Interleukin-1 Receptor Type 1 Is Essential for Control of Cerebral but Not Systemic Listeriosis

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***Listeria monocytogenes* may infect the central nervous system and several peripheral organs. To explore the function of IL-1 receptor type 1 (IL-1R1) in cerebral versus systemic listeriosis, IL-1R1^{-/-} and wild-type mice were infected either intracerebrally or intraperitoneally with *L. monocytogenes*. After intracerebral infection with various numbers of attenuated *Listeria*, IL-1R1^{-/-} mice succumbed due to an insufficient control of intracerebral *Listeria*, whereas all wild-type mice survived, efficiently restricting growth of *Listeria*. IL-1R1^{-/-} mice recruited increased numbers of leukocytes, especially granulocytes, to the brain compared with wild-type mice. In contrast, both IL-1R1^{-/-} and wild-type mice survived a primary and secondary intraperitoneal infection with *Listeria* without differences in the hepatic bacterial load. In addition, both strains developed similar frequencies of *Listeria*-specific CD4 and CD8 T cells after primary and secondary intraperitoneal infection. However, an intraperitoneal immunization before intracerebral challenge infection neither protected IL-1R1^{-/-} mice from death nor reduced the intracerebral bacterial load, although numbers of intracerebral *Listeria*-specific CD4 and CD8 T cells and levels of inducible nitric oxide synthase, tumor necrosis factor, and interferon- γ mRNA were identical in IL-1R1^{-/-} and wild-type mice. Collectively, these findings illustrate a crucial role of IL-1R1 in cerebral but not systemic listeriosis. (Am J Pathol 2007; 170:990–1002; DOI: 10.2353/ajpath.2007.060507)**

Infection of the central nervous system (CNS) by *Listeria monocytogenes* (LM), a gram-positive facultative intracellular rod, may cause severe meningitis, encephalitis, and brain abscess. The risk for development and outcome of cerebral listeriosis is dependent on the immune status of the individual; immunocompromised, elderly, and very young individuals are at increased risk for the development of cerebral listeriosis, which has a lethality of 30% despite antibiotic treatment.¹

The virulence of LM is dependent on several factors, including bacterial actin polymerase (ActA). ActA enables intracellular LM to move through the cytoplasm of the host cell by polymerization of host cell actin and to induce the formation of pseudopods, which extend from the infected cell to neighboring cells, triggering the uptake into uninfected cells.^{2–4} LM deficient in ActA-dependent direct cell-to-cell spread are highly attenuated in murine systemic listeriosis.⁵ Furthermore, intracerebral (i.c.) infection with actA-deficient LM (Δ actA LM) induces nonlethal cerebral listeriosis, whereas i.c. inoculation of wild-type (WT) LM inevitably causes death of mice within 4 to 5 days before the onset of a LM-specific T cell response.^{6,7}

Murine cerebral listeriosis caused by infection with WT LM is characterized by a strong multiplication of LM in choroid plexus epithelial cells, ependymal cells, macrophages, microglia, and some neurons resulting in a prominent meningitis, ventriculitis, and lethal necrotizing brain stem encephalitis.⁸ In addition, massive brain edema and neuronal apoptosis develop, which are reduced by intrathecally produced interleukin (IL)-10.⁹ An active systemic immunization before i.c. challenge infection induces LM-specific CD4 and CD8 T cells and reduces spread of LM from the ventricular system to the periventricular brain tissue and the brain stem, thereby preventing death in 60% of mice.^{6,10}

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In addition to T cells, a large number of macrophages and granulocytes are recruited to the LM-infected brain accompanied by an up-regulation of several cytokines including IL-1. The biological activity of IL-1 α and IL-1 β is exerted via the IL-1 receptor type 1 (IL-1R1).^{11,12} The IL-1 receptor type 2 is not involved in signal transduction but acts as a decoy receptor, which can be shed from the cell surface and inhibits binding of IL-1 to the IL-1R1. In addition, binding and signaling of IL-1 is regulated by an endogenous IL-1R antagonist (IL-1Ra), which inhibits binding of IL-1 to IL-1R1.¹³

In murine systemic listeriosis, IL-1R1^{-/-} on a mixed 129/Sv $\times$ C57BL/6 background^{11,12} as well as female B6CBAF1/J overexpressing IL-1Ra¹⁴ had an increased susceptibility with increased numbers of LM in liver and spleen and decreased survival rates, respectively, as compared with respective WT animals. However, after backcrossing to the C57BL/6 background, IL-1R1^{-/-} mice were as resistant as WT mice to systemic listeriosis,¹¹ further illustrating that C57BL/6 mice are more resistant to listeriosis than 129/Sv and CBA/J mice.¹⁵ In addition, in LM-susceptible mice a blockade of IL-1R1 resulted in an increased bacterial load on systemic infection,¹⁶ and a simultaneous inhibition of IL-1 α , IL-1 β , and IL-1R1 prevented the recruitment of neutrophils to LM-associated inflammatory foci.¹⁷

In this study, we report on the role of IL-1R1-mediated immune reactions in cerebral and systemic listeriosis of LM-resistant C57BL/6 mice and illustrate that IL-1R1 is critical for control of LM in the brain and survival of both primary and secondary cerebral listeriosis only, but not of systemic listeriosis.

Materials and Methods

Animals

Age- and sex-matched C57BL/6 WT, obtained from Harlan-Winkelmann (Borchen, Germany), and IL-1R1^{-/-} mice,¹² backcrossed to C57BL/6 mice for 10 generations, were used. All animals were kept under conventional conditions in an isolation facility throughout the experiments. Experiments were approved and supervised by local governmental institutions.

Bacteria and Infection

WT LM (serovar 1/2a, EGD, SLCC 5835), recombinant ovalbumin-expressing LM (LMova),¹⁸ $\Delta actA$ LM,¹⁹ and recombinant ovalbumin-expressing $\Delta actA$ LM ($\Delta actA$ LMova)⁷ were grown in tryptose soy broth, and aliquots of log-phase cultures were stored at -80°C . For each experiment, the respective strain of LM was thawed from the stock solution and diluted appropriately in sterile pyrogen-free phosphate-buffered saline (PBS) (pH 7.4). Anesthetized mice were infected intracerebrally with 1×10^2 WT LM or 6×10^1 , 6×10^2 , and 6×10^3 $\Delta actA$ LM, respectively, into the right caudate nucleus as described previously.⁸ For immunization, mice were infected intraperitoneally (i.p.) with 1×10^6 $\Delta actA$ LM or 5×10^4

LMova 28 days before i.c. challenge infection with 6×10^2 $\Delta actA$ LM or $\Delta actA$ LMova, respectively. For systemic listeriosis, mice were i.p. infected with 1×10^4 WT LM for primary and 1×10^6 WT LM for secondary infection. On the other hand, systemic listeriosis was induced by i.p. infection with 5×10^4 LMova and i.p. reinfection with 5×10^6 LMova. For each experiment, the bacterial dose used for infection was controlled by plating an inoculum on tryptose soy agar and counting colonies after incubation at 37°C for 48 hours.

Histopathology

For immunohistochemistry on frozen sections, mice were perfused intracardially with 0.9% NaCl in methoxyflurane anesthesia. Brains of three to six animals per group were dissected, and blocks were mounted on thick filter paper with Tissue-Tek O.C.T. Compound (Miles Scientific, Naperville, IL), snap-frozen in isopentane precooled on dry ice, and stored at -80°C . In addition to H&E staining, immunohistochemistry was performed on frozen sections as described previously.²⁰ In brief, sections were stained by an indirect immunoperoxidase protocol using rat anti-mouse CD45 (clone M1/9.3.4.HL.2), CD4 (clone G.K.1.5.), CD8 (clone 2.43), and Ly6-G (clone RB6-8C5; all antibodies from the American Type Culture Collection, Manassas, VA) as primary antibodies and peroxidase-linked sheep anti-rat IgG F(ab')₂ (Amersham Pharmacia, Freiburg, Germany) as secondary antibody. In addition, the avidin-biotin complex technique using rat anti-mouse F4/80 (clone F4/80; American Type Culture Collection) as primary antibody, biotinylated mouse serum-preadsorbed mouse anti-rat IgG F(ab')₂ (Dianova, Hamburg, Germany) as secondary antibody, and streptavidin-biotin complex (Dako, Hamburg, Germany) was used. LM was demonstrated immunohistochemically by incubating sections with a polyclonal rabbit anti-LM antiserum (Difco, Freiburg, Germany) followed by peroxidase-labeled goat anti-rabbit IgG F(ab')₂ fragments (Dianova). Brain edema was histologically analyzed on immunostained frozen sections by visualization of immunoglobulin deposits in brain tissue. Sections were stained with goat anti-mouse IgG and goat anti-mouse IgM (Vector Laboratories, Burlingame, CA) followed by biotin-labeled anti-goat antibodies and the Vectastain Elite ABC kit (Vector Laboratories). Peroxidase reaction products were visualized using 3,3'-diaminobenzidine and H₂O₂ as co-substrate. Sections were lightly counterstained with hemalum.

Determination of IL-1 α and IL-1 β in Cerebrospinal Fluid (CSF) and Serum

CSF ($\sim 7 \mu\text{l}/\text{mouse}$) was obtained from mice after intracardial perfusion with 0.9% NaCl by puncturing the cisterna cerebellomedullaris with a fine glass capillary as described previously.²¹ The CSF of five mice per experimental group was pooled and mixed with an equal volume of sterile distilled 0.1 mol/L PBS to reduce losses. CSF was stored at -80°C before being analyzed in a commercially available mouse IL-1 α and IL-1 β -ELISA

(Quantikine M kits; both from R&D Systems, Wiesbaden, Germany), which were used as recommended by the manufacturer.

Colony-Forming Units (CFU)

At the indicated time points postinfection (p.i.), brains and livers were dissected from sacrificed mice and homogenized separately with sterile tissue grinders. Tenfold serial dilutions of the homogenates were plated on tryptose-soy agar. Bacterial colonies were counted microscopically after incubation at 37°C for 48 hours.

Isolation of Leukocytes from CNS and Spleen

At the indicated days p.i., animals were anesthetized with Metofane (Janssen, Neuss, Germany) and intracardially perfused with 0.9% NaCl to remove contaminating intravascular leukocytes from the brain. Thereafter, brains were dissected, minced through a 100- μ m cell strainer (Becton-Dickinson, Heidelberg, Germany), and leukocytes separated by Percoll gradient centrifugation (Amersham Pharmacia) as described previously.²² Leukocytes from spleen were isolated by mincing spleen tissue through a 70- μ m cell strainer (Becton-Dickinson).

Flow Cytometry Analysis

Isolated cerebral leukocytes were analyzed by double or triple immunofluorescence staining followed by flow cytometry. All antibodies were rat anti-mouse antibodies from Becton-Dickinson. To block unspecific binding of antibodies to FC receptors, i.e. leukocytes were first incubated with anti-CD16/32 (clone 2.4G2) at 4°C for 10 minutes. Subsequently, the cells were stained with a cocktail of fluorochrome-labeled antibodies at 4°C for 20 minutes. CD4 and CD8 T cells were detected by staining with anti-CD4-PE (clone RM4-5) and anti-CD8-FITC (clone 53-6.7) in combination with anti-CD45-CyChrome (clone 30-F11). Macrophages and granulocytes were identified by staining with anti-Ly6G-PE (clone RB6-8C5), anti-CD11b-FITC (clone M1/70), and anti-CD45-CyChrome. Macrophages are CD11b⁺ CD45^{high}; granulocytes are CD11b⁺ CD45^{high} Ly6G^{high}.²³ Control staining was performed with isotype-matched control antibodies. Flow cytometry was performed on a FACScan (Becton-Dickinson), and the data were analyzed with WinMDI or Cell Quest software.

Enzyme-Linked Immunospot (ELISPOT) Assay

The frequency of i.c. LM-specific CD4 and CD8 T cells was determined by an interferon (IFN)- γ -specific ELISPOT as described previously.⁹ In brief, isolated i.c. and splenic leukocytes (1×10^5 /well, 1×10^4 /well, and 1×10^3 /well) were added in triplicate to 96-well ELISPOT plates coated with rat anti-mouse IFN- γ (Biosource, Camarillo, CA). Isolated leukocytes were co-incubated with

spleen cells from noninfected WT C57BL/6 mice (2×10^5 /well), which were either preloaded with listeriolysin (LLO)_{190–201} (10^{-6}) or ovalbumin (OVA)_{257–264} (10^{-8} m/mol) peptide. Controls included co-incubation of isolated leukocytes with spleen cells without peptide loading. ELISPOT plates were incubated overnight and developed with biotin-labeled rat anti-mouse IFN- γ , peroxidase-conjugated streptavidin, and aminoethylcarbazole (Sigma-Aldrich, St. Louis, MO). The spots were counted microscopically, and the frequency of antigen-specific cells was calculated from triplicate wells as the number of spots per leukocytes seeded.

RT-PCR

IFN- γ , tumor necrosis factor (TNF), inducible nitric-oxide synthase (iNOS), granulocyte chemotactic protein-2 (GCP-2), RANTES (regulated on activation, normal T cell expressed and secreted), macrophage inflammatory protein-2 (MIP-2) mRNA transcripts, and hydroxyphosphoribosyltransferase (HPRT) mRNA expression were analyzed in brain tissue homogenates following a protocol described in detail previously.⁹ Primer and probe sequences for IFN- γ , TNF, iNOS, and HPRT were as described previously.^{24,25} For the detection of MIP-2, GCP-2, and RANTES mRNA the following primers and probes were used: MIP-2 (forward): 5'-GCTGTTGTGGC-CAGTGAAC-3', MIP-2 (reverse): 5'-TTCAGGTCAAGGC-AAACTT-3', MIP-2 (probe): 5'-TCCAGAGCTTGAGTGT-GACG-3'; GCP2 (forward): 5'-GAAAGCTAAGCGGAAT-GCAC-3', GCP2 (reverse): 5'-GGGACAATGGTTTCCCT-TTT-3', GCP2 (probe): 5'-CCACCCACGGATTTTCTTTA-3'; and RANTES (forward): 5'-GGTACCATGAAGATCTC-TGC-3', RANTES (reverse): 5'-GGGTCAGAATCAAGAA-ACCC-3', RANTES (probe): 5'-CTCTCCCTAGAGCTGC-CTCG-3'. RNA was extracted from tissue homogenates using an RNA extraction kit (Dianova). After reverse transcription of mRNA using the Superscript RT kit (Life Technologies, Eggenstein, Germany), PCR reactions were performed in a volume of 30 μ l. PCR reaction conditions were optimized for each set of primers. PCR was performed at different cycle numbers to ensure that amplification occurred in the linear range. PCR products were electrophoresed through an agarose gel and the DNA was transferred to a nylon membrane (Amersham Pharmacia). Blots were hybridized using specific oligonucleotide probes, which were 3'-end-labeled with digoxigenin by use of a DIG oligonucleotide 3'-end labeling kit (Roche, Mannheim, Germany). A DIG luminescent kit (Roche) was used to visualize the hybridization products. Quantitation of RNA was performed with an imaging densitometer and Quantity One software (Bio-Rad, München, Germany) as described previously.⁹ The intensity of each band was determined and related to the intensity of the respective autoradiogram band obtained for the internal control, HPRT. The results were expressed as fold increase over the respective RNA levels in uninfected animals of the same strain.

Figure 1. Kinetics of IL-1 α and IL-1 β production in the CSF. Nonimmunized mice and mice having been i.p. immunized 28 days previously with $\Delta actA$ LM were i.c. infected with 6×10^2 $\Delta actA$ LM, and the CSF was collected at the indicated time points after infection. CSF of five mice per experimental group was pooled, and IL-1 α (A) and IL-1 β (B) levels were determined by ELISA.

Statistics

For statistical evaluation of the experimental data, the WINKS software (Texassoft, Cedar Hill, TX) was used. Survival analysis was performed with the Mantel-Haenszel log-rank test. Student's *t*-test and the Wilcoxon rank sum test were used to analyze differences in CFU and cell numbers between WT and TNF-deficient mice. *P* values <0.05 were accepted as significant.

Results

Kinetics of IL-1 α and IL-1 β Production in Cerebral Listeriosis

To determine the kinetics of IL-1 α and IL-1 β in cerebral listeriosis, both nonimmunized and immunized WT mice were infected i.c. with 6×10^2 $\Delta actA$ LM and cytokines were measured in CSF by ELISA (Figure 1, A and B). Both IL-1 α and IL-1 β rapidly increased and peaked as early as one day after i.c. infection, with higher levels in immunized than nonimmunized mice. Thereafter, both cytokines sharply declined in the CSF of both nonimmunized and immunized animals and were close or identical to baseline levels of uninfected mice at day 7 p.i. These

findings provide the basis to explore further the function of IL-1R1 in murine cerebral listeriosis.

Intracerebral Infection of Nonimmunized IL-1R1 $^{-/-}$ Mice with LM Results in Lethal Cerebral Listeriosis

After i.c. infection with 1×10^2 WT LM, all IL-1R1 $^{-/-}$ and WT mice succumbed (Figure 2A). However, IL-1R1 $^{-/-}$ mice died significantly earlier than WT animals and harbored significantly more LM in their brains at day 1 p.i. (Figure 2B).

To analyze further whether IL-1R1 $^{-/-}$ mice are more susceptible to cerebral listeriosis than WT mice, animals were infected with various amounts of attenuated $\Delta actA$ LM. After infection with 6×10^1 and 6×10^2 $\Delta actA$ LM, 90% of nonimmunized IL-1R1 $^{-/-}$ mice succumbed up to days 7 and 6 p.i., respectively, whereas all WT mice survived (Figure 2, C and E). A further increase of the dose of infection to 6×10^3 $\Delta actA$ LM resulted in death of 100% of IL-1R1 $^{-/-}$ mice until day 4 p.i., whereas all WT mice still survived (Figure 2G). In each of the experimental groups, the poor outcome of IL-1R1 $^{-/-}$ mice was paralleled by a significantly increased i.c. bacterial load as compared with WT mice (Figure 2, D, F, and H). Collectively, these findings illustrate that the IL-1R1 is essential for survival and efficient control of LM in the CNS.

Widespread Distribution of LM and Inflammation in IL-1R1 $^{-/-}$ Mice

To determine the impact of IL-1R1 on the spread and distribution of LM as well as the accompanying inflammatory leukocytes, a detailed histopathological analysis was performed after i.c. infection with 6×10^2 $\Delta actA$ LM. Remarkably, differences were conspicuous as early as day 1 p.i. Individual WT mice harbored a few meningeal CD45 $^{+}$ infiltrates, corresponding mainly to Ly6G $^{+}$ granulocytes, F4/80 $^{+}$ macrophages, and single CD4 $^{+}$ and CD8 $^{+}$ T cells. In addition, single CD45 $^{+}$ leukocytes were present in the lateral ventricle of WT mice, whereas bacteria were only exceptionally visible in the meninges and the ventricular system. In marked contrast, IL-1R1 $^{-/-}$ mice had already developed ventriculitis involving the lateral, third, and fourth ventricle in addition to meningitis with LM homing to these target sites. CD45 $^{+}$ leukocytes, consisting predominantly of Ly6G $^{+}$ granulocytes and a few F4/80 $^{+}$ macrophages as well as single CD4 $^{+}$ and CD8 $^{+}$ T and B220 $^{+}$ B cells, were present in the meninges.

Cerebral listeriosis progressed more rapidly in IL-1R1 $^{-/-}$ mice as compared with WT animals. At day 3 p.i., IL-1R1 $^{-/-}$ mice were unable to restrict bacteria to the ventricular system (Figure 3a), thereby allowing diffuse bacterial infiltration of the periventricular brain tissue, the basal ganglia, the hippocampus, the neocortex, and the cerebellum. This widespread distribution of LM was accompanied by a diffuse encephalitis affecting the same

Figure 2. Survival and CFU of WT (white bar) and IL-1R1^{-/-} (black bar) mice following infection i.c. with WT LM or $\Delta actA$ LM. **A:** After i.c. infection with 1×10^2 WT LM, IL-1R1^{-/-} mice died significantly earlier than WT mice ($P < 0.01$). **B:** Intracerebral CFU were significantly increased in IL-1R1^{-/-} mice as compared with WT animals at day 1 p.i. **C, E, and G:** After i.c. infection with 6×10^1 , 6×10^2 , and 6×10^3 $\Delta actA$ LM 90, 90, and 100%, respectively, of IL-1R1^{-/-} mice succumbed, whereas 100% of WT mice survived the infection. Survival was monitored for 10 mice per experimental group. $P < 0.01$ for all groups of IL-1R1^{-/-} versus the corresponding group of WT mice. **D, F, and H:** CFU were determined from four or five mice per experimental group at the indicated time points p.i., and data represent the mean $\pm$ SD. * $P < 0.05$ and ** $P < 0.01$ for IL-1R1^{-/-} versus WT mice. †IL-1R1 mice were dead at this time point. n.d., not determined.

anatomical structures, strong inflammation of the massively enlarged lateral, third, and fourth ventricles, in addition to meningitis (Figure 3, c and e). In contrast, both bacteria and inflammatory CD45⁺ leukocytes were

Figure 3. CNS listeriosis in nonimmunized IL-1R1^{-/-} (**a, c, e, g, i, k**) and WT (**b, d, f, h, j, l**) mice i.c. infected with 6×10^2 $\Delta actA$ LM. At day 3 p.i., in an IL-1R1^{-/-} mouse (**a, c, e**), infection of the lateral ventricle and the periventricular brain tissue are much more prominent than in a WT mouse (**b, d, f**) with massive enlargement of the ventricle due to huge numbers of leukocytes (**c**) in association with bacteria (**a**). Note that the ventricular wall is largely destroyed (**a, c, e**). In contrast, only a few bacteria are present in the lateral ventricle (**b**) in association with small inflammatory infiltrates (**d**) in a WT mouse. The ventricle is only moderately enlarged (**f**). At day 5 p.i., there is progressive encephalitis in an IL-1R1^{-/-} mouse with large numbers of bacteria (**g**) not only within the lateral ventricle but also in the adjacent hippocampus (**g, arrows**). Purulent ventricular empyema (**arrowhead in k**) with huge numbers of leukocytes also diffusely infiltrating the basal ganglia (**i, k**). Dotted lines in **i** and **k** indicate the border of the lateral ventricle. In contrast, cerebral listeriosis is regressive in a WT mouse at day 5 p.i. (**h, j, l**). Only a few bacteria (**h**) remain confined to the wall of the lateral ventricle, and some inflammatory infiltrates are present within the wall and the lumen of the normal-sized ventricle (**j, l**). **a, b, g, h:** Anti-LM immunostaining, slight counterstaining with hemalum. **c, d, i, j:** Anti-CD45 immunostaining, slight counterstaining with hemalum. **e, f, k, l:** H&E staining.

largely confined to the ventricular lumen and the subependymal brain tissue in WT mice (Figure 3, b, d, and f).

At day 5 p.i., the brains of terminally ill IL-1R1^{-/-} mice harbored huge numbers of bacteria covering the ventricular system as well as the periventricular brain tissue, and inflammation was widespread (Figure 3, g and k). CD45⁺ leukocytes were scattered throughout the brain, in particular infiltrating the basal ganglia, the hippocampus, and wide areas of the neocortex (Figure 3i). Infiltrates consisted of large numbers of Ly6G⁺ granulocytes, F4/80⁺ macrophages/microglial cells, and some CD4⁺ and CD8⁺ T cells. At this time point, ventriculitis was less severe in WT mice with the ependyma being partly intact (Figure 3, j and l), thereby restricting bacteria mainly to the lumen of the ventricular system and the ependyma (Figure 3h). Consequently, inflammatory infiltrates were mainly located in the ventricular system, only spreading to the basal ganglia immediately adjacent to the lateral ventricle (Figure 3, j and l).

Increased Recruitment of Granulocytes into the CNS of IL-1R1^{-/-} Mice with Cerebral Listeriosis

To quantitate the increased numbers of inflammatory leukocytes and to define the subtypes of inflammatory leukocytes, i.c. infiltrates were analyzed by flow cytometry after i.c. infection with 6×10^2 $\Delta actA$ LM. At days 1 and 3 p.i. with $\Delta actA$ LM, IL-1R1^{-/-} mice harbored significantly more i.c. inflammatory leukocytes than WT animals (Figure 4A). This increase was caused by an enhanced recruitment of granulocytes into the CNS of IL-1R1^{-/-} mice, whereas other subpopulations of leukocytes, ie, CD4 and CD8 T cells and macrophages, did not differ between the two strains of mice (Figure 4B). An increased recruitment of granulocytes was also observed in IL-1R1^{-/-} mice after i.c. infection with both 6×10^1 and 6×10^3 $\Delta actA$ LM compared with the corresponding WT animals (data not shown). Thus, death of IL-1R1^{-/-} mice and the insufficient i.c. control of LM cannot be attributed to an impaired recruitment of leukocytes into the CNS, but rather was associated with an increased recruitment of granulocytes.

An Active Immunization Does Not Prevent Death of IL-1R1^{-/-} Mice from Cerebral Listeriosis

Because an active i.p. immunization confers significant protection against cerebral listeriosis,⁶ we analyzed whether an active immunization may prevent death of IL-1R1^{-/-}. However, an i.p. immunization with $\Delta actA$ LM 28 days before i.c. challenge infection with 6×10^2 $\Delta actA$ did not rescue IL-1R1^{-/-} mice from death (Figure 5A). In addition, i.p. immunization did not reduce the i.c. bacterial load in IL-1R1^{-/-} mice, which harbored significantly more i.c. LM compared with WT mice at days 1, 3, and 5 after secondary i.c. infection (Figure 5B). In contrast to IL-1R1^{-/-} mice, the CFU of immunized WT mice were significantly reduced in comparison to nonimmunized WT

Figure 4. Phenotypic composition of i.c. leukocytes in WT and IL-1R1^{-/-} mice. Intracerebral leukocytes were isolated and counted from uninfected mice (d0) and animals at days 1 and 3 after i.c. infection with 6×10^2 $\Delta actA$ LM. **A:** The total number of inflammatory CD45^{high} leukocytes, ie, excluding CD45^{low} microglia (60), was determined by staining of isolated cerebral leukocytes with anti-CD45-CyCr followed by flow cytometry. **B:** The phenotypic composition of isolated i.c. leukocytes was determined by flow cytometry. At days 1 and 3 p.i., the number of i.c. granulocytes was significantly increased in IL-1R1^{-/-} compared with WT mice (* $P < 0.05$). In **A** and **B**, leukocytes of six mice per time point were analyzed and the mean $\pm$ SD is shown.

animals at day 5 p.i. (Figures 2B and 5B, $P < 0.01$), which further illustrates that an immunization improves the control of LM in the CNS of WT mice.

Histopathology showed that starting at day 3 p.i., IL-1R1^{-/-} mice were much more affected than WT animals. Huge masses of bacteria were present in the ventricles and the neighboring brain tissue of IL-1R1^{-/-} mice (Figure 6a), whereas WT animals efficiently restricted bacteria to the ventricular lumen (Figure 6b). In IL-1R1^{-/-} mice, inflammation was widespread (Figure 6e) and CD45⁺ leukocytes, consisting of large numbers of Ly6G⁺ granulocytes and F4/80⁺ macrophages/microglia, were diffusely scattered throughout the brain parenchyma including the basal ganglia, the hippocampus, and even involving large areas of the neocortex (Figure 6c). However, in WT mice inflammation was much less intense (Figure 6f), and CD45⁺ leukocytes were confined to the ventricles and the very close, adjacent brain tissue (Fig-

Figure 5. Survival and CFU of immunized WT and IL-1R1^{-/-} mice following i.c. infection with 6×10^2 $\Delta actA$ LM. **A:** 28 days after systemic immunization with $\Delta actA$ LM, WT, and IL-1R1^{-/-} mice were i.c. infected with $\Delta actA$ LM. 90% of immunized IL-1R1^{-/-} mice succumbed to the diseases, whereas 100% of immunized WT survived the infection. Survival was monitored for 10 mice per experimental group. $P < 0.01$ for IL-1R1^{-/-} versus WT mice. **B:** CFU were determined from three to five mice per experimental group at the indicated time points p.i., and data represent the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$ for IL-1R1^{-/-} versus WT mice.

ure 6d). At day 5 p.i., disease severity was increased in IL1R^{-/-} mice, and large numbers of bacteria and leukocytes were present in the massively enlarged lateral ventricle, from where purulent inflammation also involved the adjacent brain tissue (Figure 6, g, i, and k). In contrast, cerebral listeriosis did not aggravate in WT mice (Figure 6, h, j, and l).

To quantitate differences in inflammatory i.c. leukocytes between WT and IL1-R1^{-/-} mice, the total number of i.c. leukocytes and their phenotypic composition were determined. As illustrated in Figure 7A, immunized IL-1R1^{-/-} mice recruited more leukocytes to the LM-infected brain than WT animals at days 1 and 3 after i.c. challenge infection (significant at day 3 p.i. with $P < 0.05$). The increased number of i.c. leukocytes in IL-1R1^{-/-} mice was caused by an elevated number of granulocytes, whereas all other cell populations did not differ between IL-1R1^{-/-} and WT mice (Figure 7B). In conclusion, these experiments show that immuniza-

Figure 6. CNS listeriosis of immunized IL-1R1^{-/-} (a, c, e, g, i, k) and WT (b, d, f, h, j, l) mice after i.c. infection with 6×10^2 $\Delta actA$ LM. At day 3 p.i., infection of the lateral ventricle is much more severe in an IL-1R1^{-/-} mouse (a, c, e) compared with a WT mouse (b, d, f). Large numbers of bacteria are present in the enlarged lateral ventricle and the periventricular brain tissue (a) and are associated with many intra- and periventricular leukocytes (c, e) in an IL-1R1^{-/-} mouse. In contrast, only single bacteria (b) are associated with small accumulations of leukocytes (d) in the moderately enlarged (f) lateral ventricle of a WT mouse. In d, the maximum of inflammation is shown. Up to day 5 p.i., cerebral listeriosis has markedly progressed in an IL-1R1^{-/-} mouse with destruction of the wall of the lateral ventricle (g), purulent empyema (k*) and unrestricted bacterial spread into the basal ganglia (g). Diffuse encephalitis has developed with many leukocytes being scattered either diffusely or in small infiltrates throughout the hippocampus (i). In contrast, in a WT mouse, single bacteria are strictly confined to the ventricular system (h) as are leukocytes (j) at day 5 p.i. At this stage of the infection, the ventricles of WT mice are of normal size (l). a, b, g, h: Anti-LM immunostaining, slight counterstaining with hemalum. c, d, i, j: Anti-CD45 immunostaining, slight counterstaining with hemalum. e, f, k, l: H&E staining.

Figure 7. Phenotypic composition of i.c. leukocytes and frequencies of LM-specific CD4 and CD8 T cells in immunized WT and IL-1R1^{-/-} mice following i.c. infection with 6×10^2 $\Delta actA$ LM. **A:** 28 days after systemic immunization with $\Delta actA$ LM, WT, and IL-1R1^{-/-} mice were i.c. infected with $\Delta actA$ LM. At days 1 and 3 after i.c. reinfection, i.c. leukocytes were isolated and counted. The phenotypic composition of isolated i.c. leukocytes was determined by flow cytometry (**B**). At day 3 p.i., the number of i.c. granulocytes was significantly increased in IL-1R1^{-/-} mice ($P < 0.01$ for IL-1R1^{-/-} versus WT mice). In **A** and **B**, leukocytes of six mice per time point were analyzed, and the mean $\pm$ SD is shown.

tion does not protect IL-1R1^{-/-} mice and that i.c. listeriosis of these animals exhibits the same characteristics as in nonimmunized IL-1R1^{-/-} mice with respect to survival, CFU, and inflammatory infiltrates.

Normal Expression of IFN- γ , TNF, iNOS, RANTES, MIP-2, and GCP-2 in Cerebral Listeriosis of IL-1R1^{-/-} Mice

To evaluate whether the impaired control of i.c. LM in IL-1R1^{-/-} mice was associated with a reduced production of protective cytokines, i.c. mRNA expression of iNOS, TNF, and IFN- γ was determined. Individual uninfected WT and IL-1R1^{-/-} mice expressed low amounts of

Figure 8. RT-PCR analysis of i.c. iNOS, TNF, IFN- γ , GCP-2, RANTES, MIP-2, and HPRT mRNA expression in IL-1R1^{-/-} and WT mice. RT-PCR was performed on brain tissue homogenates of nonimmunized WT and IL-1R1^{-/-} mice having been i.c. infected with 6×10^2 $\Delta actA$ LM as well as on brains of mice after systemic immunization with $\Delta actA$ LM followed by i.c. infection with 6×10^2 $\Delta actA$ LM 28 days later. In each group of mice, three animals were analyzed per time point. **A:** Autoradiograms of a representative mouse per group and time point. **B:** Autoradiograms were densitometrically analyzed, and data depict the mean fold increase $\pm$ SD of the respective cytokines and chemokines over uninfected mice of the same mouse strain.

TNF mRNA, whereas iNOS and IFN- γ mRNA were not expressed (Figure 8A). At days 1, 3, and 5 p.i. after i.c. infection with 6×10^2 $\Delta actA$ LM, both mouse strains equally up-regulated iNOS, TNF, and IFN- γ mRNA without significant differences (Figure 8, A and B). In addition, immunized mice of both strains of mice expressed identical amounts of iNOS, TNF, and IFN- γ .

To analyze whether the increased recruitment of granulocytes to the brain of IL-1R1^{-/-} mice was associated with an increased expression of granulocyte attracting chemokines, mRNA expression of RANTES, MIP-2, and GCP-2 was determined (Figure 8). Both IL-1R1^{-/-} and WT mice with cerebral listeriosis up-regulated mRNA expression of RANTES, MIP-2, and, to a lesser extent, GCP-2 compared with noninfected mice. Expression of RANTES and GCP-2 mRNA was similar in LM-infected IL-1R1^{-/-} and WT animals with differences less than twofold. In addition, expression of MIP-2 was similar in nonimmunized WT and IL-1R1^{-/-} mice. Only immunized IL-1R1^{-/-} mice had a fourfold

Figure 9. Survival and CFU of nonimmunized and immunized WT and IL-1R1^{-/-} mice following systemic infection with LM. WT and IL-1R1^{-/-} mice were i.p. infected with 1×10^4 WT LM, and survival (**A**) and CFU (**B**) were determined. In **C** and **D**, WT and IL-1R1^{-/-} mice, which had been i.p. immunized with 1×10^4 WT LM 50 days before, were i.p. reinfected with 1×10^6 WT LM and the survival (**C**) and CFU (**D**) were determined. In **A** and **C** survival of eight mice each was monitored. In **B** and **D**, CFU were determined from four or five mice per time point and experimental group, and data represent the mean $\pm$ SD.

increased expression of MIP-2 compared with WT mice at day 5 p.i. Thus, IL-1R1 deficiency did not result in an impaired or increased i.c. production of iNOS, TNF, IFN- γ , RANTES, and MIP-2 with the exception of MIP-2 expression in immunized mice at late time points of cerebral listeriosis.

IL-1R1^{-/-} Mice Survive Systemic Listeriosis and Effectively Control LM in the Liver

To analyze whether IL-1R1^{-/-} mice also exhibit an aggravated course of systemic listeriosis, WT and IL-1R1^{-/-} mice were i.p. infected with WT LM. Both nonimmunized WT and IL-1R1^{-/-} mice survived primary systemic listeriosis and controlled LM with the same efficacy (Figure 9, A and B). In addition, both WT and IL-1R1^{-/-} mice survived an i.p. challenge infection with WT LM without any differences in CFU (Figure 9, C and D). These findings illustrate that IL-1R1 plays a crucial role in cerebral but not in systemic listeriosis.

Figure 10. Frequencies of LM-specific CD4 and CD8 T cells in WT and IL-1R1^{-/-} mice. **A:** The frequencies of i.c. LM-specific LLO₁₉₀₋₂₀₁-CD4 and OVA₂₅₇₋₂₆₄-CD8 T cells were determined in immunized WT and IL-1R1^{-/-} mice by an IFN- γ ELISPOT. Mice were i.p. immunized with 5×10^4 LMova and i.c. challenged with 6×10^2 Δ actA LMova 28 days later. **B:** WT and IL-1R1^{-/-} mice were i.p. infected with 5×10^4 LMova and one experimental group of each strain of mice was reinfected at day 50 p.i. with 5×10^6 LMova. At days 9 and 50 after primary infection and at day 7 after secondary infection, the frequency of splenic LM-specific LLO₁₉₀₋₂₀₁-CD4 and OVA₂₅₇₋₂₆₄-CD8 T cells was determined by an IFN- γ ELISPOT. In **A** and **B**, four or five mice per time point were analyzed, and the mean $\pm$ SD is shown.

Normal Frequencies of LM-Specific CD4 and CD8 T Cells after Systemic and Intracerebral Infection in IL-1R1^{-/-} Mice

Because the protective effect of immunization against cerebral listeriosis is mediated by CD4 and CD8 T cells,⁶ the frequencies of LM-specific CD4 and CD8 T cells were determined. In these experiments, mice were immunized with LMova and i.c. challenged with Δ actA LMova, which both contain a strong CD8 (OVA₂₅₇₋₂₆₄) and CD4 (LLO₁₉₀₋₂₀₁) T-cell epitope. As illustrated in Figure 10A,

frequencies of LM-specific CD4 and CD8 T cells were identical in IL-1R1^{-/-} and WT mice with numbers of LM-specific CD4 and CD8 T cells increasing from day 1 to 3 after i.c. infection.

In addition, a quantitation of the frequencies of LM-specific T cells after systemic infection with LMova showed that both mouse strains had the same number of LM-specific CD4 and CD8 T cells at the peak of primary infection (day 9 p.i.), in the memory phase of the T cell response (day 50 p.i.), and 7 days after reinfection at day 50 p.i. (Figure 10B). From these findings, it is concluded that 1) IL-1R1 deficiency does not affect the capacity of mice to develop pathogen-specific CD4 and CD8 T cells in response to infection with LM, and 2) i.c. infection of immunized animals results in a normal recruitment of these LM-specific T cells to the brain.

Discussion

In the present study, we took advantage of genetically attenuated LM lacking the *actA* gene. In extension of a previous report,⁷ the present study illustrates that even high amounts of $\Delta actA$ LM, which are also considered to be used in humans as a vaccine carrier,²⁶ induce a nonlethal cerebral listeriosis in WT mice and thereby allow the identification of factors crucial for the control of LM in the CNS. This is in contrast to WT LM, which inevitably induces a lethal cerebral listeriosis even in immunocompetent mice (this study and Ref. 8). In addition, these attenuated bacteria are widely used to study immune reactions in mice which succumb to infection with WT LM due to deficiencies of the immune system.^{5,27-30}

After i.c. infection with $\Delta actA$ LM, IL-1 α and IL-1 β were rapidly produced with peak levels in the CSF 24 hours p.i. Thereafter, levels of these cytokines sharply declined in both nonimmunized and immunized animals. The same kinetics of IL-1 α and IL-1 β production is also observed in systemic listeriosis with peak levels at day 1 after infection followed by a rapid decline.¹⁶ The production profile of IL-1 α and IL-1 β indicates that IL-1R1-mediated immune reactions predominantly play a role early after infection of both nonimmunized and immunized animals. It is still unknown whether the kinetics of IL-1 production in cerebral and systemic listeriosis is caused by a single pulse of IL-1 production or influenced by an induction of IL-1-neutralizing molecules including IL-1R2, which acts as a decoy receptor in its soluble and membrane-anchored form, and the IL-1R antagonist (IL-1Ra), which also inhibits binding of IL-1 to the IL-1R1.¹³

In fact, CFU were increased in nonimmunized IL-1R1^{-/-} mice as early as 1 day after i.c. infection with both WT and $\Delta actA$ LM. The important functional role of IL-1R1 is further illustrated by the significantly earlier death of IL-1R1^{-/-} mice after i.c. infection with WT LM as well as death of IL-1R1^{-/-} but not of WT mice after infection with low and high numbers of $\Delta actA$ LM. Importantly, the crucial role of IL-1R1 in cerebral listeriosis was also obvious in immunized IL-1R1^{-/-} mice, which also succumbed to the infection and had significantly increased

i.c. CFU as compared with WT animals after i.c. infection with $\Delta actA$ LM. The insufficient control of $\Delta actA$ LM in the brain of IL-1R1^{-/-} mice was accompanied by a more widespread distribution of LM in the meninges, the ventricular system, and even in the brain parenchyma. In particular, the development of necrotizing encephalitis was prevented in mice with an intact IL-1R1, illustrating that activation of this receptor inhibits both replication of LM as well as spread of LM in the brain. These findings also clearly illustrate that $\Delta actA$ LM still possesses the capacity to spread within the brain and to infect regions far distant from the site of inoculation, ie, the rostral basal ganglia. These latter findings are in agreement with experiments in TNF^{-/-} mice, which also succumb to a necrotizing encephalitis with accompanying destruction of the plexus epithelium and ependyma after i.c. infection with $\Delta actA$ LM.⁷ However, in contrast to IL-1R1^{-/-} mice, death of TNF^{-/-} animals could be prevented by an active immunization before i.c. infection.

Studies in systemic listeriosis have shown that IL-1R1-mediated immune reactions are critical for the recruitment of neutrophils into LM-infected organs.^{17,31} However, IL-1R1^{-/-} mice recruited significantly more granulocytes to the brain than WT animals in cerebral listeriosis. In fact, the increased pleocytosis of IL-1R1^{-/-} was caused by the increased recruitment of granulocytes, whereas numbers of i.c. macrophages and CD4 and CD8 T cells did not differ between nonimmunized and immunized IL-1R1^{-/-} and WT mice. In addition, inflammatory leukocytes homed to regions of LM replication, showing that i.c. movement of leukocytes is IL-1R1-independent in cerebral listeriosis. The reason for the divergent role of the IL-1R1 in the recruitment of granulocytes to the CNS and peripheral organs is yet unclear, but it may be that the increased number of LM in cerebral listeriosis compensates for IL-1R1-mediated recruitment of granulocytes, because bacterial products are chemotactic and induce chemokines in meningitis.³² However, i.c. mRNA expression of GCP-2, RANTES, and MIP-2, which all are granulocyte attracting chemokines, did not differ between IL-1R1^{-/-} and WT mice, with the exception of a slightly increased MIP-2 mRNA expression in immunized IL-1R1^{-/-} mice at late stages of cerebral listeriosis. Importantly, the increased recruitment of granulocytes in the absence of an intact IL-1R1 did not protect against cerebral listeriosis, although granulocytes contribute to the control of LM in the CNS of WT animals.³³ On the other hand, the increased number of granulocytes may contribute to damage of the brain and death of IL-1R1^{-/-} mice, because granulocytes can release neurotoxic molecules including reactive oxygen species.^{34,35} The assumption that the immune response may also contribute to death of IL-1R1^{-/-} mice is further supported by the observation that immunized IL-1R1^{-/-} mice, which mount a LM-specific i.c. T cell response, die even earlier than nonimmunized IL-1R1^{-/-} mice.

The increased i.c. CFU despite an unimpaired recruitment of leukocytes indicates that IL-1R1-dependent antibacterial effector molecules are insufficiently generated in IL-1R1^{-/-} mice. Because IL-1 β plays a role in the induction of iNOS,³⁶ and expression of iNOS has been

linked to protection in cerebral listeriosis,³⁷ we determined iNOS mRNA levels (Figure 8) and iNOS protein expression by immunohistochemistry (data not shown) in the brain. Both nonimmunized and immunized IL-1R1^{-/-} mice up-regulated and expressed iNOS mRNA and protein equivalent to WT mice, indicating that the expression of iNOS, which has been shown to play a role in killing of LM,^{38,39} is not dependent on IL-1R1 and also cannot compensate for IL-1R1 deficiency. Potentially other effector molecules including reactive oxygen intermediates,⁴⁰ small GTPases,⁴¹ or yet undefined molecules may depend on an appropriate induction by IL-1R1 in cerebral listeriosis.

In the early phase of systemic listeriosis, macrophages and dendritic cells are a major source of TNF,^{39,42} and this mediator is of critical importance in both systemic and cerebral listeriosis.^{43–46} Therefore, we evaluated whether IL-1R1-mediated immune reactions play a role in the induction of TNF in cerebral listeriosis. These experiments revealed that nonimmunized and immunized IL-1R1 up-regulated TNF mRNA expression equivalent to WT mice, illustrating that TNF production is IL-1R1-independent in cerebral listeriosis and that TNF cannot compensate for IL-1R1 deficiency in this model. These findings are in contrast to pneumococcal pneumonia, in which TNF compensates for IL-1R1 deficiency.⁴⁷ The normal TNF and iNOS expression and the identical strong expression of MHC class II molecules on macrophages and microglia of IL-1R1^{-/-} and WT mice (data not shown) also indicate that macrophage activation is mainly IL-1R1-independent in cerebral listeriosis. This is in contrast to systemic listeriosis of LM-susceptible mice, in which a combined blockade of IL-1 α , IL-1 β , and IL-1R1 resulted in a reduced expression of MHC class II molecules on macrophages.¹⁷

The critical role of IL-1R1 in bacterial infections of the CNS is not limited to listeriosis. A recent study has also demonstrated that in pneumococcal meningitis IL-1R1 is required for control of i.c. pneumococci, confinement of meningitis, and survival.⁴⁸ As observed in our experiments, cytokine production was not significantly affected by IL-1R1 deficiency. These findings and the observation that IL-1 is strongly elevated in the CSF of patients with bacterial meningitis⁴⁹ may argue for a common protective role of IL-1 in bacterial meningitis caused by different bacterial pathogens. In this context it is of note that IL-1 also has the capacity to contribute to the damage of the brain in various CNS disorders including trauma, ischemia, and neurodegeneration.⁵⁰ However, cerebral listeriosis of IL-1R1^{-/-} mice was associated with a more severe bacteria-induced destruction of the brain tissue, a more severe edema, and increased neuronal damage as compared with WT mice (data not shown). These side effects are most probably due to the more severe inflammation as well as the high numbers of LM, which secrete toxic molecules including pore-forming listeriolysin.⁵¹ In addition, the limited time period of IL-1 α and IL-1 β production in cerebral listeriosis may contribute to the prevention of cytokine-induced neurological damage in WT mice, since especially chronic exposure of the brain to IL-1 causes neurological pathology.⁵²

Active immunization of IL-1R1^{-/-} mice neither conferred protection against cerebral listeriosis nor reduced i.c. multiplication and spread of LM. Consequently, immunized IL-1R1^{-/-} mice died within a few days and after i.c. infection from necrotizing encephalitis with elevated numbers of i.c. granulocytes. In contrast to IL-1R1^{-/-} mice, immunization significantly reduced i.c. bacterial load in immunized WT mice compared with nonimmunized animals (Figures 2B and 5B, $P < 0.01$ at day 5 p.i.), illustrating that immunization conferred protection in these animals. Previous studies have shown that the protective effect of immunization is mediated by LM-specific T cells in cerebral listeriosis.^{6,10} As illustrated in Figure 10, immunization induced normal frequencies of LM-specific CD4 and CD8 T cells after primary and secondary systemic infection in IL-1R1^{-/-} mice. Thus, IL-1R1-mediated immune reactions are not critical for LM-specific T cell responses opposed to autoimmune myocarditis and murine leishmaniasis.^{53–55} In addition, the IL-1R1 is dispensable for the recruitment of LM-specific T cells to the brain, because immunized IL-1R1 and WT mice harbored similar numbers of LM-specific CD4 and CD8 T cells in their brains. This latter finding also indicates that the critical functional role of IL-1R1 cannot be compensated for by LM-specific T cells in cerebral listeriosis. In addition, the increased frequency of LM-specific T cells in combination with the increased numbers of granulocytes in immunized IL-1R1^{-/-} mice may cause an immunopathology contributing to death of these animals. In fact, immunized IL-1R1^{-/-} mice died even slightly earlier than nonimmunized IL-1R1^{-/-} mice (compare Figures 2A and 5A).

An important observation of the present study is that IL-1R1 plays a critical role in cerebral but not systemic listeriosis. Although both the route of infection as well as the infected organ influence the systemic immune response against LM,^{48,56–59} the differences were never as dramatic as observed here for brain opposed to liver. Several reasons might explain these divergent findings. One factor may be the differential dependence of various LM-infected cell populations (brain: ependymal cells, choroid plexus epithelial cells, macrophages/microglia, neurons; liver: hepatocytes) on IL-1R1-induced antibacterial effector molecules. In addition, the brain may lack mechanisms that compensate for IL-1R1 deficiency. This hypothesis is further supported by the observation that the IL-1R1 is much more important in pneumococcal meningitis⁴⁸ than in pneumococcal pneumonia,⁴⁷ pointing to a more general importance of the IL-1R1 for control and survival of acute bacterial meningitis.

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