

Differential requirements for the chemokine receptor CCR7 in T cell activation during *Listeria monocytogenes* infection

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Effective priming of T cell responses depends on cognate interactions between naive T cells and professional antigen-presenting cells (APCs). This contact is the result of highly coordinated migration processes, in which the chemokine receptor CCR7 and its ligands, CCL19 and CCL21, play a central role. We used the murine *Listeria monocytogenes* infection model to characterize the role of the CCR7/CCR7 ligand system in the generation of T cell responses during bacterial infection. We demonstrate that efficient priming of naive major histocompatibility complex (MHC) class Ia-restricted CD8⁺ T cells requires CCR7. In contrast, MHC class Ib-restricted CD8⁺ T cells and MHC class II-restricted CD4⁺ T cells seem to be less dependent on CCR7; memory T cell responses are independent of CCR7. Infection experiments with bone marrow chimeras or mice reconstituted with purified T cell populations indicate that CCR7 has to be expressed on CD8⁺ T cells and professional APCs to promote efficient MHC class Ia-restricted T cell priming. Thus, different T cell subtypes and maturation stages have discrete requirements for CCR7.

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Abbreviations used: CFSE, carboxyfluorescein succinimidyl ester; f-met, formyl-methionine; LLO, listeriolysin O; LmOVA, recombinant *Listeria monocytogenes* strain secreting a truncated OVA protein; MACS, magnetic cell sorting; TSB, tryptic soy broth.

Close contact between naive T cells and professional APCs is a prerequisite for effective T cell priming. This contact does not occur randomly, but rather is a consequence of highly coordinated migration processes which involve different adhesion molecules and chemokine/chemokine receptor systems. The chemokine receptor CCR7, and its ligands, CCL19 (EBV-induced gene 1 ligand chemokine, macrophage inflammatory protein-3β) and CCL21 (secondary lymphoid tissue chemokine, 6Ckine), seem to play a central role (1, 2). CCR7 is expressed on naive T cells, a subpopulation of memory T cells, and B cells (1). Immature dendritic cells do not express CCR7; however, during maturation, CCR7 is up-regulated on their surface (3). CCL19 and CCL21 are secreted by stromal cells that are located in the T cell zones of secondary lymphoid organs. In addition, CCL21 is expressed by high endothelial venules and the lymphatic endothelium (1, 2, 4). Standard laboratory mouse strains express at least two isoforms of CCL21—CCL21-Ser and CCL21-Leu—which are encoded by independent genes. In contrast to CCL21-Ser, expression of CCL21-Leu is restricted to the

lymphatic epithelium of peripheral tissues (4). After chemokine attraction, naive T cells and activated dendritic cells enter lymphoid tissues and migrate along the CCL19/CCL21 gradients into T cell zones, where cognate interactions eventually occur (1, 2, 5).

The concept of chemokine-controlled T cell priming is supported by observations in CCR7-deficient mice and in *plt* (paucity of lymph node T cell) mice, which fail to express CCL19 and CCL21-Ser and show impaired expression levels of CCL21-Leu (4, 6–9). In both mouse strains, T lymphocytes home poorly into lymph nodes and Peyer's patches, and inside lymph nodes, T cells are distributed aberrantly (4, 6, 8). Although T cells can still enter the spleen, these mice fail to develop distinct T cell zones in the white pulp (4, 6). Furthermore, migration of mature dendritic cells into secondary lymphoid tissues is impaired (3, 6). Consequently, both mouse strains demonstrate altered acquired immune responses. In CCR7-deficient mice, delayed-type hypersensitivity reactions are reduced, and T cell dependent antibody production is delayed (6). CCR7 mice fail to reject allogeneic tumors and reject

tion of grafted allogeneic hearts is delayed (9). In *plt* mice, acquired immune responses seem to be affected less severely. Although, *plt* mice are more susceptible to infection with

murine hepatitis virus (7), immune responses after infection with lymphocytic choriomeningitis virus, vesicular stomatitis virus, and different strains of vaccinia virus are similar to that

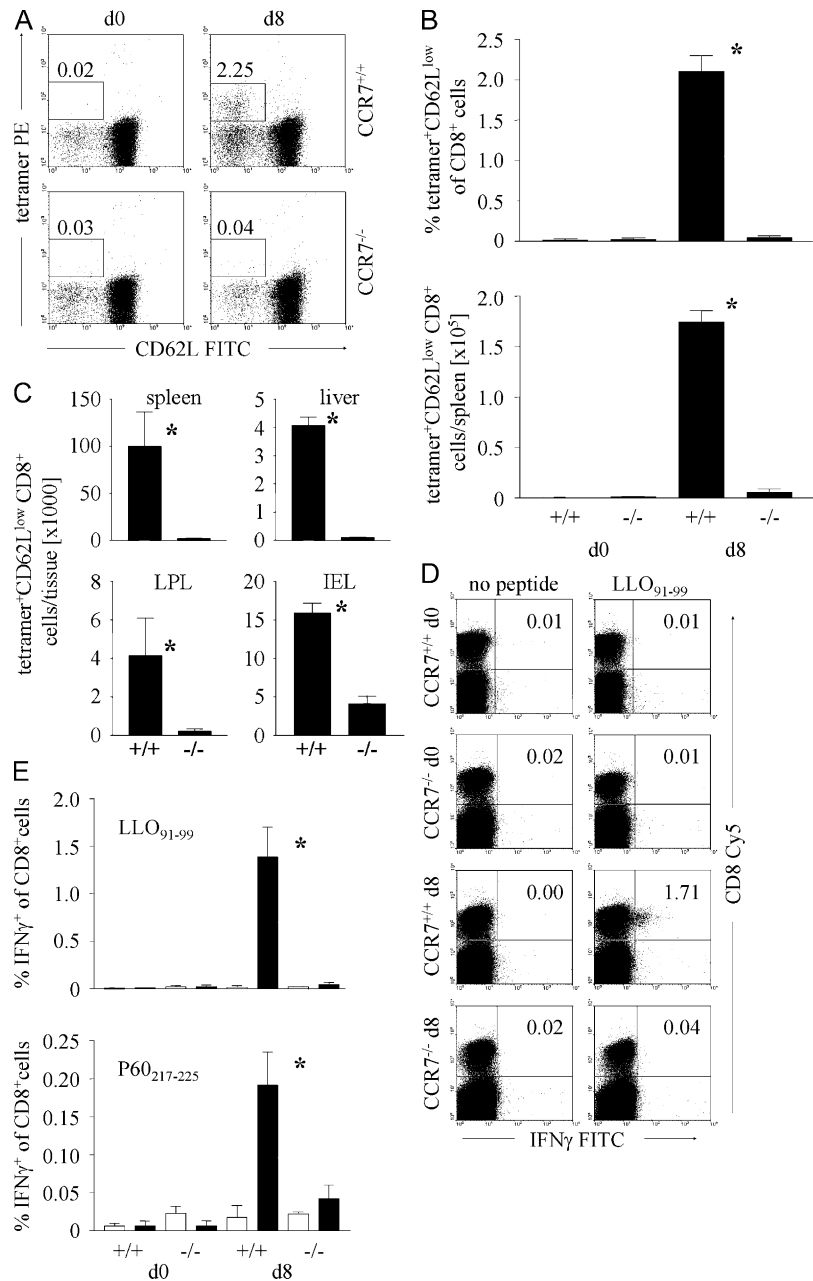


Figure 1. CD8⁺ T cell response to primary *L. monocytogenes* infection. CCR7^{+/+} and CCR7^{-/-} mice on BALB/c background were infected i.v. with 10³ (A, B, D, E) or orally with 10⁹ *L. monocytogenes* organisms (C). After 8 d, spleen cells (A, B) or cells isolated from liver, lamina propria, and epithelium of the small intestine (C) were stained with FITC-conjugated anti-CD62L mAb, Cy5-conjugated anti-CD8α mAb, and PE-conjugated LLO₉₁₋₉₉ tetramers. Cells were analyzed by flow cytometry after the addition of propidium iodide. (A) CD8-gated representative results. Numbers give the percent of cells within the region and are calculated for CD8⁺ T cells only. (B, C) The percent of LLO₉₁₋₉₉-tetramer⁺ CD62L^{low} of CD8⁺ T cells and absolute number of

LLO₉₁₋₉₉-tetramer⁺ CD62L^{low} CD8⁺ T cells per spleen (mean ± SD of three independently analyzed mice per group). In addition, spleen cells were incubated for 5 h with the peptides LLO₉₁₋₉₉ or p60₂₁₇₋₂₂₅ (D, E). Cells were stained extracellularly with anti-CD8α mAb and intracellularly with anti-IFNγ mAb and analyzed by flow cytometry. (D) Representative results. Numbers give the frequencies for IFNγ⁺ of CD8⁺ cells only. (E) Frequencies of IFNγ⁺ cells among CD8⁺ T cells after in vitro incubation without (white bars) or with peptides LLO₉₁₋₉₉ or p60₂₁₇₋₂₂₅ (black bars; mean ± SD of three individually analyzed mice per group). *Difference between cells from infected CCR7^{+/+} and CCR7^{-/-} mice was statistically significant (P < 0.05).

in control mice (8). Despite an aberrant T cell migration pattern in infected *plt* mice, antiviral T cell and antibody responses are virtually normal, and *plt* mice can control viral infections (8). Furthermore, immunization of *plt* mice with ovalbumin results in delayed, but otherwise normal or even stronger priming of CD4⁺ T cells (4). Overall, these studies question the general requirement for CCR7/CCR7 ligands interactions for T cell priming and point to more complex functions during acquired immune responses.

Infection of mice with the intracellular bacterium *Listeria monocytogenes* represents a valuable model to study T cell responses in vivo (10, 11). After infection, *L. monocytogenes* migrate to their main target organs—spleen and liver—where they invade macrophages and hepatocytes. In these cells, *L. monocytogenes* penetrate the phagosomal membrane and enter the cytoplasm. Because of their intracytoplasmic habitat, *L. monocytogenes*-derived antigens are presented efficiently via the MHC class I pathway; this results in the induction of a potent CD8⁺ T cell response. CD8⁺ T cells are crucial for the control of *L. monocytogenes* infection and are responsible for protection against reinfection. In addition, a considerable CD4⁺ T cell response is induced, although the role of this cell population is less clear (12, 13). Several *L. monocytogenes*-derived T cell epitopes have been characterized, including MHC class Ia-, MHC class Ib-, and MHC class II-presented peptides (10, 14). The respective peptides have been used extensively to study the kinetics and regulation of *L. monocytogenes*-specific T cell responses during infection. These analyses yielded extensive information on the effector and memory phases of *L. monocytogenes*-specific CD4⁺ and CD8⁺ T cell responses. However, the early events of T cell priming remain largely unknown. Only limited information is available on the sites and the APC populations that are involved in T cell priming and the requirements for T cell differentiation into effector and memory cells (15–18).

We analyzed CCR7-deficient mice using the murine *L. monocytogenes* infection model. We demonstrate that CCR7 has to be expressed on CD8⁺ T cells and professional APCs to allow efficient MHC class Ia-restricted priming. In contrast, MHC class Ib-restricted CD8⁺ T cells and MHC class II-restricted CD4⁺ T cells are less dependent, and memory T cell responses are independent of CCR7.

RESULTS

CCR7^{-/-} mice backcrossed onto the BALB/c background and CCR7^{+/+} BALB/c control mice were i.v. infected with 10³ *L. monocytogenes* organisms, and the CD8⁺ T cell response was analyzed using H-2K^d tetramers loaded with the immunodominant peptide listeriolysin O (LLO)_{91–99} (19). 8 d after infection, approximately 2% of all CD8⁺ splenocytes from CCR7^{+/+} mice were LLO_{91–99} tetramer⁺ (Fig. 1, A and B). Spleens of CCR7^{+/+} mice contained a total of 1.5–2.0 × 10⁵ LLO_{91–99} tetramer⁺ CD8⁺ T cells. In contrast, frequencies and numbers of LLO_{91–99} tetramer⁺ CD8⁺ T cells in infected CCR7^{-/-} mice were reduced and were close to

background levels that were observed in noninfected animals (Fig. 1, A and B). The impaired response in CCR7^{-/-} mice was not restricted to the spleen. After oral infection, we observed reduced cell numbers of LLO_{91–99} tetramer⁺ CD8⁺ T cells in spleen, liver, and lamina propria and epithelium of the small intestine (Fig. 1 C). Similar results were obtained at d 9, 10, and 17 after infection, excluding that the low frequencies of LLO_{91–99} tetramer⁺ CD8⁺ T cells were the result of a delayed T cell response in CCR7^{-/-} mice (unpublished data).

In parallel to the tetramer assay, splenocytes from infected CCR7^{+/+} and CCR7^{-/-} BALB/c mice were restimulated in vitro with the peptides LLO_{91–99} and p60_{217–225}, an independent immunodominant *L. monocytogenes*-derived CD8⁺ T cell epitope (19). Stimulation with both peptides induced IFNγ production in CD8⁺ spleen cells from infected control mice (Fig. 1, D and E). In contrast, only marginal frequencies of IFNγ⁺ CD8⁺ T cells were observed in spleens of CCR7^{-/-} mice; this confirmed the results from the LLO_{91–99} tetramer assay and indicated that the impaired response was not restricted to the LLO_{91–99} epitope.

Despite the marginal proportion of *L. monocytogenes*-specific CD8⁺ T cells, CCR7^{-/-} mice tolerated the *L. monocytogenes* inoculum that was used in our experiments, and susceptibility to infection was not increased substantially. CCR7^{-/-} mice showed impaired clearance of *L. monocytogenes* with slightly higher bacterial titers at late time points after infection (Fig. 2). However, at later time points (>10 d after infection), CCR7^{+/+} and CCR7^{-/-} mice usually had cleared *L. monocytogenes* from spleen and liver (unpublished data).

Infection of mice with *L. monocytogenes* results in the generation of specific CD8⁺ memory T cells, which mount an accelerated and elevated anti-*L. monocytogenes* T cell response to reinfection. To assess the role of CCR7 in the

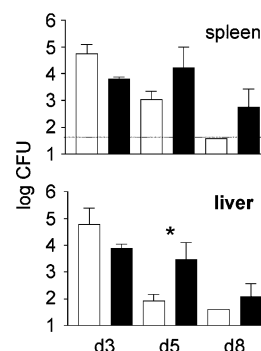


Figure 2. Bacterial burdens in spleen and liver of CCR7^{-/-} mice after *L. monocytogenes* infection. CCR7^{+/+} (white bars) and CCR7^{-/-} mice (black bars) were i.v. infected with 10³ *L. monocytogenes* organisms. On the indicated days, mice were killed and bacterial titers in spleen and liver were determined. Results represent the mean ± SD of four or five mice per group. The dotted line gives the detection limit of our assay (40 bacteria/organ). *Difference between titers from infected CCR7^{+/+} and CCR7^{-/-} mice was statistically significant (P < 0.05).

generation and maintenance of CD8⁺ memory T cells, CCR7^{+/+} and CCR7^{-/-} BALB/c mice were infected with 10³ *L. monocytogenes* organisms; 6 wk later, mice were challenged with 10⁵ *L. monocytogenes* organisms. 6 d after secondary infection, CD8⁺ T cell responses were analyzed with LLO₉₁₋₉₉ tetramers and by intracellular cytokine staining after in vitro restimulation with LLO₉₁₋₉₉ or p60₂₁₇₋₂₂₅ peptides (Fig. 3). CCR7^{+/+} control mice demonstrated en-

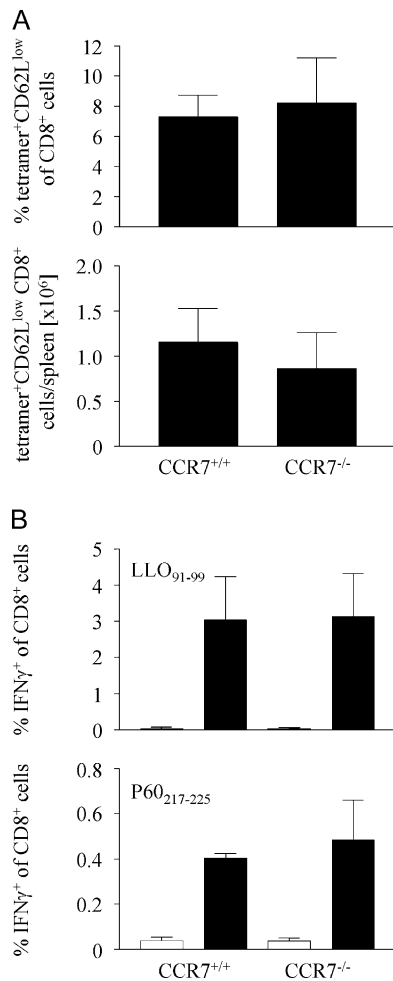


Figure 3. CD8⁺ T cell responses to LLO₉₁₋₉₉ or p60₂₁₇₋₂₂₅ after secondary *L. monocytogenes* infection. CCR7^{+/+} and CCR7^{-/-} mice on BALB/c background were infected i.v. with 10³ *L. monocytogenes* organisms. After 44 d, mice were secondarily infected (i.v.) with 10⁵ *L. monocytogenes* organisms. On day 6 after secondary infection, spleen cells were analyzed as described in Fig. 1. (A) Frequencies and absolute numbers of LLO₉₁₋₉₉-tetramer⁺ CD62L^{low} CD8⁺ T cells in CCR7^{+/+} and CCR7^{-/-} BALB/c mice (mean \pm SD of three individually analyzed mice per group). (B) Frequencies of IFN γ ⁺ cells among CD8⁺ T cells after short-term incubation without (white bars) or with peptides LLO₉₁₋₉₉ or p60₂₁₇₋₂₂₅ (black bars; mean \pm SD of three individually analyzed mice per group). There was no significant difference ($P > 0.05$) between CCR7^{+/+} and CCR7^{-/-} mice in frequencies and total cell numbers of LLO₉₁₋₉₉-tetramer⁺ CD62L^{low} CD8⁺ T cells (A), and in the frequencies of IFN γ -producing cells after peptide restimulation (B).

hanced frequencies and absolute numbers of LLO₉₁₋₉₉- and p60₂₁₇₋₂₂₅-specific CD8⁺ T cells, characteristic for secondary responses. Despite the marginal primary responses, CD8⁺ T cell frequencies and total numbers in secondary infected CCR7^{-/-} mice were similar to those in CCR7^{+/+} control animals. As could be expected from the strong secondary CD8⁺ T cell response, all CCR7^{+/+} and CCR7^{-/-} animals had cleared the infection from spleen and liver at the time point of T cell analysis. Direct measurement of LLO₉₁₋₉₉-specific CD8⁺ memory T cells 6 to 8 wk after infection was difficult, because even in CCR7^{+/+} mice, numbers of memory cells in spleen and liver were close to the detection level of our assay (unpublished data). To circumvent this problem, we used a recombinant *L. monocytogenes* strain that secretes a truncated OVA protein (LmOVA) (20). Infection of C57BL/6 mice with LmOVA results in a substantial CD8⁺ T cell response to the OVA₂₅₇₋₂₆₄-epitope and a high level of CD8⁺ memory T cells (21). As expected, we observed a strong OVA₂₅₇₋₂₆₄-specific CD8⁺ T cell response in CCR7^{+/+} C57BL/6 mice, but only a weak response in CCR7^{-/-} C57BL/6 mice (Fig. 4 A). Reinfection of mice caused an extensive secondary OVA₂₅₇₋₂₆₄-specific CD8⁺ T cell response, which was reduced only slightly in CCR7^{-/-} mice compared with CCR7^{+/+} mice (Fig. 4 B). Analysis of CD8⁺ T cells from spleen and liver of mice 2 mo after LmOVA infection revealed that both mouse strains had generated OVA₂₅₇₋₂₆₄-specific memory T cells, although the frequencies and numbers of these cells were reduced in CCR7^{-/-} mice (Fig. 4 C).

Overall, these results indicate that CCR7^{-/-} mice can generate significant CD8⁺ memory T cell populations, despite the marginal primary CD8⁺ T cell response; this population gives rise to rapid secondary responses after reinfection. In contrast to the primary response, the CD8⁺ memory T cell response seemed to be independent of CCR7.

In addition to CD8⁺ T cell responses that are restricted by conventional MHC class Ia molecules, *L. monocytogenes* induces CD8⁺ T cell responses that are restricted by MHC class Ib molecules (10). So far, three *L. monocytogenes*-derived formyl-methionine (f-met) peptides have been identified, which are recognized by H2-M3-restricted CD8⁺ T cells (10). Because the H2-M3-restricted response is far more pronounced in C57BL/6 mice than in BALB/c mice (22), CCR7^{-/-} mice backcrossed onto the C57BL/6 background were infected with 2 \times 10³ *L. monocytogenes* organisms. 8 d after infection, spleen cells were incubated for 5 h with a cocktail of the three *L. monocytogenes*-derived f-met peptides (fMIGWII, fMIVIL, and fMIVTLF); frequencies of IFN γ -producing CD8⁺ T cells were determined by flow cytometry (Fig. 5). In infected control mice, f-met peptides induced IFN γ production in approximately 0.8% of the CD8⁺ splenocytes; this corresponded to 10⁵ f-met-restricted CD8⁺ T cells per spleen. Significant frequencies of f-met-restricted CD8⁺ T cells also were present in spleens of infected CCR7^{-/-} C57BL/6 mice. Although frequencies in

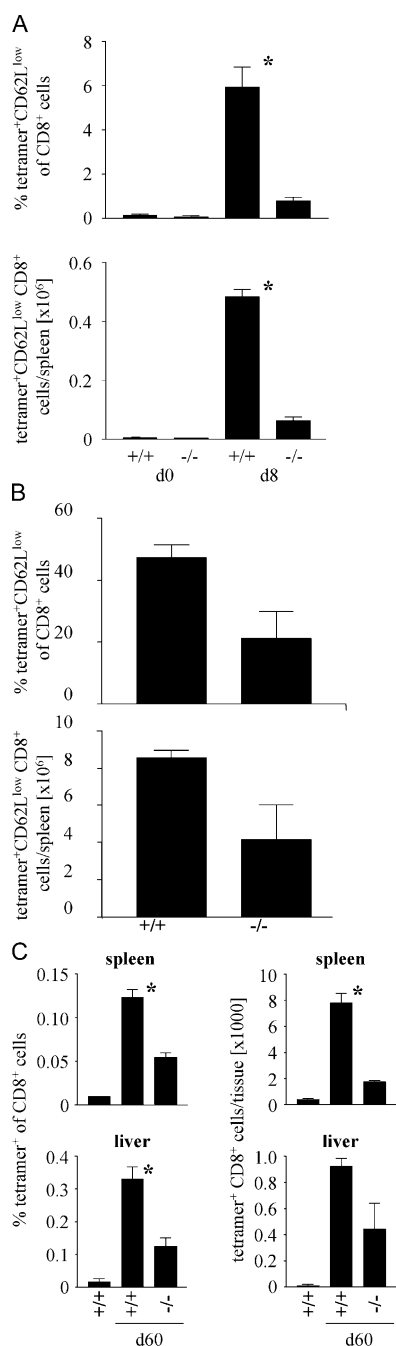


Figure 4. CD8⁺ T cell response to OVA₂₅₇₋₂₆₄ after primary and secondary infection with LmOVA. CCR7^{+/+} and CCR7^{-/-} mice on C57BL/6 background were infected i.v. with 5×10^3 LmOVA organisms and 60 d later were challenged i.v. with 10^5 LmOVA organisms. 8 d after primary infection (A) and 6 d after secondary infection (B), frequencies and numbers of OVA₂₅₇₋₂₆₄-specific CD8⁺ T cells were determined in spleens of infected mice. (C) Frequencies and numbers of OVA₂₅₇₋₂₆₄-specific CD8⁺ memory T cells in spleen and liver were measured 60 d after primary infection of mice. For comparison, we show values for naive CCR7^{+/+} animals. Mean \pm SD of three individually analyzed mice per group. *Difference between infected CCR7^{+/+} and CCR7^{-/-} mice was statistically significant ($P < 0.05$).

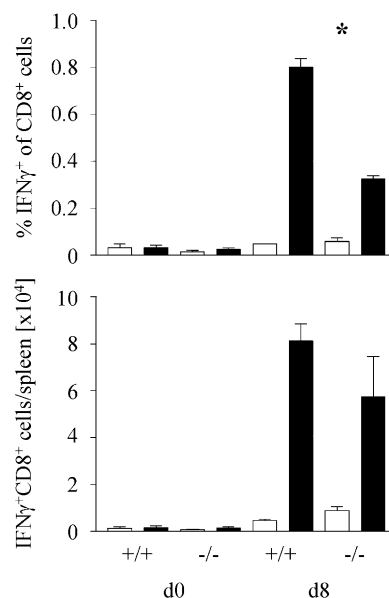


Figure 5. CD8⁺ T cell response to f-met peptides during primary infection. CCR7^{+/+} and CCR7^{-/-} mice on C57BL/6 background were i.v. infected with 2×10^3 *L. monocytogenes* organisms. After 8 d, spleen cells were incubated for 5 h with a cocktail of the f-met peptides fMIGWII, fMIVIL and fMIVTLF. Cells were stained extracellularly with anti-CD8 α mAb and intracellularly with anti-IFN γ mAb and analyzed by flow cytometry. The figure shows frequencies of IFN γ ⁺ cells among CD8⁺ T cells and total numbers of IFN γ ⁺ CD8⁺ T cells per spleen after incubation without (white bars) and with peptides (black bars; mean \pm SD of three individually analyzed mice per group). *Difference between IFN γ ⁺ cells from infected CCR7^{+/+} and CCR7^{-/-} mice after restimulation with peptide was statistically significant ($P < 0.05$).

CCR7^{-/-} mice were reduced compared with controls, total numbers did not differ significantly between both mouse strains; this mainly was due to the enlarged spleen size of CCR7^{-/-} mice (unpublished data). In two further experiments, we consistently observed similar or only slightly reduced numbers of f-met-specific CD8⁺ T cells in CCR7^{+/+} and CCR7^{-/-} mice (unpublished data). Thus, the lack of CCR7 only marginally impaired f-met peptide-specific CD8⁺ T cell responses during *L. monocytogenes* infection.

The immunodominant I-A^b-restricted epitope LLO₁₉₀₋₂₀₁ can be used for quantitative analysis of *L. monocytogenes*-specific CD4⁺ T cell responses in C57BL/6 mice (12). CCR7^{+/+} and CCR7^{-/-} mice on C57BL/6 background were infected with *L. monocytogenes*; 10 d after infection, frequencies of LLO₁₉₀₋₂₀₁-restricted CD4⁺ T cells were determined (Fig. 6). In infected CCR7^{+/+} control mice, approximately 0.6% of CD4⁺ splenocytes produced IFN γ in response to LLO₁₉₀₋₂₀₁. Infected CCR7^{-/-} mice demonstrated similar frequencies and total numbers of responding cells (Fig. 6). When we analyzed CCR7^{+/+} and CCR7^{-/-} mice 8 or 9 d after infection, results were comparable (unpublished data). T cells from CCR7^{+/+} mice showed a ho-

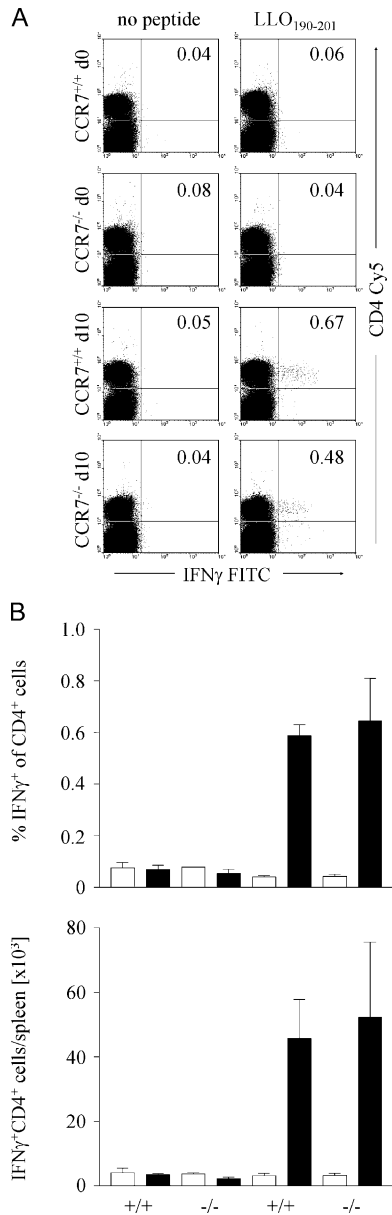


Figure 6. CD4⁺ T cell response to LLO₁₉₀₋₂₀₁ during primary infection. CCR7^{+/+} and CCR7^{-/-} mice on C57BL/6 background were i.v. infected with 2×10^3 *L. monocytogenes* organisms. After 10 d, spleen cells were incubated for 5 h with the peptide LLO₁₉₀₋₂₀₁. Cells were stained extracellularly with anti-CD4 mAb and intracellularly with anti-IFN γ mAb, and analyzed by flow cytometry. (A) Representative results. (B) Frequencies of IFN γ ⁺ cells among CD4⁺ T cells and absolute numbers of IFN γ ⁺ CD4⁺ cells per spleen after incubation without (white bars) and with peptide LLO₁₉₀₋₂₀₁ (black bars; mean \pm SD of three individually analyzed mice per group). There was no significant difference ($P > 0.05$) between IFN γ ⁺ cells from infected CCR7^{+/+} and CCR7^{-/-} mice after restimulation with peptide.

mogenous response in all six experiments, in which we analyzed CD4⁺ T cell responses. In contrast, CD4⁺ T cells from CCR7^{-/-} mice demonstrated some variation. We observed individual mice, in which the response was reduced up to

one third of the mean response of wild-type animals. However, in none of the experiments did the difference between the mean response in CCR7^{+/+} and CCR7^{-/-} mice reached a statistically significant level ($P < 0.05$). In summary, our results demonstrate that MHC class II-restricted CD4⁺ T cell responses to *L. monocytogenes* are far less dependent on CCR7 than are the corresponding MHC class Ia-restricted CD8⁺ T cell responses.

Because CCR7 is expressed on naive CD8⁺ T cells and on activated dendritic cells, we determined the role of CCR7 on CD8⁺ T cells in an adoptive T cell transfer model. CD8⁺ T cells from the TCR-transgenic mouse strain OT1 recognize OVA₂₅₇₋₂₆₄, the immunodominant CD8⁺ T cell epitope that is included in the truncated ovalbumin protein expressed by LmOVA. To determine whether CCR7 expression on CD8⁺ T cells is sufficient for the induction of a response, purified CD8⁺ T cells from OT1 donors were labeled with carboxyfluorescein succinimidyl ester (CFSE) and adoptively transferred into CCR7^{+/+} and CCR7^{-/-} recipients. After 24 h, mice were infected with LmOVA; 72 h later, proliferation of transferred CD8⁺ T cells was analyzed by flow cytometry (Fig. 7). In noninfected CCR7^{+/+} animals, transferred T cells maintained the CFSE^{high} phenotype. Infection of CCR7^{+/+} recipients induced proliferation of CD8⁺ donor cells as indicated by the stepwise loss of CFSE staining. In noninfected CCR7^{-/-} recipients, we detected a low level of donor cell proliferation for which we currently have no explanation. However, proliferation did not increase further upon LmOVA infection. As was expected from the analysis of the endogenous CD8⁺ T cell response to LmOVA infection (Fig. 4 A), the block in activation of transferred CD8⁺ T cells was incomplete in CCR7^{-/-} recipients. At day 4 after infec-

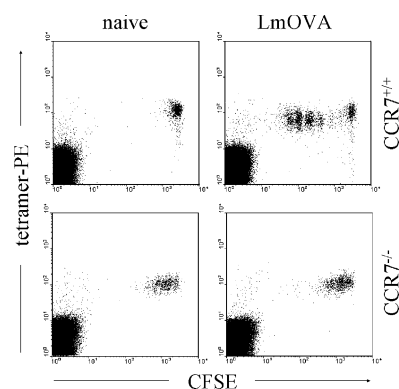


Figure 7. Expression of CCR7 on CD8⁺ T cells is insufficient for T cell priming. CCR7^{+/+} and CCR7^{-/-} mice on C57BL/6 background received 4×10^6 purified CFSE-labeled CD8⁺ T cells from OT1 mice. After 24 h, recipients were i.v. infected with 5×10^3 LmOVA organisms, or were left untreated (naive). 3 d later, spleen cells were stained with PE-conjugated OVA₂₅₇₋₂₆₄ tetramers and Cy5-conjugated anti-CD8 α mAb, and CFSE expression on transferred CD8⁺ T cells was analyzed by flow cytometry. Figures show CD8-gated T cells only and are representative for two individually analyzed mice per group.

tion, some proliferation of donor cells also was observed in CCR7^{-/-} recipients (unpublished data).

In a complementary set of experiments, we generated bone marrow chimeras, in which $\alpha\beta$ TCR⁺ T cells had a CCR7^{+/+} or a CCR7^{-/-} genotype. A similar approach recently was described to analyze mutant B cells in a nonmutant environment (23). TCR β ^{-/-} mice were irradiated and reconstituted with bone marrow cells from TCR β ^{-/-} mice. In addition, these animals received bone marrow cells from either CCR7^{+/+} or CCR7^{-/-} mice at a 5:1 ratio of TCR β ^{-/-}/CCR7^{+/+} or TCR β ^{-/-}/CCR7^{-/-}. Because of this reconstitution protocol, the majority of hematopoietic cells in the chimeras had a TCR β ^{-/-}-CCR7^{+/+} genotype. In contrast, $\alpha\beta$ TCR⁺ T cells were exclusively CCR7^{+/+} or CCR7^{-/-}. To confirm the mixed chimerism, bone marrow cells of TCR β ^{-/-} mice that were reconstituted with TCR β ^{-/-} and CCR7^{-/-} bone marrow were T cell depleted; DNA from these cells was analyzed by quantitative PCR for the ratio of CCR7^{+/+}(TCR β ^{-/-})/CCR7^{-/-} cells. 6 wk after reconstitution, we determined a ratio of 1:5.68 \pm 0.74 ($n = 5$) for CCR7⁻/CCR7⁺ DNA, a value which was

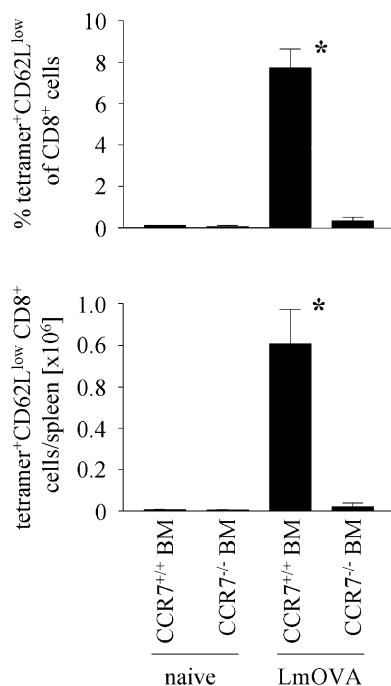


Figure 8. Expression of CCR7 on APCs is not sufficient for priming of CD8⁺ T cells. TCR β ^{-/-} mice were irradiated and reconstituted with 10×10^6 bone marrow cells from TCR β ^{-/-} mice and 2×10^6 bone marrow cells from WT (CCR7^{+/+}) or KO (CCR7^{-/-}) mice. 8 wk after the reconstitution, mice were i.v. infected with 5×10^3 LmOVA organisms. After an additional 9 d, spleen cells were stained with FITC-conjugated anti-CD62L mAb, Cy5-conjugated anti-CD8 α mAb, and PE-conjugated OVA₂₅₇₋₂₆₄ tetramers. Cells were analyzed by flow cytometry after addition of propidium iodide (mean \pm SD of three individually analyzed mice per group). *Difference between infected mice reconstituted with CCR7^{+/+} and CCR7^{-/-} bone marrow was statistically significant ($P < 0.05$).

similar to the 1:5 ratio of bone marrow cells that was used for the original reconstitution. 8 wk after reconstitution, when mice showed normal T cell levels in the peripheral blood, the chimeras were infected with LmOVA; 9 d later, CD8⁺ T cell responses were analyzed (Fig. 8). Mice that were reconstituted with bone marrow cells from CCR7^{+/+} mice showed an OVA₂₅₇₋₂₆₄-specific CD8⁺ T cell response similar to that observed in regular CCR7^{+/+} wild-type mice (as shown in Fig. 4 A). In contrast, mice that were reconstituted with CCR7^{-/-} bone marrow cells demonstrated only a marginal OVA₂₅₇₋₂₆₄-specific CD8⁺ T cell response. In conclusion, the experiments that are shown in Figs. 7 and 8 indicate that CCR7 expression on MHC class Ia-restricted CD8⁺ T cells or on APCs alone is insufficient for efficient priming. Rather, CCR7 has to be expressed on T cells and APCs for effective induction of *L. monocytogenes*-specific MHC class Ia-restricted CD8⁺ T cells.

DISCUSSION

Using *L. monocytogenes* infection of CCR7^{-/-} mice, we demonstrate differential requirements for the CCR7/CCR7 ligand system in the activation of different T cell subsets. Naive MHC class Ia-restricted CD8⁺ T cells depended markedly on CCR7; MHC class Ib-restricted CD8⁺ T cells and MHC class II-restricted CD4⁺ T cells showed some dependency; activation of MHC class Ia-restricted CD8⁺ memory T cells was virtually independent. The partial impairment of the T cell response also is reflected by the relative resistance of CCR7^{-/-} mice during primary and secondary *L. monocytogenes* infection. Although CCR7^{-/-} mice showed slightly higher *L. monocytogenes* titers at late time points of primary infection, these mice could eradicate the bacteria. Only 2 out of >100 CCR7^{-/-} mice succumbed to *L. monocytogenes* during the whole series of experiments, which is similar to the normal range for wild-type mice.

The impaired CD8⁺ T cell response was a general event that was observed for all three *L. monocytogenes*-derived MHC class Ia epitopes that were tested in our experiments (LLO₉₁₋₉₉, p60₂₁₇₋₂₂₅, and the surrogate epitope OVA₂₅₇₋₂₆₄). The reduction was observed in all tissues that were analyzed and at different time points following primary infection. Thus, the impaired CD8⁺ T cell response was neither due to changes in the tissue distribution of the activated CD8⁺ T cells, which could be anticipated from the altered distribution pattern of T cells in CCR7^{-/-} mice (6), nor due to a delayed response, which had been described for CD4⁺ T cells in *plt* mice (4). This result is in accordance with our observations in a tumor transplantation model, where CCR7^{-/-} mice failed to mount a CD8⁺ T cell response against fully allogeneic tumors (9). In contrast, Junt et al. did not detect changes of the CD8⁺ T cell responses in *plt* mice following infection with different viruses (8). Currently, we only can speculate on the reasons for the incongruent results. Differences in the cells that are involved in CD8⁺ T cell priming, the tissue localization of this process, and consequently, the

requirement for CCR7 between viral and bacterial infections can be anticipated. The discrepancy also could be due to the mouse strains that were used in the studies. The CCR7-deficiency results in a complete breakdown of the CCR7/CCR7 ligand system. In contrast, *plt* mice retain at least one functional copy of CCL21, the CCL21-Leu (4, 24). Despite its limited expression pattern, CCL21-Leu could be sufficient for effective priming of CD8⁺ T cells.

In our infection model, CCR7^{-/-} mice generated reduced numbers of MHC class Ia-restricted CD8⁺ memory T cells when compared with wild-type animals. Considering the marginal primary CD8⁺ T cell response in CCR7^{-/-} mice, even this low number of CD8⁺ memory T cells is surprising. Our results, thus, argue against a strict correlation between the strength of the primary response and the size of the memory cell population. According to current concepts, CD8⁺ memory T cells can be divided into two major subpopulations: “effector” and “central” memory T cells (25). Effector memory T cells possess a CCR7⁻CD62L^{low} phenotype, are located mainly in nonlymphoid tissues, and show rapid cytokine production and cytotoxicity following activation. In contrast, central effector cells are CCR7⁺CD62L^{high}, reside in secondary lymphoid tissues, and show delayed responses compared with effector memory T cells. Effector memory T cells most likely represent a rapidly responding cell population in the periphery, whereas central memory T cells form the basis for stable CD8⁺ T cell memory with high proliferative capacity upon reactivation (25–28). The interrelation of both memory T cell populations is not clear, and some studies even suggest a linear differentiation pathway from CD8⁺ effector T cells via effector memory intermediates to CD8⁺ central memory T cells (29). In our infection model, CCR7 was dispensable for the maintenance of CD8⁺ memory T cells and the response of these cells after challenge infection. However, this result does not challenge the current concept of central and effector memory T cells. The lack of CCR7 expression does not imply that there also is a lack of CD8⁺ central memory T cells. It is possible that even without CCR7 expression, memory T cells can locate to niches, which allows long-term survival. Furthermore, alternative mechanisms could replace the CCR7/CCR7 ligands system. Indeed, CXCL12 can facilitate homing of memory, but not naive, T cells into secondary lymphoid tissues (30).

Despite the low number of MHC class Ia-restricted CD8⁺ memory T cells, CCR7^{-/-} mice showed virtually normal secondary CD8⁺ T cell responses. CD8⁺ memory T cells are responsible for rapid elimination of *L. monocytogenes* following secondary infection. A small memory T cell population controls *L. monocytogenes* less efficiently and results in a greater abundance of bacterial antigens available for T cell activation, more profound inflammation, and, consequently, a stronger secondary T cell response. Such a compensatory mechanism could explain the normal secondary CD8⁺ T cell response that is observed in CCR7^{-/-} mice. Overall, our observations allow two general conclusions: (a) the

CCR7/CCR7 ligand system is not essential for generation and survival of memory T cells. Accordingly, CCR7-mediated migration of CD8⁺ memory T cells into lymphoid tissue is not a limiting factor for persistence of memory; and (b) to a large extent, activation of CD8⁺ memory T cells is independent of the CCR7/CCR7 ligands system. Thus, CD8⁺ memory T cells seem to have less stringent requirements on the environment of activation.

In contrast to the CD8⁺ T cell response to conventional MHC class Ia-restricted peptides, the response to f-met peptides presented in the context of H2-M3 was impaired only slightly by the absence of CCR7. H2-M3-restricted CD8⁺ T cells recognize a broad range of structurally related f-met peptides, and thus, are far more promiscuous than conventional MHC class Ia-restricted CD8⁺ T cells (31, 32). Moreover, H2-M3-restricted CD8⁺ T cells display an activated phenotype compared with conventional CD8⁺ T cells (33); in terms of magnitude and kinetics, H2-M3-restricted CD8⁺ T cell responses to *L. monocytogenes* resemble secondary responses rather than primary responses (22). Therefore, H2-M3-restricted CD8⁺ T cells could differ in their activation requirements, including a reduced dependency on mechanisms that are mediated by the CCR7/CCR7 ligand system. It also is possible that the H2-M3-restricted CD8⁺ T cell response to *L. monocytogenes* is due, at least in part, to the activation of H2-M3-restricted CD8⁺ memory T cells with specificities to related f-met peptides. This notion is consistent with our observations in CCR7^{-/-} mice. Because CD8⁺ memory T cell responses were largely independent of CCR7, H2-M3-restricted responses only should be impaired partially in CCR7^{-/-} mice.

In comparison with the CD8⁺ T cell response, the CD4⁺ T cell response against *L. monocytogenes* was affected far less in CCR7^{-/-} mice, and in some animals, even reached levels that were observed in control animals. Evidence for CD4⁺ T cell responses in the absence of the CCR7/CCR7 ligands system has been described before. CCR7^{-/-} and *plt* mice can generate T cell dependent antibody responses (6, 8, 9), and after immunization with ovalbumin, spleen and lymph node cells of *plt* mice proliferate and produce IL-2 in response to antigen restimulation (4). Although *plt* mice lack distinct T cell zones in spleen and lymph nodes, CD4⁺ T cells and dendritic cells colocalize in lymphoid tissues following immunization; this allows close interactions, and, consequently, the priming of CD4⁺ T cell responses (4). A similar scenario could be envisaged in CCR7^{-/-} mice following *L. monocytogenes* infection. Naive CD4⁺ T cells could interact with professional APCs that are loaded with *L. monocytogenes*-derived antigens outside of discrete T cell zones and result in the priming of *L. monocytogenes*-specific CD4⁺ T cells.

Expression of CCR7 is not restricted to naive T cells. Our experiments demonstrate that neither CCR7 expression on CD8⁺ T cells in a CCR7-negative background nor absence of CCR7 on CD8⁺ T cells in an otherwise CCR7-

positive environment is sufficient for effective priming. Upon activation, dendritic cells express CCR7 and dendritic cell migration is impaired profoundly in CCR7^{-/-} and *plt* mice. After immunization of these mice, dendritic cells fail to enter lymph nodes, and in the spleen, activated dendritic cells accumulate in the marginal zones rather than migrate into the white pulp (3, 4, 6–8). In a histologic analysis of spleens from *L. monocytogenes*-infected CCR7^{-/-} mice, we detected similar alterations in dendritic cell distribution. 24 to 48 h after infection, dendritic cells entered the white pulp and accumulated in the T cell zone of wild-type mice but were trapped in the marginal zones of CCR7^{-/-} mice (unpublished data). Because dendritic cells are essential for priming of CD8⁺ T cells following *L. monocytogenes* infection (16, 17), the altered migration pattern of dendritic cells could contribute to impaired priming. We conclude that limitations in CD8⁺ T cells and dendritic cells are responsible for the profound deficiency in the induction of MHC class Ia-restricted CD8⁺ T cell responses in CCR7^{-/-} mice.

In summary, our study demonstrates a discrete CCR7 requirement in the activation of different T cell subsets during *L. monocytogenes* infection. This result indicates that there is no general mechanism for the activation of T cells during *L. monocytogenes* infection; rather different T cell subpopulations have distinct requirements for the site and environment in which the contact between APCs and T cells takes place.

MATERIALS AND METHODS

Antibodies. Rat IgG antibodies, anti-CD16/CD32 mAb (clone: 2.4G2), anti-IFN γ mAb (R4-6A2, rat IgG1), anti-CD8 α mAb (YTS169), anti-CD4 mAb (YTS191.1), anti-TCR β mAb (H57-597), and anti-CD62L mAb (Mel-14) were purified from rat serum or hybridoma supernatants with protein G-Sepharose. Antibodies were Cy5- or FITC-conjugated according to standard protocols. Rat IgG1 isotype control mAb (clone: R3-34), anti-CD11c mAb (clone HL3, Armenian hamster), and alkaline phosphatase-conjugated rabbit anti-hamster Ig Ab were purchased from BD Biosciences.

Mice and *L. monocytogenes* infection. C57BL/6 and BALB/c mice were purchased from the Bundesinstitut für Risikobewertung (BfR) in Berlin. OT1 mice (34) and TCR β ^{-/-} mice (35) were bred in our facilities. CCR7^{-/-} mice were crossed for six generations into the BALB/c background and for eight generations into the C57BL/6 background (6, 9). The expression of H-2K^d in CCR7^{-/-} BALB/c mice was confirmed by FACS analysis. CCR7^{-/-} mice were genotyped by PCR as described previously (6). All animal experiments were conducted according to the German Animal Protection Law.

Mice were infected with *L. monocytogenes* strain EGD (Lm) or with LmOVA (20). Bacteria were grown overnight in tryptic soy broth (TSB), washed twice in PBS, resuspended in PBS 10% glycerol and stored at -80°C. Aliquots were thawed and bacterial titers were determined by plating serial dilutions on TSB agar plates. For i.v. infection, *L. monocytogenes* organisms were diluted appropriately and injected in a volume of 200 μ l PBS into the lateral tail veins. For i.v. infection, *L. monocytogenes* was grown overnight in TSB and washed twice in PBS. Bacterial density was determined by absorption at 600 nm and bacteria were diluted appropriately in PBS (an OD₆₀₀ value of 1 is equivalent to 10⁹ bacteria/ml). Bacteria were administered in 200 μ l PBS by gastric intubation. Bacterial inocula were controlled by plating serial dilutions on TSB agar plates. For determination of bacterial burdens in organs, mice were killed, organs were homogenized in PBS, and serial dilutions of homogenates were plated on TSB agar. Colonies were counted after 24 h of incubation at 37°C.

In vitro restimulation of cells and flow cytometric determination of cytokine expression. Spleens were removed and single cell suspensions were prepared using an iron mesh sieve. Red blood cells were lysed and spleen cells were washed twice with RPMI 1640 medium supplemented with glutamine, Na-pyruvate, β -mercaptoethanol, penicillin, streptomycin, and 10% heat inactivated FCS (complete RPMI 1640 medium). Isolation of T cells from liver and tissues of the small intestine (lamina propria lymphocyte and intraepithelial lymphocytes) was performed as described previously (12). For determination of cytokine expression, 4 \times 10⁶ cells were cultured in a volume of 1 ml complete RPMI medium. Cells were stimulated for 5 h with 10⁻⁶ M of the peptides LLO₁₉₀₋₂₀₁ (NEKYAQAYPNVS), LLO₉₁₋₉₉ (GYKDGNEYI), p60₂₁₇₋₂₂₅ (KYGVS-VQDI), OVA₂₅₇₋₂₆₄ (SIINFEKL), or a cocktail of three f-met peptides that were derived from *L. monocytogenes* proteins (fMIGWII, fMIVIL, and fMIVTLF) (10). During the final 4 h of culture, 5 μ g/ml brefeldin A (Sigma-Aldrich) were added. Cultured cells were washed and incubated for 10 min with rat IgG antibodies and anti-CD16/CD32 mAb to block non-specific antibody binding. Subsequently, cells were stained with Cy5-conjugated anti-CD4 mAb or anti-CD8 α mAb, and after 30 min on ice, cells were washed with PBS and fixed for 20 min at room temperature with PBS 4% paraformaldehyde. Cells were washed with PBS 0.1% BSA, permeabilized with PBS 0.1% BSA 0.5% saponin (Sigma-Aldrich), and incubated in this buffer with rat IgG antibodies and anti-CD16/CD32 mAb. After 5 min, FITC-conjugated anti-IFN γ mAb or isotype control mAb were added. After another 20 min at room temperature, cells were washed with PBS and fixed with PBS 1% paraformaldehyde. Cells were analyzed using a FACS-Calibur and the CELLQuest software (Becton Dickinson).

Generation of MHC class I tetramers and staining of cells with tetramers. Modified forms of the full-length cDNA of H-2K^d, H-2K^b, and human β 2-m were provided by D. Busch, Institute for Microbiology, Immunology, and Hygiene, Technische Universität, München, Germany. H-2K^d/LLO₉₁₋₉₉ tetramers and H-2K^b/OVA₂₅₇₋₂₆₄ tetramers were generated as described previously (19, 36). For flow cytometry analysis, 2 \times 10⁶ cells were incubated for 15 min at 4°C with rat IgG Ab, anti-CD16/CD32 mAb, and streptavidin (Molecular Probes) in PBS 0.5% BSA 0.01% sodium azide. After incubation, cells were stained for 60 min at 4°C with Cy5-conjugated anti-CD8 α mAb, FITC-conjugated anti-CD62L mAb, and PE-conjugated MHC class I-LLO₉₁₋₉₉ or OVA₂₅₇₋₂₆₄ tetramers. Subsequently, cells were washed with PBS 0.5% BSA 0.01% sodium azide and diluted in PBS. Propidium iodide was added before four-color flow cytometry analysis.

Cell transfer experiments. CD8⁺ spleen cells from OT-1 mice were purified by magnetic cell sorting ((MACS; Miltenyi Biotec) according to the manufacturer's protocol. Subsequently, cells were washed with PBS and incubated for 4 min with 2 μ M of CFSE (Molecular Probes). Cells were washed twice with complete medium, and 3–4 \times 10⁶ cells were injected into the lateral tail vein of CCR7^{-/-}C57BL/6 and control C57BL/6 mice. 1 d after the adoptive transfer, mice were i.v. infected with 5 \times 10³ LmOVA. 3 d after infection, mice were killed, spleen cells were stained with PE-conjugated OVA₂₅₇₋₂₆₄ tetramers and Cy5-conjugated anti-CD8 α mAb and analyzed by four-color flow cytometry after the addition of propidium iodide.

Generation of bone marrow chimeras. For the generation of mixed bone marrow chimeras, we used a protocol that was described recently by Fillatreau and Gray (23). TCR β ^{-/-} mice on the C57BL/6 background were irradiated with 600 rad. Subsequently, mice were reconstituted with 10 \times 10⁶ bone marrow cells from TCR β ^{-/-} mice. In addition, mice received 2 \times 10⁶ bone marrow cells from wild-type C57BL/6 mice or from CCR7^{-/-} mice. Before injection, the bone marrow from wild-type and CCR7^{-/-} mice was T cell depleted by MACS according to the manufacturer's protocol (CD90 depletion kit; Miltenyi Biotec). Mice were kept for

4 wk on 2 mg/ml ampicillin and 1 mg/ml neomycin sulfate in the drinking water. After 6–8 wk, reconstitution was controlled in the peripheral blood by flow cytometry. Bone marrow chimeras were infected i.v. with 5×10^3 LmOVA and analyzed 9 d later. To verify the degree of chimerism, bone marrow cells from TCR $\beta^{-/-}$ mice that were reconstituted with TCR $\beta^{-/-}$ and CCR7 $^{-/-}$ bone marrow cells were isolated, and CD90 $^+$ T cells were depleted by MACS. Genomic DNA from T cell-depleted bone marrow cells was prepared by phenol chloroform extraction and dissolved in water. Primers were designed to detect the intact CCR7 locus (CCR7 $^+$) (forward: 5'-AGAAGAACAGCGGCGAGGA-3', reverse: 5'-AGCATAGGCAC-TAGGAACCCAAA-3') or the deficient CCR7 locus (CCR7 $^-$) (forward: 5'-CAGTTTCATAGCCTGAAGAACGA-3', reverse: 5'-GCCGAT-GAAGGCATACAAGAAA-3'). 10 ng of DNA from bone marrow cells of CCR7 $^{-/-}$ animals were mixed with DNA from bone marrow cells of CCR7 $^{+/+}$ (TCR $\beta^{-/-}$) animals at the ratios 2:1, 1:1, 1:2, 1:4, 1:8, 1:12, and 1:16. These mixtures and the DNA samples from T cell depleted bone marrow cells from the chimeras were analyzed in parallel by real-time PCR (SYBR green, ABI Prism 7000, Becton Dickinson) using the primers specific for the CCR7 $^+$ and the CCR7 $^-$ locus. A curve was prepared by plotting the numbers of cycle differences (Δ CT) between the two primer pairs against the mixing factor of the two DNA samples. An exponential function was fitted onto the resulting curve using Excel (Microsoft). This function (e.g., dilution = $1.2675 \times 10^{0.8608\Delta\text{CT}}$, $R^2 = 0.9995$) was used to calculate the ratio of CCR7 $^+$ /CCR7 $^-$ DNA within T cell depleted bone marrow of reconstituted animals.

Statistical analysis. All experiments described were performed at least three times with similar results. Unless otherwise stated, experimental groups consisted of at least three mice per group, and mice were analyzed independently. For statistical analysis of frequencies and cell numbers we applied the Student's *t* test. Bacterial titers were compared using the Mann-Whitney test. Differences were considered significant with $P < 0.05$.

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