

# Protective Capacity of Memory CD8<sup>+</sup> T Cells Is Dictated by Antigen Exposure History and Nature of the Infection

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## SUMMARY

Infection or vaccination confers heightened resistance to pathogen rechallenge because of quantitative and qualitative differences between naive and primary memory T cells. Herein, we show that secondary (boosted) memory CD8<sup>+</sup> T cells were better than primary memory CD8<sup>+</sup> T cells in controlling some, but not all acute infections with diverse pathogens. However, secondary memory CD8<sup>+</sup> T cells were less efficient than an equal number of primary memory cells at preventing chronic LCMV infection and are more susceptible to functional exhaustion. Importantly, localization of memory CD8<sup>+</sup> T cells within lymph nodes, which is reduced by antigen restimulation, was critical for both viral control in lymph nodes and for the sustained CD8<sup>+</sup> T cell response required to prevent chronic LCMV infection. Thus, repeated antigen stimulation shapes memory CD8<sup>+</sup> T cell populations to either enhance or decrease per cell protective immunity in a pathogen-specific manner, a concept of importance in vaccine design against specific diseases.

## INTRODUCTION

The ability to quickly and specifically eliminate recurring infections is a hallmark of immunological memory. Thus, the generation of quality memory CD8<sup>+</sup> T cells is an appealing goal for vaccine design against a variety of infectious diseases (Harty and Badovinac, 2008; Kaech et al., 2002; Prlic et al., 2007). Although many studies have demonstrated the enhanced protective capacity of primary memory CD8<sup>+</sup> T cells compared to naive cells, much less is understood about the function and properties of memory CD8<sup>+</sup> T cells that have been exposed to additional rounds of antigenic stimulation through either recurring infections or from booster immunizations.

Recent experimental evidence has suggested that antigen restimulation can dramatically impact both the phenotype and function of the ensuing memory CD8<sup>+</sup> T cell population (Badovinac et al., 2003; Grayson et al., 2002; Jabbari and Harty, 2006;

Masopust et al., 2006; Unsoeld and Pircher, 2005). Specifically, secondary memory CD8<sup>+</sup> T cells populations express increased amounts of Granzyme B, exhibit increased cytolytic activity, and are more protective than primary memory cells against acute infection with *Listeria monocytogenes* (LM) (Jabbari and Harty, 2006). This suggests that additional antigen encounters can not only increase the overall number of antigen-specific CD8<sup>+</sup> T cells, but also result in specific biological changes that impact the per-cell protective capacity of the memory populations. However, it is unknown whether antigen restimulation increases the per-cell protective capacity of memory CD8<sup>+</sup> T cells against pathogens other than LM.

Infection with a pathogenic agent can be broadly defined as either being acute or chronic. Microbes such as LM, vaccinia virus (VacV), lymphocytic choriomeningitis virus (LCMV) Armstrong, and mouse hepatitis virus (MHV) cause acute infections, in which the pathogen is either eliminated or causes mortality. In nonlethal infections with these pathogens, antigen-specific CD8<sup>+</sup> T cell numbers peak after clearance and, after contraction, progress into long-lived primary memory populations. In contrast, infection with agents that result in chronic infection, such as LCMV clone 13, causes antigen-specific CD8<sup>+</sup> T cells undergoing a primary response to become functionally exhausted during the disease course (Wherry et al., 2007). However, it is currently unknown whether primary or secondary memory CD8<sup>+</sup> T cells are more efficient at controlling different types of acute or chronic infections. In addition, it is also unknown whether memory CD8<sup>+</sup> T cells exhibit similar characteristics of functional exhaustion compared to naive CD8<sup>+</sup> T cells during chronic infection.

Ideally, booster immunizations will result in the generation of increased numbers of memory CD8<sup>+</sup> T cells (Woodland, 2004) because this number strongly correlates with providing host protection (Badovinac et al., 2003; Harty and Badovinac, 2008; Kaech et al., 2002; Schmidt et al., 2008). In contrast to laboratory animal studies, the ability to reach large numbers of antigen-specific memory CD8<sup>+</sup> T cells in vaccinated humans has proven difficult (Hill et al., 2010; Masopust, 2009). In addition, a recent study in our laboratory demonstrated that repeated antigenic stimulations have a profound impact on the overall gene expression profile of the ensuing memory CD8<sup>+</sup> T cell populations (Wirth et al., 2010). Collectively, this suggests that antigen-restimulation-associated changes in memory CD8<sup>+</sup> T cell populations may not always accompany large increases in cell number.

Thus, it is critical to determine whether multiple antigen encounters alter the per-cell protective capacity of memory cell populations against diverse pathogens because these changes in gene expression may influence the “quality,” and, therefore, the “threshold number” of memory CD8<sup>+</sup> T cells required to provide host protection (Schmidt et al., 2008).

## RESULTS

### Secondary Antigen Encounter Impacts Pathogen-Specific Memory CD8<sup>+</sup> T Cell-Mediated Protective Immunity

Our previous studies using the OT-I CD8<sup>+</sup> TCR-tg model demonstrated that secondary memory CD8<sup>+</sup> T cells protect better against LM expressing ovalbumin (LM-OVA) than primary memory cells (Jabbari and Harty, 2006). These data raised the question of whether enhanced protection by secondary memory CD8<sup>+</sup> T cells was universal or specific for certain types of pathogens or antigens. To address this, we generated primary and secondary memory P14 TCR-tg CD8<sup>+</sup> T cells (specific for LCMV gp33-41) (Pircher et al., 1989) with adoptive transfers and LCMV Armstrong infections (Figure S1A). At 60+ days after infection, both populations of memory cells were analyzed for expression of a variety of phenotypic markers, cytokine production, and sensitivity to antigen (Figures S1B–S1F). Specifically, primary memory CD8<sup>+</sup> T cell populations contained more CD62L<sup>hi</sup> cells and produced more IL-2 than secondary memory T cells, but both populations were equal producers of IFN- $\gamma$  and TNF- $\alpha$ . Importantly, using a transfer model of bulk polyclonal CD8<sup>+</sup> T cells and LCMV Armstrong infections, we observed similar functional characteristics including cytokine production and sensitivity to antigen in endogenous primary and secondary polyclonal memory CD8<sup>+</sup> T cell populations specific for multiple LCMV-derived antigens (JCN and JTH, data not shown). Thus, primary and secondary memory P14 TCR-tg CD8<sup>+</sup> T cells recapitulate both the phenotype and functionality observed with endogenous populations of memory CD8<sup>+</sup> T cells.

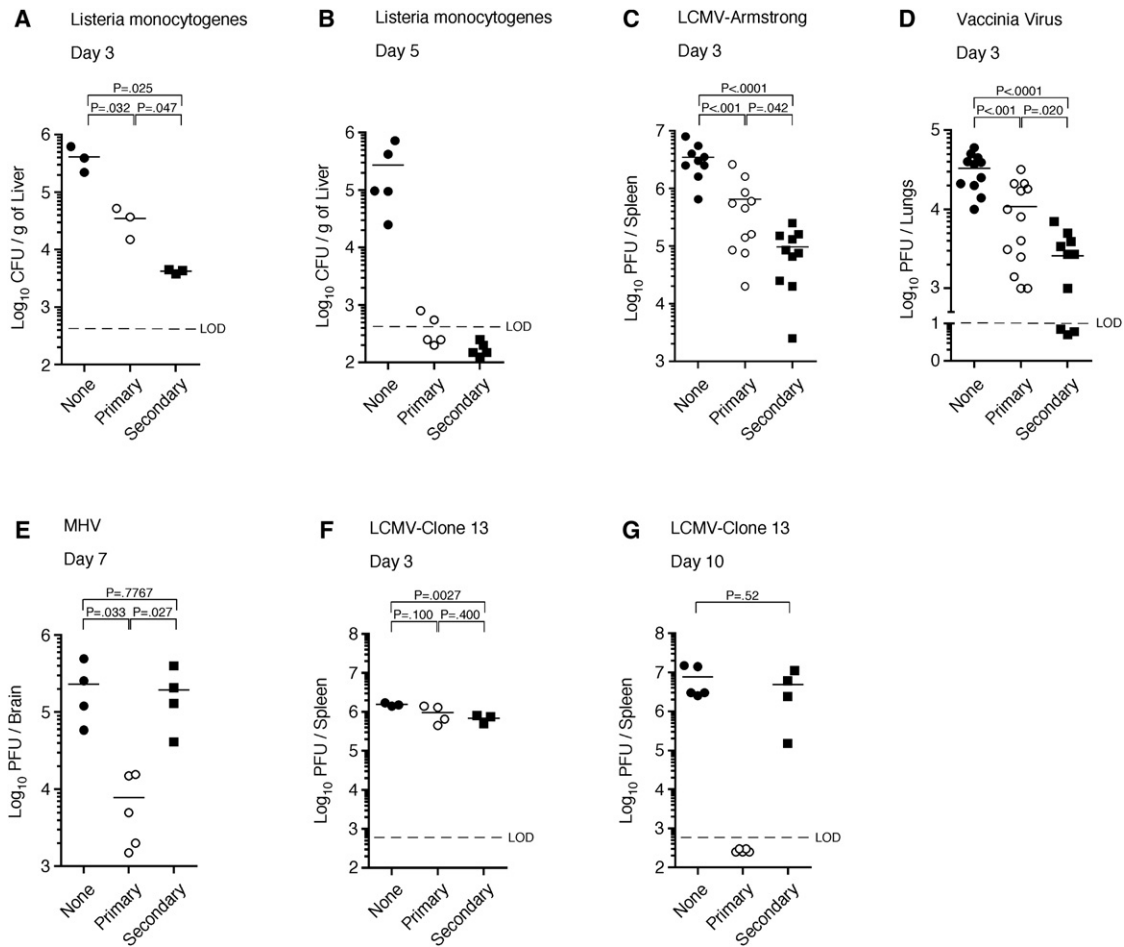
We next determined whether the enhanced protection by secondary memory OT-I CD8<sup>+</sup> T cells seen against LM-OVA could be extended to P14 CD8<sup>+</sup> T cells and the gp33 epitope from LCMV. To test this, we purified equal numbers of primary and secondary memory P14 CD8<sup>+</sup> T cells and transferred them into naive recipients prior to infection with LM-gp33. In agreement with our previous data (Jabbari and Harty, 2006), secondary memory P14 CD8<sup>+</sup> T cells were better than an equal number of primary memory CD8<sup>+</sup> T cells at reducing bacterial load after LM infection on day 3 postinfection, although both populations efficiently cleared the pathogen by day 5 (Figures 1A and 1B). To determine whether this finding could be generalized, we also compared the protective capacity of memory CD8<sup>+</sup> T cells by using three other acute infection models. Indeed, secondary memory CD8<sup>+</sup> T cells were also better at reducing viral load during a systemic, acute viral infection with LCMV-Armstrong (Figure 1C) and during an acute lung infection with VacV-gp33 (Figure 1D). In contrast, primary memory CD8<sup>+</sup> T cells protected better during a uniformly lethal infection with the neurotropic strain of MHV-gp33 on day 7 postinfection (Figure 1E), suggesting that primary memory CD8<sup>+</sup> T cells may be important for controlling acute infections at later timepoints.

These data show that secondary antigen encounter may either increase or decrease memory CD8<sup>+</sup> T cell-mediated protective immunity during acute infection.

Booster immunizations are often administered to provide enhanced protection against a number of acute infections in humans. However, attempts to create a memory CD8<sup>+</sup> T cell population capable of preventing chronic infections, such as HIV and Hepatitis C Virus, have been less successful (Autran et al., 2004; Berzofsky et al., 2004; Klenerman and Hill, 2005). To this end, we next tested whether a second antigen stimulation would also increase the per cell ability of a CD8<sup>+</sup> T cell population to prevent chronic LCMV infection. Naive mice infected with LCMV clone 13 exhibit high viral titers in the spleen on both day 3 and 10 postinfection, indicative of an established chronic infection. In agreement with our acute infection models, secondary memory CD8<sup>+</sup> T cells modestly reduced viral levels in the spleen on day 3 postinfection, whereas primary memory CD8<sup>+</sup> T cells did not (Figure 1F). However, by day 10 postinfection, primary memory CD8<sup>+</sup> T cells completely prevented the establishment of the chronic infection, whereas an equal number of secondary memory CD8<sup>+</sup> T cells failed to clear the infection and viral titers in the spleen resembled those of naive mice receiving no memory CD8<sup>+</sup> T cells (Figure 1G). These data demonstrate that secondary antigenic stimulation decreased the per-cell ability of the resulting memory CD8<sup>+</sup> T cell population to prevent chronic LCMV infection. Collectively, these results suggest the intriguing notion that qualitative changes in the memory CD8<sup>+</sup> T cell populations resulting from repeated antigen stimulation could either improve or limit pathogen-specific immunity.

### Secondary Memory CD8<sup>+</sup> T Cells Become Functionally Exhausted during Chronic LCMV Infection

The LCMV clone 13 infection model has revealed that chronic infection profoundly influences the responding T cells in ways that are also observed in chronic infections of humans (Day et al., 2006). The effects of chronic infection on a CD8<sup>+</sup> T cell population responding to LCMV clone 13 in a previously naive host include loss of cytokine production (such as TNF- $\alpha$ ), expression of inhibitory receptors, and a dependence on antigen and proliferation for CD8<sup>+</sup> T cell survival (Shin et al., 2007; Shin and Wherry, 2007; Wherry et al., 2004; Wherry et al., 2003a; Wherry et al., 2007). Because secondary memory CD8<sup>+</sup> T cells were less efficient than primary memory cells at preventing chronic LCMV infection, we next analyzed whether this cell population exhibited characteristics of functional exhaustion during the infection. On day 7 after LCMV clone 13 infection, endogenous CD8<sup>+</sup> T cells specific for gp33 and adoptively transferred naive P14 CD8<sup>+</sup> T cells undergoing a primary response exhibited functional exhaustion, with only ~35% of the IFN- $\gamma$ + population also producing TNF- $\alpha$  (Figure 2A, top two rows). In contrast, a large fraction (65%) of primary memory P14 CD8<sup>+</sup> T cells undergoing a secondary response, which cleared the virus from the spleen by day 10 (Figure 1F), were capable of producing TNF- $\alpha$  at the same time point (Figure 2A, third row). However, secondary memory CD8<sup>+</sup> T cells undergoing a tertiary response, which failed to clear the virus, were markedly impaired in TNF- $\alpha$  production (Figure 2A, bottom row). This result demonstrates that secondary memory CD8<sup>+</sup> T cells undergoing a tertiary



**Figure 1. Secondary Memory CD8<sup>+</sup> T Cells Provide Better Protection against Infections with LM, LCMV-Armstrong, and VacV, but Decreased Protection against MHV and LCMV Clone 13**

(A and B) Naive B6 mice receiving either no cells (None) or  $2.5 \times 10^5$  memory P14 TCR-tg CD8<sup>+</sup> T cells (primary or secondary) were challenged with  $1 \times 10^5$  CFU of virulent LM-gp33. Bacterial burdens were analyzed in the liver on (A) day 3 or (B) day 5 postinfection.

(C) Same as (A), except that  $5.0 \times 10^5$  memory cells were transferred and mice were challenged with  $2 \times 10^5$  PFU of LCMV Armstrong. Viral titers were analyzed in the spleen on day 3 postinfection.

(D) Same as (A), except that  $3.0 \times 10^5$  memory cells were transferred and mice were infected intranasally with  $1 \times 10^7$  PFU of VacV-gp33. Viral titers were analyzed in the lung on day 3 postinfection.

(E) Same as (C), except that mice were infected intranasally with  $1 \times 10^5$  PFU of MHV-gp33 and titers were analyzed in the brain on day 7 postinfection.

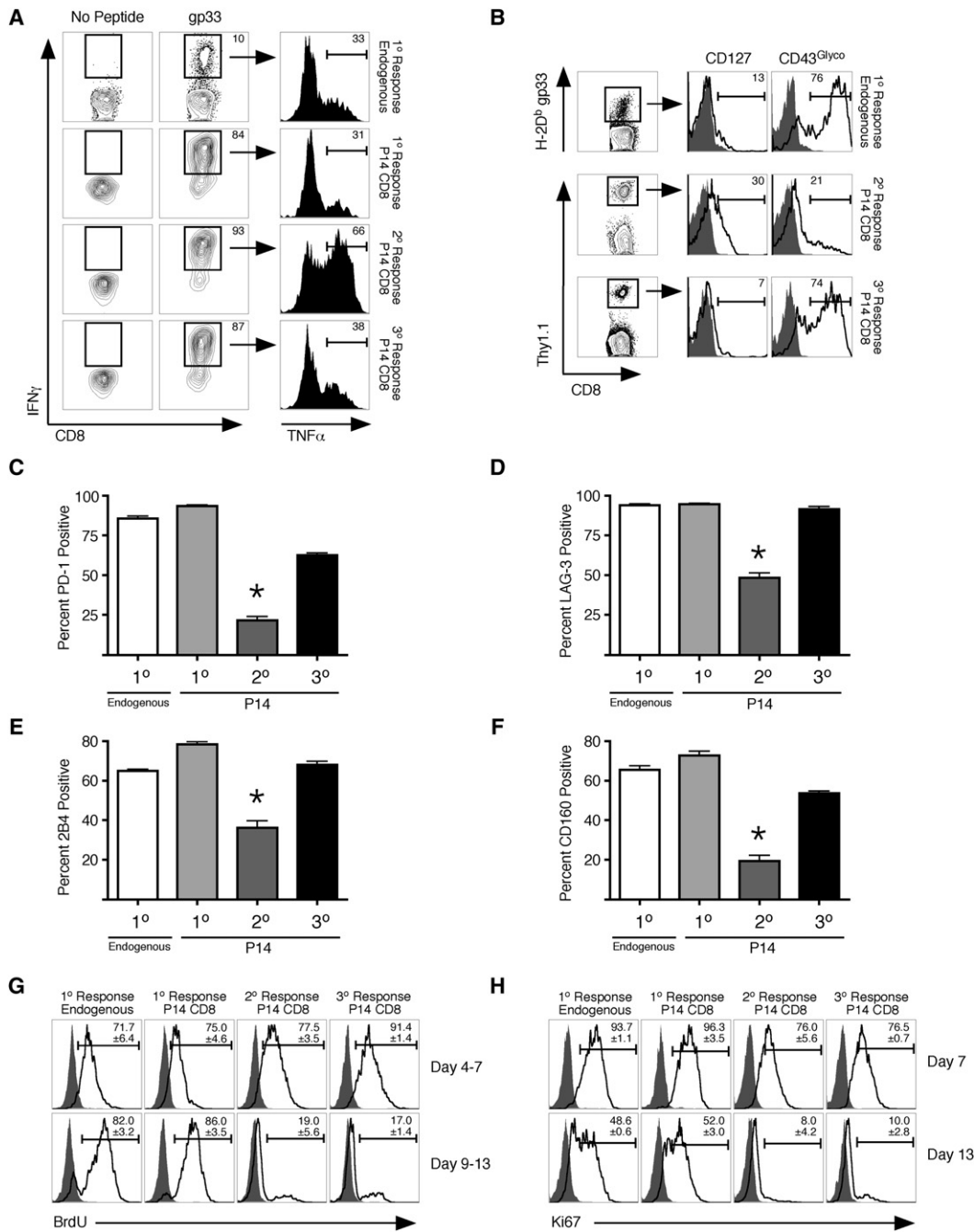
(F and G) Same as (A) except mice were challenged with  $2 \times 10^6$  PFU of LCMV clone 13. Viral titers were analyzed in the spleen on (F) day 3 and (G) day 10 postinfection. Dashed line indicates limit of detection (LOD). Statistical analyses employed the student's t test (see also Figure S1).

response become functionally exhausted during early stages of chronic viral infection.

Along with the loss of cytokine production, exhausted T cells also exhibit a phenotype that includes molecular indications of continued antigenic stimulation and increases in inhibitory receptor expression (Blackburn et al., 2009; Mueller and Ahmed, 2009). Indeed, secondary memory P14 CD8<sup>+</sup> T cells undergoing a tertiary response are impaired in re-expression of CD127 and continue to express the glycosylated isoform of CD43 (Figure 2B). In addition, a similarly high percentage of CD8<sup>+</sup> T cells expressing a variety of inhibitory receptors (Blackburn et al., 2009) including PD-1, LAG-3, 2B4, and CD160 (Figures 2C–2F and Figure S2A) were observed in tertiary responses compared with exhausted CD8<sup>+</sup> T cells undergoing a primary response. In

contrast, expression of these receptors on CD8<sup>+</sup> T cells during a secondary response is substantially reduced. Collectively, these data demonstrate that secondary memory CD8<sup>+</sup> T cells display a phenotype of functional exhaustion during a tertiary response when they fail to prevent chronic LCMV infection.

We next determined whether secondary memory CD8<sup>+</sup> T cells were also more susceptible to functional exhaustion than primary memory cells when antigen load remained constant. To test this, we cotransferred a small number of Thy1-disparate primary and secondary memory P14 CD8<sup>+</sup> T cells into naive animals and subsequently infected them with LCMV clone 13. Importantly, this number of mixed memory CD8<sup>+</sup> T cells is unable to prevent the chronic infection (Figure S2B). In this scenario, primary memory CD8<sup>+</sup> T cells underwent much greater



**Figure 2. Secondary Memory CD8<sup>+</sup> T Cells Develop a Unique Phenotype of Functional Exhaustion during Chronic LCMV Infection**

(A) Naive mice receiving either no T cells (1<sup>o</sup> Response, Endogenous), 500 naive P14 CD8<sup>+</sup> T cells (1<sup>o</sup> Response, P14 CD8<sup>+</sup>), 2.5 × 10<sup>5</sup> primary (2<sup>o</sup> Response, P14 CD8<sup>+</sup>) or secondary memory P14 CD8<sup>+</sup> T cells (3<sup>o</sup> Response, P14 CD8<sup>+</sup>) were infected with 2 × 10<sup>6</sup> PFU of LCMV clone 13. On day 7 postinfection, cells were obtained from blood of infected animals and stimulated ex vivo with gp33 peptide for 5 hr. IFN- $\gamma$ - and TNF- $\alpha$ -positive cells were identified with intracellular staining.

(B) Adoptive transfers and infections were performed as in (A). On day 25 postinfection, cells were obtained from spleen and P14 CD8<sup>+</sup> T cells, or endogenous gp33-specific T cells were analyzed for expression of CD127 and the glycosylated isoform of CD43.

(C–F) Adoptive transfers and infections were performed as in (A). On day 15 postinfection, expression of (C) PD-1, (D) LAG-3, (E) 2B4, and (F) CD160 was analyzed on the indicated CD8<sup>+</sup> T cells in spleen. Representative histograms are shown in Figure S2. For (C)–(F), \*p < 0.001 compared to all other groups with the Student's t test.

(G) Same as (A). Mice were then pulsed with BrdU on days 4–7 or 9–13. Representative BrdU incorporation profiles are shown from P14 CD8<sup>+</sup> T cells from spleen.

(H) Same as (G), except that expression of Ki67 was assessed on day 7 or 13 postinfection. For (G) and (H), numbers indicate the mean and SD from three independent mice per group (see also Figure S2).



expansion than secondary memory after infection (Figure S2C). In addition, on day 7 postinfection, the percentage of secondary memory CD8<sup>+</sup> T cells undergoing a tertiary response that were able to produce IFN- $\gamma$  and TNF- $\alpha$  was substantially lower than primary memory cells undergoing a secondary response in the same host (Figures S2D and S2E). However, both populations became functionally exhausted by day 13 postinfection. High expression of inhibitory receptors was found on all cell populations regardless of antigen exposure history (Figures S2F–2I). Thus, secondary memory CD8<sup>+</sup> T cells are inherently more “exhaustible” than primary memory cells.

### Secondary Memory CD8<sup>+</sup> T Cells Do Not Continue to Proliferate during Chronic LCMV Infection

Besides displaying increased expression of a variety of inhibitory receptors and losing the ability to produce cytokines, prior studies have demonstrated that CD8<sup>+</sup> T cells undergoing a primary response continue to proliferate during the course of chronic viral infection (Shin et al., 2007; Wherry et al., 2004). However, it remains unclear whether sustained proliferation is required for the molecular changes associated with CD8<sup>+</sup> T cell exhaustion. During the early stages of LCMV clone 13 infection (days 4–7), all CD8<sup>+</sup> T cell populations proliferated, as demonstrated by BrdU incorporation and Ki67 expression (a nuclear marker of cellular proliferation) during that time period. In agreement with previous studies (Shin et al., 2007; Wherry et al., 2004), both naive endogenous and P14 CD8<sup>+</sup> T cells continued to proliferate during the primary response after the peak day of expansion (days 9–13) (Figures 2G and 2H). In contrast, primary memory CD8<sup>+</sup> T cells undergoing a secondary response undergo minimal proliferation during that time frame because the infection has been cleared in these animals (Figure 1F). However, secondary memory CD8<sup>+</sup> T cells undergoing a tertiary response did not exhibit sustained proliferation despite failure to clear the chronic infection. Differences in sustained proliferation of memory CD8<sup>+</sup> T cells were also observed in the same host during established chronic infection (Figure S2B), in which primary memory CD8<sup>+</sup> T cells continue to proliferate more than secondary memory cells after the peak of expansion (days 9–13) (Figure S2J). These data demonstrate that although secondary memory CD8<sup>+</sup> T cells display several signs of becoming functionally exhausted during LCMV clone 13 infection, they do not continue to proliferate. These data show that CD8<sup>+</sup> T cell exhaustion in response to chronic infection is not intrinsically linked to sustained proliferation.

### CD62L<sup>hi</sup> Primary and Secondary Memory CD8<sup>+</sup> T cells Both Prevent Chronic LCMV Infection

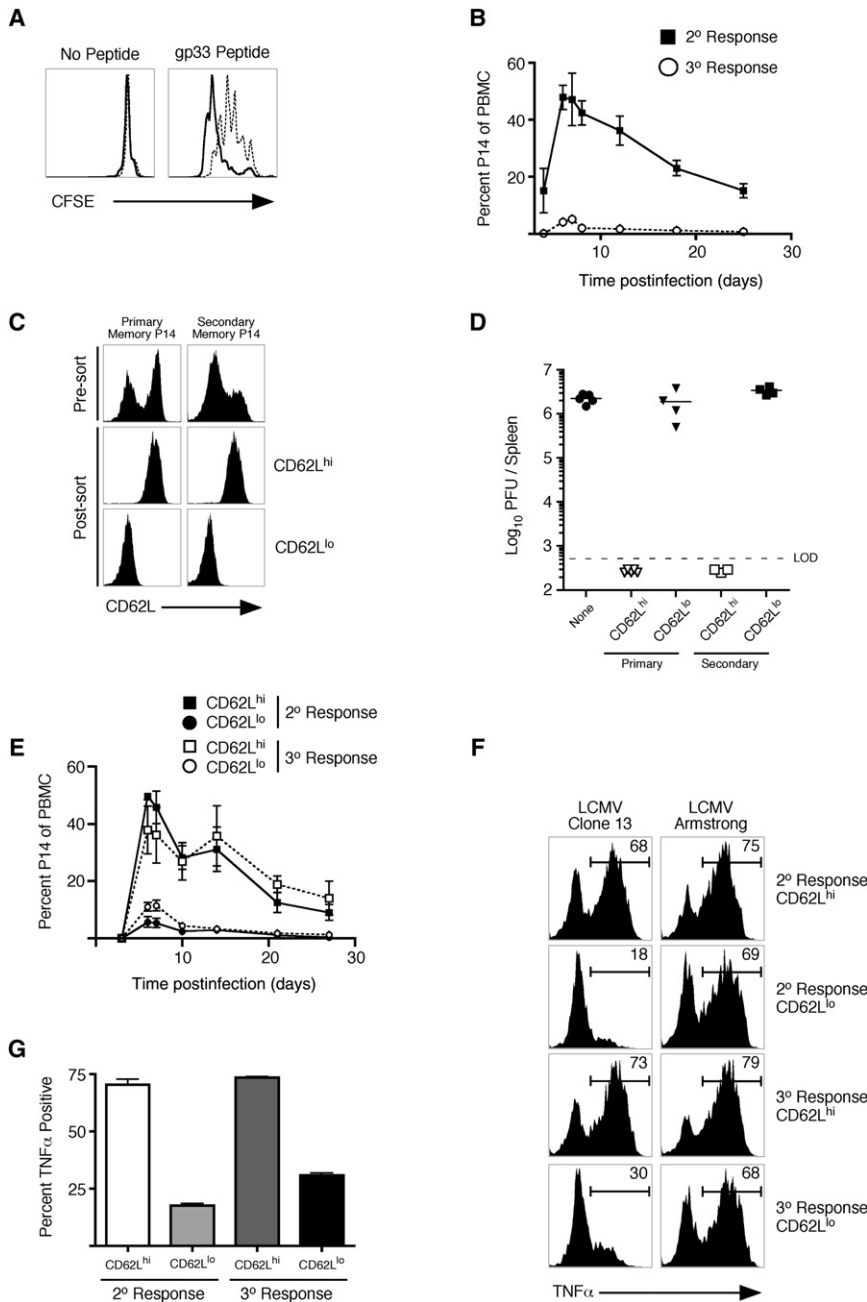
In order to determine the mechanism(s) by which primary memory CD8<sup>+</sup> T cells are more protective against LCMV clone 13 infection than secondary memory CD8<sup>+</sup> T cells, we first analyzed the proliferative capacity of each memory population. When memory P14 CD8<sup>+</sup> T cells were stimulated directly ex vivo with gp33 peptide, primary memory CD8<sup>+</sup> T cells underwent more rounds of cell division than secondary memory cells (Figure 3A). Secondary memory CD8<sup>+</sup> T cells produce less IL-2 than primary memory CD8<sup>+</sup> T cells, which could impact differences in TCR-mediated proliferation. However, addition of excess exogenous IL-2 did not rescue the differences in prolifer-

ation observed in the two populations (Figure S3A). In addition, primary memory CD8<sup>+</sup> T cells also underwent greater expansion than secondary memory CD8<sup>+</sup> T cells in response to infections with LCMV clone 13, LM, and LCMV-Armstrong (Figure 3B and Figures S3B and S3C). Collectively, these data suggest that primary memory CD8<sup>+</sup> T cells undergo more vigorous proliferation than secondary memory CD8<sup>+</sup> T cells regardless of the infectious agent. However, in contrast to some previous suggestions (Wherry et al., 2003b), but not others (Huster et al., 2006; Lauvau et al., 2001), this difference in proliferative potential does not always translate into providing greater protection by a memory CD8<sup>+</sup> T cell population because secondary memory CD8<sup>+</sup> T cells proliferate less but protect better against acute infections with LM, LCMV, or VacV (Figure 1).

It has been demonstrated that primary CD62L<sup>hi</sup> (central memory [Tcm]) CD8<sup>+</sup> T cells are better than primary CD62L<sup>lo</sup> (effector memory [Tem]) cells at clearing LCMV clone 13 (Wherry et al., 2003b). To test whether the reduced number of CD62L<sup>hi</sup> cells in the bulk secondary memory population impacted viral clearance, we purified CD62L<sup>hi</sup> and CD62L<sup>lo</sup> populations of both primary and secondary memory CD8<sup>+</sup> T cells (Figure 3C), transferred them into naive recipients, and subsequently infected them with LCMV clone 13. CD62L<sup>hi</sup> memory cells underwent robust expansion, regardless of whether they originated from primary or secondary memory, and were equally efficient in preventing chronic LCMV infection (Figures 3D and 3E). In contrast, the CD62L<sup>lo</sup> populations of both groups expanded less and failed to prevent the chronic infection. Finally, similar to what was seen with secondary memory CD8<sup>+</sup> T cells, CD62L<sup>lo</sup> populations of both primary and secondary memory cells were impaired in TNF- $\alpha$  production after LCMV clone 13 infection (Figures 3F and 3G), indicating enhanced susceptibility to exhaustion. However, this is not an intrinsic characteristic of the population, as all responding CD8<sup>+</sup> T cell populations efficiently produced TNF- $\alpha$  in response to acute LCMV Armstrong infection (Figure 3F). Collectively, these data suggest that the reduced frequency of CD62L<sup>hi</sup> cells in secondary memory CD8<sup>+</sup> T cell populations probably accounted for their failure to prevent chronic LCMV clone 13 infection.

### Memory CD8<sup>+</sup> T Cell Localization into Lymph Nodes Is Required to Prevent Chronic LCMV Infection

It has been argued that primary Tcm cells are more protective than Tem cells during chronic LCMV infection because of their increased proliferative capacity (Wherry et al., 2003b). However, it has not been addressed whether differential trafficking patterns of these subsets can also impact protective immunity, given that Tcm cells localize to lymph nodes more efficiently than Tem cells. To initially determine whether the localization of memory CD8<sup>+</sup> T cells into lymph nodes impacts the ability of these cells to prevent chronic LCMV infection, we transferred primary memory P14 CD8<sup>+</sup> T cells into WT or Lymphotoxin- $\alpha$  (*Lta*)<sup>-/-</sup> mice (which lack lymph nodes) (De Togni et al., 1994) and subsequently infected them with LCMV clone 13. As demonstrated previously, primary memory CD8<sup>+</sup> T cells transferred into WT mice prevent chronic LCMV infection. However, the same number of primary memory CD8<sup>+</sup> T cells transferred into *Lta*<sup>-/-</sup> mice did not prevent the chronic infection (Figure 4A). In contrast, both primary and secondary memory CD8<sup>+</sup> T cells



**Figure 3. CD62L<sup>hi</sup> Memory CD8<sup>+</sup> T Cells Are Equally Protective against Chronic Viral Infection Regardless of Whether They Are Primary or Secondary**

(A) Two million total splenocytes from mice containing either primary (solid line histogram) or secondary (dashed line histogram) memory P14 CD8<sup>+</sup> T cells were labeled with CFSE and were incubated with or without gp33 peptide for 60 hr. Representative CFSE profiles of Thy1.1 memory P14 CD8<sup>+</sup> T cells with and without the addition of antigen are shown.

(B) A total of  $2.5 \times 10^5$  primary and secondary memory P14 CD8<sup>+</sup> T cells (Thy1.1) were purified and adoptively transferred into naive recipients (Thy1.2), which were subsequently infected with LCMV clone 13. Kinetics of the secondary or tertiary CD8<sup>+</sup> T cell response were analyzed in the blood after infection with CD8 and Thy1.1 staining so that the adoptively transferred population could be identified. Error bars represent the SD of three to five mice at each time point.

(C) Analysis of sorted CD62L high (CD62L<sup>hi</sup>)- and low (CD62L<sup>lo</sup>)-expressing P14 CD8<sup>+</sup> T cells from bulk primary and secondary memory populations.

(D) A total of  $1.5 \times 10^5$  cells from each of the groups in (C) were transferred into naive recipients subsequently infected with LCMV clone 13. Viral titers were analyzed in the spleen on day 10 postinfection.

(E) Same as (D), except that kinetics of T cell expansion were monitored over the indicated time frame with CD8 and Thy1.1 staining so that the adoptively transferred populations could be identified. Error bars represent the SD of three samples for each time point.

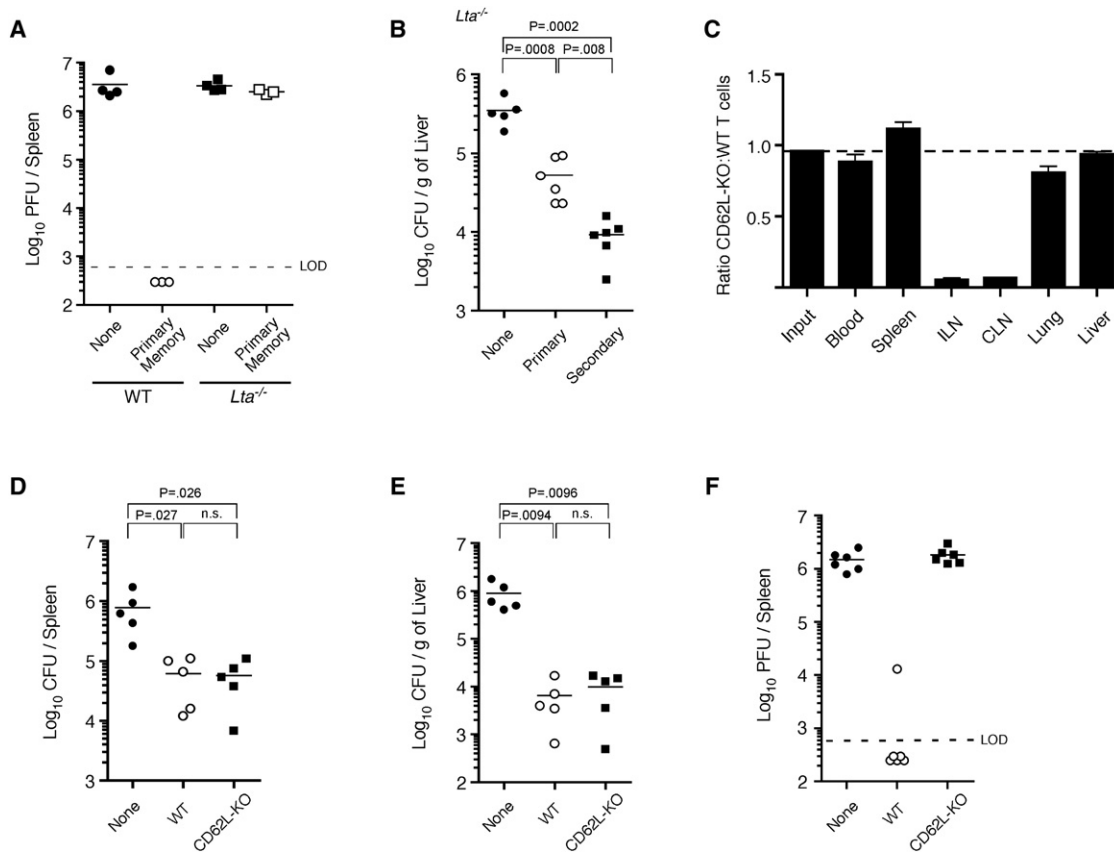
(F) Adoptive transfer of memory populations was performed as in (B), and recipient mice were subsequently infected with either LCMV clone 13 or LCMV Armstrong. On day 14 postinfection, TNF- $\alpha$  production by IFN- $\gamma$ + P14 CD8<sup>+</sup> T cells was assessed with intracellular stain after ex vivo stimulation with gp33 peptide.

(G) Cumulative data of triplicate samples shown in (F). Data are representative of two independent experiments (see also Figure S3).

were able to decrease bacterial burden in *Lta*<sup>-/-</sup> mice after LM infection (Figure 4B). These data demonstrate that lymph node-deficient *Lta*<sup>-/-</sup> mice are specifically unable to prevent chronic LCMV infection, even when a sufficient primary memory population of CD8<sup>+</sup> T cells is present.

Whereas these data suggest that lymph nodes are critical for memory CD8<sup>+</sup> T cell-mediated prevention of chronic LCMV infection, *Lta*<sup>-/-</sup> mice also exhibit other biological and anatomical defects including alterations in splenic architecture (Banks et al., 1995). To more directly address whether lymph node localization was specifically required by the memory CD8<sup>+</sup> T cell compartment in order to prevent chronic LCMV infection, we

generated primary memory P14 CD8<sup>+</sup> T cells that lacked CD62L (CD62L-deficient) (Xu et al., 1996). At 60 days postinfection with LCMV-Armstrong, CD62L-deficient P14 CD8<sup>+</sup> T cells exhibited a similar phenotype (other than CD62L expression) compared to WT cells (Figure S4A). In addition, both WT and CD62L-deficient cells were equally efficient at producing IFN- $\gamma$ , TNF- $\alpha$ , and IL-2, underwent similar ex vivo proliferation in response to gp33 peptide, and expressed equal amounts of CCR7 (Figures S4B–S4G). However, loss of CD62L dramatically impaired localization of these cells into lymph nodes, without affecting distribution into other tissues (Figure 4C and Figure S4H). Thus, naive CD62L-deficient CD8<sup>+</sup> T cells are able to



**Figure 4. Lymph Node Homing of Memory CD8<sup>+</sup> T Cells Is Required for Prevention of Chronic LCMV Infection**

(A) A total of  $2.5 \times 10^5$  primary memory P14 TCR-tg CD8<sup>+</sup> T cells were transferred into either WT or *Lta*<sup>-/-</sup> mice and subsequently infected with LCMV clone 13. Viral burden was analyzed from spleens on day 10 postinfection. Data are representative of two independent experiments.

(B) A total of  $2.5 \times 10^5$  primary or secondary memory P14 CD8<sup>+</sup> T cells were transferred into *Lta*<sup>-/-</sup> mice and subsequently infected with virulent LM-gp33. Bacterial burden was analyzed in the liver on day 3 postinfection.

(C) Equal numbers ( $2 \times 10^6$ ) of WT (Thy1.1/1.1) and CD62L-deficient (CD62L-KO) (Thy1.1/1.2) primary memory P14 TCR-tg CD8<sup>+</sup> T cells were transferred into naive (Thy1.2/1.2) recipients. Forty-eight hours later, organ-specific localization of both cell populations was determined. Representative histograms are shown in Figure S4H (ILN, inguinal lymph node; CLN, cervical lymph node).

(D and E) A total of  $2.5 \times 10^5$  WT or CD62L-deficient primary memory P14 CD8<sup>+</sup> T cells were purified and transferred into naive recipients and subsequently infected with virulent LM-gp33. Bacterial burden was measured in the (D) spleen and (E) liver on day 3 postinfection.

(F) Same as (D and E) except mice were infected with LCMV clone 13, and viral burden was analyzed in the spleen on day 10 postinfection (see also Figure S4). Statistical analyses were performed with the Student's *t* test.

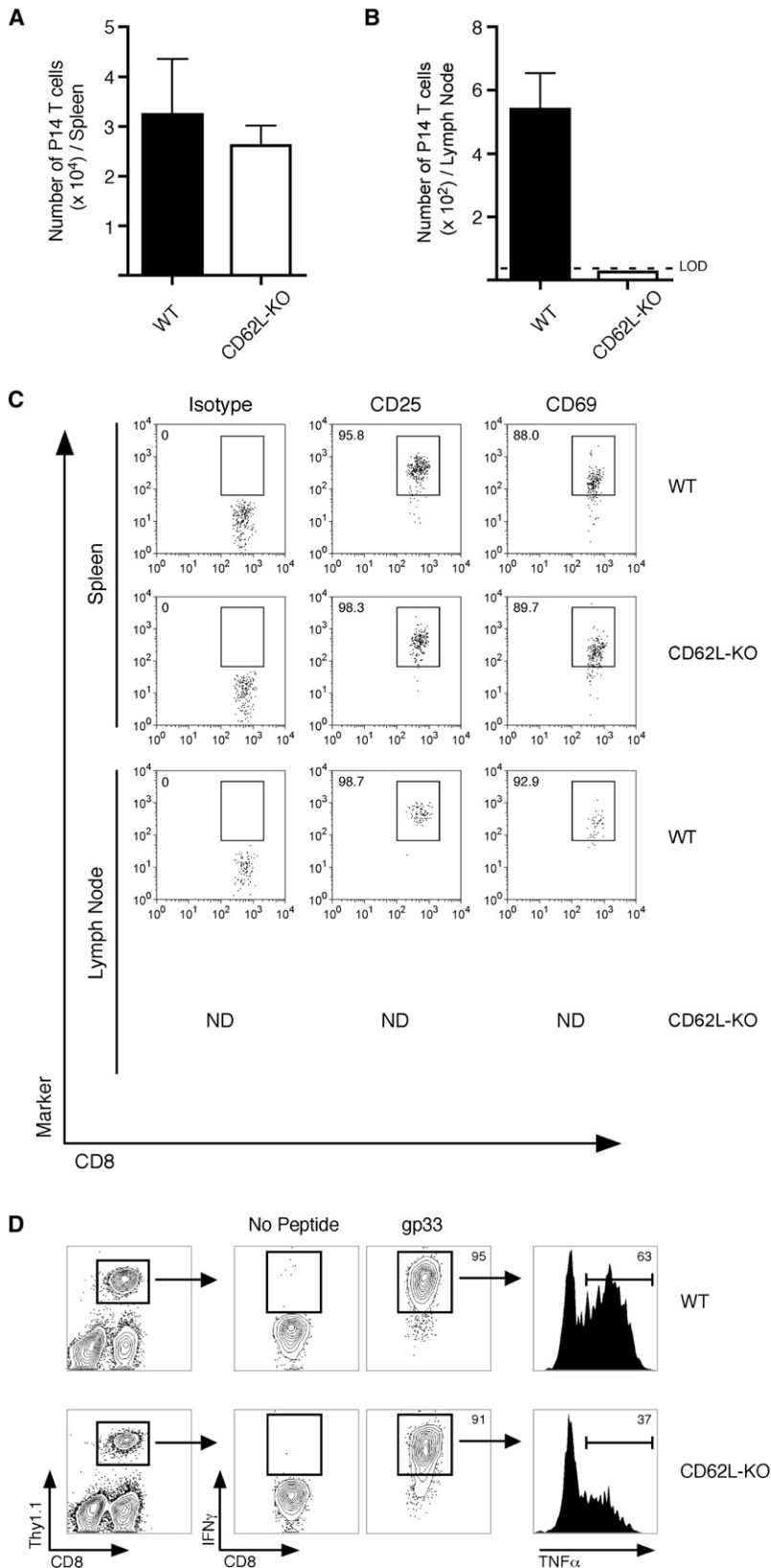
become functional memory cells after LCMV-Armstrong infection, although their ability to localize into lymph nodes is dramatically impaired.

Because CD62L is required for efficient lymph node localization (Arbonés et al., 1994; Catalina et al., 1996; Steeber et al., 1996), we next determined whether expression of CD62L provided any benefit to primary memory CD8<sup>+</sup> T cell populations with regards to protective immunity during both acute and chronic infections. To test this, we transferred equal numbers of both WT and CD62L-deficient primary memory P14 CD8<sup>+</sup> T cells into naive recipients and subsequently infected them with LM-gp33. WT and CD62L-deficient primary memory CD8<sup>+</sup> T cells provided equal protection against LM (Figures 4D and 4E), demonstrating that CD62L on memory CD8<sup>+</sup> T cells does not impact their ability to protect against this acute infection. In contrast, CD62L-deficient primary memory CD8<sup>+</sup> T cells failed to prevent chronic LCMV infection compared to an equal number

of WT cells (Figure 4F). Thus, expression of CD62L on primary memory CD8<sup>+</sup> T cells and the ensuing ability to enter lymph nodes directly impacts the ability of these cells to prevent chronic LCMV infection.

#### Lymph Node Primed Memory CD8<sup>+</sup> T cells Are Required to Prevent Chronic LCMV Infection

To determine mechanistically why lymph node localization is required to prevent chronic LCMV infection, we first analyzed organ-specific activation of both WT and CD62L-deficient primary memory CD8<sup>+</sup> T cells after LCMV clone 13 infection. On day 3 postinfection, equal numbers of both cell populations could be found in the spleen and expressed similar levels of the early activation markers CD25 and CD69 (Figures 5A–5C). In contrast, CD62L-deficient cells could not be found in the lymph nodes at this time point, whereas activated WT cells were readily detected. Furthermore, by day 7 postinfection,



**Figure 5. CD62L-Deficient Primary Memory CD8<sup>+</sup> T Cells Become Activated in the Spleen after LCMV Clone 13 Infection, but Become Functionally Exhausted**

(A and B) A total of  $2.5 \times 10^5$  WT or CD62L-deficient (CD62L-KO) primary memory P14 TCR-tg CD8<sup>+</sup> T cells were transferred into naive B6 mice and subsequently infected with LCMV clone 13. Total numbers of P14 CD8<sup>+</sup> T cells were determined in the (A) spleen or (B) inguinal lymph node on day 3 postinfection.

(C) P14 CD8<sup>+</sup> T cells from (A) were analyzed for expression of CD25 and CD69; ND, none detected.

(D) Adoptive transfers and infection was performed as in (A). On day 7 postinfection, cells were obtained from blood of infected animals and stimulated ex vivo with gp33 peptide for 5 hr. IFN- $\gamma$ - and TNF- $\alpha$ -positive cells were identified with intracellular staining. Data are representative of two independent experiments.



CD62L-deficient memory CD8<sup>+</sup> T cells in the spleen became functionally exhausted and produced less TNF- $\alpha$  than WT cells (Figure 5D). Thus, these data suggest that activation of memory CD8<sup>+</sup> T cells in lymph nodes is required for the prevention of chronic LCMV infection. Furthermore, WT primary memory CD8<sup>+</sup> T cells underwent dramatic expansion in lymph nodes after LCMV clone 13 infection (Figure 6A), which also resulted in decreased viral burden in the lymph node (Figure 6B). In addition, responding WT memory CD8<sup>+</sup> T cells produced significantly more TNF- $\alpha$  than CD62L-deficient cells that emigrated into the lymph node and became detectable on day 7 after infection (Figures 6C and 6D). Therefore, these data demonstrate that activation and expansion of memory CD8<sup>+</sup> T cells in the lymph nodes results in both decreased viral burden and generation of “quality” effector cells after LCMV clone 13 infection.

The robust expansion of WT primary memory CD8<sup>+</sup> T cells in the lymph node after LCMV clone 13 infection suggested that once activated, these cells may play an important role in combating the systemic infection. To test this, we cotransferred equal numbers of WT and CD62L-deficient primary memory CD8<sup>+</sup> T cells and infected recipient mice with LM, LCMV Armstrong, or LCMV clone 13 (Figure 6E). During early time points (day 5) after all infections, more CD62L-deficient than WT T cells could be found in the blood, further demonstrating that early activation and proliferation of CD62L-deficient memory T cells were not impaired. After LCMV Armstrong or LM infection, the representation of each population remained constant until day 10 postinfection. However, by day 10 postinfection with LCMV clone 13, nearly all of the detectable cells in the blood were WT (Figure 6E). These data show that the impaired lymph node localization of CD62L-deficient primary memory T cells prevented a sustained response from this population after LCMV clone 13 infection. This suggests that in addition to impaired viral clearance in lymph nodes, the inability of CD62L-deficient memory CD8<sup>+</sup> T cells to prevent chronic LCMV infection also resulted from the lack of a lymph node-dependent sustained CD8<sup>+</sup> T cell response.

To further evaluate the requirement for egress of lymph node primed memory CD8<sup>+</sup> T cells to prevent chronic LCMV infection, we utilized the sphingosine-1-phosphate receptor agonist FTY720, which inhibits T cell egress from lymph nodes (Brinkmann et al., 2002; Matloubian et al., 2004). Indeed, after LCMV clone 13 infection of mice seeded with primary memory P14 cells, treatment with FTY720 caused an accumulation of activated memory CD8<sup>+</sup> T cells within lymph nodes and decreased numbers in both the spleen and blood (Figures 7A–7C and Figure S5). Importantly, FTY720-mediated trapping of responding primary memory CD8<sup>+</sup> T cells in the lymph node also impaired clearance of LCMV clone 13 (Figure 7D). Although the effect of FTY720 treatment on viral clearance in mice harboring primary memory CD8<sup>+</sup> T cells was not absolute, these data demonstrate that altering the capacity of re-activated memory CD8<sup>+</sup> T cells to leave lymph nodes can dramatically impact their ability to prevent chronic viral infection. Collectively, these data suggest that memory CD8<sup>+</sup> T cell entry into lymph nodes, activation, expansion, and subsequent egress are required for the sustained CD8<sup>+</sup> T cell response necessary to prevent chronic infection with LCMV clone 13. Thus, the altered tissue localization of either CD62L-deficient primary or of boosted secondary

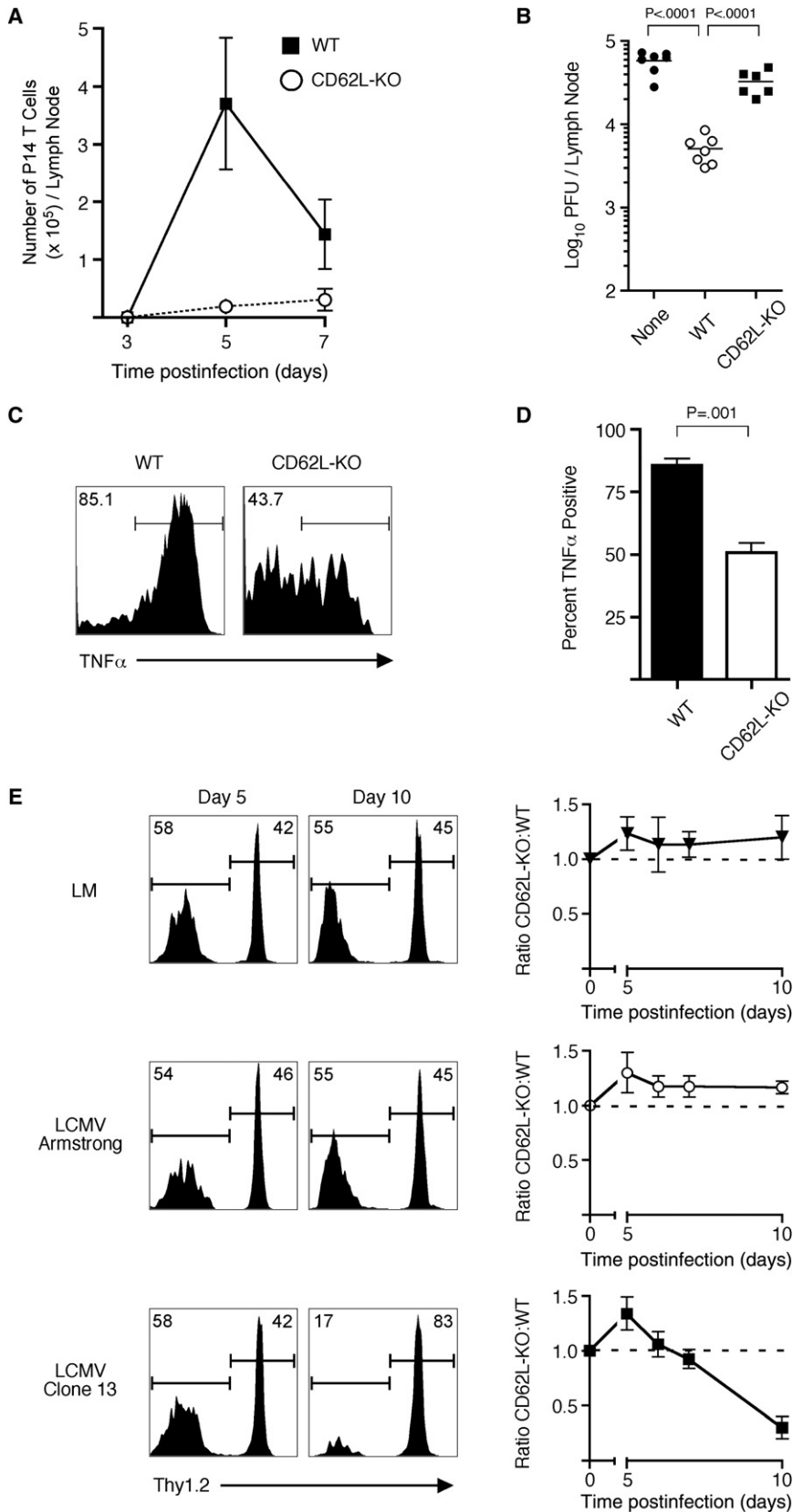
memory CD8<sup>+</sup> T cells directly impacts the per-cell protective capacity of these populations against a specific pathogen.

## DISCUSSION

Although a great deal of effort has been dedicated to the understanding of the factors and mechanisms that ultimately lead to the generation of primary memory CD8<sup>+</sup> T cells, much less is known about memory CD8<sup>+</sup> T cell populations that are generated after additional antigen restimulations. Herein, we demonstrate that antigen restimulation increases the ability for a memory CD8<sup>+</sup> T cell population to protect against acute infections with several pathogens. However, antigen restimulation decreases the per-cell ability of memory CD8<sup>+</sup> T cells to protect against acute, lethal MHV infection and to prevent chronic LCMV infection. Because the model of chronic LCMV infection has provided a wealth of knowledge to the field of experimental immunology, we went on to delineate the individual factors that contribute to both the ability for primary memory CD8<sup>+</sup> T cells to effectively control the infection and those that contribute to the impaired protection by secondary memory CD8<sup>+</sup> T cells.

Previous studies report that re-expression of CD62L is delayed in memory populations generated by multiple antigen encounters (Jabbari and Harty, 2006; Masopust et al., 2006). However, because expression of this molecule also tracks with the differentiation state of primary memory CD8<sup>+</sup> T cells (Tcm versus Tem cells), it was unknown whether localization alone or other genetic factors associated with the specific differentiation state were the driving force behind the ability for Tcm cells to efficiently prevent chronic LCMV infection (Wherry et al., 2003b). We show that CD62L-deficient primary memory CD8<sup>+</sup> T cells are similar to WT cells with regards to function, phenotype, and ability to protect against virulent LM infection. However, genetic ablation of this single molecule specifically inhibits memory CD8<sup>+</sup> T cell distribution into lymph nodes, but not to other tissues of the body. Therefore, our data strongly argue that, from a mechanistic standpoint, CD62L-dependent lymph node localization of memory CD8<sup>+</sup> T cells is critical for maximum defense (both viral control in lymph nodes and sustained recall response) against chronic LCMV infection.

In this report, we also identify three key aspects of memory CD8<sup>+</sup> T cell biology that change after a subsequent antigen encounter that impair their ability to prevent chronic LCMV infection. First, on a population level, secondary memory CD8<sup>+</sup> T cells not only become “exhausted” during chronic LCMV infection, but are inherently more “exhaustible” than a primary memory population. Second, the ability to mount a robust recall response is diminished in secondary memory CD8<sup>+</sup> T cells. Third, the ability to localize to lymph nodes decreases in secondary memory cells due to delayed re-expression of CD62L. Although we provide substantial, additional evidence that lymph node localization is critical for conferring host protection against chronic LCMV infection, this finding is complicated by the fact that hundreds of genes are differentially regulated between primary and secondary memory CD8<sup>+</sup> T cells (Wirth et al., 2010). Thus, it is highly likely that several linked mechanisms act in concert to cause the overall failure of secondary memory CD8<sup>+</sup> T cells to prevent chronic LCMV infection.



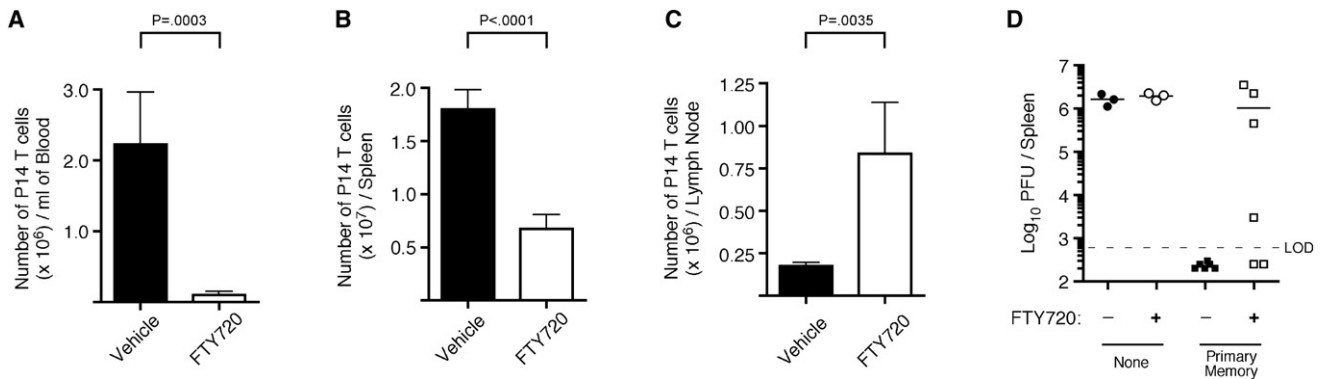
**Figure 6. Lymph Node Localization of Memory CD8<sup>+</sup> T Cells Results in Enhanced Viral Clearance and Sustained Effector Response during LCMV Clone 13 Infection**

(A) A total of  $2.0 \times 10^5$  WT or CD62L-deficient (CD62L-KO) primary memory CD8<sup>+</sup> T cells were transferred into naive recipients and subsequently infected with LCMV clone 13. On days 3, 5, and 7, the total number of transferred cells was quantified in the inguinal lymph nodes.

(B) Viral burden in the inguinal lymph node from (A) was determined on day 5 postinfection.

(C and D) Same as (A), except that on day 7 postinfection, lymph node cells were stimulated with gp33 peptide and TNF- $\alpha$  production was analyzed on IFN- $\gamma$ + cells with intracellular staining.

(E) Equal numbers ( $1 \times 10^4$ ) of WT (Thy1.1/1.2) or CD62L-deficient (Thy1.1/1.1) primary memory P14 TCR-tg CD8<sup>+</sup> T cells were transferred into naive B6 mice and subsequently infected with virulent LM-gp33, LCMV-Armstrong, or LCMV clone 13. On days 5, 6, 7, and 10, representation of each cell population was determined in the blood with Thy1.1 and Thy1.2 staining. Representative histograms from days 5 and 10 show percentages of CD62L-deficient (Thy1.2-negative) and WT (Thy1.2-positive) P14 CD8<sup>+</sup> T cells. Dashed line indicates input ratio. Error bars represent the SD of individual samples at each time point, statistical analyses were performed with the Student's T test, and data are representative of two independent experiments with three mice per group.



**Figure 7. Blocking Activated Memory CD8<sup>+</sup> T Cell Egress from Lymph Nodes after LCMV Clone 13 Infection Results in Impaired Viral Clearance**

(A–C) A total of  $1.5 \times 10^5$  primary memory P14 TCR-tg CD8<sup>+</sup> T cells were transferred into naive B6 mice and subsequently infected with LCMV clone 13. Mice were then treated with FTY720 or vehicle control on days 0, 2, and 4 postinfection. On day 6 postinfection, numbers of P14 CD8<sup>+</sup> T cells were determined in the (A) blood, (B) spleen, or (C) inguinal lymph node. Representative dot plots for each organ are shown in Figure S7. For (A)–(C), error bars represent the SD and statistical analysis was performed with the Student's *t* test. Data are representative of two independent experiments with five mice per group.

(D) Same as (A), except that mice were treated with FTY720 or vehicle control on days 0, 2, 4, 6, and 8 postinfection. On day 10 postinfection, viral burden from individual mice was determined in the spleens of infected animals. Cumulative data from two independent experiments are shown (see also Figure S5).

Current vaccination strategies have proven successful for the prevention of a variety of acute infections (de Quadros, 2002). In these cases, the establishment of a sufficient memory immune repertoire limits pathogen spread and replication, and elimination of the infectious agent occurs without onset of severe disease symptoms. However, vaccines against infectious agents that cause persistent or chronic infections have been far less successful (Berzofsky et al., 2004). Chronic viral infections are a major cause of not only a number of human diseases (HIV, hepatitis C virus), but are also thought to contribute to the development of a variety of human cancers (EBV—B cell lymphomas, human papillomavirus virus—cervical cancer). In virtually all these cases, failure to completely eliminate the pathogen leads to the generation of CD8<sup>+</sup> T cells that are functionally exhausted. In fact, it has been demonstrated that human CD8<sup>+</sup> T cells specific for antigens of HIV, EBV, and CMV express inhibitory receptors and are CD62L<sup>lo</sup> (Chen et al., 2001; Day et al., 2006). However, other aspects of pathogen biology, such as rapid mutation, also probably contribute to the establishment of chronic infection in humans. In addition, our data also suggest that the duration of antigen or pathogen load during infection (such as in MHV and chronic LCMV infection) may also be a determining factor as to which type of memory CD8<sup>+</sup> T cell population is most protective. Although it is unclear whether entry into lymph nodes will be an essential feature of protective CD8<sup>+</sup> T cells against all chronic infections, it is possible that driving memory CD8<sup>+</sup> T cells away from lymph nodes through repeated antigen encounters may be advantageous to the persistence of these types of pathogens. Clearly, the “best” memory CD8<sup>+</sup> T cell population is going to vary from pathogen to pathogen, dependent upon variables including the site of infection, microbial persistence, and cellular targets of the pathogen.

In conclusion, our data demonstrate that booster immunizations may not only increase the overall number of memory CD8<sup>+</sup> T cells, but also leads to biologically relevant functional alterations that can either increase or decrease the per-cell

protective capacity of the memory cell population for specific pathogens. Because vaccines against chronic infections have been difficult to develop, these data provide direct evidence that both the quality and quantity of a memory CD8<sup>+</sup> T cell population must be taken into account when developing vaccination strategies that are able to prevent specific diseases.

## EXPERIMENTAL PROCEDURES

### Mice and Pathogens

C57BL/6 mice (Thy1.2) were obtained from the National Cancer Institute and used for experiments at 6–10 weeks of age. P14 transgenic mice (Thy1.1) (Pircher et al., 1989) were provided by M. Bevan and B6.PL (Thy1.1) were obtained from Jackson Laboratories and maintained by sibling × sibling mating. Lymphotoxin- $\alpha$  (*Lta*)-deficient mice (De Togni et al., 1994) and CD62L (*Sei*)-deficient mice (Xu et al., 1996) have been previously described. LCMV Armstrong and LCMV clone 13 were propagated according to standard protocols. LCMV Armstrong ( $2 \times 10^5$  PFU) was injected i.p. as indicated. LCMV clone 13 ( $2 \times 10^6$  PFU) was injected i.v. Vaccinia virus expressing full-length LCMV glycoprotein (VacV-gp) was kindly provided by E. Butz and was propagated in accordance to standard protocols. VV-gp was given intranasally in 40  $\mu$ l of saline. Virulent *Listeria monocytogenes* expressing gp33 (LM-gp33) (Kaech and Ahmed, 2001) was grown and injected i.v. Recombinant MHV expressing gp33 (Kim and Perlman, 2003) was kindly provided by S. Perlman and was given intranasally. All animal experiments followed approved Institutional Animal Care and Use Committee protocols.

### Cell Purification and Adoptive Transfer

For generation of primary memory P14 CD8<sup>+</sup> T cells, naive Thy1.1 P14 CD8<sup>+</sup> T cells ( $5 \times 10^3$ ) were obtained from peripheral blood and were injected i.v. into naive Thy1.2 WT recipients. Mice were infected 24 hr later with  $2 \times 10^5$  PFU of LCMV-Armstrong i.p. For generating secondary memory P14 CD8<sup>+</sup> T cells, total splenocytes from mice containing primary memory were stained with PE-anti-Thy1.1 antibody (Clone OX-7, BD PharMingen) and purified with anti-PE magnetic bead sorting with standard AutoMacs protocols. After purification, primary memory Thy1.1 P14 CD8<sup>+</sup> T cells ( $5 \times 10^4$ ) were injected i.v. into naive Thy1.2 recipients and the recipients were infected 24 hr later with LCMV-Armstrong i.p. Primary and secondary memory P14 CD8<sup>+</sup> T cell populations were used at the same time point (60+ days) after LCMV-Armstrong infection. For sorting of CD62L high and low expressing cells, memory P14 CD8<sup>+</sup> T cells were purified with magnetic bead sorting as described above.

Purified populations were then stained with anti-CD62L antibody and sorted with a FACSDiva (BD Biosciences) cell sorter.

#### Analysis of Bacterial and Viral Burden

For analysis of bacterial burden after LM infection, primary or secondary memory P14 CD8<sup>+</sup> T cells were purified as described above and adoptively transferred into naive recipients. The recipients and naive control mice were infected 24 hr later with virulent LM-gp33. On day 3 postinfection, liver or spleen samples were obtained and bacterial content was analyzed as previously described (Harty and Bevan, 1995). For analysis of viral burdens, memory P14 CD8<sup>+</sup> T cells were adoptively transferred as indicated. The recipients and naive control mice were infected 24 hr later with LCMV Armstrong ( $2 \times 10^5$ , i.p.), LCMV clone 13 ( $2 \times 10^6$ , i.v.), VacV-gp ( $1 \times 10^7$  i.n.), or MHV-gp33 ( $1 \times 10^5$  i.n.). On the peak day of infection, the appropriate organ was obtained and homogenized, and viral titers were quantified with standard plaque assaying on VERO cells as previously described (Shen et al., 1998). For VacV-gp titers, lung homogenates underwent three rounds of freeze-thaw before being applied to VERO cells. MHV viral load was quantified as previously described (Kim and Perlman, 2003).

#### In Vitro Proliferation

Spleen samples were obtained from mice containing either primary or secondary memory P14 CD8<sup>+</sup> T cell populations. Total splenocytes were stained with 1  $\mu$ M CFSE for 15 min and thoroughly washed with RPMI containing 10% fetal calf serum. Two million total splenocytes were then incubated for 60 hr with or without 50 nM gp33 peptide. Recombinant human IL-2 was used at a concentration of 100 U/ml. Proliferation was analyzed by CFSE dilution of the Thy1.1 memory P14 CD8<sup>+</sup> T cell population.

#### Ex Vivo Cytokine Production

Analysis of IFN $\gamma$ , TNF $\alpha$ , and IL-2 production by antigen-specific CD8<sup>+</sup> T cells was performed essentially as previously described (Badovinac et al., 2002). In brief,  $2 \times 10^6$  total splenocytes were incubated for 5 hr with or without gp33 peptide in the presence of Brefeldin A. Intracellular cytokine staining was then performed with the BD Cytofix/Cytoperm kit in accordance to the manufacturer's protocol. In cases where cytokine production was analyzed from blood samples,  $2 \times 10^5$  EL4 (H-2<sup>b</sup>) cells were added for maximizing antigen presentation to responding T cells.

#### FTY720 Preparation and Treatment

FTY720 (Cayman Chemical, Ann Arbor, Michigan) was dissolved in DMSO at a concentration of 10 mg/ml and stored at  $-20^\circ\text{C}$ . Stock solutions of FTY720 were then diluted in sterile saline before i.v. administration at a dose of 1 mg/kg. After LCMV clone 13 infection, mice received FTY720 treatments on days 0, 2, 4, 6, and 8.

#### SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and Supplemental Experimental Procedures and can be found with this article online at [doi:10.1016/j.immuni.2011.03.020](https://doi.org/10.1016/j.immuni.2011.03.020).

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