

Distinct CD4⁺ helper T cells involved in primary and secondary responses to infection

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Helper T cells are critical for protective immunity, CD8⁺ T-cell memory, and CD4⁺ recall responses, but whether the same or distinct CD4⁺ T cells are involved in these responses has not been established. Here we describe two CD4⁺ T cells, LLO118 and LLO56, specific for an immunodominant *Listeria monocytogenes* epitope, with dramatically different responses to primary and secondary infection. Comparing in vivo responses, LLO118 T cells proliferate more strongly to primary infection, whereas surprisingly, LLO56 has a superior CD4⁺ recall response to secondary infection. LLO118 T cells provide more robust help for CD8⁺ T-cell responses to secondary infection than LLO56. We found no detectable differences in antigen sensitivity, but naive LLO118 T cells have much lower levels of CD5 and their T-cell receptor levels are dramatically down-regulated after their strong primary response. Thus, distinct CD4⁺ helper T cells are specialized to help either in primary or secondary responses to infection.

infectious disease | affinity | T-cell receptor transgenic mice | apoptosis

CD4⁺ T cells are essential for CD8⁺ memory-cell formation, recall responses, and protective immunity (1–3). Despite the importance of CD4⁺ T cells in immune responses, studies examining T-cell responses to *Listeria monocytogenes* have focused primarily on CD8⁺ T cells (4). CD4⁺ T cells are not required for the primary CD8⁺ response to *L. monocytogenes*, but they play a critical role in the maintenance and survival of CD8⁺ memory cells (1–5). Memory cell formation is a hallmark of the adaptive immune system and successful memory cells must navigate expansion, contraction, and maintenance (6). Much more has been learned about the pathways for formation of CD8⁺ memory cells than CD4⁺ memory cells, but it is clear that the pathways for CD4⁺ memory formation are distinct (6–8). The parameters of optimal CD4⁺ help for CD8⁺ and CD4⁺ memory formation have not been characterized and represent an opportunity to effectively improve vaccines (9).

T cells with shortened exposure to antigen do not successfully differentiate to memory cells (10). In contrast, overstimulation in a situation like chronic infection leads to T-cell exhaustion and ineffective memory cell formation or persistence (11). Thus, there appears to be a “goldilocks” level of T-cell stimulation for effective memory cell formation (6). Differences in the duration or abundance of antigen presentation or costimulatory molecules alter activation levels and T-cell receptor (TCR) levels and regulators on the T cell itself affect activation levels (12). TCR affinity and thymic development can also alter T-cell antigen sensitivity (13). For example, the surface expression of one T-cell–negative regulator of T-cell activation, CD5, correlates with the affinity of that developing TCR for self peptide (14–16). The relationship between effective CD4⁺ responses and the avidity of the TCR:pMHC interaction is not established and a polyclonal CD4⁺ T-cell population is not amenable to address the issue of TCR avidity. Therefore, we have isolated T cells that respond to *L. monocytogenes* infection and generated two CD4⁺ T-cell transgenic (Tg) mouse lines specific for the same immunodominant epitope. These transgenic mice have allowed us to

identify and analyze T cells that are infrequent and difficult to find in a polyclonal mouse and have also permitted unique identification of regulators/markers that may normally be missed in a polyclonal setting.

These TCR Tg mice, called LLO118 and LLO56, differ by only 15 amino acids in their TCR sequences. The mice have significantly different in vivo responses, with LLO118 responding more robustly to primary infection, whereas LLO56 has a dramatically better CD4⁺ recall response. Further analysis revealed that LLO56 has higher levels of CD5, causing a dampened primary response. By sorting for LLO118 cells with similar levels of CD5 as LLO56, we found a direct relationship between CD5 levels and cell proliferation in the primary response. LLO118 T cells provide better CD8⁺ help for secondary infections, but LLO118 T cells also have dramatic TCR down-regulation after infection, preventing an effective CD4⁺ recall response. These findings reveal helper T cells can specialize to perform best in a primary response, whereas others function best in the secondary response.

Results

Generation of Two CD4⁺ TCR Transgenic Mice Specific for *L. monocytogenes*. We generated two CD4⁺ TCR Tg mouse lines specific for an immunodominant *L. monocytogenes* epitope, listeriolysin (190–205) (LLO_{190–205}/I-A^b). The TCRs came from a panel of LLO_{190–205}-specific T-cell hybridomas generated from *L. monocytogenes* infected mice. We chose two T cells (LLO118 and LLO56) with identical α and β V-region use (V α 2 and V β 2), and which differed by only 15 amino acids in the complementarity determining regions (CDR) regions. Flow cytometry analysis of the thymus from both LLO118 and LLO56 *Rag1*^{−/−} mice showed a skewing toward CD4⁺ SP cells (Fig. S1A). Analysis of TCR levels shows that LLO118 and LLO56 splenic T cells have identical V α 2 levels (Fig. S1B) and in the thymus and spleen there were no detectable regulatory T (T_{reg}) cells (Fig. S1 C and D).

LLO118 Has a Significantly Stronger in Vivo Response to Primary Infection than LLO56. To examine the in vivo responses of the two *L. monocytogenes* TCR Tg lines, we used an adoptive T-cell transfer system in which 3 × 10³ LLO118-Ly5.1 or LLO56-Thy1.1 T cells were transferred into wild-type C57BL/6 recipient mice (17). One day later these mice were infected with 1 × 10⁴ CFU *L. monocytogenes* and 7 d later the total number of transferred cells were quantified by flow cytometry. On day 7 there were significantly more LLO118 cells compared with LLO56 (Fig. 1A). This finding remained consistent when we transferred 10 times the number of cells (Fig. S2A). We assessed if the

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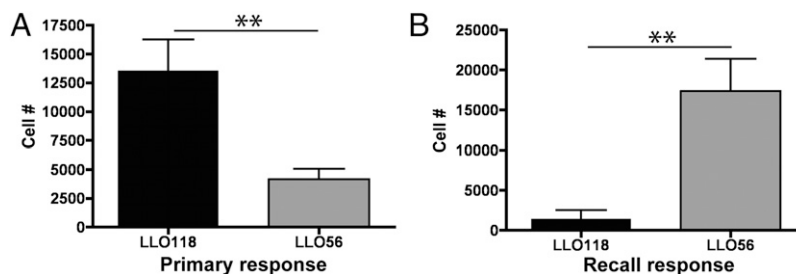


Fig. 1. LLO118 T cells have a better primary response, whereas LLO56 T cells have a better secondary response to *L. monocytogenes* infection. (A) CD4⁺ T cells from LLO118-Ly5.1 or LLO56-Thy1.1 mice were purified by negative selection and 3×10^3 cells were transferred to C57BL/6 recipient mice. The mice were subsequently infected with 1×10^4 CFU of *L. monocytogenes* and cell numbers were measured 7 d later by flow cytometry. (B) On day 35 after the primary infection recipient mice were infected with 1×10^5 CFU of *L. monocytogenes*. Four days after the secondary infection cell numbers were determined by flow cytometry. Data are representative of three independent experiments with at least three mice per group each experiment. ** $P < 0.01$ (Student *t* test).

bacteria were responsible for the differences and found the same pattern when stimulating *in vivo* with the peptide in complete Freund's adjuvant (Fig. S2B). We did not see inherent proliferative capacity differences; neither T cell had substantial levels of homeostatic proliferation, as has been observed for many TCR Tg lines (Fig. S3). Thus, LLO118 T cells respond more robustly to a primary infection or peptide stimulation *in vivo* compared with LLO56 T cells.

LLO56 Has a Stronger CD4⁺ Recall Response to Secondary Infection than LLO118. To examine the CD4⁺ cell recall response of LLO118 and LLO56, T-cell recipient mice were given a secondary infection of *L. monocytogenes* 35 d after the primary infection and on day 39 cell numbers were determined by flow cytometry. In marked contrast to what was observed in the primary response, we found that LLO56 had a much larger recall response than LLO118 T cells (Fig. 1B). The same pattern was also seen at day 6 after rechallenge (Fig. S4A). To ascertain whether this difference is a reflection of lower cell numbers generated in the primary response, we increased the number of CD4⁺ cells transferred to 3×10^4 and saw the same result (Fig. S4B). To determine if the recall differences involved competition for antigen, 3×10^3 LLO118 and 3×10^3 LLO56 cells were cotransferred into the same mouse. The same experimental procedure was followed and analysis of the cotransferred mice yielded equivalent results (Fig. S4C). To eliminate the possibility that the LLO56 recall response was the result of some indirect effect involving where the TCR transgene integrated, we performed the primary and recall experiments with a second LLO56 transgenic mouse line and the results were identical to the first mouse line (Fig. S5). Therefore, in a secondary recall response to *L. monocytogenes*, the LLO56 T cells unexpectedly responded much stronger than LLO118 T cells.

LLO118 Has Greater Magnitude of Proliferation in the Primary Response. To examine proliferation kinetics, cell numbers were tracked the first 10 d after infection. On day 4, both LLO118 and LLO56 had similar numbers of expanded cells, but by day 6 and 8 LLO118 had proliferated much more strongly than LLO56 (Fig. S6). LLO56 and LLO118 cell numbers returned near baseline by day 10. Thus, expansion and contraction kinetics are similar, but the magnitude of LLO118 proliferation is significantly more than LLO56 after *L. monocytogenes* infection.

LLO118 and LLO56 Have Identical *In Vitro* Proliferative Responses to Peptide and Infection. We wondered if the dramatic differences in the primary responses of these T cells were a result of differences in sensitivity to antigen. To address this theory, we performed experiments to examine the *in vitro* response to peptide stimulation. LLO118 or LLO56 splenocytes were stimulated by the LLO_{190–205} peptide and they had identical peptide sensitivity

in vitro (Fig. S7A). We then examined the response of LLO118 and LLO56 to *L. monocytogenes* infection. Purified CD4⁺ T cells were stimulated by splenocytes that had been infected by *L. monocytogenes* and LLO118 and LLO56 had identical proliferative responses (Fig. S7B). Thus, T cells from LLO118 and LLO56 respond identically to *in vitro* stimulation by peptide or bacteria, showing no differences in antigen sensitivity.

LLO118 Has Much Lower Levels of CD5 than LLO56. To understand why there was such a contrast in the magnitude of proliferation in the primary response, we examine a panel of T-cell surface markers before infection. LLO118 and LLO56 had dramatically different levels of CD5 and LLO118 had slightly higher levels of TCR before *L. monocytogenes* infection, whereas they had similar levels of cell-surface markers for the other markers we examined (Fig. 2 and Fig. S8A). Because CD5 is a negative regulator of T-cell activation, we determined that this was an important difference to understand. To address this finding, we

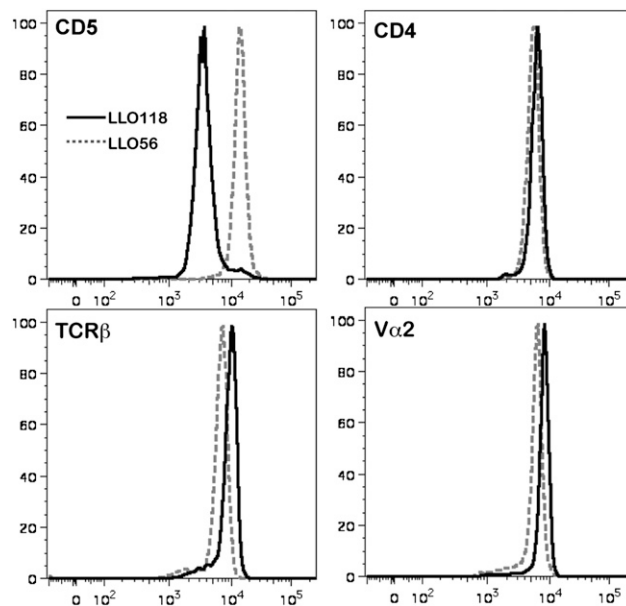


Fig. 2. LLO118 T cells have lower levels of CD5 on cell surface compared with LLO56 T cells. Flow cytometry analysis of common T-cell surface markers. T cells were isolated from the spleen and cell surface markers were directly analyzed without any stimulation and before mice had been infected with *L. monocytogenes* (see also Fig. S8A). Data are representative of three independent experiments with at least three mice per group each experiment.

measured CD5 levels over the course of 35 d after *L. monocytogenes* infection. After stimulation, LLO118 CD5 levels increased at days 5 and 8 to levels similar to LLO56 and by day 12 their levels were identical (Fig. S8B). Thus, LLO118 T cells have lower levels of CD5 initially and then increase for the first 12 d after activation, but by 12 d postinfection their CD5 levels remain identical to LLO56.

CD5^{low} T Cells Respond Best in a Primary Response. We wondered how the CD5 levels of LLO118 and LLO56 compared with the T-cell population of a C57BL/6 mouse. An overlay of LLO118 and LLO56 shows that the CD5 levels of LLO118 were at the lower end of the population and that LLO56 was at the high end (Fig. 3A). We reasoned the high level of LLO118 proliferation could be because of the low levels of CD5. To test this theory, we sorted LLO118 T cells with standard “low” levels of CD5 and also a population with “high” levels of CD5 (LLO118 CD5^{hi}). We also sorted LLO56 T cells with standard “high” levels of CD5 that were equivalent to the LLO118 CD5^{hi} population (LLO56 CD5^{hi}) (Fig. 3B). These sorted cells were cotransferred (LLO118 CD5^{hi} and LLO56 CD5^{hi}) and the recipient mice were infected with *L. monocytogenes* and cell numbers for the various populations were analyzed 8 d postinfection. Comparison of LLO118 and LLO56 with standard levels of CD5 showed the same pattern of LLO118 proliferating much better than LLO56 (Fig. 3C). When we compared LLO118 CD5^{hi} and LLO56 CD5^{hi} T cells (identical levels of CD5) they proliferated to a similar extent (Fig. 3C). Analysis of the recall response of LLO118 CD5^{hi} and LLO56 CD5^{hi} T cells showed the same divergent response as seen with LLO118 and LLO56 T cells (Fig. 3D), revealing that CD5 plays a critical role in the primary response but not the secondary response.

LLO118 Generates a Better CD8⁺ Recall Response than LLO56. CD4⁺ T cells are essential for the generation of CD8⁺ secondary responses to *L. monocytogenes* and we wondered which of the CD4⁺ T cells would provide better CD8⁺ help; LLO118 that has a higher level of proliferation after a primary infection, or LLO56, which has a better secondary response. Because there have not been immunodominant CD8⁺ epitopes identified in H-2^b mice for *L. monocytogenes*, we used the *L. monocytogenes* ovalbumin (OVA) system to evaluate the ability to LLO118 and LLO56 to help expand CD8⁺ T cells (18). We transferred 5×10^5 CD8⁺ T cells from a class II-deficient host into T-cell-deficient mice (TCR- $\alpha^{-/-}$ mice) along with 3×10^3 LLO118, 3×10^3 LLO56, or no CD4⁺ T cells. Mice received a primary infection (1×10^5

CFU) and 35 d later a secondary infection (1×10^5 CFU). We measured the level of CD8⁺ T cells in blood using the OVA-K^b tetramer. There was no difference in the number of CD8⁺ cells after the primary infection and in the first 35 d, but 4 d after the secondary infection we found the addition of LLO118 CD4⁺ cells significantly increased the CD8 recall response compared with LLO56 (Fig. 4A). Thus, the better primary LLO118 CD4⁺ response helps generate a better secondary CD8⁺ response.

LLO56 Cells Have Higher Levels of Annexin V Staining in Primary Response. We wondered why LLO118 T cells provide better T-cell help to CD8 cells. To address this question, we injected 3×10^4 T cells and examined them for 35 d postinfection and found that LLO56 T cells have fewer T cells in the primary response and at day 35 LLO56 has only slightly more cells than LLO118 (Fig. 4B). Because early timepoints are critical for T-cell help, we checked to see if LLO118 or LLO56 had differences in cytokine production. Measurements of intracellular IFN- γ and IL-2 showed that at day 7 LLO118 and LLO56 had the same per cell production levels (Fig. 4C). We also wondered if there were differences in cell survival. Analysis of early apoptosis levels by Annexin V staining at day 7 showed LLO56 cells had significantly higher levels of apoptosis compared with LLO118 (Fig. 4D). Thus, the significant T-cell help response appears to be a result of differences in T-cell numbers (ability to provide costimulation and more total cytokine production) that are a result of cell survival differences between LLO118 and LLO56.

LLO118 TCR Levels Are Dramatically Down-Regulated After Primary Infection. We wondered, why do the LLO118 T cells respond poorly in comparison with LLO56 in a secondary challenge? We analyzed numerous T-cell surface markers on LLO118 T cells at day 35 and, surprisingly, we found that most surface markers were identical, but LLO118 had dramatically lower TCR levels compared with LLO56 (Fig. 5 and Fig. S9). This finding is in contrast to the identical levels measured before exposure to *L. monocytogenes* (Fig. 2).

TCR levels were measured over 35 d after infection and, starting on day 8, LLO118 TCR levels dramatically decreased and remained lower than LLO56 (Fig. 5B). We also measured TCR levels after the rechallenge and found the same phenotype: LLO56 has much higher levels of TCR compared with LLO118 (Fig. S9B). Decreased TCR levels have been shown to correlate with diminished responses to antigen (19) and are the likely cause for the poor LLO118 secondary response. To make sure TCR down-regulation was not affected by the LLO118 TCR

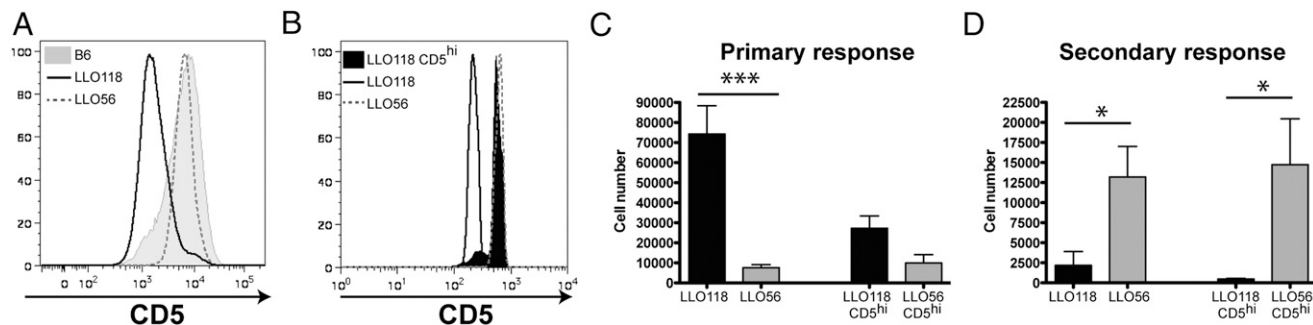


Fig. 3. CD5 levels determine the magnitude of the primary response to *L. monocytogenes*. (A) CD5 levels of T cells from a C57BL/6 mouse (shaded) overlaid with the CD5 levels from LLO118 (black) and LLO56 mice (gray). (B) Postsort analysis of CD5 populations showing LLO118 standard (black) and LLO118 CD5^{hi} population (shaded black), and LLO56 standard population (gray). (C and D) Cell numbers of primary (C) or secondary response (D). The two bars on the left show LLO118 and LLO56 T cells with standard levels of CD5; the two bars on the right show the proliferation levels of LLO118 and LLO56 cells sorted for equivalent levels of CD5 (LLO118 CD5^{hi} and LLO56 CD5^{hi}). In experiments measuring the primary and secondary responses the LLO118 and LLO56 cells were cotransferred into recipient mice, and the LLO118 CD5^{hi} and LLO56 CD5^{hi} were cotransferred into separate recipient mice. Data are representative of three independent experiments with at least three mice per group each experiment. * $P < 0.05$ and *** $P < 0.001$ (Student *t* test).

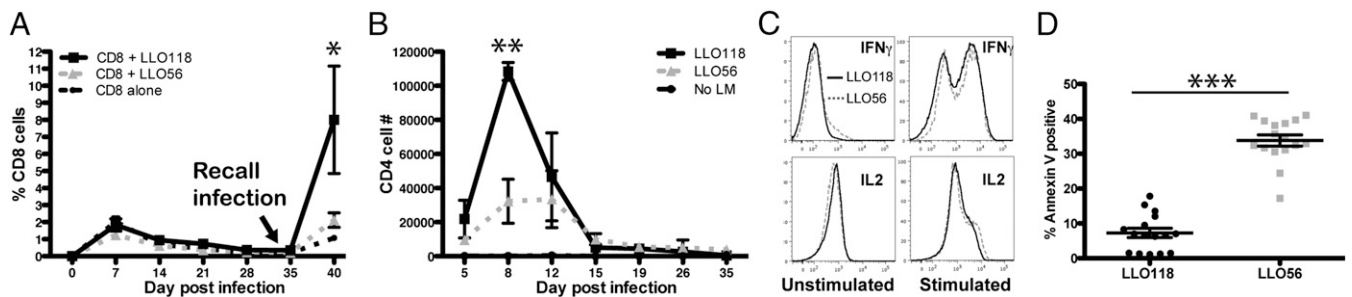


Fig. 4. The LLO118 T-cell is significantly better at CD8⁺ help than LLO56 in a recall response to *L. monocytogenes*. (A) 5×10^5 CD8 T cells from a class II deficient host were transferred into TCR- $\alpha^{-/-}$ mice along with 3×10^3 LLO118, 3×10^3 LLO56, or no CD4⁺ T cells. Mice received a primary infection (1×10^3 CFU) and 35 d later a secondary infection (1×10^5 CFU). T cells were examined 4 d later. Data are representative of one experiment with at least six mice per group. * $P < 0.05$ (ANOVA). (B) 3×10^4 LLO118-Ly5.1 or LLO56-Thy1.1 cells were transferred into B6 mice and they were subsequently infected with 1×10^4 CFU *L. monocytogenes*. T-cell numbers were monitored by flow cytometry over the course of 35 d. (C) IFN- γ and IL-2 intracellular cytokine production of LLO118 and LLO56 isolated 7 d after primary response. (D) Annexin V staining of LLO118 and LLO56 isolated 7 d after primary response. Data are representative of two independent experiments with at least three mice per group each experiment. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Student *t* test).

integration site, we used a separate LLO118 transgenic mouse and confirmed that the same primary response and TCR down-regulation was seen (Fig. S10). Thus, the LLO118 T cells that have a poor recall response have down-regulated TCR levels. This finding is intriguing compared with the previous *in vivo* result of LLO118 responding better to a primary infection. A lower initial response for LLO56 provides a better environment for a more robust recall response. A strong initial proliferation, such as LLO118, results in a better CD8⁺ recall response, but a decreased CD4⁺ recall because of down-regulated TCR levels.

Discussion

An unresolved question is what characteristics of CD4⁺ T cells provide optimal CD8⁺ and CD4⁺ memory. Using TCR Tg mice specific for an immunodominant *L. monocytogenes* epitope and physiological levels of transferred T cells provides a unique means for understanding of the characteristics for CD8⁺ and CD4⁺ memory formation. When examining CD4⁺ memory, unnaturally high levels of transferred cells can lead to anomalous results (20). In our experiments the estimated “parked” T-cell

population, ~300 epitope specific cells, is within the normal physiological range for a mouse and have revealed helper T cells can specialize to perform best in a primary response, whereas others function best in the secondary response.

LLO56 has markedly higher levels of CD5 compared with LLO118, resulting in a dampened primary response and more robust CD4⁺ recall responses. During thymic development CD5 is a negative regulator of TCR signal transduction, and thymocytes with high affinity for self-antigen up-regulate CD5 expression (14). Cell-surface expression of CD5 is proportional to TCR signaling capacity for self-peptide MHC and CD5 acts to fine-tune the T-cell response (14, 21). High levels of CD5 are associated with resistance to activation in peripheral T cells and blocking CD5 restores their responsiveness (22). The negative regulatory ability of CD5 has also been shown to play a critical role in preventing hyper-proliferation and activation-induced cell death (AICD) when examining CD8⁺ T cells in a tumor model and in CD4⁺ and CD8⁺ cells in experimental autoimmune encephalomyelitis (23–25). We find that CD5 does prevent hyperproliferation in LLO56 and possibly AICD at later stages.

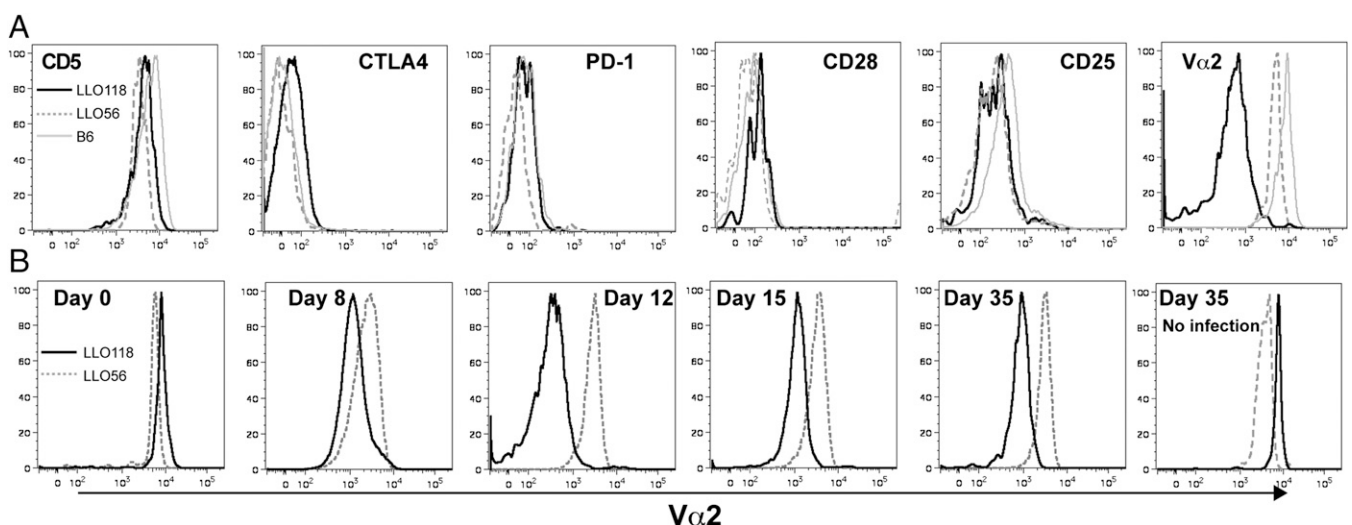


Fig. 5. LLO118 T cells have increased TCR down-regulation compared with LLO56. (A) Flow cytometry analysis of common T-cell surface markers at day 35 (see also Fig. S9). LLO56 and LLO118 levels are shown as well as baseline surface marker levels using endogenous T cells of the recipient B6 mouse. (B) 3×10^4 LLO118-Ly5.1 or LLO56-Thy1.1 cells were transferred into B6 mice and subsequently infected with 1×10^4 CFU *L. monocytogenes*. TCR levels were monitored by flow cytometry over the course of 35 d. The baseline TCR levels are constant in noninfected animals at all time points and representative day 0 and day 35 histograms are included for reference. Data are representative of two (B) or three (A) independent experiments with three mice per group each experiment.

Table 1. LLO118 and LLO56 α and β V-region usage

	CDR3 V α	CDR3 V β	CDR2 V β
LLO118	A A S D T G N Y K Y V	T C S G G G I S Q N T L Y F G A G T R L S V L	L R S P G D
LLO56	A A K D T G N Y K Y V	T C S - - - A D V G V F F G K G T R L T V V	L R N P G D

Boldface indicates differing amino acids in each region.

Surprisingly we also find higher levels of Annexin V-expressing LLO56 cells at day 7 postinfection. Recent work has shown that the level of T-cell apoptosis in primary responses is more complex, involving the T-cell affinity for antigen in a critical balance with Mcl-1, Bim, and Noxa proteins (26). Developing CD5-deficient LLO118 and LLO56 mice and analyzing their primary and secondary responses to *L. monocytogenes* infection will allow this question to be more fully addressed.

The strong primary response by LLO118 results in prolonged TCR down-regulation and poor CD4⁺ memory cell formation. T-cell exhaustion is common in situations of chronic exposure to antigen, but in immunocompetent mice *L. monocytogenes* is rapidly cleared (27). Although LLO118 cells do have slight up-regulation of PD-1 and CTLA-4, they do not exhibit classic signs of exhaustion, such as dramatically increased levels of PD-1 (28). LLO118 T cells have TCR levels that are down-regulated significantly and for an extended period. In the case of LLO118, the strong primary response to *L. monocytogenes* leads to T-cell dysfunction, sustained TCR down-regulation, and inhibited CD4⁺ memory cell formation. T-cell help is critical for the maintenance of CD8⁺ memory cells and T-cell help is generated by the T-cell interactions with the CD8⁺ cells over the course of the primary response. Interestingly, we found that LLO118 is better at providing help not because individual cells are producing more cytokines, but because there are more cells in the population and the total amount of cytokine production and costimulation is increased because of the increased cell survival in the primary response. The greater number of LLO118 T cells produce identical levels of cytokines on a per cell level and can provide better CD8⁺ help in the maintenance phase.

Work done using CD8⁺ T cells has found that T cells with low affinity to antigen proliferate and generate effector and memory cells, but their expansion stops at earlier time points (13). Using a 2D binding assay, CD4⁺ T cells too weak to be stained by peptide-MHC tetramers were as prevalent as high-affinity responders in polyclonal responses (29). CD4⁺ cells with reduced levels of Lck and decreased functional avidity actually had better memory CD4⁺ responses to chronic antigen (30). These studies provide evidence that both CD8⁺ and CD4⁺ with differing affinities can expand to in response to infection.

In summary, we find that LLO56 T cells expand worse than LLO118 T cells in a primary response, but are actually better at generating CD4⁺ memory. A robust primary response provides the best CD8⁺ recall response, whereas a moderate primary response results in the best CD4⁺ memory cell generation. Thus, helper T cells can specialize to work best in a primary or the secondary response, potentially providing improved immunity. These findings shed new light on the role of CD4⁺ T cells in

primary and secondary responses to infectious disease and provide a new avenue to consider in vaccine design.

Materials and Methods

Mice. The LLO118 and LLO56 TCR Tg lines, specific listeriolysin (190-205) (LLO₁₉₀₋₂₀₅/I-A^b), were made from T-cell hybridomas generated from *L. monocytogenes* infected mice. LLO118 and LLO56 have identical α and β V region use (V α 2 and V β 2), which differed by only 15 amino acids (Table 1): one in the CDR3 region of the α chain; 10 of them span the CDR3 region of the β chain; three of them reside between the CDR3 and the constant region of the β chain; and one is in the CDR2 region of the β chain (the origin of which is unclear because it is not germ-line encoded).

See *SI Materials and Methods* for the details of transgenic mouse generation.

Class II^{-/-} mice and T-cell-deficient (C α ^{-/-}) mice are on a C57BL/6 background and were purchased from The Jackson Laboratory. Experiments were started when mice were 5–8 wk of age. All mice were bred and housed in specific pathogen-free conditions of the animal facility at the Washington University Medical Center. All of the use of laboratory animals was approved and done in accordance with the Washington University Division of Comparative Medicine guidelines.

Bacterial Infections. The *L. monocytogenes* strain 1043S used in this study was generously provided by D. Portnoy (University of California, Berkeley, CA). For details of *L. monocytogenes* preparation, see *SI Materials and Methods*.

Proliferation Measurements and Adoptive Transfer of T Cells. See *SI Materials and Methods*.

Flow Cytometry. All samples were analyzed on BD FACSCalibur, BD FACSCanto, or BD LSR II cytometers and data were analyzed with FlowJo software (TreeStar). Sample from in vivo studies were prepared using the previously published protocol for low frequency adoptive transfers (17). See *SI Materials and Methods* for antibody details.

Intracellular Cytokine Assay. See *SI Materials and Methods*.

Statistics. All data were analyzed by the unpaired two-tailed Student *t* test using Prism 4 software (GraphPad). *P* values of less than 0.05 were considered significant.

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