associated with MHC-class I-dependent CD8 T cells, the key effectors against pre-erythrocytic stage infection. We have adopted the *P. berghei* γ-spz mouse model to study memory CD8 T cells that are specific for antigens expressed by Pb liver-stage (LS) parasites and are found predominantly in the liver. On the basis of phenotypic and functional characteristics, we have demonstrated that liver CD8 T cells form two subsets: $CD44^{hi}CD62L^{lo}$KLRG-$1^{+}CD107^{+}CD127^{-}CD122^{lo}$CD8 T effector/effector memory ($T_{E/EM}$) cells that are the dominant IFN-γ producers and $CD44^{hi}CD62L^{hi}$KLRG-$1^{-}CD107^{-}CD127^{+}CD122^{hi}$CD8 T central memory ($T_{CM}$) cells. In this review, we discuss our observations concerning the role of CD8 $T_{E/EM}$ and CD8 T_{CM} cells in the maintenance of protracted protective immunity against experimental malaria infection. Finally, we present a hypothesis consistent with a model whereby intrahepatic CD8 T_{CM} cells, that are maintained in part by LS-Ag depot and by IL-15-mediated survival and homeostatic proliferation, form a reservoir of cells ready for conscription to CD8 $T_{E/EM}$ cells needed to prevent re-infections.

Keywords: memory T cells, malaria, *Plasmodium*, liver, IL-15, CD8 T cells, mouse model

INTRODUCTION

One of the cardinal features of Ag-specific immune responses elicited by infections or vaccinations is the persistence of optimally effective memory T cells that are inextricably linked to long-lasting protection (Ahmed and Gray, 1996). Adequately maintained memory T cells assure a fast, effective, and specific response against reoccurring infections. Studies of protective immunity amongst residents of malaria endemic areas indicate that protective immunity to *Plasmodia* antigens develops gradually after multiple exposures over many years and, although associated with a decline in clinical manifestations of the disease, it decays rapidly once exposure to the parasite ceases (Langhorne et al., 2008). However, it is not clear why protection does not persist after malaria infection. We hypothesized (Krzych et al., 2000) that the absence of adequately developed immunologic memory, which stems from the tolerant milieu of the liver (Crispe, 2011), sequestration of the liver-stage antigens (LS-Ags) within hepatocytes, and relatively short duration of the liver-phase infection, is mainly responsible for the lack of lasting protection. Others suggested that a phenomenon known as altered peptide ligand, resulting from polymorphisms at CD8 T cell sites, induces antagonistic effects that interfere with the priming and the survival of memory T cells (Plebanski et al., 1999). Poor immunogenicity may also stem from inadequate immunizing doses or immunologic interferences from blood-stage parasites (Good et al., 2005; Urban et al., 2005). Interestingly, currently conducted studies with the RTS,S vaccine, which is based on *Plasmodium falciparum* circumsporozoite protein (CSP), indicate that protection is conferred to infants and small children but it lasts for a relatively short period of time (Abdulla et al., 2008).

In contrast, exposure of laboratory rodents (Nussenzweig et al., 1967), monkeys (Nussenzweig et al., 1970), and humans (Clyde et al., 1973) to radiation-attenuated (γ) *Plasmodia* sporozoites (γ-spz) induces sterile and long-lasting protection. *Plasmodia* γ-spz-induced protection is multifactorial (Nardin and Nussenzweig, 1993), involving antibody (Egan et al., 1993), CD4 (Nardin et al., 1989), and CD8 T cell (Wizel et al., 1995) responses directed primarily to CSP. However, blood-stage antigens also recall IL-4 producing memory CD4 T cells in protected subjects (Palmer and Krzych, 2002) and LS antigen-1- (LSA-1) specific proliferative T cell responses correlate with protection (Krzych et al., 1995).

CD8 T cells have been considered key effectors against pre-erythrocytic stage infection. Evidence supporting the effector function of CD8 T cells is based on studies in human (Malik

et al., 1991) and animal (Schofield et al., 1987; Weiss et al., 1988; Berenzon et al., 2003) models of γ-spz-induced protection, protection induced by genetically attenuated parasites (GAP) (Jobe et al., 2007; Mueller et al., 2007; Tarun et al., 2007; Trimnell et al., 2009) as well as on more recent observations made in models of protection induced by wild type *Plasmodia* sporozoites administered under drug coverage (Nganou-Makamdop et al., 2012). Studies conducted in malaria endemic areas confirm the involvement of effector CD8 T cells in protection (Bejon et al., 2007). The effector function is associated mainly with the production of inflammatory cytokines such as IFN-γ or TNF-α that mediate elimination of the parasite within the hepatocytes by the nitric oxide (NO) pathway (Seguin et al., 1994). CD8 T cells also exhibit cytolytic activity against targets that express antigens belonging to pre-erythrocytic stage parasites (Hill et al., 1991; Malik et al., 1991; Trimnell et al., 2009).

Our lab has adopted the *P. berghei* γ-spz (Pb γ-spz) mouse model to study memory CD8 T cells against experimental malaria infection. CD8 T cells that arise in Pb γ-spz-immunized B6 mice are specific for antigens expressed by the developmentally aborted LS parasites (Berenzon et al., 2003) and antigen experienced $CD44^{hi}$CD8 T cells are found predominantly in the liver (Guebre-Xabier et al., 1999). In this review, we mainly focus on memory CD8 T cells in the maintenance of protracted protection in the Pb γ-spz model. We also comment on studies in other model systems of sterile protection that extend our hypothesis by demonstrating the necessity for late LS-Ags and the induction of effector and memory CD8 T cells for lasting protection (Butler et al., 2011; Teirlinck et al., 2011). We hypothesize that long-term protection to pre-erythrocytic stage infection can be induced and maintained. As such, it requires the formation and persistence of a central memory (T_{CM}) CD8 T cell reservoir, which is maintained in part by LS-Ag depot and by IL-15 and which gives rise to IFN-γ producing effector/effector memory ($T_{E/EM}$) CD8 T cells during re-infections.

PLASMODIA PARASITES IN THE MAMMALIAN HOST; THE IMPORTANCE OF THE LIVER-STAGE

Plasmodia sporozoites are inoculated into a mammalian host from the salivary glands of an Anopheles mosquito during its blood meal. Sporozoites then quickly travel to the liver via the circulatory and/or lymphatic systems. In the liver, sporozoites undergo further development before they emerge as blood-stage merozoites that infect the red blood cells. Hence, in the mammalian host the *Plasmodium* parasite exhibits three morphologically distinct phases of development: sporozoite-, liver-, and blood-stage. During each stage, the parasite expresses, to some extent, unique protein profiles. The sporozoite-associated proteins facilite parasite invasion of hepatocytes (Rogers et al., 1992; Frevert, 1994; Robson et al., 1995; Fidock et al., 1997) and, under certain conditions, are potent antigens for the induction of cellular and antibody responses. For example, CSP-specific responses correlate with protection in humans (Hoffman et al., 2002) and mice (Schofield et al., 1987). LS-Ags are expressed by the developing parasite within the parasitophorous vacuole in hepatocytes (Mueller et al., 2005; Prudencio et al., 2006) and they are considered to be the major inducers of protective cellular immune responses against the pre-erythrocytic stage parasite (Fidock et al., 1994; Bucci et al., 2000). Proteins that characterize the erythrocytic stage are thought to play a role in the invasion of red blood cells (Holder, 1996).

The mammalian liver plays a key role in the life cycle of the *Plasmodium* parasite as the LS is not only pivotal for survival of the parasite, but it also represents a significant period for the induction, effector phase, and the maintenance of immune memory responses. Understanding immune events that occur in the liver in model systems of protective immunity, as well as during natural infection, will expand our knowledge of organ-specific immune responses to *Plasmodia* antigens and hence facilitate exploitation of these responses to expedite progress in vaccine development against this serious disease.

A MODEL OF PROTRACTED PROTECTION INDUCED BY *PLASMODIA* γ-SPZ

Plasmodia γ-spz-induced sterile and protracted protection is considered the gold standard of anti-malaria vaccines. Like infectious sporozoites, γ-spz carrying CSP and other sporozoite-associated proteins, invade the liver where they undergo aborted development and express LS-Ags (Zechini et al., 1999; Hollingdale and Krzych, 2002). It is believed that antigens expressed by the underdeveloped liver schizonts remain in the liver forming a LS-Ag depot (Scheller and Azad, 1995), which is critical for induction and persistence of Ag-specific protracted protective immunity (Krzych et al., 2000). Treatment of animals with primaquine, a drug which disrupts LS development, concurrently with the γ-spz-immunizations abolishes lasting protection (Scheller and Azad, 1995; Berenzon et al., 2003).

The prevailing state of tolerance in the liver allows for infectious sporozoites to expand and continue their life cycle. However, immunization with γ-spz reverses this tolerance to inflammation, which is needed for the induction as well as persistence of adaptive immune responses (Krzych, 1999). Before the invasion of hepatocytes, Pb γ-spz, like infectious sporozoites, pass through Kupffer cells (KC) (Pradel and Frevert, 2001) changing them to become high IL-12 producers (Steers et al., 2005). In contrast, infectious sporozoites do not activate naïve KC to produce IL-12, and instead downregulate IL-10 (Steers et al., 2005). The importance of IL-12 was demonstrated as an inducer of IFN-γ and iNOS (Sedegah et al., 1994; Seguin et al., 1994) and as a critical cytokine for the development of CD8 T cell responses to pre-erythrocytic stage malaria (Doolan and Hoffman, 1999).

The molecular form of the sporozoites might influence the mode of sporozoite entry into KC, which, in turn, might dictate intracellular localization of sporozoites, as has been recently shown for DC interacting with other parasites (Cervi et al., 2004). On the basis of *in vitro* conducted studies (Pradel and Frevert, 2001; Silvie et al., 2004), the entry of infectious sporozoites is mediated by membrane:membrane fusion and parasites localize in a vacuole that does not co-localize with lysosomes so that sporozoites avoid metabolic degradation before reaching hepatocytes. Conceivably, γ-spz could be internalized by phagocytosis and channeled to phagosomes for metabolic degradation and export by MHC-class II and I molecules. A significant upregulation of MHC-class I is evident on KC after sporozoite challenge of γ-spz-immune mice. In sharp contrast, MHC-class I molecules are downregulated

on KC during infection of naïve mice and their APC function is severely reduced (Steers et al., 2005). Inflammatory cytokines increase the expression of MHC-class I-peptide complexes on APC by inducing immune proteosomes for more efficient generation of antigenic peptides for entry into the ER and loading onto empty MHC-class I molecules (Khan et al., 2001). Accordingly, KC from γ-spz-immune/challenged mice present peptides and protein antigens to specific T cells. We propose that a cascade of pro-inflammatory cytokines released during the innate immune response induced by γ-spz leads to temporary local inflammation, which is perceived as a "danger signal" needed to trigger proper responses from the adaptive immune system and lead to long-lasting immune memory (Matzinger, 1994).

CD8 T CELLS MEDIATE PROTECTIVE IMMUNITY

Work based on *in vivo* depletion of CD8 T cells (Weiss et al., 1988) unequivocally established CD8 T cells as key effectors in a rodent model of protection against malaria. We confirmed these results by demonstrating a failure to protect β_2 microglobulin knockout (β_2m KO) mice (White et al., 1996) and K^bD^b KO mice (Krzych and Schwenk, 2005) immunized with Pb γ-spz. Protection induced by GAP spz is also CD8 T cell dependent as shown by us in β_2m KO mice immunized with Pb GAP spz (Jobe et al., 2007) and by others in Py GAP protected mice after depletion of CD8 T cells (Trimnell et al., 2009). We also established that effector CD8 T cells are MHC-class I-restricted/dependent because protection is not transferred by γ-spz-immune wt cells into β_2m KO recipients as CD8 T cells must recognize LS-Ag peptides presented by MHC-class I on APC in the liver. The necessity for cognate peptide recognition by CD8 T_E cells was confirmed in experiments using MHC-class I mismatched effector and target hepatocytes (Chakravarty et al., 2007). The need for proximity between effector lymphocytes and target hepatocytes was demonstrated (Rodrigues et al., 1992), as has the ability of hepatocytes to present CSP to CD8 T cells (Bongfen et al., 2007). Target LS-Ags that induce CD8 T cells are being currently defined by us, as well as others (Doolan et al., 2003), using the combination of genomic and proteomic approaches. For example, targeted gene deletion reveals that *Plasmodium* sporozoite low-complexity asparagine-rich protein is essential for early LS development (Aly et al., 2011), whereas FabB/F gene (Vaughan et al., 2009) is essential for late-LS development. Immunization of mice with GAP spz with deletions of each of these genes induces protective immunity. Interestingly, immunization with the late-arresting fabb/f$^-$ parasites induces durable protection in several mouse strains and across *Plasmodia* species, presumably because these parasites express a broader repertoire of potential antigens that activate a wider spectra of effector T cells (Butler et al., 2011).

The initial site of induction of liver-resident CD8 T cells remains unclear. It is possible that these cells arise in the liver after interaction with liver APC such as KC or DC that present LS-Ags from developmentally aborted parasites. In support of this hypothesis, we have observed that the number of cCD8α$^+$ DC increases in the liver concurrently with the number of Pb γ-spz immunizations, while those in the spleen do not change. Additionally, liver cDCs are very efficient at inducing CD8 $T_{E/EM}$ phenotype and expression of IFN-γ, a process that is both MHC-class I- and IL-12-dependent (Jobe et al., 2009). The possibility of local activation of CD8 T cells by LS-Ags is quite attractive for several reasons, but especially because it enables a rapid response during re-infection. Although it has been shown that activated CD8 T cells home to the liver to be eliminated as a way of purging their destructive effector mechanism (Mehal et al., 1999), there is also evidence that direct activation of naïve CD8 T cells occurs in the liver (Bertolino et al., 2001; Klein and Crispe, 2006). Alternatively, CD8 T cells might be induced in a draining LN by LS-Ag-loaded DCs and during sporozoite challenge migrate to the liver, where they might undergo further expansion. Although quite attractive, migratory DC do not normally express the CD8α$^+$ phenotype (Belz et al., 2004) and the cCD8α$^+$ DC are the major activators of CD8 T cells in our system (Jobe et al., 2009). Although lack of firm evidence supporting either scenario favors the prevailing view that T cell activation occurs as a result of interaction with DC in the LN, the possibility of an organ-specific activation of CD8 T cells remains very attractive and should be explored further.

The development of TCR transgenic CD8 T cells specific for the *P. yoelii* CSP immunodominant peptide SYVPSAEQI (CS-Tg) has enabled the tracking of early immune responses (Sano et al., 2001). After exposure to bites from Py γ-spz infected mosquitoes, IFN-γ producing CS-Tg T cells were detected in the skin draining LN as early as 48 h after immunization, while spleen and liver responses were not detected until 72 h (Chakravarty et al., 2007). According to these results, CD8 T_E cells are generated in the draining LN near the infection (site of mosquito bite) (Chakravarty et al., 2007), and then migrate to liver to kill infected hepatocytes, a process that has been shown to be TAP-dependent, but endosome independent (Cockburn et al., 2011). It is possible that the induction of CD8 T_E cells depends on their fine specificity and the site of parasite inoculation. Consequently, CSP-specific CD8 T cells would be induced in the skin draining LN, as sporozoites that are trapped in the skin shed CSP, which could be presented by DCs to T cells. In contrast, LS-Ags, being expressed exclusively in the liver, might activate T cells in the liver, or be transferred either directly or through liver-resident APCs to the liver-draining LNs, to activate T cells there. Owing to the paucity of antigen-specific cells naturally present at the time of immunization or infection, it may not be possible to precisely determine the location of initial priming events.

EFFECTOR LIVER CD8 T CELLS

Pb γ-spz-immune intrahepatic mononuclear cells (IHMC) contain CD4 and CD8 T cells with inducible CD44^{hi} CD25^{hi} and CD45RB^{lo} phenotypic markers (Guebre-Xabier et al., 1999). Expression of CD45RB, an activation/memory marker, changes from CD45RB^{hi} to CD45RB^{lo} with increased antigen exposure and the state of cellular maturation (Lee et al., 1990; Seder and Ahmed, 2003). Although CD8 CD45RB^{lo} T cells are present in naïve liver, multiple immunizations with γ-spz stabilize and increase the CD45RB^{lo} (or CD62L^{lo}) phenotype as well as other phenotypic and functional attributes. For example, enhanced frequencies of CD8 CD45RB^{lo} T cells that secrete IFN-γ and express KLRG-1^{hi}CD107$^+$ phenotype coincide with the induction of sterile protection (Krzych et al., 2010). These observations are in agreement with the transient expansion of T cells (Ahmed and Gray, 1996; Badovinac et al., 2002) and the role of CD8 T cells as effectors.

Liver CD8 T cells from Pb γ-spz-immunized mice produce enhanced IFN-γ 6 h after challenge and the response peaks around day 7 after challenge (Berenzon et al., 2003). CD8 T cells that produce IFN-γ followed by the induction of nitric oxide synthetase (NOS) (Klotz et al., 1995; Doolan and Hoffman, 1999) might be physiologically relevant to the process of elimination of LS parasites: IFN-γ inhibits the hepatic stages of rodent and human malaria both *in vitro* and *in vivo* (Mellouk et al., 1987); injection of IFN-γ protects mice against sporozoite challenge (Ferreira et al., 1986); and immunization with γ-spz fails to generate protection in IFN-γR KO mice (Tsuji et al., 1995). Moreover, a reduced IFN-γ response from liver CD8 $CD44^{hi}$ T cells correlates with decreased protection in mice (Nganou-Makamdop et al., 2012); and Py CS-Tg T cells eliminate the parasite by a mechanism that depends upon rapid IFN-γ production (Sano et al., 2001).

Secretion of IFN-γ by liver CD8 T cells would preclude the need for direct lysis of hepatocytes, as IFN-γ could suppress parasite growth by the few CD8 T cells that encounter infected hepatocytes. IFN-γ could also contribute to protection indirectly by upregulating MHC-class I and class II molecules and B7-1 and B7-2 co-stimulatory molecules on KC, DC, and hepatocytes. This, in turn, would further promote activation of CD8 T_E cells.

The release of IFN-γ, which coincides with the activation of CD8 T cells, is preceded by elevated production of IL-4, which declines when IFN-γ reaches its peak (Krzych et al., 2000). The reciprocal regulation between these two cytokines reflects the precise orchestration of functional activities among T cell subsets induced by γ-spz. It is likely that IL-4 in the liver is produced by NK T cells, whereas IFN-γ is produced primarily by CD8 T cells (Berenzon et al., 2003). This view is in agreement with the observation that CD8 T_E cells decline after inflammation has subsided (Badovinac et al., 2002), whereas memory CD8 T cells persist, if they are supported by lymphokine-secreting cells.

In our view, sustained protection requires various CD8 T cell specificities, particularly those belonging to proteins expressed during pre-erythrocytic LS development. It could be envisaged that CSP-specific CD8 T cells initiate the effector stage of protection because they are the first cells to produce IFN-γ upon encountering infectious sporozoites. Protracted protection might require the subsequent activation of a second wave of CD8 T cells specific for epitopes other than CSP, as they would have to target hepatocytes by recognizing LS-Ags. Such concerted and functionally integrated activity provided by CD8 T_E cells with multiple specificities might be necessary to provide sustained protection. In their recent study, Butler et al. (2011) propose that GAP parasites arrested during late-LS development induce stronger CD8 T cell responses and durable protection presumably because these parasites contain a richer repertoire of antigens able to induce effector T cells. Similar observations regarding the availability of a more abundant level of late-LS-Ags have been made in a model system of protection induced by *P. bergehi* sporozoites delivered by the intravenous route under a drug coverage (Nganou-Makamdop et al., 2012).

MEMORY CD8 T CELLS

The formation of optimally effective memory T cells is an essential feature of an adaptive immune response elicited by infections and it is inextricably linked to long-lasting protective immunity (Ahmed and Gray, 1996). Intrahepatic memory CD8 T cells generated by immunization with Pb γ-spz segregate into at least two distinct but developmentally related subsets: the IFN-γ-producing $CD44^{hi}CD45RB^{lo}CD122^{lo}CD62L^{lo}$ phenotype, or $T_{E/EM}$ cells, which can be further subdivided based on the expression of IL7Rα (CD127) and KLRG-1 into T_E ($CD127^{-}KLRG\text{-}1^{+}$) or T_{EM} ($CD127^{+}KLRG\text{-}1^{-}$); and the indolent IFN-γ producing $CD44^{hi}CD45RB^{hi}CD122^{hi}CD62L^{lo/hi}$ phenotype, hence CD8 T_{CM} cells. The elevated expression of CD122 (IL-15Rα) on CD8 T_{CM} cells suggests that, in contrast to CD8 $T_{E/EM}$ cells, they are IL-15-dependent (Berenzon et al., 2003; Krzych et al., 2010).

Recently, various phenotypic and functional attributes have been evaluated in an effort to understand the differentiation of memory CD8 T cells (Joshi et al., 2011). In addition, asymmetric division (Ciocca et al., 2012), duration and strength of the TCR signal (D'souza and Hedrick, 2006), and inflammatory cytokines (Obar and Lefrancois, 2010) have been examined as requirements for memory T cell development and differentiation. Nonetheless, many questions remain regarding the regulation of memory cell formation and in the case of organ-specific infections, like malaria, additional aspects of memory CD8 T cell development and differentiation need to be considered. We propose that these functionally and phenotypically unique subsets of liver memory CD8 T cells form an interactive network involving different phases of dynamic cell activation and differentiation (Berenzon et al., 2003). The co-presence of distinct subsets within the intrahepatic memory CD8 T cell pool in mice protected against malaria is consistent with an earlier view that virally induced memory CD8 T cells are organized into subsets on the basis of distinct functional activities and the maturation/activation status (Sallusto et al., 1999; Kaech et al., 2002; Wherry et al., 2003).

Similar to the rapid responses mediated by influenza- and Sendai-specific CD8 $T_{E/EM}$ cells (Hogan et al., 2001), intrahepatic CD8 $T_{E/EM}$ cells from Pb γ-spz-immunized mice produce a copious amount of IFN-γ within 1–6 h after infection. Although the pool of CD8 T_E cells eventually contracts and the IFN-γ response diminishes, the IFN-γ-producing memory T cells persist in the livers of mice that maintain protracted protection against a re-challenge (Berenzon et al., 2003; Nganou-Makamdop et al., 2012). A decay of protection is typically accompanied by the decline of IFN-γ-producing $KLRG\text{-}1^{hi}CD107^{hi}$ CD8 T cells (Krzych et al., 2010).

CD8 T_{CM} cells also produce IFN-γ but the responses are low and relatively short-lived. Therefore, these cells do not appear to be directly involved in the elimination of the parasite. Instead, by acquiring the $CD122^{hi}$ phenotype, liver CD8 T_{CM} cells engage in homeostatic proliferation, which qualifies them to function as a reservoir to maintain the size of memory CD8 T cell pools (Judge et al., 2002; Berenzon et al., 2003; Krzych and Schwenk, 2005). The maintenance of memory pools is one of the prerequisites of a memory T cell response because attrition, particularly of CD8 T_E cells, is inevitable during any infection (Badovinac et al., 2002).

MECHANISMS FOR MAINTENANCE OF PROTECTION INDUCED BY γ-spz

Evidence from our laboratory indicates that the persistence of memory CD8 T cells correlates with the maintenance of protective immunity. Interestingly, the persistence of memory phenotype CD8 T cells is restricted to liver lymphocytes, because splenic T cells from the same group of γ-spz-immunized mice display a phenotype similar to splenic T cells from naïve mice (Guebre-Xabier et al., 1999).

The persistence of memory CD8 T cells in the liver can be accounted for by several co-existing mechanisms. First, some of the memory T cells detected after immunization may be long-lived memory cells derived from CD8 T_E cells that survived the contraction phase after sporozoite challenge and have not lost their replicative abilities (Bannard et al., 2009). Second, the $CD44^{hi}CD45RB^{lo}$ CD8 T cells in the livers of long-term immune mice may be derived from cells that constantly ingress to the liver in response to the liver repository of *Plasmodia* antigens. The persisting memory may also be derived from naive CD8 T cells that do not quite acquire effector function during priming and subsequent boost immunizations with Pb γ-spz. It appears unlikely that they traffic to the liver from the spleen, because $CD44^{hi}$ CD8 T cells are not present in the spleens during protracted immunity; it is possible, however, that they traffic from the draining celiac LNs. Irrespective of whether maintenance of protection relies on long-lived intrahepatic memory T cells or T cells that constantly ingress to the liver, both require a repository of *Plasmodia* antigens (Berenzon et al., 2003).

THE LS-Ag DEPOT IS REQUIRED FOR THE MAINTENANCE OF PROTRACTED PROTECTION

There is ample contradictory evidence with respect to antigen requirement for the persistence of memory T cells (Gray and Matzinger, 1991; Murali-Krishna et al., 1999; Jelley-Gibbs et al., 2005; Zammit et al., 2006). On the basis of results from our laboratory, the persistence of a threshold of accumulated LS-Ags is critical for the maintenance of protective immunity. Administration of primaquine at the time of immunization with Pb γ-spz does not affect protection at primary challenge, but results in a loss of protracted protection, which correlates with a decrease of CD8 $T_{E/EM}$ cells in the liver. The disruption of the intrahepatic-stage parasite development prevents the formation of a local antigen depot, which impedes the conscription of T_{CM} into $T_{E/EM}$ CD8 T cells upon re-challenge. In contrast, delayed administration of primaquine has no effect on lasting protective immunity (Berenzon et al., 2003). These results are indeed expected, as the primary action of primaquine is against LS development, without affecting the sporozoite stage, represented by CSP-specific CD8 T cells (Hafalla et al., 2002; Cockburn et al., 2010).

Although most of the results from viral systems argue against the need for antigen to maintain long-lived memory CD8 T cells (Lau et al., 1994; Murali-Krishna et al., 1999), there is evidence that T cell memory persists if a protracted restimulation of effector T cells is maintained, either by persisting or by cross-reacting environmental antigens (Zinkernagel et al., 1996; Jelley-Gibbs et al., 2005). We suggest that antigen requirements might be quite different in malaria because the parasite exhibits tropism to the liver, which is characterized by immunologic tolerance. The liver antigen repository may be sufficient to play a unique role in distinguishing the "locally" activated liver memory T cells from those found in the spleen or LN.

The precise location of the LS-Ag depot has not been established. In principle, hepatocytes can function as APC. Although there is no evidence that hepatocytes present LS-Ags to activate or to maintain memory T cells, because so few hepatocytes become infected by the invading sporozoites and they also are inefficient as APC (Steers et al., 2005), hepatocytes may provide LS-Ag for cross-presentation by either KC or cDC. It could be envisaged that liver APCs internalize infected hepatocytes and engage in cross-presentation of LS-Ag that gained entry from phagosomes into the MHC-class I pathway. Exogenous particulate Ags were shown to enter the MHC-class I pathway via phagosome-ER fusion (Gagnon et al., 2002) or, as in the case of *Toxoplasma gondii*, fusion of the parasitophorous vacuole with the ER (Goldszmid et al., 2009).

The mechanism of LS-Ag processing and presentation has not been fully investigated; however, we demonstrated that adoptively transferred liver $CD11c^+$ DCs isolated from Pb γ-spz-immunized mice confer sterile protection to naive mice during a primary Pb sporozoite challenge (Jobe et al., 2009). In a recent study, it was shown that proliferation of Py CS-Tg CD8 T cells transferred into Py γ-spz-immunized mice can be maintained in the liver for up to 60 days post immunization. Depletion of CD11c prior to immunization abolishes this response, which indicates a role for DC as a possible source of CSP depot (Cockburn et al., 2010).

MAINTENANCE OF CENTRAL MEMORY CD8 T CELLS BY IL-15

It has been established that IL-15 promotes the survival of long-term memory CD8 T cells by maintaining their homeostatic proliferation, whereas IL-2 stimulates both the initial expansion and subsequent contraction of T lymphocytes (Ku et al., 2000; Li et al., 2001; Waldmann et al., 2001; Sprent and Surh, 2002). Although the CD8 T_{CM} cell subset represents a much smaller fraction of the liver CD8 T cells, twice as many CD8 T_{CM} cells are $CD122^{hi}$ than $T_{E/EM}$ cells, which are primarily $CD122^{lo}$ (Berenzon et al., 2003). On the basis of results from *in vitro* studies, only CD8 T_{CM} cells proliferate in the presence of IL-15 and these cells are severely reduced in IL-15KO mice (Krzych et al., 2009). The enhanced sensitivity of the CD8 T_{CM} cells to reduced levels of IL-15 suggests that this subset preferentially expand upon exposure to elevated levels of IL-15 in the liver. It also implies that an optimal protective response requires a developmental compartmentalization of CD8 T cells, with each subset performing not only a unique role, but also relying on distinct regulatory mechanisms (Krzych et al., 2009).

We explored the issue of IL-15 as a signal required for the maintenance of memory CD8 T cells in IL-15 deficient mice (Krzych et al., 2009). Like wt mice, IL-15 KO mice are protected against a primary challenge administered shortly after the last boost immunization with Pb γ-spz. Protection is short-lived, however, as at re-challenge 2 months later, the IL-15 deficient mice become parasitemic. Analysis of the CD8 T cell subsets at primary challenge show an accumulation of CD8 T_{EM} cells and a very small pool of CD8 T_{CM} cells. It appears, therefore, that in the absence of IL-15, CD8 T_{EM} cells might have developed directly from naïve CD8 T

cells as has been shown in other systems (Decaluwe et al., 2010). Without the provision of IL-15, the critical reservoir of memory CD8 T cells is severely reduced and hence unable to sustain a sufficient number of cells needed during re-challenge. We observed a near absence of CD8 T_{CM} cells in IL-15 KO mice prior to secondary challenge. The majority of CD8 T_{CM} cells undergo severe attrition as evidenced by the level of apoptosis mainly within the CD8 T_{CM} cells. Consequently, only ∼1% of the cells remain in IL-15 deficient mice in relation to ∼7% in wt mice. Upon 2° challenge, the majority of $CD127^{hi}$CD8 T_{CM} cells transition to $CD127^{lo}$ phenotype in wt mice, but in IL-15KO mice few cells become $CD127^{lo}$. These observations strongly support our hypothesis that CD8 $T_{E/EM}$ cells are conscripted from the CD8 T_{CM} cells in a continuous, albeit slow, process that occurs in the liver as a result of an increased antigen load after repeated immunizations with γ-spz. An association between sterile protection and increased antigenic load of late-LS parasites has also been demonstrated in another model system using Pb sporozoites (Nganou-Makamdop et al., 2012). The conscription of T_E cells from the T_{CM} reservoir also occurs during infection, when large numbers of CD8 T_{EM} cells would be most needed to combat the parasite. For example, it has been shown that a large number of Pb $CS_{252–260}$-specific CD8 T cells is needed to to maintain sterile protection in Balb/c mice challenged with Pb sporozoites (Schmidt et al., 2011). Compared to many bacterial or viral infections, sterile immunity against malaria infection requires 100–1000 fold higher numbers of CD8 T_E cells (Schmidt et al., 2008). The requirement for these large numbers of antigen-specific CD8 T cells may be, in part, due to the short time frame of the LS when the parasite is most vulnerable to immune intervention.

In either case, CD8 T_{CM} cell pool is maintained in the liver by IL-15. IL-15 is produced by a variety of cell types (although not by T cells) in response to signaling via TLRs or exposure to type I IFN (Mattei et al., 2001). Pb γ-spz cause upregulation of IL-15 mRNA in KC (Krzych et al., 2009) and liver cDC (Jobe et al., 2007). Upon encounter with specific antigen from the liver repository or upon re-infections, the CD8 T_{CM} cells would be driven to differentiate into the $CD44^{hi}CD45RB^{lo}CD122^{lo}$ phenotype that is easily triggered by infectious sporozoites to produce IFN-γ. Recently, it was demonstrated that CD8 T cell survival during influenza infection is promoted in the lung by trans-presentation of IL-15 by pulmonary $CD8\alpha^{+}$DCs (McGill et al., 2010). There is evidence (Dubois et al., 2002) that APC retain IL-15 bound to the IL-15Rα chain to transactivate CD8 T cells expressing the IL-15Rβγc complex. On the basis of our previously published results that liver cDC activate CD8 T cells in a MHC-class I-dependent manner to express $CD44^{hi}$, up-regulate IL-15 mRNA (Jobe et al., 2009) and express detectable IL-15 protein (Krzych et al., 2010), together with KC they can function as APCs of LS-Ags and as trans-presenters of IL-15 that target only liver CD8 T_{CM} cells. Our hypothesis is supported by observations from *in vitro* conducted studies that only CD8 T_{CM} cells require trans-presentation of IL-15 in the context of a concurrent signaling via TCR for optimal recall response, as responses by CD8 T_{EM} cells are not augmented by IL-15 (Kokaji et al., 2008).

SUMMARY

MHC-class I-restricted CD8 T cells have been established as key effectors in protective immunity against pre-erythrocytic-stage malaria infection. The effector function is associated mainly with the production of inflammatory cytokines such as IFN-γ or TNF-α that mediate elimination of the parasite within the hepatocytes by the NO pathway. The success of protection induced by γ-spz depends upon the long-lived intrahepatic memory CD8 T cells that consist of developmentally related subsets as CD8 T_{CM} and CD8 T_{EM} cells. While the CD8 T_{EM} cells are maintained by the antigen-driven conscription of the CD8 T_{CM} cells, the latter representing a very broad spectrum of antigen-specific T cells, is maintained by IL-15 and possibly the LS-Ag depot. This arrangement assures a steady availability of antigen-specific T cells should they be required to combat re-infection. The dependence on specific antigen essentially controls the balance between the two phenotypes and the differential expression of IL-15R prevents the CD8 T_{EM} cells from becoming activated in the event of sporadic co-infections. However, it is the activated status of the intrahepatic memory CD8 T cells that really distinguishes them from the memory CD8 T cells in the spleen and LN as it represents the sentinel of a local, organ-specific infection.

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Development of CD4 T cell dependent immunity against *N. brasiliensis* infection

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Of all the microbial infections relevant to mammals the relationship between parasitic worms and what constitutes and regulates a host protective immune response is perhaps the most complex and evolved. *Nippostrongylus brasiliensis* is a tissue migrating parasitic roundworm of rodents that exemplifies many of the salient features of parasitic worm infection, including parasite development through sequential larval stages as it migrates through specific tissue sites. Immune competent hosts respond to infection by *N. brasiliensis* with a rapid and selective development of a profound Th2 immune response that appears able to confer life long protective immunity against reinfection. This review details how the lung can be the site of migrating nematode immune killing and the gut a site of rapid immune mediated clearance of worms. Furthermore it appears that *N. brasiliensis* induced responses in the lung are sufficient for conferring immunity in lung and gut while infection of the gut only confers immunity in the gut. This review also covers the role of IL-4, STAT6, and the innate cytokines IL-25, IL-33, and thymic stromal lymphopoietin in the generation of CD4-mediated immunity against *N. brasiliensis* reinfection and discusses what cytokines might be involved in mediated killing or expulsion of helminth parasites.

Keywords: regulation, CD4 Th2, protective immunity, helminth

NIPPOSTRONGYLUS BRASILIENSIS INFECTION INDUCES CD4 T CELL MEDIATED TH2 CYTOKINE IMMUNITY

In immune competent rodent hosts primary invasion by *Nippostrongylus brasiliensis* presents as a self limiting infection which is resolved within 9–14 days (Camberis et al., 2003). *N. brasiliensis* initiates infection at the skin site, migrating in a matter of hours through the vasculature to the lungs where maturation occurs. Through exploitation of host mucus/ciliary lung clearance mechanisms, worms are transported out of the lung to be ingested. The L4/L5 become localized in the duodenum where final maturation to adults occurs, leading to reproductive activity and subsequent egg production before the adult worms are expelled. As a consequence of migration through the host *N. brasiliensis* stimulates immune responses at three distinct peripheral sites; skin, lung, and gut which builds to a maximum expression of effector cells and molecules associated with Th2 immunity 9–14 days following infection. This "Th2 cytokine storm" includes the production of cytokines IL-3, IL-4, IL-5, IL-9, IL-10, IL-13 (Conrad et al., 1990; Kopf et al., 1993; Urban et al., 1998; Finkelman et al., 2000, 2004; Holland et al., 2000, 2005; Liu et al., 2002; Min et al., 2004; Mohrs et al., 2005; van Panhuys et al., 2008; Harvie et al., 2010), and the effector mechanisms mediated by IgE antibodies and inflammatory mucus (Lebrun and Spiegelberg, 1987; Katona et al., 1988) and inflammatory cells such as eosinophils (Shin et al., 1997; Daly et al., 1999; Knott et al., 2007), basophils (Conrad et al., 1990; Min et al., 2004), and the newly characterized nuocytes (Neill et al., 2010).

IL-4 has been a Th2 cytokine of interest in *N. brasiliensis* infection since the discovery that disruption of the IL-4 gene prevents Th2 cytokine responses (Kopf et al., 1993). The developments of new technologies such as IL-4 gene reporter mice have enabled careful and thorough investigation of the Th2 immune response elicited by *N. brasiliensis* infection (Hu-Li et al., 2001; Mohrs et al., 2001, 2005). Experiments using the G4/IL-4 reporter mice (Hu-Li et al., 2001), which express green fluorescent protein (GFP) under the control of the IL-4 promoter, have allowed a detailed quantitative analysis of the generation of Th2 immune responses following *N. brasiliensis* infection (van Panhuys et al., 2008; Harvie et al., 2010) to be performed. Interestingly, it was shown that the IL-4 producing T cell response at the skin, lung, and gut sites of infection peaked at a time when parasites had already migrated through the tissue (Harvie et al., 2010) and were at their egg producing adult phase in the gut. It is unknown whether the expulsion of the worms by the Th2 response at this time is a mechanism to aid in egg survival and distribution in the environment or a host protective mechanism to reduce the burden of gut parasites. Our ability to sensitively detect the generation and maintenance of the Th2 immune response *in vivo* has enabled us to undertake a more detailed study of the Th2 immune response in protective immunity against *N. brasiliensis*, and identify the cellular and molecular cues produced by the parasite which coordinate this response.

IMMUNITY TO REINFECTION BY *N. BRASILIENSIS* OCCURS IN THE LUNG

Previous exposure to infection with *N. brasiliensis* induces potent CD4 T cell immunity in the murine host and upon subsequent reinfection with the parasite, results in 90% fewer worms being able to migrate, mature, and develop to adults in the duodenum. Using this reinfection experimental protocol, *N. brasiliensis* has

been used as a model for identifying the cells and molecules that regulate CD4 T cell and Th2 dependent protective immunity.

The skin is the initial site of *N. brasiliensis* infection where infective L3 (iL-3) invade and enter the host. Infective L3 are the only stage capable of penetrating through otherwise intact skin. Normally, worms are able to leave the skin infection site and migrate through the vasculature to the lungs. Interestingly, experiments comparing wild type, IL-5 transgenic and IL-5 deficient animals in an ear air pouch model have shown that worms can be trapped and killed in the skin, due to the action of eosinophils (Daly et al., 1999). These results could provide the context for either innate resistance of various mouse strains to primary infection, or represent the effector stage of an overwhelming adaptive immune response. However experiments by Harvie et al. (2010) comparing migration through skin, found no difference in larval migration through naïve or immune skin using a Balb/c model of *N. brasiliensis* infection. Although it should be noted that in natural epicutaneous infection through the skin experimental protocols may reveal a role for the skin site and skin localized immune cells in immune mediated immunity.

Of recent significance is the finding that the lung can be a site of protective immunity against reinfection with *N. brasiliensis*. Normally, iL-3 burst through into the lung parenchyma as early as 16 h post-infection. In a primary infection, the migration to the lung is traumatic for the host as the migrating worms cause damage to the delicate lung tissue (Marsland et al., 2008). This damage can easily be seen by gross pathological analysis as red petechial spots within the lung parenchyma. It is here in the lung that the iL-3 mature into L4 stage parasites before being transported by the lung mucus/ciliary ladder to the esophagus and swallowed.

Although often not considered, there have been a few reported experiments investigating lung immune responses in *N. brasiliensis* infection. Knott et al. (2009) proposed that damage during the lung phase, or pre-lung phase is responsible for reduced worm burdens in FVB/N mice, a strain found to be resistant to *N. brasiliensis* infection. A further study by Harvie et al. (2010) showed that priming of CD4 T cells in the lung tissue was sufficient to confer protection against reinfection with *N. brasiliensis* at both the lung and gut sites. These investigations definitively demonstrate that CD4 and STAT6 dependent responses at the lung site are required for immunity to *N. brasiliensis* as a significant increase in numbers of migrating larvae are recovered from lung tissue upon reinfection with *N. brasiliensis*.

In a large number of previously reported studies, there has been a major focus on investigating immune responses in the gut in the *N. brasiliensis* infection model. These studies have viewed protective immunity as the ability to expel worms from the gut. Gut expulsion is also profoundly dependent on CD4 T cells and STAT6. IL-4 and IL-13 production by immune cells acting on goblet cells to increase mucous secretion and also cause contraction of smooth muscle, leading to the expulsion of the worms (Lawrence et al., 1996; Urban et al., 1998; Voehringer et al., 2006; Neill et al., 2010). IL-5 and IL-9 are also thought to play a role in worm expulsion (Fallon et al., 2002). The newly described innate lymphocyte subtypes have been proposed to have the potential to also affect worm survival through their expression of IL-13 and IL-5. Although the experimental models of *Nippostrongylus*, infection in RAG1 deficient mice would suggest that nuocytes are not able to directly mount a protective response to *N. brasiliensis* by themselves, they could represent a relevant effector arm of the CD4 T cell response playing a key role in gut immunity (Neill et al., 2010).

In conclusion it has become appreciated that although Th2 cells get generated or differentiate in the lymph nodes following parasite infection or allergen challenge their subsequent sequestration to tissues such as the lung, gut and skin can lead to them developing potentially different types of activation dynamics and functional phenotype (Harris et al., 2002). Although this functional plasticity is much better appreciated in studies of CD8 T cells and viral infections, new data and models are emerging for CD4 T cells and innate cells in the context of helminth infections and allergic responses. The studies reviewed above point to the lung being a key tissue involved in generating and being a site of Th2 immune responses. In experimental models the Th2 responses in the lung appear able to significantly arrest the development and function of helminth parasites through the CD4 T cell mediated production of cytokines and effector molecules.

IMMUNITY TO *N. BRASILIENSIS* REINFECTION IS GENERATED IN THE LUNG

Infection with helminths such as *N. brasiliensis* presents a unique challenge to the immune system in that over a period of several days the parasite moves to quite different tissue sites including, dermis, blood, lung, and duodenum and at each of these sites presents as an antigenically and biologically different form. The CD4 T cell response that mediates protection against reinfection could be generated at either one of these immune sites or the same one at all sites. Our recent study of *N. brasiliensis* infection in G4/IL-4 reporter mice attempted to determine which site is the most relevant to generating the Th2 cells which mediate immunity against this parasite (Harvie et al., 2010). Surprisingly, these studies found in an intradermal infection model that the skin is not a site of immune protection or immune priming for protection against *N. brasiliensis* infection. These conclusions were drawn from studies where the primary immune response to worms was induced by injecting dead worms in the skin which obviously could not migrate to other tissue sites and prime Th2 responses elsewhere and these did not confer protection against reinfection in the lung (Harvie et al., 2010). However, it could be imagined that under special circumstances of high infection rates or vaccination boosting that skin infection could lead to generation of protective immunity in the lung (Daly et al., 1999; Girod et al., 2003; Fujiwara et al., 2006; Knott et al., 2007). One point to note is that these protocols bypassed the epicutaneous infection stage of worm infection by direct injection into the dermis and it may be that immunity could be both generated and effected at this site if a natural infection model of epicutaneous infection through the skin was employed.

In the same study using an experimental protocol where priming of CD4 T cells against *N. brasiliensis* was confined to gut tissues using gavaged L5/adult worms lung protective immunity against *N. brasiliensis* was not generated. However, mice whose immune priming was confined to the lung site via intranasal priming with L3/L4 stage *N. brasiliensis* were protected at both the lung site and also the gut. Using the G4 reporter mice which revealed the priming of CD4+ T cells it could be shown that this generation of protective immunity was closely associated with the appearance of significant numbers of IL-4 producing CD4

positive cells. Collectively, these results demonstrated that CD4 T cell mediated protection against reinfection could be conferred when animals were primed at the lung site.

An interesting possibility is that the Th2 response in the lung modifies the environment of the lung so that it is no longer supportive of *N. brasiliensis* development. Experiments by Reece et al. (2008) investigate the impact of *N. brasiliensis* infection on the lung environment using mRNA transcript level analysis of cytokines found that *N. brasiliensis* infection induces the development of alternately activated macrophages (AAMs) in a STAT6 dependent manner. This finding builds on the earlier work of Marsland et al. (2008) who also investigated lung pathology and saw the induction of alternately AAMs after *N. brasiliensis* infection. Both of these groups focused on the physical pathological changes in the lung and related the CD4 T cell role in activating AAMs to the ongoing repair of tissue damage, it remains to be determined whether they may also mediate an anti-parasite role upon reinfection.

THE CELLS, CYTOKINES, AND SIGNALS REQUIRED FOR GENERATING CD4 T CELL MEDIATED IMMUNITY AGAINST *N. BRASILIENSIS* REINFECTION

The most compelling argument for the role of CD4 T cells in mediating protective immunity against *N. brasiliensis* infection comes from studies with MHC class II deficient and STAT6 deficient mice. When infected, these immune deficient mouse strains fail to mount Th2 effector responses, fail to clear worms in primary infection and lose the ability to prevent reinfection. In immune competent mice the protective CD4 T cell immunity appears to last for at least 9 months following primary *N. brasiliensis* infection. The observation that CD1 deficient mice show no ablation of protective immunity would suggest that there is no major role for NKT cells in immunity against *N. brasiliensis*. Infection studies with B cell deficient mice reveal perhaps somewhat surprisingly, that antibodies play little role, despite the often-reported association between high IgE levels and immunity to parasites.

The attempts of investigators to identify in more satisfactory detail, which elements of a Th2 mediated STAT6 dependent responses confer immunity have proven elusive to date. It is well established that the cytokine IL-4 is necessary for protection against *N. brasiliensis*, however it does not appear to be linked to its role in driving IgE production but rather a mechanism also able to be mediated by IL-13. Although the exact effector mechanisms employed by Th2 cell mediated protective immunity remain undefined, it has been recently demonstrated that a diverse T cell receptor repertoire is required for protective immunity at the gut site of infection (Seidl et al., 2011). This finding would imply that simply having high numbers of effector cells in the locality of a parasite is insufficient and that intimate MHC restricted, CD4 T activation is also required for conferring protection.

ROLE OF INNATE CYTOKINES AND INNATE HELPER CELL TYPES IN TH2 IMMUNITY

An intriguing feature of the immune response induced by *N. brasiliensis* infection is the selective and specific induction of Th2 cells producing a "cytokine storm" involving all the Th2 cytokines. As a consequence the *N. brasiliensis* infection model has become the "de rigueur" physiological model for evaluating the upstream mechanisms for the induction and regulation of Th2 cells. The recent identification and cloning of candidate innate cytokines either produced in the skin or identified by their gene sequence homology to other CD4 T cell activating factors has given this research field many candidates to evaluate and test with thymic stromal lymphopoietin (TSLP), IL-25, and IL-33 perhaps being the most pertinent candidates. We would argue that to date the results are still somewhat contradictory and much still remains to be done. As an example although TSLP induced DCs have been shown to initiate Th2 immune responses *in vitro* (Sokol et al., 2008), TSLP deficient mice have unimpaired protective responses against *N. brasiliensis* (Massacand et al., 2009). Similarly, recent data appears to suggest a key role for the Th2 cytokine IL-25 in respect to induction of Th2 responses and Th2 mediated worm expulsion (Fallon et al., 2006; Neill et al., 2010). However, the recent discovery of the effect of IL-25 on innate cell lineages of nuocytes (Neill et al., 2010), innate type 2 helper (ih2) cells (Price et al., 2010), and multipotent progenitor type 2 cells (Saenz et al., 2010) to produce Th2 cytokines such as IL-13 now suggest the view that IL-25 has an accessory role in augmentation of a CD4 Th2 response. In considering the role of IL-25 in CD4 T cell mediated protective immunity against *N. brasiliensis* protective immunity several points emerge. Although IL-25 may feature in *N. brasiliensis* expulsion it is independent of T cell mechanisms as injection of IL-25 into RAG deficient mice leads to the expulsion of adult worms (Fallon et al., 2006; Price et al., 2010). In addition, induction of nuocytes and ih2 cells can be achieved in the presence of IL-33 (Neill et al., 2010; Price et al., 2010), but this cytokine is not required to mount a protective response against *N. brasiliensis* (Senn et al., 2000). Moreover, recent studies indicate that IL-33 can drive CD4-mediated IL-13 production in the *N. brasiliensis* infection model although the majority of the effect is at the level of IL-33 induced ILC2 producing IL-13 in the gut environment leading to worm expulsion (Hung et al., 2012).

An interesting set of studies recently resurrected the issue that innate cells able to produce IL-4 could play a role in Th2 differentiation (Le Gros et al., 1990). Specifically, the recent studies identified that IL-4 producing basophils also expressed Class ll MHC antigens and associated machinery for antigen processing and thus were capable of driving Th2 differentiation (Perrigoue et al., 2009). However, in the setting of *N. brasiliensis* infection Th2 immune responses were found to be unimpaired in the absence of basophils (Kim et al., 2010). These seemingly disparate observations highlight the dearth of knowledge that is critical to our understanding of the innate cellular and cytokine mechanisms driving CD4 T cell mediated protective immunity in helminth infections. In conclusion, to date, no conclusive mechanism linking innate cytokines or innate cells to the *in vivo* differentiation and regulation of Th2 cells and CD4 T cell mediated protective immunity has yet to emerge.

IDENTIFICATION OF TH2 EFFECTOR MECHANISMS INVOLVED IN IMMUNITY AGAINST HELMINTHS

Evidence reviewed here shows there are differences in the parameters of lung and gut immunity against *N. brasiliensis*. Using the

G4/IL-4 reporter mice it could be shown that this generation of protective immunity was closely associated with the appearance of significant numbers of IL-4 producing CD4 positive cells. A requirement for IL-4 in lung protection that is not necessary for protection within the gut has been shown (Harvie et al., 2010). This may be a reflection of the redundancy in the roles of IL-4 and IL-13 in gut expulsion of worms, where the as yet unknown mechanisms of protection within the lung must be more dependent on IL-4. The role of eosinophils and IL-5 remains unclear, with some reports suggesting eosinophilia is critical for parasite clearance (Shin et al., 1997; Daly et al., 1999; Knott et al., 2007) and others finding that IL-5 deficiency did not affect infectivity (Harvie et al., 2010). These differences could be due to the use of different mouse model systems, but the role of eosinophils remains unresolved.

Other studies looking at protective mechanisms able to affect *N. brasiliensis* have led to the discovery of novel cell types and molecules; in particular a role for RELMβ has been put forward (Herbert et al., 2009) with RELMβ expression coincident with cytokine production and expulsion of *N. brasiliensis* from the gut (Artis et al., 2004).

The recently discovered class of innate lymphocytes such as the nuocyte has also been shown to play a role in IL-13 mediated expulsion of *N. brasiliensis* from the gut (Neill et al., 2010), although their activity is still dependent on CD4 T cells being maintained at this tissue site. The significance of these innate cells in a *N. brasiliensis* infection is unclear as studies in RAG1 deficient mice that have strong innate immune responses, but defective adaptive response, are unable to clear infection, arguing that innate cells have roles only within the context of CD4 T cell mediated responses.

SUMMARY

In summary, the regulation of CD4 T cell mediated protective immunity against the parasite *N. brasiliensis* is necessarily complex due to the multiple larval stages and different tissues that are involved during migration through the rodent host. There are increasing numbers of studies that seek to tease apart the CD4 T cell components of this fascinating process. Of particular interest is that the development of long-lived CD4 T cell mediated Th2 effector responses are induced at specific tissue sites of infection by the migrating parasite leading to subsequent Th2 mediated effector responses that can confer local immunity at that tissue site. Also it appears some mucosal sites are linked as indicated by the observation that immune priming to migrating worms in the lung was sufficient to subsequently confer CD4 T cell mediated immunity in the gut. The development and maintenance of protective immunity varies by tissue and/or larval stage of the *N. brasiliensis* parasite, with the requirements of immunity being different within lung and gut tissues (Harvie et al., 2010; Neill et al., 2010). At the gut site it seems that while innate type 2 responses have an important role to play in worm expulsion (Fallon et al., 2006; Voehringer et al., 2006; Neill et al., 2010), it appears that the maintenance of these responses are still dependent on CD4 T cells (Neill et al., 2010). Although the Th2 responses mediating protective immune responses at the lung site are less well defined it is clear that activated CD4 T cells can have a key role in protecting against reinfection or reducing significantly the worm burden carried by the host (Harvie et al., 2010).

Identification of the processes, cells and cytokines that regulate the Th2 responses to tissue parasites such as *N. brasiliensis* is proving exciting but controversial. The overexpression transgenic studies of the cytokines IL-33, TSLP, and IL-25 reveal a potential role for each cytokine while the corresponding reverse genetics gene deletion studies reveal either no role or redundant roles in the models of *N. brasiliensis* infection. The role of innate cells such as basophils able to present antigen, produce IL-4 and drive Th2 development has recently challenged the current paradigm that all antigen presentation to CD4 T cells must go via the dendritic cell. However the relevance of this pathway to *N. brasiliensis* infection induced Th2 responses has yet to be confirmed. The recent identification of the other innate lymphocyte subsets such as nuocytes and ih2 that may be able to regulate CD4 T cells has not yet been fully developed. The discovery or correct assignment of other cytokines and effector molecules to the so-called Th2 subset of CD4 T cell responses is an area of active investigation. It is hoped future studies will be able to unravel this fascinating and highly involved process that is relevant to all vertebrate species.

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Keeping memories alive

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Memory T cells confer immune protection against a diverse range of pathogens. The quantity and quality of responding memory T cells depends on a number of factors including cytokines, recognition of pathogen-derived MHC complexes, and costimulatory molecules. Integration of these signals ensures that T cell responses are tightly regulated. Delineating the mechanisms that regulate the differentiation, establishment, and maintenance of memory T cells is fundamental to life-long immune protection and for engineering of effective T cell-based vaccines.

Despite extensive research dissecting the features of T cell memory, many aspects of this process remain incompletely understood. This is in part due to a realization that integral to protective memory is the existence of multiple T cell subsets with diverse distributions to lymphoid, mucosal, and non-lymphoid sites. While there is a consensus that T cell memory is essential, which cells provide effective memory in different infections and at different anatomical sites evokes considerable debate.

This volume brings together 10 articles that are intended to summarize the current thinking on the development of immunological memory and to highlight important areas of investigation for the future in teasing apart the ability of the immune system to preserve the knowledge of a previously encountered antigen or pathogen and to use this to vigorously defend against a second or subsequent infection.

The first article (Hamilton and Jameson, 2012) introduces the feature of $CD8^+$ T cell memory and the cellular and transcriptional factors that influence memory T cell formation. This review introduces new subsets that have begun to populate the memory T cell landscape such as the self-renewing "memory stem cells" and effector-like memory cells and raises the important question of which cells within the memory compartment (effector, effector-like, and central memory) actually provide immune protection? In general, the broad picture painted of an immune response arises from viewing outcomes at a population level. However, as shown by Buchholz et al. (2013), drilling down on the decision processes of a single cell is now technically feasible, offering unparalleled opportunity to determine how the population relates to the choices of individual cells and the ability to search deeply for putative "memory stem cells" and how they contribute to shaping the memory compartment.

The next article by Gebhardt and Mackay (2012) discusses in depth the role of non-lymphoid tissue-resident memory $CD8^+$ T cells. Although experimental models often tackle questions of T cell memory using systemic models of infection, vast exposure of organisms occurs through contact of mucosal and cutaneous tissues, placing how the body deals with localized infections in the peripheral tissues as a major and critical arm of protective immunity. Evolving concepts of memory T cell formation that include the subpopulation of memory T cells that express the integrin CD103 are described, as are human $CD8^+CD103^+$ memory T cells that share a multitude of characteristics with tissue-resident memory T cells described in mice.

This leads on to a cluster of three articles that address how differentiation affects survival and persistence of memory T cells and the impact of these features to vaccination approaches (Kedzierska et al., 2012; Kurtulus et al., 2012; Vasconcelos et al., 2012). The traditional view of T cell memory has revolved around the development of a small residual stable memory pool (~5% of expanded cells) of T cells following massive expansion and extensive contraction or death of many pathogen-specific cells in response to infection. This pattern of development promoted the idea of a linear model of T cell development that has been widely accepted. Nevertheless, other studies provided non-concordant insights suggesting that alternate models might account for the fate decisions undertaken by T cells. Indeed it is now clear that the signals received by T cells early in infections are likely to be highly instructive in dictating the more immediate outcome of a response. These early cues also appear to imprint the T cells with a program that ensures their longevity through the establishment of a cell survival program. This contrasts with the diminished response elicited from "old" naïve T cells when confronted by a pathogen in an immune response and provides important insights to how we might shape the immune response at the point of initial challenge through vaccination approaches.

Co-ordinate regulation of surface molecules and the internal machinery fine tune the transferral of external signals to the molecular apparatus controlling T cell fate decisions. This is initiated by engagement of the T cell antigen receptor (TCR). Using super high-resolution microscopy, Rossy et al. (2012) discuss new spatiotemporal models that regulate TCR organization and the impact of these new concepts on our understanding of T cell activation and differentiation. TCR activation ultimately induces a signaling cascade that results in the induction of a transcriptional program that results in terminal differentiation of T cells (Russ et al., 2012; Kim and Suresh, 2013). Kim and Suresh (2013) discuss in detail the role of phosphatidylinositol 3-kinase (PI3K)/Akt/mTor signaling pathway which is positioned to coordinate the convergence of TCR and costimulatory signals and influence $CD8^+$ T cell effector/memory fate decisions principally by regulating cellular metabolism Superimposed on these activatory programs are epigenetic changes that fine-tune gene transcription or silencing (Russ et al., 2012).

The volume concludes with two articles that examine the development of T cell memory following malaria (Krzych et al., 2012) and helminth (Harvie et al., in review) infection. Using the *P. berghei* γ-spz mouse model, Krzych et al. (2012) have identified two populations of intrahepatic memory $CD8^+$ T cells: IFN-γ-producing $CD8^+$ T effector/effector memory cells and $CD8^+$ T central memory cells. They follow with a model that proposes that liver T central memory cells are maintained by IL-15, and that $CD8^+$ T effector/effector memory cells are "conscripted" from this population to prevent re-infection. In contrast to *P. berghei* infection, the adaptive immune response to parasitic roundworm infection is mediated mainly by the effector cytokines produced by $CD4^+$ T cells. Harvie et al. (in review) discuss a number of interesting features of this response including the capacity to generate long-term local protection, particularly in the lung—a site where priming may be sufficient to confer protection.

In summary, these articles present a range of aspects of immune memory formation that are currently under investigation. They bring together current knowledge and models that transport us from the initial signaling events at the cell surface to the internal workings that guide the fate decisions of memory T cells in dealing with different types of infections. Furthermore, they highlight the challenges that still confront the field in unraveling what defines memory and how these characteristics are optimized during infection or designing strategies for vaccination.

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