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*J Immunol* 2001; 167:5620-5627; doi: 10.4049/jimmunol.167.10.5620

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Role of CD28 for the Generation and Expansion of Antigen-Specific CD8⁺ T Lymphocytes During Infection with Listeria monocytogenes

Hans-Willi Mittrücker, Mischo Kursar, Anne Köhler, Robert Hurwitz, and Stefan H. E. Kaufmann

Infection of mice with the intracellular bacterium Listeria monocytogenes results in a strong CD8⁺ T cell response that is critical for efficient control of infection. We used CD28-deficient mice to characterize the function of CD28 during Listeria infection, with a main emphasis on Listeria-specific CD8⁺ T cells. Frequencies and effector functions of these T cells were determined using MHC class I tetramers, single cell IFN-γ production and Listeria-specific cytotoxicity. During primary Listeria infection of CD28⁻/⁻ mice we observed significantly reduced numbers of Listeria-specific CD8⁺ T cells and only marginal levels of specific IFN-γ production and cytotoxicity. Although frequencies were also reduced in CD28⁻/⁻ mice during secondary response, we detected a considerable population of Listeria-specific CD8⁺ T cells in these mice. In parallel, IFN-γ production and cytotoxicity were observed, revealing that Listeria-specific CD8⁺ T cells in CD28⁻/⁻ mice expressed normal effector functions. Consistent with their impaired CD8⁺ T cell activation, CD28⁻/⁻ mice suffered from exacerbated listeriosis both after primary and secondary infection. These results demonstrate participation of CD28 signaling in the generation and expansion of Ag-specific CD8⁺ T cells in listeriosis. However, Ag-specific CD8⁺ T cells generated in the absence of CD28 differentiated into normal effector and memory T cells. The Journal of Immunology, 2001, 167: 5620–5627.

Infection of mice with Listeria monocytogenes is a well-characterized model for analyzing cellular immunity against intracellular bacteria (1, 2). After systemic infection, L. monocytogenes gains access to the liver and the spleen and infects macrophages and hepatocytes. Within these cells, bacteria leave the phagosome and enter the cytoplasm where they replicate. The intracytoplasmic habitat results in processing and presentation of the phagosome and enter the cytoplasm where they replicate. The macrophages and hepatocytes. Within these cells, bacteria leave

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0022-1767/01/$02.00

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4 Abbreviations used in this paper: LLO, listeriolysin O; LCMV, lymphocytic choriomeningitis virus; VSV, vesicular stomatitis virus; TSB, tryptic soy broth.
requirement on CD28 costimulation was influenced by the differentiation state of CD8⁺ T cells, because preactivated CD8⁺ T cells were less dependent on CD28 (13, 18).

The contribution of CD28 to the activation of CD8⁺ T cells in vivo is even more controversial. Treatment of mice with nonimmunogenic tumors that had been transfected with one of the B7 molecules resulted in antitumor responses and protection against subsequent challenge with untransfected tumors (24–28). Blocking of CD28 signaling with a soluble CTLA4 protein prolonged graft survival and induced donor specific tolerance (29–31). However, more recent experiments using different B7-transfected tumors failed to demonstrate a protective antitumor response (32–35). Furthermore, CD28−/− mice respond to syngeneic or induced tumors (36) and reject allografts (37). In different viral infection models the requirement for CD28 signaling to generate a virus-specific CD8⁺ T cell response also varied significantly. Whereas the CD8⁺ T cell response of mice against infection with lymphocytic choriomeningitis virus (LCMV) was completely independent of CD28 signaling (7, 38, 39), CD8⁺ T cell responses against vesicular stomatitis virus (VSV), influenza virus, and certain strains of vaccinia virus were drastically impaired (7, 38–42). For viral infections, several mechanisms have been suggested to explain the distinct CD28 requirements, including differences in viral load, viral persistence, and the degree of inflammation (43, 44).

We infected CD28-deficient mice with L. monocytogenes and analyzed their immune responses, with the main emphasis on the generation and differentiation of Listeria-specific CD8⁺ T cells as the major mediators of acquired resistance. We show that CD28 is important for the protective antilisterial response, as demonstrated by higher bacterial titers in the liver and the spleen, delayed clearance of bacteria, and increased susceptibility to infection in CD28−/− mice. During primary infection, Listeria-specific cytotoxicity and frequencies of IFN-γ-secreting Listeria-specific CD8⁺ T cells were markedly diminished. A closer analysis with MHC class I tetramers loaded with dominant listerial peptides revealed that the lack of CD28 costimulation is in fact CD8⁺ T cells, because preactivated CD8⁺ T cells as effectors were completely dependent on CD28 costimulation (7, 38, 39), CD8⁺ T cells and, in close correlation, a substantial population of CD8⁺ effector T cells. Thus, the lack of CD28 impairs the generation, and particularly the expansion, of specific CD8⁺ T cells during L. monocytogenes infection; however, it does not prevent differentiation into CD8⁺ effector and memory T cells.

Materials and Methods

Mouse strains

CD28−/− mice, backcrossed eight times onto the BALB/c background, were kindly provided by Dr. T. W. Mak (Toronto, Canada) (7). CD28−/− BALB/c control mice were bred in our facility at the Federal Institute for Health Protection of Consumers and Veterinary Medicine (Berlin, Germany) and experiments were conducted according to the German animal protection law.

Bacteria and bacterial infection of mice

Mice were infected with L. monocytogenes strain EGD. Bacteria were grown overnight in tryptic soy broth (TSB), washed twice in PBS, aliquoted in PBS 10% glycerol, frozen, and stored at −80°C. Aliquots were thawed and bacterial titers were determined by plating serial dilutions on TSB agar plates. For infection, aliquots were thawed and appropriately diluted in PBS. Bacteria were injected in a volume of 200 μl PBS into the lateral tail veins of mice. The bacterial dose was controlled by plating dilutions of the inoculum on TSB agar plates. For determination of bacterial burdens in organs, mice were killed at the time points indicated. Livers and spleens were homogenized in PBS, serial dilutions of homogenates were plated on TSB agar plates, and colonies were counted after incubation at 37°C overnight. Unless otherwise indicated, mice were first infected with 10⁸ bacteria. After 2–3 mo, mice were secondarily infected with 10⁹ bacteria. In our hands, L. monocytogenes were 8.5 × 10⁸ and 4.0 × 10⁸ Listeria for CD28−/− and CD28−/− mice, respectively.

Antibodies

Polyclonal rat Abs, anti-CD16/CD32 mAb (clone, 2.4G2), anti-IFN-γ mAb (clone, R4-6A2, rat IgG1), anti-CD3 mAb (clone, 145 2C11), anti-CD4 mAb (clone, YTS191.1), anti-CD8α mAb (clone, YTS169), and anti-CD62L mAb (clone, Mel-14) were purified from rat serum or hybridoma supernatants with protein G-Sepharose. Abs were Cy5- or FITC-conjugated according to standard protocols. PE-conjugated anti-IL-2 mAb (clone, JS6-54h, rat IgG2b), FITC-conjugated rat IgG1 isotype control mAb (clone, R3-34), and PE conjugated rat IgG2b isotype control mAb (clone, A95-1) were purchased from BD PharMingen (San Diego, CA).

In vitro restimulation of spleen cells and flow cytometric determination of IFN-γ expression

At the time points indicated, spleens from infected mice were removed and single cell suspensions were obtained by teasing spleens through stainless steel meshes. Erythrocytes were lysed and spleen cells (3 × 10⁶/well) were cultured in 48-well plates in RPMI medium supplemented with glutamine, Na-pyruvate, 2-ME, penicillin, streptomycin, and 10% heat inactivated FCS. Spleen cells were stimulated for 6 h with 5 μg/ml anti-CD3 mAb or with 10⁻⁶ M of the peptides LL091-99 (GYKDGNEYI) or p60217-225 (KYGVSVMQDI), both purchased from Jerini Bio Tools (Berlin, Germany). During the final 4 h of culture, 10 μg/ml brefeldin A (Sigma-Aldrich, St. Louis, MO) were added.

Cultured cells were washed and incubated with polyclonal rat Abs and anti-CD16/CD32 mAb to block unspecified Ab binding. After 10 min, cells were stained with Cy5-conjugated anti-CD4 mAb or anti-CD8α mAb. After 30 min on ice, cells were washed with PBS and fixed for 20 min at room temperature with PBS 4% paraformaldehyde (Sigma-Aldrich). 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 polyclonal rat Abs or anti-CD16/CD32 mAb. After 5 min, FITC-conjugated anti-IFN-γ mAb or FITC-conjugated isotype control mAb was added. After an additional 20 min at room temperature, cells were washed with PBS and fixed with PBS 1% paraformaldehyde. Cells were analyzed using a FACSCalibur and the CellQuest software (BD Biosciences, Mountain View, CA).

Generation of MHC class I tetramers and staining of cells with tetramers

Modified forms of the full-length cDNA of H-2Kk and human β2m were kindly provided by Drs. E. Pamer and D. Busch. Tetrameric H-2Kk/β2m peptide complexes were generated as described by Busch et al. (5). Briefly, human β2m and the extracellular domains of H-2Kk fused with a peptide containing a specific biotinylation site were expressed as recombinant proteins in E. coli. Proteins were purified, dissolved in 8 M urea, and diluted into a refolding buffer containing the peptides LLO91-99 or p60217-225 to generate recombinant soluble MHC class I-peptide complexes. These complexes were purified by gel filtration and enzymatically biotinylated using the biotin protein ligase BirA (Aviity, Denver, CO). Free biotin was removed and MHC-peptide complexes were purified by gel filtration. To generate tetrameric MHC-peptide complexes, PE-conjugated streptavidin (Molecular Probes, Eugene, OR) was added to the monomers at a molar ratio of 4:1. Tetramers were purified by gel filtration and stored at 4°C.

For flow cytometry analysis, 1 × 10⁶ cells were incubated for 15 min at 4°C with polyclonal rat Abs, anti-CD16/CD32 mAb, and streptavidin (Molecular Probes) in PBS containing 0.05% BSA and 0.01% sodium azide. After incubation, cells were stained for 60 min at 4°C with Cy5-conjugated anti-CD8α mAb, FITC-conjugated anti-CD62L mAb, and PE-conjugated MHC class I-LLO91-99 or MHC class I-p60217-225 tetramers. Subsequently, cells were washed with PBS 0.5% BSA, 0.01% sodium azide, and diluted in PBS. Propidium iodide was added before 4-color flow cytometry analysis.

IFN-γ production after restimulation in vitro

Spleen cells from infected mice were removed and 5 × 10⁶ cells/well were cultured in 96-well plates in triplicate per experimental value and mouse. Cells were stimulated with 5 μg/ml anti-CD3 mAb or with 10⁻⁶ M of the peptides LLO91-99 or p60217-225. After 2 days, IFN-γ was determined in the supernatants by ELISA as described previously (45).

Cytotoxicity assay

At the time points indicated, spleens from infected mice were removed and 3 × 10⁶ cells were cultured in 1 ml complete medium supplemented with
30 U of recombinant human IL-2. Cells were restimulated with 3 × 10^6 naive BALB/c splenocytes that had been either loaded with 10^{-7} M of the peptides LLO_{91-99} or p60_{217-225} or were left untreated. After 3 days, cells were washed, counted, and incubated with 5000 51Cr-labeled P815 target cells at the effector/target ratios indicated in V-bottom plates in a total volume of 200 µl. Peptides were added at 10^{-6} M. After 4 h, supernatants (100 µl) were counted with a gamma counter. Each value was determined as triplicate. Cytotoxicity is given as the percentage of specific lysis which was calculated with the standard formula: percentage of specific lysis = (experimental 51Cr release − spontaneous 51Cr release)/(detergent induced 51Cr release − spontaneous 51Cr release).

Statistical analysis

The statistical significance of the results was determined with the statistics program included in the GraphPad Prism program (GraphPad, San Diego, CA). Mean bacterial titers are given as the geometric mean and differences in titers were determined with the unpaired t test from log-transformed values: *, p < 0.05; **, p < 0.005.

Results

**CD28^{−/−} mice show increased sensitivity to L. monocytogenes infection**

CD28^{−/−} and control mice were i.v. infected with 2 × 10^5 L. monocytogenes and bacterial titers in spleens and livers were determined (Fig. 1). Control mice had high bacterial titers on day 3 of infection but then readily cleared bacteria. In contrast, CD28^{−/−} mice had 10- to 100-fold higher bacterial titers in spleens and livers on days 3 and 6 of infection. By day 9, most CD28^{+/+} mice had cleared the infection, whereas CD28^{−/−} mice still suffered from considerable bacterial loads. L. monocytogenes infection did not result in chronic disease in CD28^{−/−} mice and on day 13 most CD28^{−/−} mice were free of bacteria. Increased listerial titers caused death in a significant proportion of CD28^{−/−} mice. Although, we lowered the dose to 10^3 Listeria in all additional experiments, still 20–25% of the CD28^{−/−} mice succumbed to infection whereas none of the control mice died from this dose.

Elevated bacterial titers were also observed during secondary infection of CD28^{−/−} mice (Fig. 2). Two days and particularly 3 days after challenge with 10^3 L. monocytogenes we observed elevated bacterial titers in spleens and livers of CD28^{−/−} mice compared with control mice. However, there was significant protection in challenged CD28^{−/−} mice when compared with naive CD28^{−/−} mice, indicating that CD28 is not absolutely required for protective immunity against L. monocytogenes.

**Improved Listeria-specific cytotoxic T cell response in CD28^{−/−} mice**

T cells are central for acquired immunity against infection with L. monocytogenes. Particularly CD8^+ T cells are critical for control and eradication of this pathogen (2). For BALB/c mice several immunodominant CD8^+ T cell epitopes from L. monocytogenes have been characterized (5), which allowed us to analyze the generation of the Ag-specific CD8^+ T cell response against L. monocytogenes in detail. For our additional experiments, we used the peptides LLO_{91-99} and p60_{217-225} which are both presented by H-2K^d.

In a first series of experiments, we investigated whether CD28^{−/−} mice were able to mount a Listeria-specific CTL response. Spleen cells from infected mice were restimulated with LLO_{91-99} or p60_{217-225} for 3 days. Cultures contained IL-2 to supplement the reduced cytokine production of CD28-deficient T cells (7). Peptide-specific cytotoxicity was tested in a 4 h 51Cr release assay (Fig. 3). In CD28^{−/−} mice, infection with L. monocytogenes induced a CTL response against both listerial peptides during the primary response and cytotoxicity was enhanced during the secondary response. In CD28^{−/−} mice, only very low levels of peptide-specific cytotoxicity were measured during the primary response. During the secondary response, specific cytotoxicity was detected although at a reduced level compared with CD28^{+/+} mice.

**Reduced frequencies of peptide-specific CD8^+ T cells in L. monocytogenes-infected CD28^{−/−} mice**

Our cytotoxic assay depends on in vitro restimulation of specific CD8^+ T cells. Although we cultured these cells for only 3 days and added IL-2, we cannot formally exclude that differences in the CTL assays were due to altered behavior of CD28-deficient T cells in culture. Furthermore, the CTL assay does only allow limited frequency estimates of responding CD8^+ T cells. Therefore, we generated MHC class I-peptide tetramers containing the peptides LLO_{91-99} or p60_{217-225} to quantify Listeria-specific CD8^+ T cells directly ex vivo. With both tetramers we detected activated CD8^+CD62L^low T cells in CD28^{−/−} control mice infected with L. monocytogenes (Fig. 4). Peptide-specific T cells were first identified at days 4 and 5 and the response reached a maximum at days

![FIGURE 1](http://example.com/figure1.png)

**FIGURE 1.** Course of primary L. monocytogenes infection in CD28^{−/−} mice. CD28^{+/+} and CD28^{−/−} mice were i.v. infected with 2 × 10^5 Listeria and titers in spleens and livers were determined at the time points indicated. The arrow indicates the inoculum. Data are given as mean ± SD of 5 mice per group and time point. Results are representative of two independent experiments.
Although *Listeria*-specific CD8+ T cells were generated in CD28−/− mice, their numbers were markedly reduced, particularly at the peak of response on days 9 and 10. Only at later time points of infection were levels of specific CD8+CD62Llow T cells comparable in CD28+/+ and CD28−/− mice. These results imply that the lack of CD28 does not principally block induction of *Listeria*-specific CD8+ T cells. However, generation, and particularly expansion, of these cells during the peak of response is significantly impaired.

After 2 mo, mice were challenged with *L. monocytogenes* and the specific CD8+ T cell response was analyzed. At this time point we noticed that in some, though not all, experiments numbers of tetramer+ CD8+ T cells were reduced in CD28−/− mice compared with CD28+/+ mice. However, this difference never reached statistically significant levels (in all experiments \( p > 0.1 \)). Furthermore, this result has to be interpreted with care, because 2 mo after primary infection frequencies of specific CD8+ T cells were close to the detection limit of our assay.

In CD28−/− mice, secondary *Listeria* infection caused a marked expansion of the *Listeria*-specific CD8+ T cell population. At the peak of secondary response, 20% of the CD8+ T cells were LLO91−/− tetramer+ and 4−5% were p6017−225 tetramer+, correlating with \(-2 \times 10^5\) and \(-4 \times 10^5\) of LLO91−/− and p6017−225 specific CD8+ T cells per spleen, respectively (data not shown and Fig. 6). In CD28−/− mice, the secondary response of specific CD8+ T cells showed a kinetic equivalent to that observed in CD28+/− mice. Similar to the primary response, the magnitude of the secondary response was reduced in CD28−/− mice compared to CD28+/− mice. However, CD28−/− mice developed a memory response because numbers of *Listeria*-specific CD8+ T cells were...

**FIGURE 3.** Reduced CTL response in CD28−/− mice infected with *L. monocytogenes*. For determination of primary responses, CD28+/+ and CD28−/− mice were i.v. infected with \(10^3\) *Listeria* on days −10 and −7 or were left untreated. For secondary responses, mice were i.v. infected with \(10^5\) *Listeria* and after 2 mo, mice were challenged at days −7 or −5 or were left untreated. On day 0, spleen cells were restimulated for 3 days with the peptides LLO91−/− or p6017−225 and specific CTL were determined with peptide-loaded target cells in a 51Cr release assay. Results shown are representative of three and two independent experiments for primary and secondary responses, respectively. Open symbols, CD28+/+ effector cells; closed symbols, CD28−/− effector cells; circles, target cells without peptide; diamonds, peptide loaded target cells.

9 and 10. At the peak of response, spleens from infected mice contained \(-3 \times 10^7\) LLO91−/− specific CD8+CD62Llow T cells and \(-1 \times 10^5\) p6017−225 specific CD8+CD62Llow T cells (Fig. 5).

**FIGURE 4.** Reduced frequencies of LLO91−/− and p6017−225 specific CD8+ T cells during primary *L. monocytogenes* infection of CD28−/− mice. CD28+/+ and CD28−/− mice were i.v. infected with \(10^3\) *Listeria*. At the time points indicated, spleen cells were stained with Cy5-conjugated anti-CD8a mAb, FITC-conjugated anti-CD62L mAb, and PE-labeled LLO91−/− or p6017−225 MHC class I tetramers and analyzed by 4-color flow cytometry after the addition of propidium iodide. Figures show live-gated, propidium iodide-negative CD8+ cells. Numbers represent the percentage of values of tetramer+ CD62Llow cells of live CD8+ T cells of single mice.

**FIGURE 5.** Reduced numbers of LLO91−/− and p6017−225 specific CD8+ T cells during primary *L. monocytogenes* infection of CD28−/− mice. CD28+/+ and CD28−/− mice were i.v. infected with \(10^3\) *Listeria*. At the time points indicated, spleen cells were counted and analyzed as described in the legend of Fig. 4. Symbols give the absolute numbers of LLO91−/− (A) and p6017−225 (B) tetramer+ CD8+CD62Llow live cells/spleen and represent the mean value ± SD of three mice per group and time point. Results are representative of three independent experiments.
considerably increased compared to numbers in the primary response. Thus, frequencies of Ag-specific CD8+ T cells were diminished in the absence of CD28 in both the primary and secondary responses to L. monocytogenes.

**Reduced peptide-specific IFN-γ secreting CD8+ T cells in L. monocytogenes-infected CD28-/- mice**

In addition to MHC class I tetramer staining, we measured IFN-γ production after short term in vitro restimulation with peptides to determine frequencies of Listeria-specific CD8+ T cells. In contrast to tetramer staining, this assay is based on cytokine production and therefore allows an estimate of the frequencies of specific CD8+ effector T cells. CD28+/+ and CD28-/- mice were infected with L. monocytogenes. On days 7 and 10 of primary infection and on days 5 and 7 of secondary infection (Fig. 7), frequencies of IFN-γ producing CD8+ T cells were determined by intracellular cytokine staining after short-term restimulation with LLO91-99. During both primary and secondary responses, we observed Ag-specific CD8+ T cells in both CD28+/+ and CD28-/- mice. However, the frequencies of IFN-γ producing T cells were markedly reduced in CD28-/- mice, particularly during the primary response. Restimulation with p60217-225 resulted in 4- to 5-fold lower frequencies compared to restimulation with LLO91-99 but a similar difference of response between CD28+/+ and CD28-/- mice (data not shown). When compared at a single cell level, IFN-γ+ CD8+ T cells from CD28+/+ and CD28-/- mice showed the same intensity of anti-IFN-γ mAb staining, indicating that single IFN-γ-positive CD28+/+ and CD28-/- CD8+ T cells produced similar amounts of IFN-γ (Fig. 8 and data not shown).

Reduced peptide-specific IFN-γ production was also observed by ELISA. Spleen cells of mice infected for 8 days with L. monocytogenes were restimulated in vitro for 2 days with peptides and IFN-γ in the supernatants was determined. Cells from infected CD28+/+ mice produced 14.8 ± 2.6 and 7.3 ± 5.3 U IFN-γ/ml after restimulation with LLO91-99 or p60217-225, respectively. IFN-γ production of cells from naive mice and infected CD28-/- mice was below the detection limit of 1 U/ml (mean ± SD from 3 mice per group; three independent experiments).

**IFN-γ and IL-2 secretion of CD4+ and CD8+ T cells from CD28+/+ and CD28-/- mice infected with L. monocytogenes**

It could be argued that the reduced frequencies of Listeria-specific CD8+ T cells was due to limited production of growth factors resulting in impaired expansion of the specific CD8+ T cell population. IL-2 production was analyzed in CD8+ T cells from L. monocytogenes infected mice after short-term peptide restimulation (Fig. 8). Although a high frequency of CD8+ T cells from CD28+/+ mice secreting IFN-γ after peptide restimulation, there was hardly any IL-2 secretion by this cell population in our 6 h assay. As described above, frequencies of IFN-γ secreting CD8+ T cells were significantly reduced in spleens from CD28-/- mice. Polyclonal restimulation with soluble anti-CD3 mAb lead to similar results. We observed IFN-γ secreting CD8+ T cells in infected CD28+/+ and CD28-/- mice, but there were only very low frequencies of IL-2 secreting CD8+ T cells in spleens of these mice (data not shown).

IL-2 and IFN-γ secretion by CD4+ T cells was also analyzed. Because strong immunodominant CD4+ T cell epitopes from L.
monocytogenes have not been characterized for BALB/c mice, we used polyclonal anti-CD3 mAb restimulation for induction of cytokine secretion in CD4+ T cells after short term restimulation (Fig. 8). In spleen cells from naive CD28+/+ mice, anti-CD3 stimulation induced a high frequency of cells secreting IFN-γ and/or IL-2. After infection of mice, cytokine-positive populations were enlarged and this effect was most prominent for the IFN-γ/IL-2 double-positive population. In contrast, there was reduced IL-2 and IFN-γ production in spleen cells from CD28−/− mice and frequencies did not increase after infection of these mice with L. monocytogenes. Reduced IFN-γ production in infected CD28−/− mice was also evident when spleen cells from naive and infected CD28+/+ and CD28−/− mice were restimulated for 2 days with anti-CD3 mAb and IFN-γ production was determined by ELISA (data not shown).

Discussion
Our studies demonstrate the importance of CD28 costimulation for the protective immune response of mice against L. monocytogenes infection. CD28−/− mice suffered from augmented listerial titers in livers and spleens, delayed bacterial clearance, and mortality. This result is in contrast to two recent reports. Shen et al. stated that CD28-deficiency had only a “limited effect” on L. monocytogenes infection in mice (46). Chan and Chees (47) used anti-B7 mAb to block CD28 costimulation during L. monocytogenes infection. Restimulation of T cells from these mice with heat-killed Listeria resulted in reduced production of IL-2 and IFN-γ. However, Ab treated and control mice showed equal listerial titers in livers and spleens during primary and secondary infection. We observed markedly diminished IFN-γ production after in vitro restimulation of spleen cells from infected CD28−/− mice, but lack of CD28 signaling impaired resistance to L. monocytogenes infection in our experiments. There are several explanations for these conflicting results. Compared with a genetic CD28-deficiency, injection of anti-B7 mAb could lead to only partial inhibition of CD28 signaling and anti-B7 mAb could affect not only CD28 but also CTLA4 signaling. Furthermore, differences in virulence of L. monocytogenes strains and susceptibility of mouse strains (C57BL/6 vs BALB/c) cannot be excluded.

In CD28+/+ mice, analysis of LLO91−/− and p60017−225-specific CD8+ T cells with MHC class I tetramers gave results similar to those described by Busch et al. (5), although in our experiments, the response was slightly delayed which can be explained by differences in the virulence of the L. monocytogenes strains used. In CD28−/− mice, we detected Ag-specific CD8+ T cells but at significantly reduced levels, particularly during the primary response. Furthermore, the profile of the primary response was altered in that CD8+ T cell frequencies did not decline at late time points of infection as was the case in control mice. The delayed clearance of L. monocytogenes in CD28−/− mice should result in increased amounts of Ag available for prolonged time periods, which could in turn induce continued T cell stimulation. In addition, the sustained bacterial persistence could maintain an inflammatory environment that could support prolonged survival and expansion of Listeria-specific CD8+ T cells in CD28−/− mice.

How could the lack of CD28 signaling affect the generation and expansion of Ag-specific CD8+ T cells? Several reports indicate that the deficiency of CD28 costimulation does not impair early events of T cell activation in vitro and in vivo, but without costimulation T cell activation is incomplete and cannot be sustained resulting in reduced proliferation, induction of unresponsiveness, and increased levels of apoptosis. These defective responses could be related to reduced production of growth factors and impaired induction of antiapoptotic molecules such as BCL-xL (7, 8, 44–48–51). So far, we have no evidence for increased T cell apoptosis in our infection model and the prolonged listerial persistence and consequently sustained T cell stimulation does not allow valid comparisons on T cell survival in our model. We regard reduced growth factor production as a major factor responsible for the impaired generation, and especially expansion, of CD8+ T cells. In this context, it is important to consider that the lack of CD28 costimulation affects not only CD8+ T cells but also CD4+ T cells and other cell types that normally express CD28. Flow cytometric analysis of T cells that had been restimulated with anti-CD3 mAb revealed reduced frequencies of IFN-γ and IL-2 secreting CD4+ T cells in infected CD28−/− mice, indicating that generation and expansion of Listeria-specific CD4+ T cells is impaired, as well. In our assays, CD8+ T cells produce only marginal amounts of IL-2. In contrast, CD4+ T cells are a major source of this growth factor and reduced IL-2 production by CD4+ T cells could at least in part explain the impaired expansion of the Listeria-specific CD8+ T cell population. It has been shown that CD4+ T cells are involved in protection against L. monocytogenes. However, their role in the generation of Listeria-specific CD8+ T cells needs further investigation.

Characterization of the CD8+ T cell response in CD28−/− mice during secondary infection demonstrated that in terms of kinetics and magnitude, this response showed all the hallmarks of a secondary T cell response. Similar to the situation in CD28+/+ mice, the kinetic of the secondary response in CD28−/− mice was accelerated and, at the peak of the secondary response, the numbers of peptide-specific CD8+ T cells were 5- to 10-fold higher when compared to the numbers at the peak of the primary response. In addition, we were able to directly identify Listeria-specific CD8+ T cells in both CD28+/+ and CD28−/− mice 2–3 mo postinfection. In summary, we demonstrate that CD28−/− mice can generate CD8+ memory T cells at a level that is not drastically reduced compared with CD28+/+ mice. This result also indicates that the capacity of CD28 costimulation to prolong T cell survival is not
essential for the generation and maintenance of CD8+ memory T cells. The results of our _L. monocytogenes_ infection model are consistent with data from viral models showing that impaired CD28 signaling does not abrogate generation of virus-specific CD8+ memory T cells (38–40).

To evaluate the role of CD28 costimulation in the differentiation of Ag-specific CD8+ T cells into effector cells, we analyzed cytotoxicity against peptide-loaded cells and IFN-γ production by single cells. We detected significant _Listeria_-specific cytotoxicity in CD28−/+− spleen cells only after secondary _L. monocytogenes_ infection. Considering the low frequencies of _Listeria_-specific CD8+ T cells identified with the tetramer analysis, this observation is consistent with the interpretation that CD28 signaling is not essential for the differentiation of _Listeria_-specific CD8+ T cells into CTL and only during secondary infection do frequencies of these cells exceed the threshold of our CTL assay. However, this result should be evaluated with care because spleen cells were cultured for 3 days in the presence of IL-2. IL-2 could bypass the requirement for CD28 costimulation and thereby allow the differentiation of CD8+ T cells into CTL during in vitro culture. The analysis of IFN-γ production on the single-cell level is more conclusive because we used only short-term culture without additional exogenous cytokines. Frequencies of IFN-γ secreting CD8+ T cells were in close correlation to frequencies of T cells determined with tetramers. This result confirms our assumption that CD28 costimulation is not a prerequisite for the differentiation of CD8+ T cells and that the low numbers of CD8+ effector T cells observed in CD28−/+− mice are due to the lowered frequency of _Listeria_-specific T cells.

When directly compared, frequencies of tetramer+ CD8+ T cells were 2- to 3-fold higher than frequencies of IFN-γ+ CD8+ T cells responding to the specific peptide and this difference was maintained during the course of infection in both CD28+/+ and CD28−/+− mice. One explanation is that only a fraction of the LL031-99 and p60217–225-specific CD8+ T cells secreted IFN-γ. It is also possible that the lower frequencies of IFN-γ+ CD8+ T cells are due to the limits of our assay. Although peptide restimulation lasted for only 6 h, we cannot exclude the fact that a proportion of specific T cells died as a consequence of activation-induced cell death. Current experiments are aimed to clarify this difference in detail.

When compared with different viral infection systems, our results from the _Listeria_ system are comparable to VSV, influenza virus, and low virulence strains of vaccinia virus, but different to LCMV and high virulence strains of vaccinia virus (7, 38–42). In the former group of viral infections, blocking of CD28 costimulation results in diminished CTL responses and reduced numbers of virus-specific IFN-γ-secreting CD8+ T cells. In contrast, the response to LCMV and high virulence strains of vaccinia virus appeared to be not impaired or only marginally impaired. It has been suggested that differential requirements for CD28 costimulation in these models were at least in part due to the level of Ag load and Ag persistence during infection (43, 44). Viruses such as LCMV and virulent strains of vaccinia virus that replicate widely in mice produce abundant amounts of viral Ags for a sustained time period. In contrast, VSV replicates abortively and viral Ags are only available in small amounts for a limited time period. As a consequence, CD28 costimulation would be more important for the generation and expansion of VSV-specific CD8+ T cells (43, 44). Our results imply that this hypothesis cannot be generalized to all types of infections. CD28−/+− mice suffered from high lifestial titers over an extended period of time. Therefore, large amounts of Ag should be available for sustained activation of CD8+ T cells. However, the CD8+ T cell response was still impaired, indicating that factors other than abundance and availability of Ag influence the dependence on CD28 costimulation.

As an alternative explanation it has been proposed that pattern recognition by the innate immune system creates an environment that supports activation and expansion of Ag-specific T cells in the absence of costimulatory molecules (43). _L. monocytogenes_ infection causes a massive inflammation; lifestial cell wall components such as lipoteichoic acids have been shown to activate macrophages via Toll-like receptors and to induce the production of proinflammatory cytokines (52). Together with additional costimulatory molecules such as inducible costimulatory molecule, heat stable Ag, or members of the TNFR family (reviewed in Ref. 53), inflammatory cytokines and other factors of the innate immune system are certainly involved in the residual generation, expansion, and differentiation into effector or memory CD8+ T cells that occurs in CD28−/+− mice. Yet, the impaired CD8+ T cell response to _L. monocytogenes_ implies that CD28 fulfills unique functions that cannot be compensated for by other mechanisms of the innate or acquired immune system.

**Acknowledgments**

We thank Drs. Tak W. Mak, Eric Pamer, Dirk Busch, and John Altman for providing material and valuable suggestions and Ralf Winter, Karin Bordash, and Manuela Stüber for their excellent technical assistance.

**References**


