Evolution of a Complex T Cell Receptor Repertoire during Primary and Recall Bacterial Infection

By Dirk H. Busch, Ingrid Pilip, and Eric G. Pamer

From the Section of Infectious Diseases and the Section of Immunobiology, Yale University School of Medicine, New Haven, Connecticut 06520

Summary

The mechanisms underlying the genesis and maintenance of T cell memory remain unclear. In this study, we examined the evolution of a complex, antigen-specific T cell population during the transition from primary effector to memory T cells after *Listeria monocytogenes* infection. T cell populations specific for listeriolysin O $(LLO)_{91-99}$, the immunodominant epitope recognized by H2-K^d–restricted T lymphocytes, were directly identified in immune spleens using tetrameric H2-K^d–epitope complexes. The T cell receptor (TCR) Vβ repertoire of specific T cells was determined by direct, ex vivo staining with a panel of mAbs. We demonstrate that LLO_{91-99}-specific, primary effector T cell populations have a diverse TCR Vβ repertoire. Analyses of memory T cell populations demonstrated similar TCR diversity. Furthermore, experiments with individual mice demonstrated that primary effector and memory T cells have indistinguishable TCR repertoires. Remarkably, after reinfection with *L. monocytogenes*, LLO_{91-99}-specific T cells have a narrower TCR repertoire than do primary effector or memory T cells. Thus, our studies show that the TCR repertoire of primary effector T lymphocytes is uniformly transmitted to memory T cells, whereas expansion of memory T cells is selective.

Key words: T cell receptor repertoire • cytotoxic T lymphocytes • *Listeria monocytogenes* • effector/memory T cells • recall

The adaptive immune response to infectious agents is characterized by initial priming and expansion of complex, pathogen-specific T cell populations. The elicited effector T cells participate in the host defense by controlling the infection and eradicating the pathogen. Interestingly, the in vivo dynamics of antigen-specific T cell responses during the course of infection are very similar, even when the pathogens are very different: initial expansion of effector T cells is followed by a rapid contraction phase, leaving a relatively stable pool of memory T cells that provide long-term immunity (1, 2). The mechanisms that determine and regulate this transition from effector to memory T cells are not known. How are memory T cells generated and maintained? When do memory T cells become distinct from effector T cells? Are there qualitative differences between these two populations that might be reflected by differences in their TCR repertoire? These questions are fundamental to our understanding of protective immunity and have important implications for vaccine design and development.

The differences between naive, unprimed T cells and memory T cell populations are dramatic. Memory T cells require less antigen, do not require costimulation for activation, and expand more rapidly than naive T cells (3, 4). Additional phenotypic differences, such as higher surface expression of adhesion molecules, have also been described (2, 5–7). However, the distinction between effector T cells and memory T cells is less clear. Thus, it is still unknown whether memory T cells are a distinct cell lineage generated during antigen challenge, or if they are directly selected from activated effector T cells (1). If there is selection of memory T cells, the avidity of the TCR–MHC-peptide interaction might be of special importance. Consistent with this notion, recent studies showed that maintenance of naive or memory T cells had distinct requirements, but both required the presence of MHC molecules in the periphery (8).

One approach to determine the differences or similarities between effector and memory T cell populations is to characterize and compare their TCR repertoire. Because of the difficulties identifying small numbers of epitope-specific T cells among much larger populations of nonspecific cells, most of our knowledge of TCR repertoire evolution after immunization comes from systems where a highly restricted T cell population responds to a dominant T cell epitope (6, 9–12). In these systems, the predominance of a particular TCR Vβ segment was used to detect antigen-specific T cells for further analyses of TCR Vα chains and CDR3 sequences. These experiments demonstrated very similar TCR repertoires in effector and memory T cell populations, although in one system some selection for cer-

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tain CDR3 regions was described (9). However, most effector T cell responses during infectious diseases are highly diverse (13–16), and it remains unknown whether memory T cell populations maintain this level of TCR repertoire diversity. We have used murine infection with *Listeria monocytogenes* to study complex T cell responses to infection. Intravenous infection of mice with a sublethal dose of *L. monocytogenes* causes rapid clearance of the pathogen and the development of very effective, long lasting immunity, which is mainly mediated by MHC class I–restricted CTLs (17, 18). Unlike many viral infections, which cause prolonged or chronic infections, *L. monocytogenes* is cleared from infected mice (19, 20). Four different *Listeria* epitopes are presented to $CD8^+$ T lymphocytes by the MHC class I molecule H2-K^d and the in vivo kinetics of T cells responding to these epitopes have been determined (21–24). The H2-K^d–restricted immunodominant epitope listeriolysin O $(LLO)_{91-99}$[1] induces the largest number of CTLs (22). Interestingly, in vitro–expanded, LLO_{91-99}-specific T cells express a highly diverse TCR Vβ repertoire (25).

In this study we have used tetrameric H2-K^d–LLO_{91-99} complexes to characterize the TCR Vβ repertoire of specific effector, memory, and recall T cells after *L. monocytogenes* infection. Primary effector T lymphocytes specific for the *Listeria* epitope LLO_{91-99} are characterized by a diverse TCR Vβ repertoire. This diversity is maintained in memory T cell populations. Remarkably, rechallenge with *L. monocytogenes* induces changes in the epitope-specific TCR repertoire, with focus on a narrower range of TCR Vβ segments. These findings suggest that the breadth of the primary effector TCR repertoire is transmitted to and maintained in the memory compartment. However, expansion of the memory T cell pool narrows the repertoire of recall effector T cells. We propose that contracting or static T cell populations after primary infection maintain TCR diversity, whereas rapidly expanding T cells lose diversity.

Materials and Methods

Mice and Bacteria. BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME). *L. monocytogenes* strain 10403s was obtained from Daniel Portnoy (University of California Berkeley, Berkeley, CA) and grown in brain–heart infusion broth.

Immunization with Listeria and Harvesting of Spleen Cells. Mice were immunized by intravenous injection of 2×10^3 *L. monocytogenes* 10403s into the tail vein. Spleens were removed 7 d after immunization and splenocytes were harvested by dissociation through a wire mesh and lysis of erythrocytes with ammonium chloride, and subsequently resuspended in $RP10^+$, which consists of RPMI 1640 (GIBCO BRL, Gaithersburg, MD) supplemented with 10% FCS, l-glutamine, Hepes (pH 7.5), β-mercaptoethanol, penicillin (100 U/ml), streptomycin (100 μg/ml), and gentamicin (50 μg/ml).

Enrichment for $CD8^+$ T Cells. Splenocytes were enriched for $CD8^+$ T cells by positive separation using the magnetically activated cell separation system (MACS; Miltenyi, Bergisch Gladbach, Germany). Splenocytes were incubated with anti–mouse CD8α microbeads for 20 min in separation buffer (PBS, pH 7.45, 0.5% BSA, and 2 mM EDTA), and after two washes cells were applied on a type LS column (Miltenyi) and $CD8^+$ T cells were separated using the MidiMACS (Miltenyi) following the manufacturer's recommendations.

Tetrameric H2-K^d–Peptide Complexes. MHC–peptide tetramers for staining of epitope-specific T cells were generated as recently described (24, 26). In brief, a specific biotinylation site (27) was added to the COOH terminus of truncated H2-K^d heavy chain (no transmembrane region, truncation after the amino acid in position 284). This fusion protein and β_2 microglobulin (β_2m) were expressed in large amounts as recombinant proteins in *Escherichia coli* using the isopropyl-β-d-thiogalactopyranoside (IPTG)-inducible pET3a vector system (Novagen, Inc., Madison, WI) and BL21(DE3) as an expression host. Purified heavy chain and β_2m were dissolved in 8 M of urea and diluted into refolding buffer containing high concentrations of synthetic peptide LLO_{91-99} (60 μM; Research Genetics Inc., Huntsville, AL) to generate monomeric, soluble H2-K^d–peptide complexes (24, 28). MHC–peptide complexes were purified by gel filtration over a Superdex 200HR column (Pharmacia Biotech AB, Piscataway, NJ), and in vitro biotinylated for 12 h at 20°C in the presence of 15 μg biotin operon repressor protein A (BirA) (AVIDITY, Boulder, CO), 80 μM biotin, 10 mM ATP, 10 mM MgOAc, 20 mM bicine, and 10 mM Tris-HCL (pH 8.3). To remove free biotin, MHC complexes were again purified by gel filtration, and then tetramerized by addition of PE-conjugated streptavidin (Molecular Probes, Eugene, OR) at a molar ratio of 4:1. Tetramers were purified by gel filtration over a Superdex 200HR column and stored at 3–5 mg/ml at 4°C in PBS (pH 8.0) containing 0.02% sodium azide, 1 μg/ml pepstatin, 1 μg/ml leupeptin, and 0.5 mM EDTA.

In vitro Peptide Restimulation of LLO_{91-99}-specific T Cell Lines. T cell lines were established by in vitro peptide restimulation as recently described (25). In brief, $3–4 \times 10^7$ spleen cells from immunized mice were incubated in the presence of 3×10^7 irradiated, syngeneic spleen cells that were peptide pulsed for 1 h at 37°C with 10^{-9} M LLO_{91-99} peptide. The low peptide concentration is required for optimal in vitro expansion of LLO_{91-99}-specific T cell lines (25). T cell lines were generated by restimulating responder cells every week with 3×10^7 peptide-coated stimulator cells. After the second restimulation, the medium was supplemented with 5% rat Con A supernatant. For short-term in vitro expansion of LLO_{91-99}-specific $CD8^+$ T lymphocytes from peripheral blood, 0.5–0.7-ml blood samples were taken by eye bleeding from mice 7 d after infection with *L. monocytogenes.* Erythrocytes of heparinized blood samples were lysed with ammonium chloride, and remaining cells were placed in a well of a 24-well plate containing 6×10^6 LLO_{91-99}-coated stimulator cells (see above) and 5% rat Con A supernatant (total volume, 2 ml in $RP10^+$). Cells were analyzed 3 d after a second restimulation.

Staining and Flow Cytometry Analysis. For flow cytometry analysis, $\sim 3 \times 10^5$ cells were added per staining to a well of a 96-well plate. After incubation at 4°C for 20 min with unconjugated streptavidin (0.5 mg/ml, Molecular Probes) and Fc-block (PharMingen, San Diego, CA) in FACS® staining buffer (SB; PBS, pH 7.45, 0.5% BSA, and 0.02% sodium azide), cells were triple stained with Cy-Chrome–conjugated anti-CD8α (clone 53-6.7, PharMingen), PE-conjugated H2-K^d–LLO_{91-99} tetramers (0.25–0.5 mg/ml), and FITC-conjugated mAbs specific for TCR-α/β

[1]*Abbreviations used in this paper:* β_2m, β_2 microglobulin; BirA, biotin operon repressor protein A; LLO, listeriolysin O; SB, staining buffer.

(clone H57-597; PharMingen), or with 13 different TCR Vβ segments (Vβ 2, 3, 4, 5.1/2, 6, 7, 8.1/2, 8.1-3(pan), 9, 10, 11, 12, 13, and 14 (all obtained from PharMingen) in SB for 60 min at 4°C. Subsequently, cells were washed three times in SB and then fixed in 1% paraformaldehyde/PBS (pH 7.45). Three-color flow cytometry was performed using a FACSCalibur® flow cytometer and data were further analyzed with CELLQuest software (Becton Dickinson, Mountain View, CA).

Results

Generation of H2-K^d–LLO$_{91-99}$ Tetramers. The interaction of T cell receptors with their cognate MHC–peptide complexes is characterized by relatively low affinity and a high dissociation rate. Therefore, it has not been possible to use monomeric MHC–peptide complexes to identify antigen-specific T cells. However, a recently described approach using tetrameric MHC class I–peptide complexes (26) increases the affinity sufficiently to allow cell staining. We therefore generated tetrameric H2-K^d complexes stabilized with the immunodominant *Listeria* epitope LLO$_{91-99}$. Truncated H2-K^d heavy chain (no transmembrane region) containing a genetically engineered biotinylation site at the COOH terminus (Fig. 1 *A*), and β$_2$m were expressed as recombinant proteins in *E. coli*, and were refolded in the presence of high concentrations of LLO$_{91-99}$ peptide. Monomeric H2-K^d–peptide complexes were purified by gel filtration (Fig. 1 *B*) and subsequently enzymatically biotinylated with BirA. H2-K^d–peptide complexes could be immunoprecipitated with the conformation-dependent, H2-K^d–specific mAb SF1-1.1.1 (Fig. 1 *C*), indicating that complexes were properly refolded, and precipitation experiments with streptavidin agarose beads demonstrated essentially complete biotinylation after treatment with BirA (Fig. 1 *C*). Since streptavidin has four binding sites for biotin, incubation of the biotinylated complexes in the presence of streptavidin at a molar ratio of 4:1 results in formation of MHC–peptide tetramers (Fig. 1 *D*). Streptavidin conjugated with PE was used for flow cytometric detection of LLO$_{91-99}$-specific T lymphocytes.

Direct Determination of TCR Vβ Usage Profiles of Complex Epitope-specific T Cell Populations. We have recently shown that tetrameric H2-K^d–peptide complexes specifically detect *Listeria* epitope-specific T cells in vitro and ex vivo (24). LLO$_{91-99}$ is an immunodominant epitope inducing relatively high numbers of specific T cells during the course

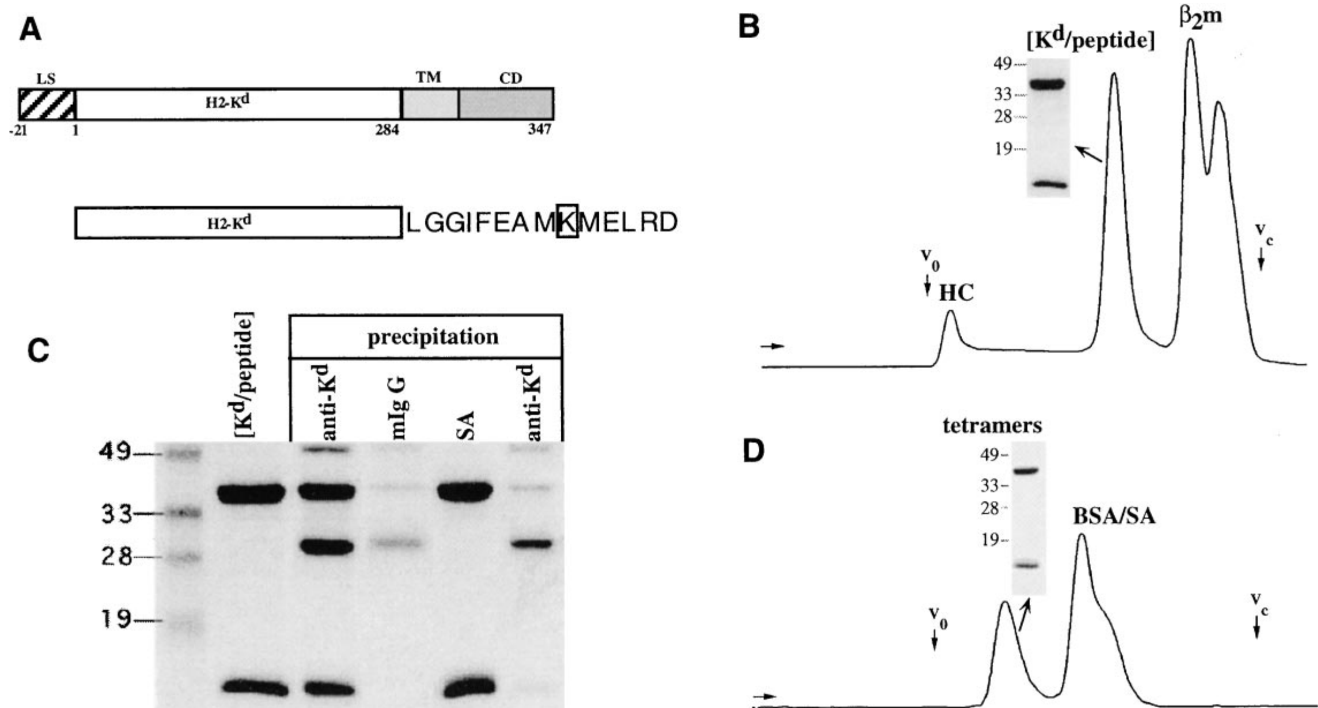

Figure 1. Folding and biotinylation of soluble H2-K^d and generation of tetramers. (*A*) The cDNA for murine H2-K^d was mutagenized by PCR to delete the leader sequence (*LS*), the transmembrane (*TM*), and the cytosolic domain (*CD*), and to extend the COOH terminus with a biotinylation sequence (indicated in single letter amino acid code) recognized by the *E. coli* BirA enzyme. The biotinylated lysine residue is enclosed in a box. (*B*) Recombinant H2-K^d and human β$_2$m were overexpressed in *E. coli* and inclusion bodies were purified, resolubilized, and folded with LLO$_{91-99}$ peptide as indicated in Materials and Methods. A typical fast protein liquid chromatography (FPLC) gel filtration absorbance profile demonstrates a large peak consisting of folded H2-K^d, β$_2$m (seen in the gel inset), and peptide. *HC* indicates a small peak of aggregated, unfolded H2-K^d heavy chains. (*C*) Refolded, FPLC-purified, and biotinylated H2-K^d complexes were either directly subjected to PAGE (first labeled lane) or precipitated with conformation-dependent anti–H2-K^d-specific antibody SF1-1.1.1 (*anti-K^d*), control mouse IgG (*mIgG*) or streptavidin-agarose beads (*SA*). After SA precipitation, only a very small amount of folded H2-K^d could be precipitated with SF1-1.1.1 (*anti-K^d*, right lane). (*D*) Biotinylated H2-K^d complexes were mixed with streptavidin and again subjected to FPLC gel chromatography. The absorbance profile demonstrates a high molecular weight complex consisting of tetramerized H2-K^d–β$_2$m–peptide complexes (gel inset shows H2-K^d heavy chain, β$_2$m, and a faint band of streptavidin). The large peak consists of free streptavidin and carrier BSA (*BSA/SA*).

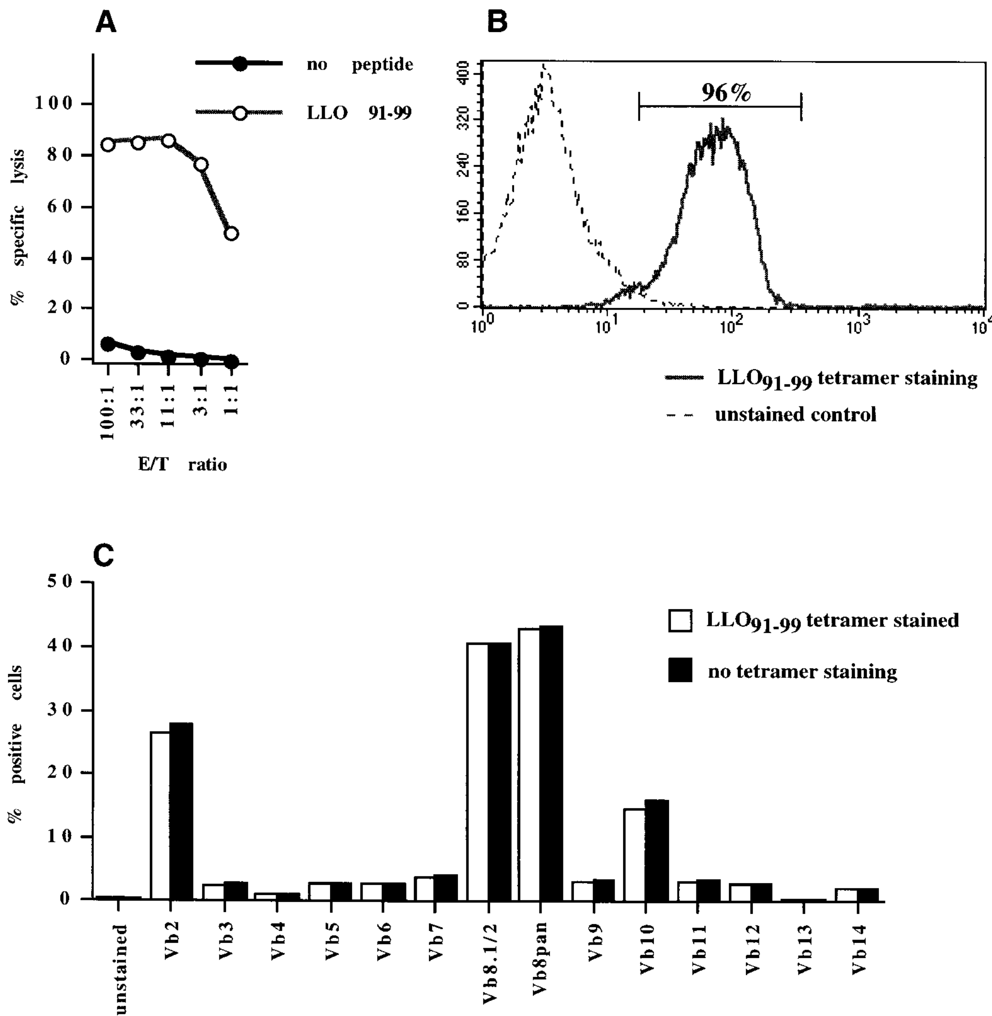

Figure 2. Costaining with LLO_{91-99}–H2-K^d tetramers and TCR Vβ mAbs. A LLO_{91-99}-specific CTL line was generated from an *L. monocytogenes*–immunized BALB/c mouse by in vitro peptide restimulation. (*A*) P815 ($H2^d$) target cells were labeled with ^{51}Cr and incubated in the presence (*open circles*) and absence (*closed circles*) of 10^{-6} M LLO_{91-99} and decreasing numbers of LLO_{91-99}-specific CTL. The percentage of specific lysis and the E/T ratio are indicated. (*B*) The CTL line was stained for CD8 (anti-CD8α Cy-Chrome) and LLO_{91-99} tetramers (PE-conjugated). Gating for $CD8^+$ blasts revealed that nearly all T cell blasts stained with LLO_{91-99} tetramers. (*C*) Lymphoblasts were stained with a panel of FITC-conjugated, Vβ-specific antibodies in the presence (*white bars*) and absence (*black bars*) of LLO_{91-99} tetramers.

of infection with *L. monocytogenes,* which can be easily detected by tetramer staining as primary effector T lymphocytes, as well as after establishment of a memory T cell pool (24). LLO_{91-99} tetramers stain essentially all antigen-specific lymphocytes within the complex T cell population (24). Analysis of in vitro expanded LLO_{91-99}-specific T cell lines revealed a diverse TCR Vβ repertoire (25). To examine whether the TCR Vβ repertoire of LLO_{91-99}-specific T cell populations can be directly determined by costaining T cells with tetramers and TCR Vβ–specific mAbs, we compared TCR Vβ staining of in vitro expanded LLO_{91-99}-specific T cell lines in the presence and absence of LLO_{91-99}–H2-K^d tetramers. First, a T cell line specific for LLO_{91-99} was generated from an *L. monocytogenes*–immunized mouse by in vitro peptide restimulation (Fig. 2 *A*). Essentially all $CD8^+$ lymphoblasts in the cell culture are stained by LLO_{91-99} tetramers (Fig. 2 *B*). Staining with a panel of different TCR Vβ–specific mAbs demonstrated a diverse TCR Vβ profile, which is identical to that obtained when cells were double stained with LLO_{91-99} tetramers (Fig. 2 *C*, gated on $CD8^+$, tetramer-positive lymphoblasts). This experiment indicates that double staining with these TCR Vβ mAbs does not interfere with the LLO_{91-99} tetramer staining. Similar results were obtained for other LLO_{91-99}-specific T cell lines (data not shown).

Direct Ex Vivo Determination of TCR Vβ Usage Profiles. Next, we examined whether double staining with TCR Vβ mAbs and LLO_{91-99} tetramers would allow direct ex vivo TCR repertoire analyses. BALB/c mice were immunized with a sublethal dose of *L. monocytogenes,* and spleen cells were harvested and enriched for $CD8^+$ T cells 7 d later. As shown in Fig. 3, LLO_{91-99} tetramers stain a distinct population of $CD8^+$ T cells. Double staining with a TCR-α/β–specific mAb demonstrates high level TCR-α/β surface expression on all tetramer-positive T cells (Fig. 3 *A*). Double staining with the anti–TCR Vβ 8.1-3 mAb F21.3 identifies a distinct subpopulation within the LLO_{91-99}-specific T cell population expressing this particular TCR Vβ segment (Fig. 3 *B*).

We performed direct ex vivo analyses of LLO_{91-99}-specific, primary effector T cells using 14 different TCR Vβ-specific mAbs, which usually cover >90% of all T cells within this population. Representative histograms of TCR Vβ stainings of $CD8^+$/ LLO_{91-99} tetramer-positive T cells are shown in Fig. 4. In almost all mice analyzed, substantial subpopulations within the LLO_{91-99}-specific T cell popula-

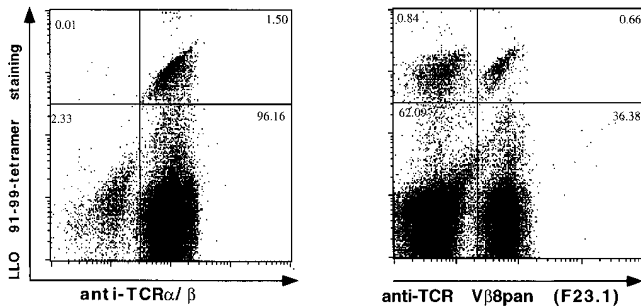

Figure 3. Direct ex vivo TCR staining of LLO_{91-99}-specific T cells. $CD8^+$ T cells from the spleen of a BALB/c mouse immunized 7 d previously with a sublethal dose of *L. monocytogenes* were stained with LLO_{91-99} tetramers (PE-conjugated) and FITC-conjugated antibody specific for TCR-α/β (*left*) and the TCR Vβ8 chain (*right*).

tion could be identified for the TCR Vβ segments Vβ2, 4, 5, 8.1/2, 8.1-3, and 10, whereas for other TCR Vβ segments (Vβ6, 7, 9, 11, or 14) larger subpopulations could only be identified in some individual mice (see also Figs. 6 and 7).

TCR Vβ Profiles of Primary Effector and Memory T Cells. The relatively high and reproducible frequencies of T cells specific for the immunodominant *Listeria* epitope LLO_{91-99} (day 7 primary responders: 1.2–1.5%; 5-wk memory cells: 0.4–0.6% within $CD8^+$ splenocytes) allowed us to determine TCR Vβ profiles using all 14 different TCR Vβ mAbs among immune splenocytes. In Fig. 5, TCR Vβ profiles of LLO_{91-99}-specific T cell populations are shown for six individual mice, three analyzed at the peak of the primary response and three analyzed 5 wk after primary infection with *L. monocytogenes* (memory phase). Primary LLO_{91-99}-specific effector T cell populations show TCR Vβ diversity, similar to the results obtained with in vitro expanded T cell lines (25, Fig. 2). The predominant Vβ segments that are used are Vβ 2, 4, 8, and 10, whereas other segments are represented at relatively low frequencies. However, there is some variability in the TCR Vβ profiles between individual mice. In the memory pool, LLO_{91-99}-specific T cell populations are also characterized by diverse TCR Vβ repertoires, overall showing an extent of diversity similar to that of primary effector T cell populations. However, there is variability between individual mice, making it difficult to determine if the TCR repertoire of LLO_{91-99}-specific memory T cells directly reflects the repertoire of primary effector T cells.

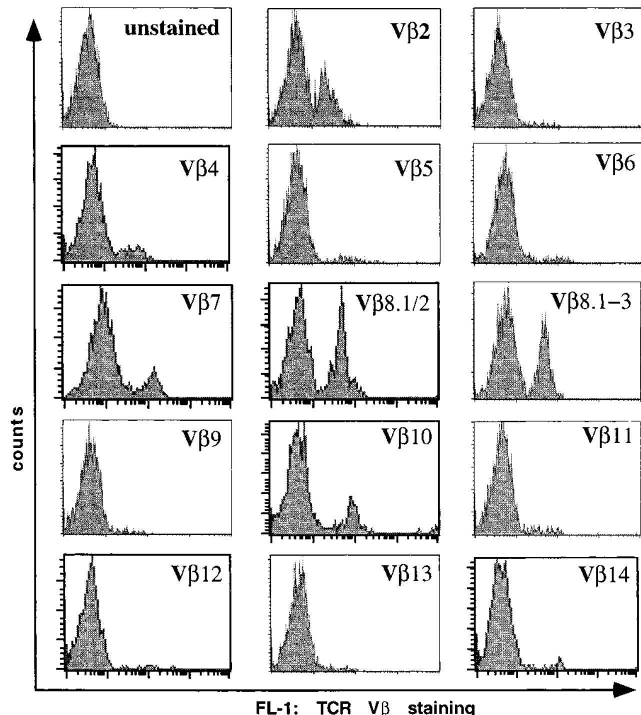

Figure 4. TCR Vβ staining reveals multiple subpopulations of LLO_{91-99}-specific T cells. Immune BALB/c $CD8^+$ splenocytes obtained 7 d after *L. monocytogenes* infection were stained with LLO_{91-99}-specific tetramers and a panel of 14 different FITC-conjugated, Vβ-specific mAbs. These histograms demonstrate the proportion of cells that are stained with each of the antibodies.

Direct ex vivo staining with MHC–peptide tetramers allowed us to compare the TCR Vβ profiles of an epitope-specific T cell population with the overall TCR Vβ usage by $CD8^+$ T cells in the same mouse (Fig. 5, *black bars*). The overall TCR Vβ repertoire of $CD8^+$ T cells in the individual mice tested are remarkably similar, suggesting that intermouse variability in LLO_{91-99}-specific TCRs cannot be attributed to general differences in the frequencies of different TCR Vβ populations. TCR Vβ stainings of naive, BALB/c splenocytes demonstrated very similar overall TCR Vβ repertoires (data not shown). Thus, there is no detectable skewing in the overall TCR Vβ repertoire during primary responses to *L. monocytogenes.*

Taken together, our data show that LLO_{91-99}-specific primary effector and memory T cell populations have diverse TCR Vβ repertoires. The prevalence of TCR Vβ chains among LLO_{91-99}-specific T cells and the general population of $CD8^+$ T cells is remarkably similar.

Effector and Memory TCR Vβ Repertoire Analyses in Individual Mice. As mentioned above, the mouse to mouse variability in the TCR Vβ profiles does not permit precise correlations between memory and primary effector TCR repertoires. To address this issue, we decided to determine the TCR Vβ repertoire of LLO_{91-99}-specific effector and memory T cells in the same mouse. Therefore, we expanded LLO_{91-99}-specific T cell lines from blood samples taken at day 7 during the primary response by short term in vitro peptide stimulation. The TCR Vβ usage of LLO_{91-99}-specific T cell lines was determined by double staining with LLO_{91-99} tetramers and TCR Vβ mAbs, and compared with the TCR Vβ profiles of the LLO_{91-99}-specific memory T cell population 5 wk after primary infection in the spleen of the same mouse. Fig. 6 shows TCR Vβ profiles of LLO_{91-99}-specific effector and memory T cell populations determined in individual mice. The TCR Vβ repertoires determined at the two time points are very similar, if not identical. In particular, even relatively small T cell subpopulations persist among memory T cells (e.g., Vβ 7, 9

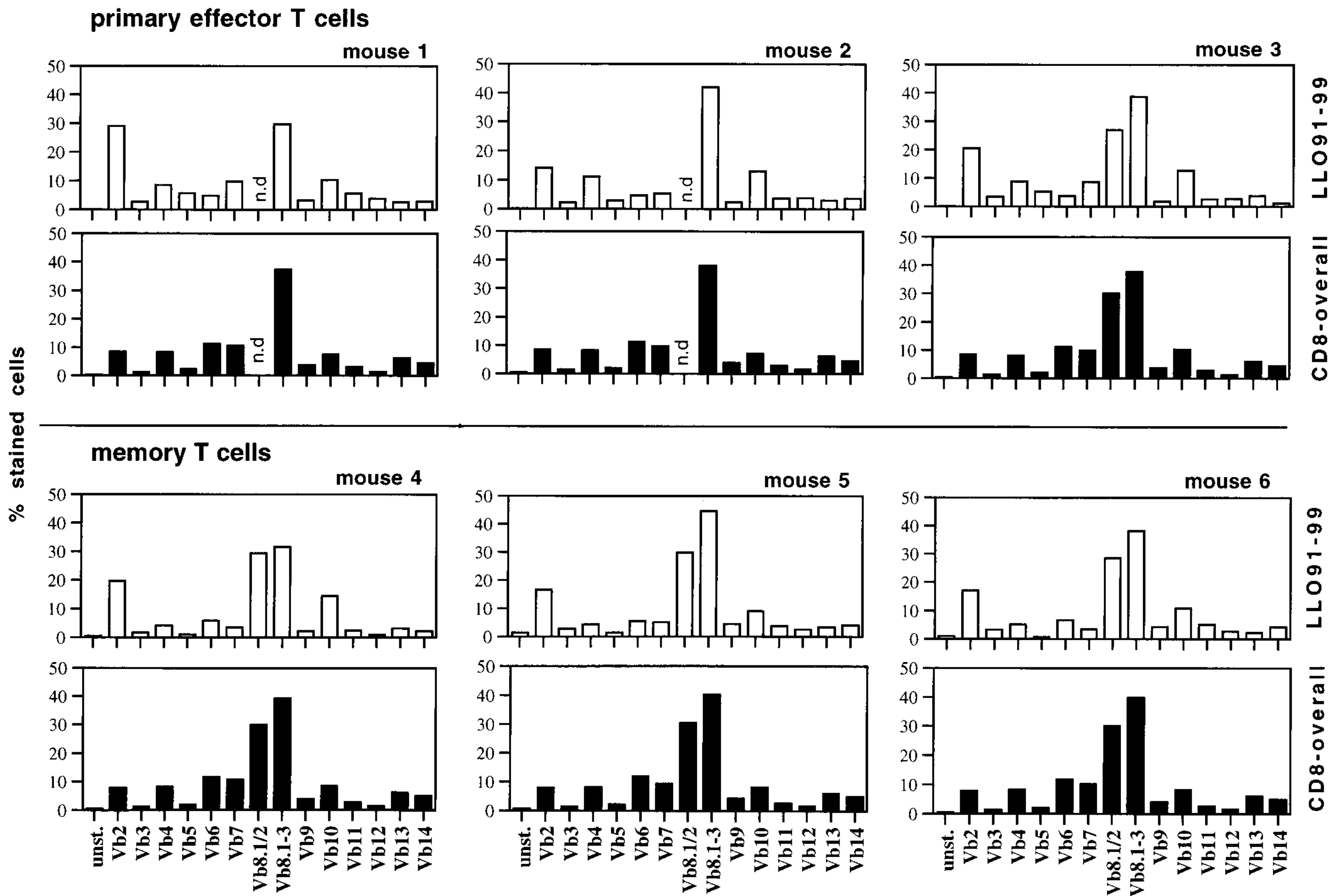

Figure 5. LLO_{91-99}-specific primary and memory T cell repertoires closely reflect the general TCR repertoire of BALB/c $CD8^+$ T cells. Six BALB/c mice were infected with a sublethal dose of *L. monocytogenes,* and $CD8^+$ T cells from three mice were stained for TCR Vβ expression 7 d after infection (*top,* primary effector T cells) and from the remaining three mice 35 d after infection (*bottom,* memory T cells). White bars indicate the percentage of LLO_{91-99} tetramer-positive cells that stain with the individual TCR Vβ specific antibodies. Black bars indicate the percentage of overall $CD8^+$ T cells that stain with the TCR Vβ-specific antibodies. Minimum number of gated $CD8^+$ and tetramer-positive T cells for each TCR Vβ staining was 2,000 for primary effector T cells and 1,000 for memory T cells, respectively. n.d. = not done.

and 10). T cell populations that are distinct for individual mice, such as the unusual TCR Vβ profile in mouse 386, with an unusually large TCR $V\beta9^+$ subpopulation, is maintained in the memory pool and underlines the importance of performing serial repertoire analyses in individual mice. These data indicate that the TCR repertoire of acute effector T cells is transmitted to the memory T cell compartment.

Recall TCR Repertoires Differ from the Primary Effector T Cell Population. We used the same approach to determine the TCR Vβ repertoire of LLO_{91-99}-specific, primary effector T cells from peripheral blood samples and compared this to the repertoire in mice rechallenged with *L. monocytogenes.* As shown in Fig. 7, the recall TCR Vβ repertoires of LLO_{91-99}-specific T cell populations differ substantially from the repertoires of primary effector T cell populations. For all four mice, we found a more restricted TCR Vβ repertoire in the recall population, suggesting a focusing on certain TCR Vβ segments. In particular, less prevalent subpopulations decreased in frequency while the frequency of dominant Vβ populations generally increased. Although focusing could be detected in all four mice, specific changes were not uniform and differed from mouse to mouse. Whereas mice 390, 391, and 392 show focusing mostly onto the TCR $V\beta8^+$ subpopulation (in mouse 391, 70% of all recall effector T cells are positive for the anti–TCR Vβ8 panantibody), in mouse 393, TCR Vβ2 becomes the predominant subpopulation. Focusing of the recall TCR repertoire on other TCR Vβ segments was found for Vβ10 in mouse 390 and for Vβ4 in mouse 392. Mouse 390 showed an unusually high TCR Vβ7 subpopulation in the effector T cell population. Unlike mouse 386 in Fig. 6, where the "fingerprint" was also found in the memory pool, after reimmunization the TCR Vβ7 subpopulation in mouse 390 disappeared almost completely. Taken together, the recall TCR Vβ profiles appear to be more restricted when compared with the corresponding primary effector T cell repertoires.

Discussion

Our studies comparing the TCR repertoires of effector and memory T cell populations responding to the immu-

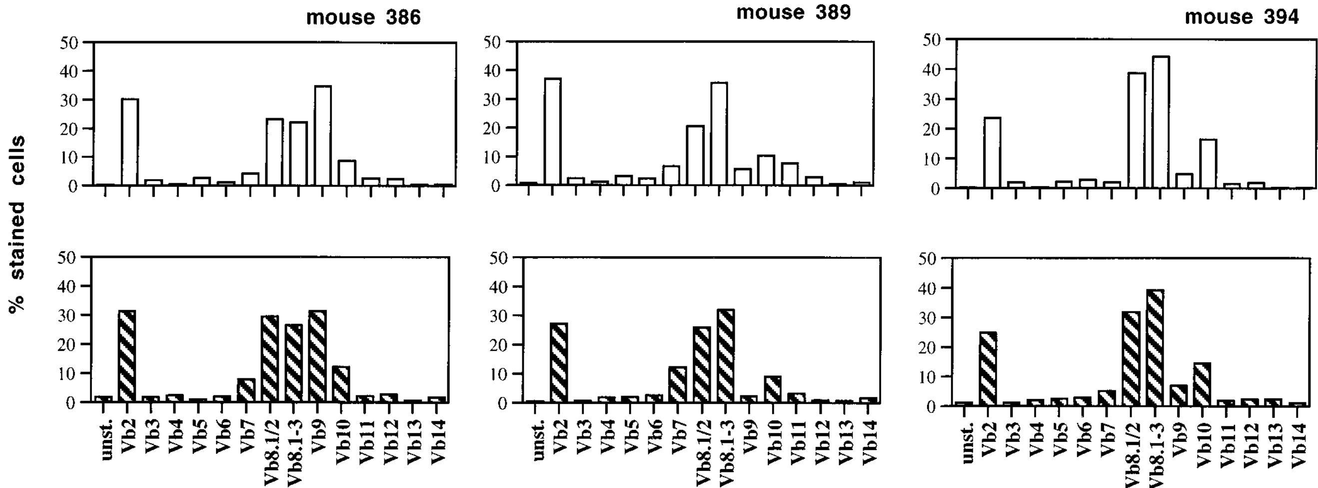

Figure 6. Primary and memory, LLO_{91-99}-specific T cells from individual mice have indistinguishable ratios of TCR Vβ chains. (*top*) Three BALB/c mice were immunized with a sublethal dose of *L. monocytogenes* and 7 d later peripheral blood lymphocytes were restimulated with LLO_{91-99}-coated splenocytes. 10 d later these T cell lines were stained with LLO_{91-99} tetramers and the panel of TCR Vβ–specific antibodies (*white bars; primary effector T cells*). (*bottom*) 35 d after infection, these three BALB/c mice were killed and $CD8^+$ T cells were isolated from spleens and stained with LLO_{91-99} tetramers and the panel of TCR Vβ–specific antibodies (*hatched bars; memory T cells*). The percentage of cells stained with each of the TCR antibodies is indicated.

nodominant *Listeria* epitope LLO_{91-99} show the following: (*a*) the memory TCR Vβ repertoire is similar to the repertoire of primary effector cells; and (*b*) focusing of the repertoire on certain TCR Vβ segments occurs during rechallenge with the antigen. Furthermore, our studies demonstrate the use of tetrameric MHC class I–peptide complexes for direct ex vivo TCR repertoire analyses, and for the first time show the TCR repertoire evolution of a complex T cell population responding to bacterial infection.

Previous studies investigating the evolution of TCR repertoires during in vivo T cell responses have resulted in the following two models. In the first model, memory T cell receptor repertoires directly reflect those selected during the primary response, remain stable over time, and are not influenced by repetitive antigen exposure (11, 12). The second involves selection for particular T cell receptors during the transition from effector to memory T cells, which results in a more restricted memory TCR repertoire (9). Both models are based on findings in experimental systems where T cell responses are directed at single, highly dominant epitopes, expanding relatively uniform T cell populations that express a predominant TCR Vβ chain. With our studies we wanted to investigate whether the diversity of a highly complex effector T cell population responding to an infectious agent is maintained in the memory pool. Our findings support a third model, which combines aspects from both of the above-mentioned models (Fig. 8). Although the TCR repertoire of memory T cells appears to directly reflect the repertoire selected during the primary response, the composition of the repertoire can be substantially modified during reexposure with the antigen.

Although there is some mouse to mouse variability in the TCR repertoires of T cell populations selected for the immunodominant *Listeria* epitope LLO_{91-99}, the TCR profile elicited by primary infection is highly conserved in the memory T cell population of the same individual mouse. This is consistent with observations in other experimental systems (11). In our studies, we determined the TCR Vβ repertoire of epitope-specific T cell populations, which provides a general picture of the overall diversity of the epitope-specific response. Although this approach does not determine the complete diversity of the T cell response, its advantage is that TCR Vβ staining allows us to characterize the majority of T cells within the antigen-specific T cell population (usually >90% of LLO_{91-99}-specific T cell populations are covered by staining with the 14 different TCR Vβ mAbs) directly ex vivo without the need of further in vitro propagation. The validity of this approach is supported by the recent finding that the degree of TCR repertoire diversity of EBV-specific T cell populations is maintained on the level of the TCR Vβ segments, indicating that TCR Vβ usage directly correlates with the overall complexity of a given T cell population (29). The complexity of primary effector T cells specific for LLO_{91-99} is precisely maintained in the memory T cell population. This finding suggests that selection of memory T cells is either very similar to or completely overlaps the selection of primary effector T cells during priming. Our results are interesting in the context of recent findings, which show that the maintenance of memory T cells is far less dependent on the presence of the restricting MHC molecule than is the maintenance of naive, unprimed T cells (8). Thus, individual T cell subpopulations within the broad repertoire of the T cells specific for LLO_{91-99}, which may consist of TCRs with different affinities for $H2\text{-}K^d$–LLO_{91-99} complexes, are treated equivalently in the memory compartment. However, our experiments do not rule out the possibility that further maturation and qualitative changes of memory T cell populations occur very slowly

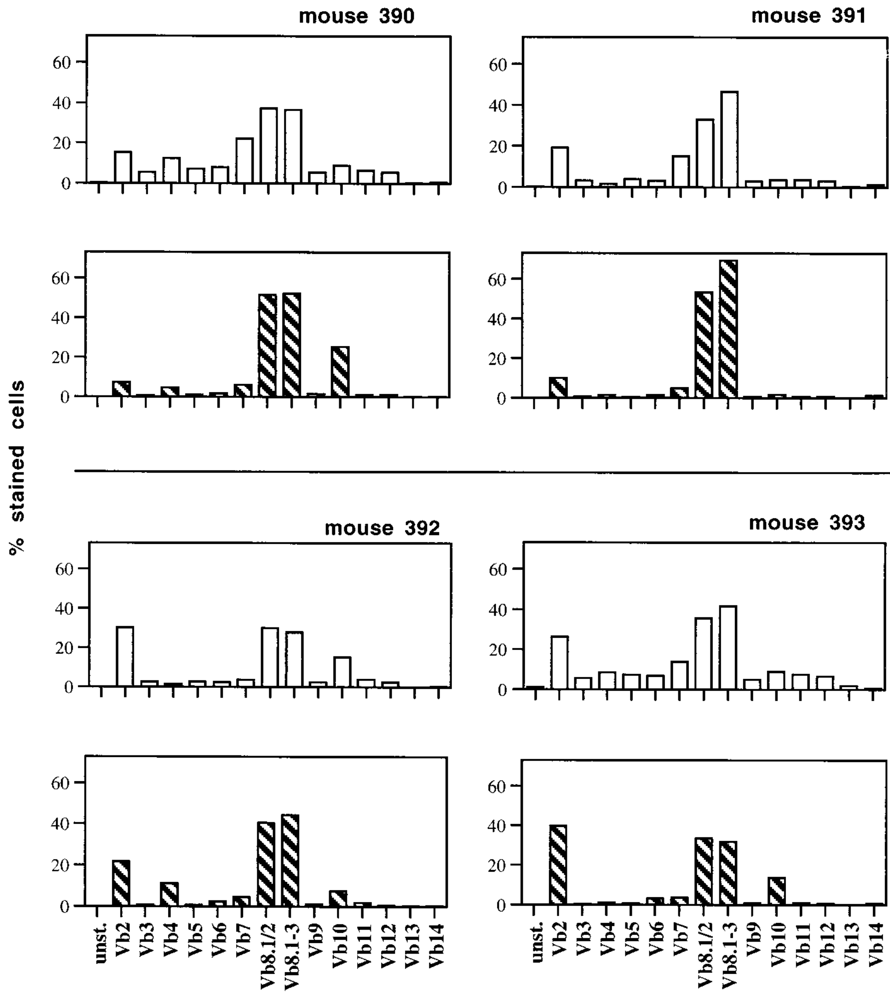

Figure 7. After a recall response, LLO_{91-99}-specific T cells express a more limited TCR repertoire than do primary effector T cells. Four BALB/c mice were infected with a sublethal dose of *L. monocytogenes* and 7 d later peripheral blood lymphocytes were used to generate LLO_{91-99}-specific T cell lines, as described for Fig. 6. These T cell lines were stained with LLO_{91-99} tetramers and the panel of TCR Vβ-specific antibodies (*white bars; primary effector T cells*). 35 d after primary infection, these four BALB/c mice were reinfected with a 50-fold higher dose (100,000 bacteria) and 5 d later $CD8^+$ splenocytes were isolated and stained with LLO_{91-99} tetramers and the TCR Vβ panel (*hatched bars; recall effector T cells*). The percentage of cells stained with each of the TCR antibodies is indicated.

over prolonged periods of time. Thus, it will be of particular interest to monitor TCR repertoires of complex memory T cell populations over longer periods of time.

The TCR Vβ repertoire of LLO_{91-99}-specific T cells becomes narrower in immune mice after rechallenge with *L. monocytogenes.* This observation differs from findings in other experimental systems, where antigen reexposure had no influence on the TCR repertoire (11). However, those experiments were characterized by a highly restricted primary T cell response, a factor that may limit further restriction during a recall response. The basis for repertoire focusing in our system is not known. Although the mice were rechallenged with a much larger infecting dose of *L. monocytogenes* (100,000 bacteria for recall infection compared with 2,000 bacteria for primary infections), bacterial clearance occurs much more rapidly in immune mice than in naive mice (48–72 h compared with 1 wk). It is possible that the kinetics of bacterial clearance and, consequently, the differences in the overall antigen quantity may account for qualitative changes in the expanded T cell population. Thus, T cell clones that are capable of responding to lower amounts of epitope, perhaps on the basis of a higher avidity for the cognate MHC–peptide complexes, might have a se-

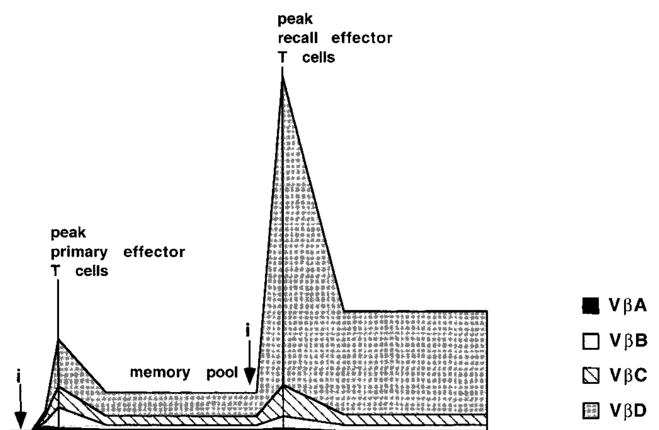

Figure 8. Model for TCR repertoire evolution during primary and recall infection with *L. monocytogenes.* The diversity of a pathogen-specific T cell population expanded during primary infection (*arrows* indicate time points of infection) is maintained in the memory pool. After rechallenge with the pathogen, the recall TCR repertoire is more restricted compared with the primary effector and memory T cell populations. These differences might be due to different in vivo expansion rates of T cells within the epitope-specific population.

lective advantage. However, preliminary experiments analyzing recall TCR repertoires in response to 20-fold lower infecting doses of *L. monocytogenes* demonstrated similar expansion of LLO_{91-99}-specific T cells with an identical extent of TCR repertoire focusing (Busch, D.H., and E.G. Pamer, unpublished data).

Determining the cellular and molecular basis for repertoire focusing after reexposure to antigen will require further investigation. Studies of the relative affinities of more focused T cell populations for their cognate peptide–MHC complex may be particularly informative.

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Address correspondence to Eric G. Pamer, Sections of Infectious Diseases and Immunobiology, Yale University, New Haven, CT 06520. Phone: 203-785-3561; Fax: 203-785-3864; E-mail: eric.pamer@yale.edu

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