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Protective Immunosurveillance of the Central Nervous System by Listeria-Specific CD4 and CD8 T Cells in Systemic Listeriosis in the Absence of Intracerebral Listeria

Lai-Yu Kwok,* Hrvoje Miletic,† Sonja Lütjens,* Sabine Soltek,* Martina Deckert,† and Dirk Schlüter‡*‡

The invasion of the CNS by pathogens poses a major risk for damage of the highly vulnerable brain. The aim of the present study was to analyze immunological mechanisms that may prevent spread of infections to the CNS. Intraperitoneal application of Listeria monocytogenes to mice induced infection of the spleen, whereas pathogens remained absent from the brain. Interestingly, Listeria-specific CD4 and CD8 T cells homed to the brain and persisted intracerebrally for at least 50 days after both primary and secondary infection. CD4 and CD8 T cells resided in the leptomeninges, in the choroid plexus, and, in low numbers, in the brain parenchyma. CD4 and CD8 T cells isolated from the brain early after infection (day 7) were characterized by an activated phenotype with spontaneous IFN-γ production, whereas at a later stage of infection (day 28) restimulation with Listeria-specific peptides was required for the induction of IFN-γ production by CD4 and CD8 T cells. In contrast to splenic T cells, T cells in the brain did not exhibit cytotoxic activity. Adoptively transferred T cells isolated from the brains of Listeria-infected mice reduced the bacterial load in cerebral listeriosis. The frequency of intracerebral Listeria-specific T cells was partially regulated by the time of exposure to Listeria and cross-regulated by CD4 and CD8 T cells. Collectively, these data reveal a novel T cell-mediated pathway of active immunosurveillance of the CNS during bacterial infections.

Cerebral infections, in general, develop by spread of the pathogen from the periphery to the brain. During the initial peripheral phase of infection, the ensuing immune response aims at the local restriction of the pathogen to peripheral organs and to its elimination. A failure of the immune system to control peripheral infections substantially increases the risk for development of a cerebral infection.

This scenario is also observed in infection with Listeria monocytogenes, which is orally acquired by consumption of contaminated food. In immunocompromised patients, the pathogen may cause severe meningoencephalitis and/or brain abscess (1). Survival and spread of Listeria in the host are critically dependent on several bacterial virulence factors including the actA protein, which is required for the intracellular movement of Listeria (2, 3). The actA molecule is essential for the accumulation of actin, and Listeria propels through the host cell cytoplasm via its actin tail (2, 3). L. monocytogenes mutants lacking actA are highly attenuated and impaired in their ability to spread from cell to cell, although they still elicit protective CD4 and CD8 T cell responses (4, 5). It is experimentally important that very high numbers of actA-deficient Listeria can be applied to mice, which would rapidly succumb to infection caused by an identical dose of wild-type (WT) L. monocytogenes, and even immunodeficient mice, which inevitably succumb to low dose infection with WT L. monocytogenes, survive infection with the attenuated ΔactA mutant (6, 7). Studies in murine listeriosis have revealed that control and elimination of L. monocytogenes in peripheral organs including spleen and liver as well as in the CNS are critically dependent on L. monocytogenes-specific CD4 and CD8 T cells (8, 9). In mice, CD4 and CD8 T cells are mainly directed against listeriolysin (LLO) and the p60 protein of L. monocytogenes. Immunodominant CD4 (LLO190–201) and CD8 (LLO91–99) as well as subdominant CD4 (p60367–376) and CD8 (p60217–225) epitopes against these proteins have been identified (10).

In addition to control of the peripheral infection, active immunosurveillance of the CNS may provide an elegant mechanism by which to prevent bacteria-induced damage of the highly vulnerable brain, which has long been considered an immune privileged site due to its anatomical confinement beyond the blood-brain barrier, the lack of a conventional lymphatic drainage, and the low level of MHC Ag expression (11–13). The concept of an active immunosurveillance of the brain during infection is supported by adoptive T cell transfer experiments, which have shown that in normal animals i.v. injected activated T cells home to the CNS irrespective of their Ag specificity (14). To address the question of an active CD4 and CD8 T cell-mediated immunosurveillance of the brain during systemic infection, we studied intracerebral immune reactions in a model of murine systemic listeriosis, in which the pathogen is strictly confined to peripheral organs in the absence of CNS infection. Both WT L. monocytogenes as well as the ΔactA mutant were used to explore the impact of both virulence of Listeria and dose of infection on the induction of immunosurveillance of the

*Institut für Medizinische Mikrobiologie und Hygiene, Universitätsklinikum Mannheim, Universität Heidelberg, Mannheim, Germany; and †Abteilung für Neuropathologie, Klinikum der Universität zu Köln, Cologne, Germany

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2 Address correspondence and reprint requests to Dr. Dirk Schlüter, Institut für Medizinische Mikrobiologie und Hygiene, Universitätsklinikum Mannheim, Theodor-Kutzer-Ufer 1-3, D-68167 Mannheim, Germany. E-mail address: dirk.schlueter@imh.ma.uni-heidelberg.de

3 Abbreviations used in this paper: WT, wild type; LLO, listeriolysin; p.i., postinfection; LCA, leukocyte common Ag.
brain. These experiments identified a novel pathway of T cell-mediated immunosurveillance of the CNS and shed new light on the integration of the CNS in immunological circuits intended to protect the highly vulnerable brain from life-threatening infections.

**Materials and Methods**

**Mice**

(iC57BL/6 × BALB/c-H2b)F1, (B6C) mice (Janvier, Le Genest St. Isle, France), 6–10 wk old, were kept under specific pathogen-free conditions throughout the experiments.

**Bacteria and infection**

WT *L. monocytogenes* (serovar 1/2a, EGD, SLCC 5835) and the *L. monocytogenes* ΔactA mutant (15) were grown in tryptone soy broth, and aliquots of log phase cultures were stored at −80°C. For infection of mice, frozen aliquots were thawed and diluted appropriately in sterile pyrogen-free PBS (pH 7.4). Mice were infected i.p. with 1 × 10^6 CFU of WT or ΔactA *L. monocytogenes*, 1 × 10^6 CFU of WT or ΔactA *L. monocytogenes*, or 1 × 10^6 CFU of the ΔactA mutant for primary infection. For secondary infection, 1 × 10^5 ΔactA *L. monocytogenes* were injected i.p. in each experiment, the numbers of *Listeria* injected were controlled by plating the inoculum on tryptose soy agar.

**Determination of the bacterial load in brain and spleen**

For determination of bacterial load in brain and spleen, organs were dissected from sacrificed mice and homogenized with tissue grinders; 10-fold serial dilutions of the homogenates were plated on tryptose-soy agar. Bacterial colonies were counted after incubation at 37°C for 24 h.

**Polymerase chain reaction**

For determination of bacterial DNA in brain tissue, mice infected i.p. with either WT or ΔactA *L. monocytogenes* were anesthetized with Metofane (Janssen, Neuss, Germany) and intracardially perfused with 0.9% NaCl solution to remove intravascular leukocytes from the brain. Thereafter, brains were dissected and snap-frozen in isopentane precooled by dry ice. Sagittal frozen sections of representative areas of the brain including forebrain covered by meninges, basal ganglia, ventricular system with choroid plexus, brain stem, and cerebellum were used for isolation of DNA. DNA was isolated by use of a DNA isolation kit (Nucleospin; Clontech Laboratories, Palo Alto, CA). DNA was also isolated from representative areas of the brain of mice intracerebrally infected 3 days before with either WT or ΔactA *L. monocytogenes*. DNA (100 ng) was used as template per PCR.

Integrity of DNA was controlled by amplification of the murine β-actin gene. The 586 bp gene of *L. monocytogenes* was amplified using the following primers: forward primer 5′-CAA ACT GCT AAG ACA GCT ACT-3′; reverse primer 5′-GCA CCT GAA TTG CTT TTG-3′. PCR products were visualized on ethidium bromide-stained 2% agarose gels. PCR products of homogenized liver and spleen tissue.

**Tissue preparation and immunohistochemistry**

At the indicated days postinfection (p.i.), animals were anesthetized and intracardially perfused with 0.9% NaCl solution. Thereafter, brains were dissected, embedded in Tissue Tek OCT compound (Miles Scientific, Naperville, IL), snap-frozen in isopentane (Fluka, Neu-Ulm, Germany) precooled on dry ice, and stored at −80°C.

For immunohistochemistry, 5- to 7-μm cryostat sections were cut from the brain and stained for leukocyte common Ag (LCA; CD45), CD4, CD8, and Ly-6G (Gr-1) in an indirect peroxidase protocol using rat anti-mouse LCA (clone M1/9.3.4.HL.2), rat anti-mouse CD4 (clone G.K.1.5), rat anti-mouse CD8 (clone 53-2.1; BD Biosciences, Heidelberg, Germany), peroxidase-conjugated streptavidin (Dianova) and aminoethylcarbazole dye solution (Sigma). Spots were counted microscopically. The number of *Listeria*-specific T cells against each peptide was expressed as the number of spots formed in each well per 10⁵ leukocytes.

**Flow cytometry**

Intracerebral leukocytes were isolated, and the percentage of CD4 and CD8 T cells was assessed by staining with rat anti-mouse CD4-PE and rat anti-mouse CD8-PE. To analyze the activation state of *Listeria monocytogenes*-specific CD8 and CD4 T cells, brain was either stimulated with LLO90-91 (CD8 specific) and LLO90-201 (CD4 specific) in the presence of GolgiPlug (1 μM; BD PharMingen, San Diego, CA) at 37°C for 6 h or left unstimulated. Thereafter, cells were washed and stained with rat anti-mouse CD4-FITC or rat anti-mouse CD62 ligand-FITC in combination with either anti-mouse CD4-CyChrome (BD PharMingen) or rat anti-mouse CD8-CyChrome. Finally, cells were fixed with Cytofix/Cytoperm (BD PharMingen) solution and stained intracellularly with rat anti-mouse IFN-γ-PE (clone XMG1.2). In addition, cerebral leukocytes were stained with mouse anti-rat-mouse CD11c-PE and rat anti-mouse Ly6G-PE combined with rat anti-mouse F4/80-FITC and rat anti-mouse LCA-CyChrome. Moreover, cells were stained with mouse anti-mouse NK.1.1-PE and rat anti-mouse CD3-FITC or rat anti-mouse B220-PE and rat anti-mouse LCA-CyChrome. Flow cytometric analysis was performed using a FACSscan (BD Biosciences, San Jose, CA). Data were analyzed with CellQuest software (BD Biosciences).

**Ampicillin treatment**

To clear *L. monocytogenes* from infected mice, the drinking water of mice was supplemented with 2 mg/ml ampicillin (Sigma) starting 24 h p.i. Ampicillin treatment was performed for 5 days. Mice receiving ampicillin were sacrificed at day 7 p.i. The number of *Listeria*-specific CD8 (LLO90-91) and CD4 (LLO90-201) T cells in brain and spleen was determined by ELISPOT assay. The clearance of *L. monocytogenes* by ampicillin was monitored by agar plating of homogenized liver and spleen tissue.

**T cell depletion**

For depletion of CD4 and CD8 T cells, mice were treated with either rat anti-mouse CD4 (clone GK.1.5) or rat anti-mouse CD8 (clone 2.43). Abs were purified from tissue culture supernatant by protein G chromatography and adjusted to concentrations of 2.5 mg/ml in 0.1 M PBS, sterile filtered, and stored at −30°C until use. Control mice were treated with rat IgG (Sigma). Ab were injected i.p. at a concentration of 0.5 mg/ml per mouse. Three days before i.p. infection with *L. monocytogenes*, mice received Ab for 3 consecutive days. Thereafter, Ab were injected at days 2 and 5 p.i. The efficiency of T cell depletion was monitored by flow cytometry. The number of *Listeria*-specific CD8 (LLO90-90) and CD4 (LLO90-201) T cells in the spleen and brain was determined by ELISPOT assay at day 7 p.i.

**Adaptive transfer experiments**

At day 7 p.i., intracerebral leukocytes were isolated and resuspended in PBS for adoptive transfer. Three groups of recipient mice receiving the whole leukocyte population isolated from the brain (group 1), receiving cerebral leukocytes depleted of T cells (group 2), and receiving PBS only (group 3) were included in this experiment. T cell depletion was performed by 95% of Thy-1.2-expressing T cells were depleted. Each recipient mouse was infected intracerebrally with 1 × 10⁵ ΔactA *L. monocytogenes* and, immediately thereafter, received either 3 × 10⁸ cerebral leukocytes in 200 μl PBS (i.v.) or 200 μl PBS instead of cells.
Three days thereafter, recipient mice were sacrificed, and the bacterial load in brains and spleens was determined.

Cytotoxicity assay
At day 7 p.i., leukocytes were isolated from brains and spleens. P815 (H2b) cells were used as target cells and were coated with $10^{-6}$ M LLO$_{91-99}$ at 37°C, 5% CO$_2$ in MEMa supplemented with 10% FCS. During the last hour of peptide incubation, P815 cells were labeled with $^{51}$Cr (Amersham Pharmacia). Thereafter, target cells were washed three times with MEMa to remove unbound peptide and extracellular $^{51}$Cr. Isolated leukocytes and target cells were incubated at E:T ratios of 200:1, 100:1, 30:1, 3:1, and 0.3:1 in triplicate. After incubation at 37°C in 5% CO$_2$ for 5 h, 100 µl of the supernatant from each well were collected, and released $^{51}$Cr was counted in a gamma counter. The specific release was calculated according to the following formula: 100°(test release – spontaneous release)/ (maximal release – spontaneous release), where test release was in the presence of effector cells, spontaneous release was in the presence of medium alone, and maximal release was in the presence of detergent.

Statistics
Student’s t test was used for comparison of the means between sample groups. Values of p of SD.

Results
Recruitment of CD4 and CD8 T cells to the brain after i.p. infection
To study whether a systemic infection with *L. monocytogenes* induces recruitment of *Listeria*-specific T cells to the brain, leukocytes of uninfected and *Listeria*-infected mice were isolated from the brain and analyzed by flow cytometry. At day 7 after i.p. infection with WT and ΔactA *L. monocytogenes*, the percentage of both intracerebral CD4 and CD8 T cells was increased (Fig. 1, B–F) as compared with uninfected animals (Fig. 1A). This increase was correlated with the number of applied *Listeria*; maximal numbers were observed in mice infected with $1 \times 10^8$ ΔactA *L. monocytogenes*, a dose that is lethal for mice when the WT strain is used for infection. Using identical numbers of *Listeria* for infection, the increase of intracerebral CD4 and CD8 T cells was slightly more pronounced in mice infected with the WT strain than in those infected with ΔactA *L. monocytogenes* (compare Fig. 1, B and C, with Fig. 1, D and E).

To analyze whether *L. monocytogenes*-specific CD4 and CD8 T cells contributed to the increased number of intracerebral T cells, cerebral leukocytes from either uninfected mice or animals infected i.p. with WT or ΔactA *L. monocytogenes* (day 7 p.i.) were isolated and restimulated in an ELISPOT assay with *Listeria* peptides specific for either CD8 (LLO$_{91-99}$) or CD4 T cells (LLO$_{100-201}$). In general, the WT strain induced a stronger intracerebral *Listeria*-specific CD4 and CD8 T cell response than did the ΔactA mutant. This difference was statistically significant after infection with $1 \times 10^8$ WT and ΔactA *L. monocytogenes* ($p < 0.01$ for LLO$_{91-99}$; $p < 0.001$ for LLO$_{100-201}$). The highest number of intracerebral *L. monocytogenes*-specific CD4 and CD8 T cells was induced by infection with $1 \times 10^8$ ΔactA *L. monocytogenes*. In both WT- and ΔactA-infected mice, the LLO$_{91-99}$ CD8 T cell epitope induced a stronger response as compared with the LLO$_{100-201}$ CD4 T cell epitope (Fig. 1G).
Systemic listeriosis is not associated with entry of bacteria into the CNS

To analyze whether the recruitment of Listeria-specific T cells to the brain was due to an invasion of the brain by Listeria, mice were infected i.p. either with \(1 \times 10^4\) WT or \(1 \times 10^6\) ΔactA L. monocytogenes, i.e., the bacterial doses that induced the maximal numbers of intracerebral Listeria-specific T cells. In both groups of mice, systemic listeriosis developed with detection of the respective strains of L. monocytogenes in the spleen. Although a higher number of ΔactA L. monocytogenes was used for infection as compared with the WT strain (1 × 10^6 bacteria vs 1 × 10^4 bacteria), the CFU of ΔactA L. monocytogenes was lower in the spleen as compared with infection with WT Listeria at day 5 p.i. (Fig. 2A).

In addition, the ΔactA mutant was completely eliminated from the spleen at day 7 p.i., whereas clearance of WT L. monocytogenes was delayed up to day 14 p.i. (Fig. 2A). Both strains of L. monocytogenes did not spread to the brain up to day 50 p.i. as evidenced by assessment of CFU. Moreover, the absence of Listeria from the brain was confirmed by PCR (Fig. 2B). In addition, L. monocytogenes was not identified either in the brain parenchyma or in the ventricular system of mice infected i.p. by immunohistochemistry (data not shown).

Thus, i.p. infection with both WT and ΔactA L. monocytogenes did not result in invasion of the brain by the pathogen, but, nevertheless, induced an intracerebral Listeria-specific CD4 and CD8 T cell response. Because infection with \(1 \times 10^6\) ΔactA L. monocytogenes induced the strongest Listeria-specific CD4 and CD8 T cell response in the brain; in addition, the ΔactA mutant was cleared from mice at the onset of intracerebral Listeria-specific T cell responses (Figs. 1G and 2A), the ΔactA mutant was used for additional experiments.

**Kinetics of Ag-specific CD4 and CD8 T cells in the brain after primary and secondary infection**

The kinetics of CD4 and CD8 T cell homing to the brain was monitored after primary and secondary i.p. infection with ΔactA L. monocytogenes. After primary infection, the frequencies of both CD4 and CD8 T cells peaked at day 7 p.i. (Fig. 3A). Thereafter, the frequency of both T cell populations slowly declined, reaching baseline levels of uninfected mice at day 50 p.i. Interestingly, the kinetics of the individual Listeria-specific CD4 and CD8 T cell populations appeared to be synchronized and paralleled the kinetics of CD4 and CD8 T cell frequency (Fig. 3B). Like the kinetics of T cell frequency, LLO_91−99 and p60_217−225 CD8 T cells as well as LLO_90−201-specific CD4 T cells peaked on day 7 of primary infection and decreased thereafter (Fig. 3B). A lower number of Listeria-specific T cells, corresponding to the epitopes LLO_91−99, p60_217−225, LLO_90−201, persisted intracerebrally until at least day 50 p.i. The magnitude of Listeria-specific T cell responses to individual epitopes showed a clear hierarchy. At day 7 p.i., the T cell response against the dominant CD8 T cell epitope LLO_91−99 was >2-fold increased as compared with the subdominant CD8 T cell epitope p60_217−225, and ~3-fold higher than that of the dominant CD4 T cell epitope LLO_90−201. A p60_367−378-specific CD4 T cell response was not detectable throughout primary infection (Fig. 3B).

Secondary systemic i.p. infection initiated at day 50 of primary infection caused an increase of both the frequency of intracerebral CD4 and CD8 T cells (Fig. 3C) and the amount of intracerebral Listeria-specific T cells (Fig. 3D), which exceeded the number induced by primary infection. The frequencies of both CD4 and CD8 T cells as well as Listeria-specific T cells rose in the CNS at day 3 p.i., with a peak at day 7 p.i. and a slow decline thereafter (Fig. 3, C and D). Although the percentage of CD4 and CD8 T cells decreased to baseline levels at day 50 after secondary infection (Fig. 3C), a substantially increased number of LLO_91−99-specific T cells persisted up to day 50 p.i. after secondary infection (Fig. 3D). In secondary infection, the hierarchy of T cell responses to the four Listeria epitopes was even more pronounced. At day 7 after secondary infection, the number of CD8 T cells specific for the dominant LLO_91−99 epitope was 6-fold increased as compared with the number of CD8 T cells specific for the subdominant p60_217−225 epitope, whereas this difference was only 2-fold after primary infection. This distinct hierarchy was further illustrated by the much lower rate of decrease of CD8 T cells specific for LLO_91−99 than of CD8 T cells specific for p60_217−225.

**Homing of activated CD4 and CD8 T cells to specific compartments of the CNS after i.p. infection**

To investigate whether these T cells were recruited to distinct compartments of the brain on systemic Listeria infection, immunohistochemical studies were conducted (Fig. 4). On primary infection, both CD4 and CD8 T cells homed to the leptomeninges and the choroid plexus but did not cross the blood-brain barrier to enter the brain parenchyma. Secondary infection led to an increase in the number of both CD4 and CD8 T cells predominantly in the leptomeninges (Fig. 4, A and B). Moreover, a few CD4 and CD8 T cells homed to the choroid plexus (Fig. 4, C and D) and were even observed in the brain parenchyma (Fig. 4, E and F). In addition, some macrophages were detected.
A flow cytometric analysis of the activation state of intracerebral CD4 and CD8 T cells showed that CD4 and CD8 T cells of L. monocytogenes-infected mice produced IFN-\(\gamma\) after restimulation with LLO190-201 and LLO91-99, respectively, whereas intracerebral CD4 and CD8 T cells of uninfected animals did not produce IFN-\(\gamma\) after peptide restimulation (Fig. 5). Interestingly, at day 7 p.i., both intracerebral CD4 and CD8 T cells produced IFN-\(\gamma\) without peptide restimulation, although restimulation with peptides further increased the IFN-\(\gamma\) production. In contrast, at day 28 p.i., restimulation with Listeria-specific peptides was required to induce IFN-\(\gamma\) production of CD4 and CD8 T cells. Both at days 7 and 28 p.i., intracerebral IFN-\(\gamma\)-producing CD4 and CD8 T cells had an activated CD44highCD62 ligand- phenotype (data not shown). These findings indicate that i.p. infection induced the recruitment of IFN-\(\gamma\)-producing CD4 and CD8 T cells to the brain, and that Listeria-specific T cells persisting in the brain terminated their IFN-\(\gamma\) production but were able to rapidly restart production of this cytokine after stimulation with Listeria-specific peptides. In contrast to T cells and macrophages, intracerebral NK cells, dendritic cells, B cells, and granulocytes were not detected by flow cytometry after primary and secondary infection (data not shown).

Adoptively transferred intracerebral T cells induced by i.p. infection with L. monocytogenes reduced the number of Listeria in cerebral listeriosis but did not show cytotoxic activity

Having established that activated Listeria-specific T cells were recruited to and persisted in the brain, an adoptive transfer experiment and a cytotoxic T cell assay were conducted to further identify the functional role and activity of these intracerebral T cells. A flow cytometric analysis of the activation state of intracerebral CD4 and CD8 T cells showed that CD4 and CD8 T cells of L. monocytogenes-infected mice produced IFN-\(\gamma\) after restimulation with LLO190-201 and LLO91-99, respectively, whereas intracerebral CD4 and CD8 T cells of uninfected animals did not produce IFN-\(\gamma\) after peptide restimulation (Fig. 5). Interestingly, at day 7 p.i., both intracerebral CD4 and CD8 T cells produced IFN-\(\gamma\) without peptide restimulation, although restimulation with peptides further increased the IFN-\(\gamma\) production. In contrast, at day 28 p.i., restimulation with Listeria-specific peptides was required to induce IFN-\(\gamma\) production of CD4 and CD8 T cells. Both at days 7 and 28 p.i., intracerebral IFN-\(\gamma\)-producing CD4 and CD8 T cells had an activated CD44highCD62 ligand- phenotype (data not shown). These findings indicate that i.p. infection induced the recruitment of IFN-\(\gamma\)-producing CD4 and CD8 T cells to the brain, and that Listeria-specific T cells persisting in the brain terminated their IFN-\(\gamma\) production but were able to rapidly restart production of this cytokine after stimulation with Listeria-specific peptides. In contrast to T cells and macrophages, intracerebral NK cells, dendritic cells, B cells, and granulocytes were not detected by flow cytometry after primary and secondary infection (data not shown).

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Having established that activated Listeria-specific T cells were recruited to and persisted in the brain, an adoptive transfer experiment and a cytotoxic T cell assay were conducted to further identify the functional role and activity of these intracerebral T cells. At day 7 post-i.p. Listeria infection, leukocytes were isolated from the brain and used for adoptive transfer.Recipient mice were infected intracerebrally with \(1 \times 10^5\) actA L. monocytogenes, and immediately thereafter, recipient mice received either \(3 \times 10^6\) cerebral leukocytes including CD4 and CD8 T cells, macrophages, and microglia (group 1), cerebral leukocytes depleted of T cells (group 2), or PBS only (group 3) in the absence of cells. Three days p.i., significantly reduced numbers of Listeria were recovered from the brains and spleens of recipient mice having received the whole population of cerebral leukocytes as compared with mice having received either T cell-depleted cerebral leukocytes or PBS only (Fig. 6).

CD8 T cells isolated from the brain were not able to lyse target cells loaded with LLO91-99 (Fig. 7). In contrast, splenic CD8 T cells exhibited a cytotoxic activity (Fig. 7), confirming previous results from Huleatt et al. (17). These data suggest that Listeria-specific T cells reduced bacterial titers without exerting cytotoxic activity.

Role of the time point of exposure to Listeria for the induction and recruitment of CD4 and CD8 T cells to the brain

Having established that protective Listeria-specific T cells were recruited to the brain, factors that might regulate the size of the intracerebral T cell pool were analyzed. To investigate whether the induction and expansion of Listeria-specific CD4 and CD8 T cells in the spleen and their recruitment to the brain were dependent on the period of bacterial presence in the host, mice were treated with ampicillin from day 2 to 7 p.i. This regimen cleared Listeria within 48 h from spleen and liver (data not shown) but did not effect the
frequency of *Listeria*-specific CD8 T cells in the spleen and the brain (Fig. 8A). However, ampicillin treatment significantly reduced the frequency of *Listeria*-specific CD4 T cells in spleen and brain (Fig. 8A). These results suggest that the induction and expansion of *Listeria*-specific CD8 T cells in the spleen as well as their recruitment to the brain are programmed very early during infection, whereas an optimal induction of *Listeria*-specific CD4 T cells in the spleen requires a prolonged exposure to the pathogen.

To assess whether the magnitude of the splenic T cell response is a major factor determining the frequency of intracerebral *Listeria*-specific CD4 and CD8 T cells, the brain-spleen ratios of the frequency of *Listeria*-specific CD4 and CD8 T cells, respectively, in ampicillin-treated and untreated mice were calculated. There were no significant differences in the brain-spleen ratios (Fig. 8B) illustrating that the frequency of intracerebral *Listeria*-specific CD4 and CD8 T cells is correlated to the magnitude of the splenic T cell response.

**Cross-regulation of CD4 and CD8 T cells for the induction of *Listeria*-specific CD4 and CD8 T cells in the spleen and their recruitment to the brain**

To evaluate the role of CD4 and CD8 T cells for the induction and expansion of *Listeria*-specific CD4 and CD8 T cells in the spleen and their recruitment to the brain, T cell depletion experiments were performed. Depletion of peripheral CD8 T cells did not affect the frequency of *Listeria*-specific CD4 T cells in the spleen, but significantly reduced the frequency of these cells in the brain (Fig. 9A). The functional importance of CD8 T cells for the recruitment of LLO\textsubscript{190–201} CD4 T cells to the brain is illustrated by the reduced brain-spleen ratio of specific CD4 T cells in CD8-depleted mice as compared with control Ab-treated animals (Fig. 9B). Depletion of peripheral CD4 T cells before the infection reduced the frequency of *Listeria*-specific CD8 T cells in both the spleen and the brain (Fig. 9C). A calculation of brain-spleen ratios revealed that CD4 depletion strongly reduced the recruitment of LLO\textsubscript{190–201}-specific CD8 T cells to the brain (Fig. 9D). Thus, for *Listeria*-specific CD8 T cells, both the induction in the spleen and the recruitment to the brain were largely dependent on CD4 T cells. In contrast, for *Listeria*-specific CD4 T cells only, the recruitment to the brain, but not the induction in the spleen, was dependent on CD8 T cells. These

**FIGURE 4.** Histopathology of intracerebral CD4 and CD8 T cells 7 days after secondary i.p. infection with ΔactA *Listeria*. A, Some CD4 T cells reside in the meninges. Anti-CD4 immunostaining. B, Compared with A, an increased number of CD8 T cells homed to the leptomeninges. Anti-CD8 immunostaining. C, A few CD4 T cells (arrowhead) are present in the choroid plexus of the lateral ventricle (*). Anti-CD4 immunostaining. D, Some CD8 T cells in the lumen and wall of the lateral ventricle. *., The choroid plexus within the lateral ventricle. Anti-CD8 immunostaining. E, In the brain parenchyma, a few CD4 T cells are discernible. Anti-CD4 immunostaining. F, Single CD8 T cell in the brain parenchyma. Anti-CD8 immunostaining. A–F, ×400.

**FIGURE 5.** IFN-γ production of intracerebral *Listeria*-specific CD4 and CD8 T cells. At day (d) 7 after primary infection, brains of six mice were pooled, and intracerebral leukocytes were isolated and restimulated with CD8-specific (LLO\textsubscript{91–99}) or CD4-specific (LLO\textsubscript{190–201}) *Listeria* epitopes or left unstimulated. Cells were stained for CD4 or CD8 and for intracellular IFN-γ. Dot plots show data for either gated CD8 or CD4 T cells. In each dot plot, IFN-γ producing cells are encircled and the percentage of IFN-γ producing cells is included.
were intracerebrally infected with 10^5 L. monocytogenes, and at day 7 p.i., intracerebral and splenic leukocytes were isolated from 12 mice and pooled. The percentages of CD8^+ T cells in the brain and spleen were 6.2% and 8.1%, respectively, as determined by flow cytometry (data not shown). Pooled cells were incubated with LLO^+ or LLO^- 51Cr-labeled P815 target cells. The specific release of 51Cr was determined; data represent the mean value of triplicates.

**FIGURE 7.** Cytotoxicity assay. Mice were infected i.p. with ΔactA L. monocytogenes, and at day 7 p.i., intracerebral and splenic leukocytes were isolated from mice infected i.p. at day 7 after primary infection. Recipient mice immediately thereafter received an i.v. injection of either 3 × 10^6 unseparated intracerebral leukocytes, cerebral leukocytes depleted of T cells, or PBS only without cells; 72 h after cerebral infection, brains and spleens were isolated and homogenized, and CFU were determined. Data show the mean of five mice ± SD per group. * Significantly reduced CFU (p < 0.05). In a repeat experiment, similar data were obtained.

findings illustrate different requirements for an optimal induction of Listeria-specific CD4 and CD8 T cells in the spleen and their recruitment to the brain. Additionally, there was clear evidence for a cross-regulation of CD4 and CD8 T cells in these processes.

**Discussion**

The present study identifies and analyzes the phenomenon of immunosurveillance of the brain in the course of a systemic infection, during which the pathogen is strictly confined to peripheral organs. Pathogen-specific T cells home to the brain and are able to slow down bacterial replication in cerebral listeriosis.

Infection i.p. with L. monocytogenes induced homing of activated Listeria-specific CD4 and CD8 T cells to the brain. The extent of invasion of Listeria-specific T cells was correlated to the dose of infection, and a lower number of as few as 100 WT or actA-deficient L. monocytogenes injected i.p. were sufficient to induce homing of Listeria-specific CD4 and CD8 T cells to the brain. Thus, the induction of T cell surveillance of the brain requires only exposure to a low dose of Listeria, even small numbers of highly attenuated L. monocytogenes, severely impaired in their intracellular movement and their capacity to spread from cell to cell, still induced this process. Nevertheless, the frequency of intrace-
of Listeria

**FIGURE 8.** Role of time point of exposure to *Listeria* for the induction of *Listeria*-specific T cells in the spleen and their recruitment to the brain. Mice were infected i.p. with ΔactA *L. monocytogenes*. Mice were either treated with ampicillin initiated 24 h after infection or left untreated. At day 7 p.i., intracerebral and splenic leukocytes were isolated from six mice per group, and leukocytes of each group and organ were pooled. A. The frequency of CD4 and CD8 T cells specific for CD4 (LLO<sub>190–201</sub>) and CD8 (LLO<sub>91–99</sub>) epitopes was determined in an IFN-γ ELISPOT assay. Data represent the mean value of triplicates + SD. B. The ratio of the frequency of intracerebral and splenic *Listeria*-specific CD4 and CD8 T cells, respectively, was calculated for ampicillin-treated and untreated mice. *, significantly reduced T cell frequencies of ampicillin-treated mice as compared with untreated mice (p < 0.01).

The number of intracerebral *Listeria*-specific CD4 and CD8 T cells peaked at day 7 after primary and secondary infection, thereby corresponding to the kinetics of *Listeria*-specific CD4 and CD8 T cell responses in the spleen (23) (D. Schlüter, unpublished data). In addition, the hierarchy of LLO<sub>190–201</sub> and p60<sub>201</sub>–225-specific CD4 T cells and LLO<sub>91–99</sub> and p60<sub>173–179</sub>-specific CD8 T cells was conserved in the brain as compared with the spleen (10, 23), indicating that the microenvironment of the brain does not alter the hierarchy of T cell responses. Beyond day 7 of primary and secondary infection, the number of intracerebral *Listeria*-specific T cells declined; nevertheless, *Listeria*-specific T cells persisted for at least 50 days after primary and secondary infection in the brain. Such a long-lasting persistence of *Listeria*-specific T cells may be due to either a persistence of these cells in the brain or by their continuous recruitment to the brain. Because *Listeria* did not infect the brain in the course of infection, it is highly unlikely that intracerebral *Listeria* Ag was the driving factor maintaining *Listeria*-specific T cells in the brain. Whereas the preferential persistence of pathogen-specific CD8 T cells in nonlymphoid organs is well documented for extracerebral viral and bacterial peripheral infections including listeriosis (24–26), a few studies have shown that T cells are able to persist in the brain. On intracerebral infection with neurotropic influenza virus, virus-specific CD8 T cells persisted for at least 320 days after viral clearance from the brain (27). This latter experiment and our findings illustrate that under both experimental conditions, i.e., a systemic and extracerebrally confined bacterial infection and a viral CNS infection, pathogen-specific T cells are able to persist in the brain for an extended period of time in the absence of a pathogen. Such persistence of intracerebral T cells is in sharp contrast to autoimmune CNS disorders, during which autoactive T cells are rapidly eliminated from the brain by apoptosis (22, 28).

Importantly, intracerebral *Listeria*-specific T cells were functionally active and reduced bacterial load after cerebral infection with *L. monocytogenes*. Thus, intracerebral *Listeria*-specific T cells can be considered as one important line of defense, which may protect the host together with systemic *Listeria*-specific T cells and other cell populations including macrophages, granulocytes, and NK cells against cerebral listeriosis. Because both intracerebral CD4 and CD8 T cells produced IFN-γ, a cytokine of key importance in listeriosis (29), but intracerebral CD8 T cells did not exhibit cytotoxic activity, protection is most probably conferred by production of protective cytokines. This assumption is in agreement with the observation that CD8 T cells from perforin-deficient mice are able to confer protection against *Listeria* (6). The finding that cytotoxicity was confined to splenic LLO<sub>91–99</sub>-specific CD8 T cells indicates an organ-specific regulation of the phenotype and function of *Listeria*-specific CD8 T cells and may be protective to the highly vulnerable brain. Remarkably, the missing cytotoxic activity of intracerebral CD8 T cells is distinct from the preferential localization of *Listeria*-specific cytotoxic effector memory cells in extracerebral nonlymphoid tissues (24) and further illustrates a CNS-specific regulation of the intracerebral T cell response. An organ-specific regulation of CD8 T cells in peripheral lymph node, liver, spleen, and intestinal lamina propria has also been observed in oral infection with *Listeria* (17, 30).

Recently, it has been shown that the magnitude and kinetics of *Listeria*-specific CD8 T cell responses in the spleen are determined as early as within the first 24 h of bacterial infection (31). The present study confirms and extends these findings by the observation that after CD8 T cell induction in the spleen, subsequent recruitment of the cells to the brain was also determined within the first 48 h of infection. In contrast, ampicillin treatment caused a decrease of the frequency of *Listeria*-specific CD4 T cells in the spleen, indicating that an optimal induction of Ag-specific CD4 T cells required a prolonged exposure to *Listeria*. The frequency of both intracerebral *Listeria*-specific CD4 and CD8 T cells was largely dependent on the magnitude of the splenic T cell responses and the brain-spleen ratios of *Listeria*-specific CD4 and CD8 T cells were not influenced by antibiotic abridgement of Ag exposure.

In addition to the magnitude of the peripheral T cell response, the frequency of intracerebral *Listeria*-specific T cells was regulated by a CD4 and CD8 T cell-dependent recruitment of T cells to the brain: depletion of CD4 T cells reduced the recruitment of *Listeria*-specific CD8 T cells; and vice versa depletion of CD8 T cells reduced the recruitment of *Listeria*-specific CD4 T cells to the brain. How CD4 and CD8 T cells regulate the recruitment of *Listeria*-specific T cells to the brain is unknown but may include the induction of cell adhesion molecules on brain endothelial cells by cytokines. In the normal brain, only low levels of cell adhesion molecules are expressed; however, in infectious and autoimmune diseases they are rapidly up-regulated by cytokines including...
IFN-γ and critically regulate the recruitment of T cells to the brain (21, 32–37). Thus, a depletion of cytokine-producing T cells may result in an impaired recruitment of T cells to the brain via the reduced expression of cell adhesion molecules on brain vessel endothelial cells. The assumption that IFN-γ reduced expression of cell adhesion molecules on brain vessel endothelial cells paves the way for intracerebral CD4 and CD8 T cells to the brain is further supported by the observation that intracerebral CD4 and CD8 T cells specifically for LLO (A) and CD8 T cells specific for LLO (B) were determined in an IFN-γ ELISPOT assay. Data represent the mean value of triplicates ± SD. B and D, The ratio of the frequency of Listeria-specific CD4 and CD8 T cells in brain and spleen was calculated for rat IgG-, anti-CD4-, and anti-CD8-treated mice.

In conclusion, the present study identifies a novel pathway how the brain is surveilled and protected from pathogens during a systemic infection, which is strictly confined to peripheral organs. The active, Ag-specific T cell-mediated immunosurveillance of the brain illustrates that the brain is well integrated into immunological circuits intended to protect the host from life-threatening cerebral infections.

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References


