Depletion of CD4+ T Cells during Immunization with Nonviable Listeria monocytogenes Causes Enhanced CD8+ T Cell-Mediated Protection against Listeriosis

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Depletion of CD4\(^+\) T Cells during Immunization with Nonviable *Listeria monocytogenes* Causes Enhanced CD8\(^+\) T Cell-Mediated Protection against Listeriosis\(^1\)

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Immunization of mice with nonviable *Listeria monocytogenes* generates an insufficient CD8\(^+\) T cell response and consequently only limited protection against subsequent *L. monocytogenes* infection. We have recently demonstrated that depletion of regulatory CD4\(^+\) T cells during immunization significantly enhances CD8\(^+\) T cell responses. In the present study, we determined the impact of CD4\(^+\) T cell depletion on the CD8\(^+\) T cell response against heat-killed *Listeria*. Treatment of mice with anti-CD4 mAb during boost immunization with heat-killed *Listeria* significantly increased numbers of *Listeria*-specific CD8\(^+\) T cells and improved protection against subsequent infection with *L. monocytogenes*. During challenge infection, numbers of *Listeria*-specific CD8\(^+\) T cells were enhanced, and these cells expressed effector functions in terms of IFN-\(\gamma\) production. In summary, we demonstrate that combining nonviable *L. monocytogenes* vaccination and CD4\(^+\) T cell depletion improves generation of long-lasting and functional *Listeria*-specific CD8\(^+\) memory T cells. *The Journal of Immunology*, 2004, 172: 3167–3172.

Experimental infection of mice with *L. monocytogenes* is a well-established model for the analysis of acquired immunity against intracellular bacteria and for the evaluation of vaccination strategies against this type of pathogen (1). *L. monocytogenes* provokes a profound T cell response including both CD4\(^+\) and CD8\(^+\) T cells (2–4). Due to the intracytoplasmic habitat of *L. monocytogenes*, CD8\(^+\) T cells are particularly important for the control of infection and are major mediators of protection against reinfection (1, 5). In BALB/c mice, a large fraction of CD8\(^+\) T cells is directed against a few dominant *Listerial* proteins (2, 6). The most dominant CD8\(^+\) T cell epitope is listeriolysin O (LLO)\(_{91–99}\) (3), a peptide derived from the secreted pore-forming toxin LLO. At the peak of a primary anti-*Listerial* response, 3–4% of the CD8\(^+\) splenocytes are specific for LLO\(_{91–99}\), and during secondary infection, LLO\(_{91–99}\)-specific T cells reach levels as high as 15% of all splenic CD8\(^+\) T cells (2).

Vaccination approaches using nonviable *Listeria* have generally proven ineffective in inducing long-lasting protection against subsequent challenges with viable *L. monocytogenes* (7–9). Only repeated injections of heat-killed *Listeria* (HKL) in short intervals or the combination of HKL with IL-12 or anti-CD40 mAb elicited protection (10–13). Closer analysis revealed that vaccination-induced protection was mediated by both CD4\(^+\) and CD8\(^+\) T cells (10, 11). Since nonviable *Listeria* fail to egress from phagosomes into the cytoplasm, it was assumed that the failure of HKL to induce protection was mainly due to insufficient induction of *Listeria*-specific CD8\(^+\) T cells. A recent study by Lauvau et al. (14) challenged this assumption by demonstrating that immunization with HKL generates *Listeria*-specific CD8\(^+\) T cells. However, in this study, specific CD8\(^+\) T cells were functionally impaired in terms of IFN-\(\gamma\) production and cytotoxicity, and therefore, failed to confer protection (14).

In a recent study, using a DNA vaccine coding for LLO or immunization with the LLO\(_{91–99}\) peptide, we observed that depletion of CD4\(^+\) T cells during boost immunization significantly enhances memory CD8\(^+\) T cell responses against LLO\(_{91–99}\) (15). A more detailed analysis revealed that the enhanced response was most likely due to the removal of CD25\(^+\)CD4\(^+\) T cells, a T cell subpopulation, which has been described to contain a major pool of suppressor or regulatory T cells (16, 17). Overall, these results suggest that memory CD8\(^+\) T cell responses are controlled by regulatory T cells, and that removal of these T cells enhances CD8\(^+\) T cell responses.

In the present study, we analyzed the impact of CD4\(^+\) T cell depletion on the generation of a protective CD8\(^+\) T cell response against listeriosis. We demonstrate that depletion of CD4\(^+\) T cells during boost immunization with HKL enhanced the generation of long-lasting *Listeria*-specific CD8\(^+\) memory T cells and improved protection against subsequent challenge with viable *L. monocytogenes*.

**Materials and Methods**

**Bacterial infection of mice**

BALB/c 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. Mice were infected with *L. monocytogenes* strain EGD. Bacteria were injected in a volume of 200 \(\mu\)l of PBS into the lateral tail vein of mice. The bacterial dose was controlled by plating dilutions of the inoculum on tryptic soy broth (TSB) agar plates. For determination of bacterial burdens in organs, mice were killed, 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 (18).

\(^1\) Abbreviations used in this paper: LLO, listeriolysin O; HKL, heat-killed *Listeria*; TSB, tryptic soy broth.
CD4⁺ T CELLS REGULATE GENERATION OF PROTECTIVE CD8⁺ T CELLS

Immunization with HKL

For the production of HKL, an overnight culture of L. monocytogenes was washed twice and incubated at 80°C for 2 h. Bacterial numbers were determined by absorption at 600 nm (OD of 1 is equivalent to 1 × 10⁹ bacteria). Effective killing was validated by plating HKL onto TSb agar plates. Mice were injected into the lateral tail vein with 3 × 10⁹ HKL in a volume of 200 μl of PBS.

Antibodies

Rat Ig, anti-CD16/CD32 mAb (2.4G2), anti-CD8α mAb (YTS169), anti-CD4 mAbs (YTS191.1 and GK1.5), anti-CD62L mAb (Mel-14), and anti-IFN-γ mAb (clone: R4-6A2, IgG1) were purified from rat serum or hybridoma supernatants with protein G-Sepharose. Abs were Cy5- or FITC-conjugated according to standard protocols. FITC-conjugated rat-IgG1 isotype control mAb (R3-34) was purchased from BD Pharmingen (San Diego, CA).

In vivo mAb application and adoptive transfer experiments

CD4⁺ T cells were depleted by i.p. injection of 300 μg of anti-CD4 mAb YTS191.1 at intervals of 5 days starting 3 days before immunization. Efficacy of depletion was controlled with the anti-CD4 mAb GK1.5 and was always >95% (15).

For adoptive transfer experiments, donor mice were left untreated or were prime-boost immunized or infected as indicated. Seven days after the boost immunization, mice were killed. Single-cell suspensions of pooled spleens were prime-boost immunized or infected as indicated. Seven days after the boost immunization, mice were killed. Single-cell suspensions of pooled spleens were analyzed.

Flow cytometric determination of cytokine expression and MHC class I tetramer staining

Intracellular cytokine staining after short-term in vitro restimulation was performed as described (18). Briefly, spleen cells were stimulated for 5 h with 10⁻⁶ M of the peptide LLO91-99. During the final 4 h of culture, 10 μg/ml brefeldin A (Sigma-Aldrich, St. Louis, MO) were added. Cytometric analysis was performed as described (18). Cytometric analysis was performed as described (18).

Statistical analysis

Statistical analysis was performed as described (18).

Results

Depletion of CD4⁺ T cells during boost immunization with HKL enhances frequencies of Listeria-specific CD8⁺ T cells

Immunization of mice with nonviable L. monocytogenes is insufficient in inducing protection against L. monocytogenes infection. Limited protection is probably in large part due to inefficient induction of Listeria-specific CD8⁺ T cells (10, 14). Recently, we have demonstrated that depletion of regulatory CD4⁺ T cells during boost immunization with a DNA vaccine coding for LLO or with the LLO91-99 peptide significantly increased LLO91-99-specific CD8⁺ T cells (15). Moreover, the majority of the LLO91-99-specific CD8⁺ T cells generated in this way appeared to be functional CD8⁺ effector T cells in terms of IFN-γ and TNF-α production and, to some degree, cytoxicity (15).

To determine whether depletion of CD4⁺ T cells could enhance the Listeria-specific CD8⁺ T cell response upon administration of HKL, mice were immunized twice with 3 × 10⁹ HKL i.v. During the boost immunization, one group of mice was treated with anti-CD4 mAb (YTS191.1) to deplete CD4⁺ T cells. Depletion efficacy was controlled with a second anti-CD4 mAb (GK1.5), which recognizes an independent epitope on the CD4 molecule. Depletion efficacy was always >95% (data not shown). At different days after boost immunization, frequencies and numbers of LLO91-99-specific CD8⁺ T cells were determined with MHC class I tetramers (LLO91-99 in the context of H-2Kd) and CD62L staining (Fig. 1). CD62L is a surface molecule of CD8⁺ T cells that is down regulated following T cell activation. Therefore, costaining with tetramers and CD62L allows the precise determination of LLO91-99-specific CD8⁺ T effector cells. Before secondary HKL immunization, we detected only low frequencies and numbers of LLO91-99-specific CD8⁺ T cells and immunization with HKL without any further treatment did not result in a visible enlargement of this cell population. In contrast, depletion of CD4⁺ T cells induced a significant increase in frequencies and numbers of specific CD8⁺ T cells, which reached a maximum at day 7 after boost immunization and then slowly declined.

Following HKL immunization, frequencies of LLO91-99-specific IFN-γ-producing CD8⁺ T cells were determined as a measurement for a specific effector function of these cells. Spleen cells were incubated for 5 h with 10⁻⁶ M of the peptide LLO91-99 and IFN-γ production was analyzed after intracellular cytokine staining (Fig. 2). Before boost immunization, frequencies of IFN-γ⁰⁰⁰⁰⁺⁺ CD8⁺ T cells were below the detection level of our assay. In control mice (immunized with HKL), HKL immunization resulted in a small number of LLO91-99-specific IFN-γ secreting CD8⁺ T cells. Depletion of CD4⁺ T cells significantly increased the number of these cells. Similar to the tetramer assay, we observed the maximum response 7 days after boost immunization.

Our results indicate, that treatment of mice with anti-CD4 mAb causes enhanced CD8⁺ T cell activation. One explanation for this observation could be a removal of a CD4⁺ T cell-mediated restriction of the CD8⁺ T cell response. However, it is also possible that depletion of CD4⁺ T cells leads to an increased expression of co-stimulatory molecules on antigen-presenting cellsresulting in an enhanced CD8⁺ T cell response.

FIGURE 1. Frequencies and numbers of LLO91-99-specific CD8⁺ T cells after secondary immunization with HKL. BALB/c mice were prime-boost immunized with 3 × 10⁹ HKL i.v. in an interval of 35 days. During the boost immunization, mice were left untreated (□) or received 300 μg of anti-CD4 mAb i.p. at days −3 and +2 of immunization (■). At the days indicated, spleen cells were counted and stained with FITC-conjugated anti-CD8α mAb, Cy5-conjugated anti-CD8α mAb, and PE-labeled LLO91-99/MHC class I tetramers. Cells were analyzed by flow cytometry after the addition of propidium iodide. Bars represent mean values ± SD for spleen cells of three individually analyzed mice. Results are representative for two independent experiments. *, Difference between anti-CD4 mAb-treated and untreated groups; p < 0.05.
that anti-CD4 mAb treatment could mediate its effects independently from CD4+ T cell depletion. Destruction of extensive numbers of CD4+ T cells or simply the infusion of a large amount of Abs could result in unspecific stimulation of the CD8+ T cell response. To circumvent this problem, mice were depleted 7 days before the boost immunization with HKL and the LLO91-99-specific CD8+ T cell response was determined 7 days after HKL application (Fig. 3). Similar to the anti-CD4 mAb treatment in parallel to the HKL immunization, treatment 7 days before the boost immunization induced a significant increase in the number of LLO91-99-specific CD8+ T cells. Thus, enhanced the CD8+ T cell response is most likely due to the removal of CD4+ T cells with suppressive function.

Depletion of CD4+ T cells during boost immunization with HKL results in increased protection against L. monocytogenes
Since depletion of CD4+ T cells during boost immunization induced an enhanced Listeria-specific CD8+ T cell response, we tested whether this treatment caused enhanced cell-mediated protection against L. monocytogenes infection. Mice were prime-boost immunized with 3 × 10^9 HKL i.v. One group of mice received in addition anti-CD4 mAb during boost immunization. Seven days later, spleen cells from immunized mice were adoptively transferred into naive BALB/c recipients. In parallel, groups of mice received spleen cells from naive mice and from mice infected with L monocytogenes 5 wk before the transfer. All recipient mice were infected with 1 × 10^4 Listeria (~1 × LD50), and 4 days postinfection, Listeria titers in the spleen were determined (Fig. 4). Transfer of spleen cells from Listeria-primed mice caused protection in recipient mice with highly reduced Listeria titers. Transfer of cells from HKL and HKL + anti-CD4-treated mice lowered the Listeria titers in spleens of recipient mice. Notably, reduction in titers in spleens of mice transferred with HKL + anti-CD4 mAb-treated mice was stronger than that observed in mice receiving spleen cells from HKL-treated mice.

To test whether our immunization protocol also induced long-term protection and whether anti-CD4 mAb treatment during boost immunization improved protection, mice were prime-boost immunized using the protocol described above. Mice were rested for 6–12 wk to allow recovery of the CD4+ T cell population, and then infected with 2 × 10^9 Listeria. As controls, we used naive mice and mice infected previously with 2 × 10^9 viable Listeria. Five days after the challenge infection, mice were killed and bacterial burdens in spleens and livers were determined (Fig. 5). At this time point, mice secondary infected with L. monocytogenes had completely cleared bacteria from their organs. In naive mice, up to 10^7 L. monocytogenes organisms were detected in the spleen and liver. Immunization with HKL resulted in a small, but not significant reduction of L. monocytogenes titers in the spleen and liver. In contrast, depletion of anti-CD4+ T cells during boost immunization markedly enhanced protection. In the spleen, this treatment caused a profound reduction in the bacterial burden in some mice close to or even below the threshold level of our assay. In the liver, reduction of bacterial titers was not as strong. Although there was a significant difference between HKL + anti-CD4 mAb-treated mice and naive mice, the difference was not significant compared with mice treated with HKL only. Overall, the transfer
peptide LLO_{91,99}. After 5 h, IFN-γ production was determined by intracellular cytokine staining (Fig. 7). In correlation with the results obtained with the LLO_{91,99} tetramer assays, we detected only marginal frequencies of LLO_{91,99}-induced IFN-γ CD8+ T cells in primary infected mice but high frequencies of these cells in secondary infected mice. The L. monocytogenes challenge of HKL-immunized mice induced high frequencies of LLO_{91,99}-specific IFN-γ CD8+ T cells and anti-CD4 mAb treatment during the boost immunization further increased the frequencies of these cells significantly. Overall, the frequencies of CD8+ T cells responding to LLO_{91,99}-restimulation with IFN-γ production correlated well with the frequencies of LLO_{91,99} tetramer-positive CD8+ T cells, indicating that in our experimental model, HKL immunization induced functional CD8+ effector T cells in terms of IFN-γ production.

**Discussion**

In the absence of adjuvant measures, vaccination with nonviable L. monocytogenes is inefficient in inducing protection against L. monocytogenes infection (7–13). Our results confirm this observation. Using two consecutive HKL immunizations, we induced only limited protection against challenge infection with L. monocytogenes. Depletion of CD4+ T cells during the boost immunization significantly enhanced protection against listeriosis. Yet, protection never reached levels generated with a low-dose infection with viable L. monocytogenes. A similar observation was made when we transferred cells from HKL-vaccinated or L. monocytogenes-primed mice into naive mice. Cells from HKL-immunized mice transferred only limited protection to recipients. Anti-CD4 mAb treatment enhanced protection, however, protection did not reach levels of protection following transfer of cells from L. monocytogenes-primed mice. Several observations correlate vaccination-induced protection with the generation of Listeria-specific CD8+ memory T cells. Seven days after boost immunization and anti-CD4 mAb treatment, we detected LLO_{91,99}-tetramer+ CD8+ T cells and CD8+ T cells producing IFN-γ upon restimulation with LLO_{91,99}. When we analyzed the CD8+ T cell response after challenge infection, we found high numbers of LLO_{91,99}-specific CD8+ T cells already 5 days after infection and these cells were...
potent IFN-γ producers. During primary *L. monocytogenes* infection, a significant LLO_{91-99}-specific CD8^+ T cell response is usually not detected before days 6–7 of infection (2, 18). Only after a secondary infection, frequencies reach such levels at day 5 of the response (2, 18). Thus, high frequencies of *Listeria*-specific CD8^+ effector T cells demonstrate the generation of fully functional specific CD8^+ memory T cells, which rapidly mount an effective response upon challenge infection. Overall, our results support the concept of a priming effect of anti-CD4 mAb treatment (15). Even though the detailed mechanisms underlying the enhanced CD8^+ T cell response following HKL immunization and anti-CD4 mAb treatment were not further investigated in the current study, we propose that similar mechanisms are involved. In conclusion, our results suggest that by interfering with a negative T cell-mediated mechanism, pathogen-specific CD8^+ T cell responses and the generation of pathogen-specific CD8^+ memory T cells can be improved.

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**References**


