Innate Responses to Systemic Infection by Intracellular Bacteria Trigger Recruitment of Ly-6C high Monocytes to the Brain

Douglas A. Drevets, Jennifer E. Schawang, Marilyn J. Dillon, Megan R. Lerner, Michael S. Bronze and Daniel J. Brackett

*J Immunol* 2008; 181:529-536; doi: 10.4049/jimmunol.181.1.529

http://www.jimmunol.org/content/181/1/529

References

This article cites 50 articles, 27 of which you can access for free at: http://www.jimmunol.org/content/181/1/529.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Innate Responses to Systemic Infection by Intracellular Bacteria Trigger Recruitment of Ly-6C<sup>high</sup> Monocytes to the Brain<sup>1</sup>

Douglas A. Drevets,* Jennifer E. Schawang,* Marilyn J. Dillon,* Megan R. Lerner,† Michael S. Bronze,* and Daniel J. Brackett†

Blood borne <i>Listeria monocytogenes</i> enter the CNS via migration of parasitized Ly-6C<sup>high</sup> monocytes, but the signals that trigger this migration are not known. To understand more completely events leading to monocyte recruitment, experiments presented here combined microarray analysis of gene expression in the brains of experimentally infected mice with measurements of bacterial CFU and serum cytokines following i.v. infection with <i>L. monocytogenes</i>. At 24 and 48 h postinfection, the brain was sterile but there were significant changes in transcriptional activity related to serum proinflammatory cytokines. Real-time PCR confirmed mRNA up-regulation of genes related to IFN-γ, IL-1, and TNF-α, although IFN-γ itself was not up-regulated in the brain. Infection with <i>S. acta</i>, but not <i>Delyl</i> mutants, increased serum concentrations of IFN-γ, IL-6, and to a lesser extent TNF-α. The brain was not infected but there was widespread mRNA up-regulation in it and an influx of Ly-6C<sup>high</sup> monocytes in <i>S. acta</i>-infected mice. Moreover, <i>S. acta</i>-infected IFN-γ<sup>−/−</sup> mice had no brain influx of Ly-6C<sup>high</sup> monocytes despite normal monocyte trafficking from bone marrow to blood and spleen. Additionally, IFN-γ<sup>−/−</sup> mice showed diminished mRNA expression for monocyte-attracting chemokines, and significantly less CXCL9 and CXCL10 protein in the brain compared with normal mice. These data demonstrate that monocyte recruitment to the brain is independent of bacterial invasion of the CNS and is triggered by proinflammatory cytokines, in particular IFN-γ, produced by the innate immune response to intracellular infection in peripheral organs. The Journal of Immunology, 2008, 181: 529–536.

<i>Listeria monocytogenes</i> is a Gram-positive, facultative intracellular bacterium that causes bacteremia and CNS infections in humans and animals (1). CNS infections assume a variety of presentations such as meningitis, meningoc-encephalitis, rhombencephalitis, and brain abscess and are present in 29–79% of patients with invasive listeriosis (2). Interestingly, once an invasive infection has been established in the periphery, <i>L. monocytogenes</i> is nearly 10-fold more efficient at entering the CNS than are other commonly neuroinvasive Gram-positive bacteria including <i>S. pneumoniae</i> and Group B <i>Streptococci</i> (3). Current data from experimentally infected mice suggest that migration of parasitized monocytes is the predominant means by which <i>L. monocytogenes</i> establish CNS infection (4–6). Given this paradigm for how <i>L. monocytogenes</i> first enters the CNS, it is axiomatic that factors other than bacterial invasion of the brain or subarachnoid space must trigger monocyte recruitment into them.

In clinical situations microbes typically enter the CNS from the bloodstream in the context of a systemic infection. This is true for <i>L. monocytogenes</i> as well as for other neuroinvasive bacteria, and is readily recapitulated in experimental <i>L. monocytogenes</i> infection of mice. In this model the liver and spleen rapidly remove injected bacteria from the bloodstream, whereas the brain remains uninfected (7–9). Infection is initially confined to peripheral organs, in particular the liver and spleen, and later also involves the bone marrow. In the marrow, bacteria parasitize CD11b<sup>+</sup>Ly-6C<sup>high</sup> monocytes and monocyte precursors coincident with a skewing of the homeostatic balance between Ly-6C<sup>high</sup> and Ly-6C<sup>−</sup> monocytes in the bloodstream in favor of Ly-6C<sup>high</sup> cells (5, 6, 10, 11). As the infection progresses, there is a secondary bacteremia composed of intracellular and cell-free bacteria and it is during this phase that bacteria enter the CNS within parasitized Ly-6C<sup>high</sup> monocytes (5, 6). Although the mechanisms that enable monocyte entry into the CNS in this situation are not yet defined, it is possible that innate immune responses to infection in peripheral organs trigger the influx of monocytes into the CNS, thereby enabling microbial neuroinvasion.

Recent studies suggest that innate immune responses to systemic infection can activate the CNS by at least two general pathways before bacterial invasion. The first is through direct interactions between microbes or microbial products with cells of the CNS as demonstrated by recognition of peripherally injected LPS by TLR 4 on resident cells in the CNS (12, 13). A second general pathway is for microbes or their products to stimulate production of proinflammatory mediators in the periphery that then interact with their cognate receptors on resident CNS cells. This situation is modeled by i.p. injection of TNF-α, which interacts with the p75 TNF-receptor on the endothelium to up-regulate expression of E- and P-selectin on brain microvasculature and stimulate neutrophil recruitment into the brain (14, 15). Previous data support the notion that systemic <i>L. monocytogenes</i> infection can activate resident CNS cells and stimulate influxes of blood leukocytes in the absence of bacterial invasion. For example, infected mice demonstrate increased expression of the adhesion molecules E-selectin,
P-selection, ICAM-1, and VCAM-1 on brain capillary endothelial cells before recovery of bacteria from the brain (16–18). Similarly, Listeria-specific CD4 and CD8 T cells home to the CNS in the absence of brain infection (19).

The experiments presented here were undertaken to define more precisely the dynamic relationship between systemic infection and the CNS with particular attention to the mechanisms by which peripheral infection triggers recruitment of Ly-6C<sup>high</sup> monocytes into the brain. For this we analyzed gene expression in the brain during a lethal systemic L. monocytogenes infection as it progressed from visceral organs, to bloodstream, and then to the brain. The data show that proinflammatory cytokines released during the innate immune response to intracellular bacterial replication in peripheral organs rather than bacterial invasion of the brain, have a major role in activating gene transcription in the brain and triggering a central influx of Ly-6C<sup>high</sup> monocytes. Additionally, IFN-γ has a key role in mediating early events in the brain critical for monocyte recruitment.

Materials and Methods

Animals
Specific pathogen-free mice female C57BL/6J, and IFN-γ-deficient B6.129S7-H<sup>−</sup>H<sub>−</sub>H<sub>−</sub>H<sub>−</sub>-<sup>−</sup>/H<sup>−</sup> (IFN-γ<sup>−/−</sup>) along with age- and gender-matched genotype control mice, were purchased from The Jackson Laboratory. Mice were group housed in micro-isolator cages and given food and water ad lib. Animal experiments were reviewed and approved by the Animal Care and Use Committees of the Oklahoma City Veterans Affairs Medical Center and the University of Oklahoma Health Science Center.

Bacteria
Bacteria were stored in brain-heart infusion broth (Difco) at 10<sup>6</sup> CFU/ml at −70°C. Bacteria used included wild-type L. monocytogenes strain EGD, and gene deletion mutants deficient in listeriolysin O (Δhol) DP-L2161 and actA (ΔactA) DP-L1942, which were gifts from D. Portnoy (University of California, Berkeley, CA) (20, 21). Δhol mutants neither escape phagosomes nor replicate intracellularly (22). ΔactA mutants escape phagosomes and replicate intracellularly, but cannot spread cell-to-cell (23). Before injection, 0.5 ml of stock culture was diluted in 4 ml of broth, cultured for 4.5 h at 37°C, and then was diluted in sterile PBS.

Mouse infection and tissue collection
Mice were injected i.v. with 0.1 ml containing 4.0–4.3 log 10 CFU (1–3 LD<sub>50</sub>) strain EGD, or 6.0 log 10 CFU of the mutant bacteria, or with sterile PBS (control). The animals were euthanized at the indicated time postinfection (PI) by i.p. injection with ketamine/xylazine (Vedco). Blood was collected and cultured, then was allowed to clot and was centrifuged to collect serum for cytokine analysis. The spleen was aseptically removed then homogenized in sterile dH<sub>2</sub>O and CFU bacteria were quantified by serial dilution and plating. Animals were perfused transcardially with 30 ml sterile PBS containing 2 U/ml heparin (Sigma-Aldrich), and then the brains were divided along the sagittal sulcus. CFU bacterial were quantified from brain tissue by homogenization and plating on agar. Specimens for cellular analysis, microarray studies, and real-time PCR (qPCR) were processed as described below.

Flow cytometry
Leukocytes were isolated from brains of perfused mice by enzymatic digestion with 0.1% collagenase D (Roche) and 10 μg/ml DNase I (Sigma-Aldrich) followed by immunomagnetic collection of CD45<sup>+</sup> cells on a miniMACS column (Miltenyi Biotec) as previously described (5). Cells from individual mice were labeled with directly conjugated mAb against Ly-6C (ER-MP20) (24), CD11b (M1/70) (BD Pharmingen), and Ly-6G (1A8) (BD Pharmingen). Flow cytometry was performed on a FACSCalibur (BD Pharmingen) and the data were analyzed with WinMDI 2.8 software (J. Trotter).

Cytokine and chemokine analysis
In microarray experiments, serum concentrations of TNF-α and IFN-γ were measured by Cytometric bead array (BD Pharmingen) whereas IL-6 and IL-10 were quantified using OptEIA ELISA kits (BD Pharmingen). Subsequent experiments used only Cytometric Bead Array. Concentrations of CXCL9 and CXCL10 in brain were quantified by ELISA (R&D Systems). For this, perfused brains were sectioned along the sagittal sulcus and 1/2 brain was homogenized in 1.0 ml RPMI 1640 medium containing 1% CHAPS (Sigma-Aldrich) as described (25). Homogenates were centrifuged for 20 min at 2000 × g, and the supernatants were frozen at −70°C until used.

Microarray analysis
Mice were injected with wild-type L. monocytogenes or with sterile PBS (control), then seven animals from each group were harvested daily for 4 days. From each day, groups of five infected animals were selected for microarray analysis based upon similarities in CFU bacteria in the spleen (day 1), blood (day 2), and brain (days 3 and 4) and were randomly paired with brains harvested from controls. RNA was extracted from the brain using the Atlas Total Total RNA labeling system then hybridized to plastic slides with 32P-labeled probes using Atlas Plastic Mouse 5K Microarray (Clontech) according to the manufacturer’s directions. Hybridization and phosphorimaging analysis were performed in the OUHSC microarray facility. Phosphorimaging analysis was performed using a Storm optical scanner (Molecular Dynamics), and initial background corrections were performed using the Array Vision software (Imaging Research).

FIGURE 1. Altered gene expression in the brains of L. monocytogenes-infected mice precedes bacterial invasion and coincides with high serum concentrations of proinflammatory cytokines. Mice were infected i.v. with L. monocytogenes then were harvested at the indicated time. A, Serum cytokine concentrations were determined by cytometric bead array (IFN-γ, TNF-α) or by ELISA (IL-6, IL-10). Results presented are the mean ± SD concentration (pg/ml) of the indicated cytokine from 4 to 6 mice/point. *p < 0.05 compared with steady-state. B, RNA was extracted from brains and used for microarray. Data were analyzed using Genespring GX v. 7.3 software with a Mann-Whitney Wilcoxon nonparametric test using a p-value cutoff of 0.05. Results presented are numbers of significantly changed genes/day based on cut-off values of ≥2-fold (open bars), ≥3-fold (closed bars), or ≥5-fold (hatched bars) changed from control values.
Data analyses were performed using Genespring GX v. 7.3 (Agilent Technologies). Raw signal data were normalized to the median of the entire chip for each sample, and all samples were further normalized per gene to the medians of data from the uninfected samples. Data for each day were analyzed by Mann-Whitney Wilcoxon nonparametric test with a p-value cutoff of 0.05. Lists of genes were generated to reflect 2-, 3-, or 5-fold up-regulation and down-regulation based on significance. Expression data were incorporated into Ingenuity Pathways Analysis software (Ingenuity Systems) to enable further understanding of complex relationships as well as provide further information about canonical pathways in which genes of interest function.

Real-time PCR
Perfused brain tissue was stored in RNALater (Ambion) at −20°C until RNA extraction was performed. RNA was extracted using Trizol LS (Invitrogen) and residual genomic DNA was removed using TURBO DNA-free (Ambion) according to the manufacturers’ instructions. Reverse transcription was performed with the iScript cDNA Synthesis kit (BioRad) and qPCR was performed with SYBR green PCR Master Mix (Applied Biosystems) and custom-made primers (Integrated DNA Technologies). Primer sequences were obtained using Beacon Designer 4 (Premier Biosoft International), or previously published literature (Supplemental Data Table IA). qPCR was performed on a Stratagene MX3005P system. Data were analyzed by the standard curve method and t tests confirmed statistical significance. Hypoxanthine phosphoribosyl transferase (HPRT) was used as the housekeeping gene (26).

Results
Microarray analysis shows altered gene expression in the brain precedes bacterial invasion
Mice were infected i.v. with L. monocytogenes then were harvested at 24-h intervals for analysis of bacterial CFU, serum cytokine concentrations, and to analyze gene expression in the brain.

Table I. Pathway analysis of gene expression data reveals largely unique patterns of significantly changed signaling pathways on different days of infection

<table>
<thead>
<tr>
<th>Pathwaya</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-protein coupled receptor signaling</td>
<td>Xb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycosaminoglycan degradation</td>
<td>Xc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JAK/STAT signaling</td>
<td>Xb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NF-κB signaling</td>
<td>Xb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serotonin receptor signaling</td>
<td>Xc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apoptosis signaling</td>
<td>Xc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complement and coagulation cascades</td>
<td>Xc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epidermal growth factor signaling</td>
<td>Xc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endoplasmic reticulum stress pathway</td>
<td>Xc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GM-CSF signaling</td>
<td>Xc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Huntington’s disease signaling</td>
<td>Xb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parkinson’s signaling</td>
<td>Xc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sterol biosynthesis</td>
<td>Xb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aminophosphonate metabolism</td>
<td>Xc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-alanine metabolism</td>
<td>Xb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium signaling</td>
<td>Xc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dopamine receptor signaling</td>
<td>Xc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerophospholipid metabolism</td>
<td>Xc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin receptor signaling</td>
<td>Xc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synaptic long-term potentiation</td>
<td>Xc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amyotrophic lateral sclerosis signaling</td>
<td>Xb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Death receptor signaling</td>
<td>Xc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PI3K/AKT signaling</td>
<td>Xc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vascular endothelial growth factor signaling</td>
<td>Xc</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Lists significantly changed genes defined by p ≤ 0.05 and ≥ 2-, 3-, or 5-fold change (up or down) compared with control were analyzed by Ingenuity Pathways Analysis software to identify significantly changed (p ≤ 0.05) signaling pathways.
b Pathway significantly changed in 2 of 3 analyses.
c Pathway significantly changed in 3 of 3 analyses.

FIGURE 2. Confirmation of gene up-regulation in the brain by qPCR. Mice were euthanized 24 and 48 h after i.v. injection with L. monocytogenes, then were perfused and the brains harvested and mRNA was collected. Gene expression was measured in individual animals by qPCR and normalized to expression of HPRT in the same animal. Results presented are the mean ± SD fold-increase of normalized expression of the indicated gene in mice infected 24 h (A), 48 h (B), or both (C), compared with the normalized expression in steady state mice (n = 4). Ingenuity Pathways Analysis software was used to identify canonical interactions between genes and proinflammatory cytokines. Related cytokines are shown in the left column as IFN (Type I or Type II), IFN plus TNF or IL-1, TNF or IL-1, or other (e.g., IL-6). * p < 0.05, ** p < 0.01, compared with steady state. § p ≤ 0.05 compared with control on microarray data.
by microarray. By 24 h PI, bacteria were present in the spleen, but the blood and brain were sterile as previously reported (5). Bacteria were first recovered from the blood 48 h PI (3.03 ± 0.93 log10 CFU bacteria/ml), and from the brain beginning 72 h PI (3.84 ± 0.01 log10 CFU bacteria/brain). There was a large increase in serum IFN-γ levels 24 h PI as well as statistically significant, but less dramatic, increases of TNF-α and IL-6 (Fig. 1).

As the infection progressed, IFN-γ levels declined but increased again in a preterminal fashion, whereas TNF-α and IL-6 increased progressively. IL-10 levels were increased over steady-state by 48 h PI then continued to rise. Although not specifically analyzed in these experiments, serum cytokines represent the collective responses of a variety of cells in infected organs particularly the liver and spleen (27). In addition to cytokines produced by infected macrophages, the early peak of IFN-γ is largely due to NK cells and Ag-independent CD8+ T cells (28), whereas TNF-α/inducible NO synthase producing dendritic cells have a major role TNF-α production (29).

Microarray data were analyzed with GeneSpring 7.3 software using cut-off p-value of 0.05 and lists of significantly changed genes were created based upon changes of ≥2-, ≥3-, and ≥5-fold (increase or decrease) in infected animals compared with controls (Fig. 1 and Supplemental Data Table IB-E). Interestingly, gene expression was altered 24 and 48 h PI, which was before bacterial invasion of the brain. Results from 24 h PI differed from other time points in several ways. First, the majority of changes in gene expression were due to up-regulation whereas at subsequent time points most changed genes were down-regulated. Second, more genes were changed at 24 h PI than at any other time point regardless of the cut-off applied. For example, using the ≥2-fold changed criteria, there were 339, 124, 99, and 37 changed genes 24, 48, 72, and 96 h PI, respectively. By comparison, there were
Infections with ΔactA and Δhly mutants of L. monocytogenes elicit differential gene up-regulation in the brain. Perfused brains were harvested from mice infected 48 h with ΔactA (●) or Δhly (□) mutants of L. monocytogenes. Expressions of mRNA for the indicated genes were measured by qPCR and normalized to the expression of HPRT in the same animal. Results presented are the mean ± SD fold-increases of normalized gene expression in infected mice compared with steady state mice (n = 4). * p < 0.05, ** p < 0.01 compared with steady state. ## p < 0.01 ΔactA-infected vs Δhly-infected animals.

137, 118, 93, and 37 changed genes using ≥5-fold changed criteria at the same time points. Third, a large percentage of genes up-regulated 24 h PI were changed modestly, between 2- and 5-fold. At subsequent time points, the vast majority of flagged genes were changed by 5-fold or greater. Ingenuity Pathways Analysis of significantly changed genes showed that largely unique sets of signaling pathways were changed each day of the experiment (Table I). Interestingly, only two pathways, NF-κB and apoptosis, were significantly changed on more than 1 day.

qPCR was used to confirm up-regulation of genes identified by microarray on days 1 and 2 PI. In total, qPCR showed significant changes in 17% (3/18) and 82% (14/17) of genes flagged as up-regulated by microarray using the ≥2-fold cut-off at 24 and 48 h PI, respectively (Fig. 2 and data not shown). Based on pathways analysis and the presence of high systemic levels of proinflammatory cytokines, e.g., IFN-γ and TNF-α, we used qPCR to test the extent to which cytokine-related genes that were not identified by microarray, were in fact up-regulated. These results showed widespread up-regulation of IFN-related genes 24 and 48 h PI. In addition, qPCR showed up-regulation of genes related to TNF-α or IL-1β confirming activation of the NF-κB pathway. Interestingly, genes for IFN-β1, IL-1α, IL-1β, and TNF-α were each up-regulated in the brain 24 and 48 h PI, suggesting these mediators could be produced locally as well as systemically. In contrast, IFN-γ was not up-regulated, suggesting peripheral sources of this cytokine were of primary importance for triggering IFN-γ responses in the CNS.

Next, we tested the extent to which infection with L. monocytogenes ΔactA and Δhly mutants could trigger Ly-6C<sup>high</sup> monocyte influxes to the brain. ΔactA mutants escape phagosomes and reproduce intracellularly but are incapable of spreading cell-to-cell, whereas Δhly mutants neither escape phagosomes nor replicate intracellularly (22, 23). Both mutants are highly attenuated and do not cause bacteremia or invade the CNS, but trigger different immune responses (30). Thus, they are useful for analyzing the extent to which intracellular parasitism of peripheral organs and the immune responses to it trigger CNS inflammation in the absence of CNS infection. As expected, neither mutant was recovered from the blood or brain 48 h PI (data not shown). Serum concentrations of IFN-γ and IL-6 increased dramatically in ΔactA-infected animals 24 h PI, but not in Δhly-infected mice (Fig. 3). TNF-α concentrations were elevated in both groups 24 h PI, but were

![FIGURE 4](https://example.com/figure4.jpg)

**FIGURE 4.** Infections with ΔactA and Δhly mutants of L. monocytogenes elicit differential gene up-regulation in the brain. Perfused brains were harvested from mice infected 48 h with ΔactA (●) or Δhly (□) mutants of L. monocytogenes. Expressions of mRNA for the indicated genes were measured by qPCR and normalized to the expression of HPRT in the same animal. Results presented are the mean ± SD fold-increases of normalized gene expression in infected mice compared with steady state mice (n = 4). * p < 0.05, ** p < 0.01 compared with steady state. ## p < 0.01 ΔactA-infected vs Δhly-infected animals.

![FIGURE 5](https://example.com/figure5.jpg)

**FIGURE 5.** IFN-γ<sup>-/-</sup> mice demonstrate selective impairment of Ly-6C<sup>high</sup> monocyte influxes to the brain. Age- and gender-matched C57BL/6 (control) or IFN-γ<sup>-/-</sup> mice were not infected (steady state) or were infected with ΔactA L. monocytogenes mutants and then were euthanized 48-h PI. Leukocytes were collected from bone marrow, blood, spleen, and brains and then analyzed by flow cytometry. Results are presented as the number (mean ± SEM) of Ly-6C<sup>high</sup> monocytes in bone marrow (tibia plus femur) (A), per spleen (C), and per brain (D). Results from blood (B) are presented as the mean ± SEM % monocytes expressing high amounts of Ly-6C. * p < 0.05 compared with same genotype steady state, n = 5–6 per group.
FIGURE 6. Muted gene up-regulation in the brains of infected IFN-γ−/− mice. Perfused brains were harvested from IFN-γ−/− or IFN-γ+/+ mice infected 48 h with ΔactA mutants of L. monocytogenes. Expressions of mRNA for the indicated genes were measured by qPCR and normalized to the expression of HPRT in the same animal. A. Results presented are the mean ± SD fold-increases of normalized gene expression in infected mice compared with same genotype steady state mice (n = 4). * p < 0.05, ** p < 0.01 compared with same genotype steady state. B. Chemokine concentrations in brains were measured by ELISA. Results presented are the mean (± SD) chemokine concentration (pg/ml) n = 4. # p < 0.05, ## p < 0.01 infected IFN-γ−/− vs infected IFN-γ+/+ animals.

significantly higher in Δhly-infected mice than in Δhly-infected mice. IL-10 was not increased in either group (not shown).

Importantly, there was an influx of Ly-6Chigh monocytes into the brains of ΔactA-infected mice 48 h PI but not in Δhly-infected mice (Fig. 3, B and C). Previous data showed Δhly mutants do not expand the Ly-6Chigh monocyte subset in the peripheral circulation 72 h PI (5). Nonetheless flow cytometry performed 48 h PI showed numbers of Ly-6Chigh monocytes in the bone marrow increased from 5.22 × 10^5 ± 0.67 cells/tibia plus femur at steady-state (mean ± SD) to 10.91 × 10^5 ± 0.30 48 h PI (p < 0.001) in Δhly-infected mice. By comparison, monocyte numbers increased to 15.07 × 10^5 ± 1.32 cells/tibia plus femur in ΔactA-infected mice 48 h PI (p < 0.001 compared with steady-state and with Δhly-infected mice). Thus, a lack of available monocytes does not explain their declining numbers in the brains of Δhly-infected mice. Analysis of gene expression focused on IFN- and IFN/TNF-α-related genes, including those encoding monocyte-attracting chemokines (Fig. 4A). There was robust up-regulation of 14 of 15 genes analyzed in ΔactA-infected mice compared with slight up-regulation in only 2 of 15 genes analyzed in Δhly-infected mice. Additionally, IFN-γ, IL-1α and β, and TNF-α were not up-regulated at the mRNA level in brains of Δhly-infected mice (data not shown). Collectively, these data show that bacterial infection of the brain is not required for activating CNS events that lead to monocyte influxes.

A specific role for IFN-γ in triggering central monocyte influxes

Next, the extent to which IFN-γ triggered these responses was tested by infecting IFN-γ−/− or normal mice with ΔactA L. monocytogenes then analyzing bacterial CFU, monocyte trafficking, and gene up-regulation in the brain 48 h PI. IFN-γ−/− mice harbored significantly more bacteria than did normal mice in bone marrow, 2.60 ± 0.20 (mean ± SEM, n = 6) vs 0.93 ± 0.44 log10 CFU bacteria/10^6 cells (p = 0.006), and spleen, 3.25 ± 0.22 vs 1.85 ± 0.21 log10 CFU bacteria/10^6 cells (p = 0.001). However, blood and brain remained sterile in both groups. There were no differences between IFN-γ−/− and IFN-γ+/+ mice in steady-state Ly-6Chigh monocyte populations in the bone marrow, blood, spleen, or brain (Fig. 5). Similarly, infected IFN-γ−/− and normal mice did not differ from each other with regard to numbers of monocytes in the bone marrow, percentages of blood monocytes that were Ly-6Chigh, or numbers of monocytes recruited into the spleen. In contrast, numbers of Ly-6Chigh monocytes in the brain significantly increased in normal mice but there was no measurable influx into the brains of infected IFN-γ−/− mice. qPCR showed absent or reduced up-regulation of most genes analyzed in IFN-γ−/− mice, the exceptions were ISG15 and SELE (Fig. 6). In addition, genes for IL-1α, IL-β, and TNF-α were up-regulated by 5.9 ± 1.5-, 4.6 ± 1.8-, and 2.8 ± 1.1-fold (mean ± SD, p < 0.05) over steady-state levels in infected IFN-γ+/+ mice but were not significantly changed in infected IFN-γ−/− mice.

Recent data from our laboratory show infection-induced influxes of Ly-6Chigh monocytes to the brain are unaffected in CCL2−/−, CX3CR1−/−, and CCR5−/− mice, whereas the influx is transiently delayed in CCR2−/− mice due to bone marrow retention of these cells (our manuscript in preparation). Results presented above suggest that CXCL9 and CXCL10 could have a role in recruiting monocytes to the brain because they are up-regulated under conditions with monocyte recruitment, i.e., ΔactA infection in IFN-γ−/− mice, and not when there is not monocyte influx, i.e., Δhly infection and ΔactA infection in IFN-γ−/− mice. In support of this notion, data in Fig. 6 show infection with ΔactA mutants stimulated robust protein production in normal mice, whereas brain concentrations were barely detectable in infected IFN-γ−/− mice. Thus, CXCL9 and CXCL10 are candidate chemoattractants for inducing Ly-6Chigh monocyte influxes to the brain.

Discussion

Data presented in this study demonstrate that the innate immune response to systemic L. monocytogenes infection has a profound influence on gene expression in the brain and triggers the initial influx of blood Ly-6Chigh monocytes into it. These events are independent of bacterial invasion of the CNS and depend in large part on IFN-γ. Microarray analysis of genes expressed in the brain during systemic L. monocytogenes infection showed changes beginning as early as 24 h PI, most of which reflected up-regulation
with a magnitude of <5-fold. In contrast, changes at subsequent time points were more robust and with greater numbers of down-regulated genes than up-regulated ones. Pathways Analysis was particularly useful and indicated that each time point analyzed had a signature set of activated pathways that were largely unique to that time point. In fact only two pathways, NF-κB signaling and apoptosis signaling, were identified more than once. Although a detailed analysis of each pathway at each day was not performed, these data are consistent with current paradigms of sepsis that include stage-specific mediators and processes as it progresses from the systemic inflammatory response syndrome to the compensatory anti-inflammatory response syndrome (31, 32).

Experiments performed using ΔactA and Δhly mutants of *L. monocytogenes* show that CNS events including gene up-regulation and monocyte influxes are not generic responses to infection of live bacteria and are independent of bacterial invasion/infec tion of the CNS. Our results showed infection with ΔactA mutants lead to significantly higher serum concentrations of IFN-γ, TNF-α, and IL-6 than did infection with Δhly mutants, and triggered robust gene up-regulation in the brain. This suggests that intracellular replication and/or cytosolic entry are critical steps in microbial infection of peripheral organs that lead to CNS activation (33). Kayal et al. (18) demonstrated that infection with wild-type *L. monocytogenes* but not listeriolysin O-deficient mutants triggered nuclear translocation of NF-κB in brain capillaries. Recent data show that listeriolysin O is rapidly inactivated at physiologic pH making it unlikely that circulating toxin, if present in the bloodstream during in vivo infection, could activate endothelial cells directly (34). Thus, the key contribution of listeriolysin O to remote activation of the brain in our experiments is more likely due to its role in promoting phagosomal escape and triggering release of secondary mediators, i.e., proinflammatory cytokines, from infected cells (29, 33).

The molecular mechanisms that underlie recruitment of Ly-6Chigh monocytes to the brain are not yet defined. Thus, understanding how it is initiated is a critical step toward defining relevant chemokines and other required molecules. Our results indicate that peripheral IFN-γ is a critical trigger. This is underscored by its abundance in plasma early during infection, the lack of up-regulation of IFN-γ mRNA in the brain, and its ability to induce transcription of genes for chemokines and endothelial cell adhesion molecules that could participate in monocyte recruitment. This hypothesis is supported by results in IFN-γ−−/− mice showing impaired Ly-6C+ monocyte trafficking to the brain despite normal eflux from the bone marrow and preserved recruitment to other organs. Rather, it was more likely due to the failure to activate key responses and mediators in the brain. Two of these, CXCL9 and CXCL10, were also measured on the protein level and were found only in marginal amounts in infected IFN-γ−−/− mice compared with normal animals. CXCL9 is able to recruit monocytes into lymph nodes during sterile inflammation and into the CNS in SIV-infection of rhesus macaques (35, 36). Nonetheless, it will be necessary to test more directly the roles of these chemokines and their receptor, CXCXR3, in recruiting Ly-6C+ monocytes in *Listeria*-infected mice.

In addition to proinflammatory cytokines, TLRs are present on a variety of cells in the CNS and trigger inflammatory cell recruitment to the CNS in response to injection of bacteria or microbial products (37–40). *L. monocytogenes* interacts with multiple extracellular pattern recognition receptors including TLR 2, 5, and 6 as well as intracellular receptors including TLR 9 and activates the RICK/RIP2/NOD pathway (30, 41, 42). Extracellular receptors could interact with live bacteria during bacteremia, or possibly with bacterial degradation products such as peptidoglycan or flagellin released into the circulation. Although not directly tested in our experiments, our results suggest that TLR-mediated activation of CNS resident cells by bacteria/microbial products is not required for triggering the CNS influx of monocytes. Specifically, there was no monocyte recruitment and very little gene up-regulation in mice injected with 10^9 CFU live Δhly mutants. Additionally, because IFN-γ−−/− mice had significantly higher bacterial loads than did normal mice, it is logical to expect that their brains would have had greater exposure to any bacterial products released during infection. Even so, there was no monocyte influx in the brains of these animals. Intracellular pattern recognition receptors clearly have a key role in initiating key systemic responses, but also are unlikely to contribute to the initial CNS activation in the absence of bacterial invasion.

Precise knowledge of which cells in the brain are responding to a particular stimulus in vivo requires further study and is complicated in our experiments by the fact that several different cell types can respond to the same stimuli. Nonetheless it is possible to identify likely relationships early in infection. For example, serotonin receptors are G-protein coupled receptors that have widespread distribution on neurons in the CNS (43). Neuronal activity was suggested by Pathways Analysis identification of serotonin and G-protein coupled receptors signaling. Activation of endothelial cells is demonstrated as early as 24 h PI by up-regulation of mRNA for E-selectin (SELE), and is consistent with immunohistochemistry showing increased expression of these proteins on brain vessels in *Listeria*-infected mice (17). Additionally, expression of genes such as MHC class II and C1q likely represent activation of microglia (44).

Direct injection of bacteria or their components into the CNS is an experimental technique commonly used to study bacterial meningitis (45). These models have yielded many insights about the host response to the presence of bacteria in the CNS and have revealed a variety of potential therapeutic avenues (46–48). In contrast, the extent to which systemic infection contributes to the pathophysiology of CNS infections is less well-defined but is gaining attention (49). Interesting data in West Nile virus-infected mice show that TLR 3-mediated innate immune responses in the periphery induce TNF-α production that weakens the blood-brain barrier and facilitates viral entry into the CNS (50). Together with our results, these data suggest that subversion of innate immune responses is a novel strategy used by neuroinvasive pathogens to gain entry into the CNS. They also underscore the added value that studying CNS infections as part of a systemic disease brings to the understanding of microbial pathogenesis.

**Acknowledgments**

We thank Jim Henthorn of the William K. Warren Medical Research Institute for assistance with flow cytometry, Dan Carr (Oklahoma University Health Sciences Center, Oklahoma City, OK) for critical reading of the manuscript, and Yuriy Gusev and Lydgia Jackson (Oklahoma University Health Sciences Center, Oklahoma City, OK) for assistance with bioinformatics.

**Disclosures**

The authors have no financial conflict of interest.

**References**


