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Lymphocytes serve as a reservoir for *Listeria monocytogenes* growth during infection of mice

Denise S. McElroy, Taylor J. Ashley, and Sarah E.F. D'Orazio*

Department of Microbiology, Immunology, and Molecular Genetics, University of Kentucky, Lexington, KY

Abstract

It is widely reported that *Listeria monocytogenes* can infect virtually all cell types, however, the degree to which this facultative intracellular pathogen can infect lymphocytes has not been well characterized. Previous studies have shown that a subset of lymphocytes, including activated T cells, are susceptible to apoptosis following exposure to *L. monocytogenes*, but the ability of the bacteria to replicate in the cytosol of lymphocytes prior to cell death was not examined. In this report, we demonstrate that intracellular *L. monocytogenes* can survive and multiply in vitro in a variety of transformed cell lines of lymphocytic origin. Intracellular *L. monocytogenes* were also recovered from splenic B cells, T cells, and NK cells following intravenous infection of mice. In fact, lymphocyte-associated *L. monocytogenes* comprised a substantial portion of the total bacterial burden in the spleen throughout the course of murine infection and B cell deficient mice had significantly lower titers of bacteria present in the spleen following intravenous infection. These results suggest that lymphocytes can be a reservoir for *L. monocytogenes* growth in vivo.

Keywords

intracellular pathogen; B cells; T cells

1. Introduction

Listeria monocytogenes is a facultative intracellular bacterial pathogen that causes human disease following the ingestion of contaminated food products. The bacterium codes for an extensive network of regulatory proteins to allow for survival in a wide variety of environments both inside and outside the host cell [1]. In the mammalian host, *L. monocytogenes* are readily taken up by professional phagocytes such as macrophages or dendritic cells. The bacteria can also mediate their own uptake into non-phagocytic cells using a using an internalin-mediated “zipper” mechanism [2]. Once inside the cell, *L. monocytogenes* escape from either the phagocytic or endocytic vacuole into the host cell cytosol and multiply with a doubling time that is approximately the same as that observed for bacteria growing in broth culture in vitro [3].

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*Corresponding author. Mailing address: Sarah E.F. D'Orazio, Ph.D., Department of Microbiology, Immunology, & Molecular Genetics, University of Kentucky, 800 Rose Street – MS415, Lexington, KY 40536. Phone: (859) 323-8701. Fax: (859) 257-8994. sarah.dorazio@uky.edu.

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Early reports indicated that *L. monocytogenes* was found mainly in macrophages or macrophage-like cells with dendritic processes in the spleen and in hepatocytes in the liver during systemic infection of mice [4-6]. Numerous *in vitro* studies have since shown that *L. monocytogenes* can also infect many other adherent cell types, including epithelial cells, fibroblasts, endothelial cells, astrocytes, and even neurons [7-11]. Thus, it is often reported that *L. monocytogenes* can infect virtually all cell types. However, the ability of *L. monocytogenes* to infect lymphocytes and other non-adherent cell types has not been studied in detail [12]. Westcott et al. recently showed that bone marrow derived dendritic cells are not productively infected with *L. monocytogenes* [13], a finding which suggests that not all cell types can support the exponential growth of these bacteria.

Within 10-15 minutes of intravenous inoculation, macrophages and dendritic cells in the marginal zone of the spleen and a specialized subset of macrophages in the liver known as Kupffer cells remove most *L. monocytogenes* from the bloodstream [5,14]. It has been suggested that splenic macrophages are more permissive for growth of *L. monocytogenes* than Kupffer cells [5]. Thus, the prevailing thought has been that the primary reservoirs for *L. monocytogenes* during systemic infection are macrophages in the spleen and hepatocytes in the liver. Neuenhahan et al. recently showed that 3 hours after intravenous infection, 70% of the *L. monocytogenes* that could be recovered from the spleen were found in dendritic cells [14]. However, 12 hours later, despite the fact that the total bacterial burden had increased in the spleen, only 30% of the recovered bacteria were found in either macrophages or dendritic cells. This observation strongly suggests that other cell types in the spleen must harbor intracellular *L. monocytogenes* during the early stages of the infection. In this report, we tested the hypothesis that lymphocytes could support the survival and intracellular growth of *L. monocytogenes*. Using both *in vitro* and *ex vivo* approaches, we showed that both B cells and T cells act as reservoir for *L. monocytogenes* growth during murine infection.

2. Results

2.1. Adaptation of the bacterial intracellular growth assay (IGA) for non-adherent cell types

The standard approach for measuring the intracellular growth of a bacterial pathogen is to infect adherent cells that have been seeded on glass coverslips. To facilitate contact between *L. monocytogenes* and the cell monolayer, the culture dish is typically subjected to centrifugation after addition of the bacteria. At the end of the infection period (30 minutes for phagocytic cells or 1 hour for non-phagocytic cells), gentamicin is added to kill extracellular bacteria. The coverslips can be harvested at various time points after infection, washed extensively and then either stained to visually inspect the cells, or the cells can be lysed in sterile water and the number of intracellular bacteria determined by plating the lysates on agar plates. We modified this assay to measure the intracellular growth of *L. monocytogenes* in non-adherent cell lines by infecting the cells in untreated culture dishes for 1 hour, then transferring the cells to centrifuge tubes to wash the cells in pre-warmed buffer (PBS) before re-plating in media containing gentamicin. At each time point, a portion of the cells was collected and counted, and then the cells were lysed by suspension in sterile water and the lysates were plated on BHI agar to determine the total number of intracellular *L. monocytogenes*.

One potential concern with an IGA using cells grown in suspension is that a higher multiplicity of infection (MOI) might be needed to promote contact between the bacteria and the cells in order to achieve the same level of infection as seen when using adherent cells. To address this issue, we first compared the results obtained using non-adherent J774 macrophage-like cells with the results from a traditional IGA using J774 cells seeded on glass coverslips. J774 cells were chosen because they adhere only loosely to non-tissue

culture treated dishes; the cells can be gently removed from the dish with pipetting alone. However, J774 cells adhere tightly to either glass or tissue culture-treated plastic. As shown in Fig. 1, both adherent and non-adherent J774 cells infected with *L. monocytogenes* 10403s at a MOI of 0.1 showed a similar increase in bacterial load over an 8 hour time period. These data indicated that cells grown in suspension could be infected with *L. monocytogenes* at maximal efficiency and validated the use of our non-adherent IGA protocol.

2.2. *L. monocytogenes* survives and replicates within a variety of transformed cell lines of lymphocytic origin

To test the ability of *L. monocytogenes* to multiply within lymphocytes, we initially infected transformed cell lines of either T cell (EL-4, D011.10, RMA), B cell (A20) or lymphoblast-like mastocytoma (P815) origin. Since none of these cell types were known to be phagocytic, we infected the cells for 1 hour at MOI ranging from 6 to 100. For three of these cell lines (EL-4, P815, and A20), the number of intracellular *L. monocytogenes* increased steadily (10 to 200-fold) over the 8 hour time period (Fig. 2). For both D011.10 and RMA cells, the number of intracellular *L. monocytogenes* increased for the first 5 hours of infection, and then the number of intracellular bacteria recovered began to decrease. Thus, all of the lymphocytic cell lines we tested allowed for at least a few doublings of bacteria.

One notable feature of the intracellular growth curves shown in Fig. 2 is the relatively low number of bacteria recovered from lymphocytes at the earliest time point compared with J774 macrophage-like cells (Fig. 1). To directly test the efficiency of infection for lymphocytes, we infected all five of the cell lines with *L. monocytogenes* at a MOI of 10 for 1 hour. The cells were then washed, suspended in media containing gentamicin, and incubated at 37°C. Thirty minutes later, the cells were harvested, a portion was lysed, and the total number of live intracellular *L. monocytogenes* was determined. On average, the lymphocyte cell lines contained 50-100 bacteria per 10⁵ cells, for an apparent efficiency of infection of approximately 0.1% (Fig. 3A). To directly visualize the infected cells, we also prepared Cytospin slides of EL-4 and A20 cells harvested 1.5 hours post-infection. The slides were stained with Diff-Quick and then manually inspected to calculate both the percentage of infected cells and the number of bacteria per infected cell. Only 1.7% of the A20 cells and 0.89% of the EL-4 cells we observed were associated with *L. monocytogenes*. For each cell line, the majority of the infected cells contained only 1 bacterium (Fig. 3B), and a few cells contained 2 bacteria. As a control, we also infected J774 cells and showed that 82% of the cells were infected with an average of 11 bacteria per cell. Together, these results suggested that *L. monocytogenes* invaded lymphocytes inefficiently, but once inside a lymphocyte, the bacteria could survive and replicate for several hours.

2.3. Viable *L. monocytogenes* can be recovered from splenic lymphocytes following intravenous infection of mice

The experiments described above indicated that cells of lymphocytic origin could support the growth of *L. monocytogenes*. To determine if lymphocyte infection occurred in vivo following intravenous inoculation of mice, we isolated subsets of splenocytes from *L. monocytogenes*-infected BALB/c mice, lysed the cells, and plated the lysates on BHI agar to determine the total number of bacteria that could be recovered from each cell type. For the pilot experiment, we harvested splenocytes 24 hours post-infection and then stained the cells with a monoclonal antibody that recognizes the B cell specific marker CD22.2. We used a cell sorter to collect two splenocyte subsets: B cells (CD22.2⁺ cells) and CD22.2-negative cells (all other splenocyte subsets). As a control, a sample of the stained, unsorted cells was also lysed and plated on BHI to determine the total number of intracellular *L. monocytogenes* per 10⁶ splenocytes (6.85×10^3). In this experiment, 72.9% of the bacteria

present in the pre-sort sample were recovered in either the CD22.2⁺ (9.08×10^2 CFU per 10^6 cells) or the CD22.2⁻ (4.09×10^3 CFU per 10^6 cells) sorted populations. Therefore, the number of *L. monocytogenes* recovered from the sorted B cells represented 18.2% of the total intracellular bacteria recovered from the sorted splenocytes.

The data obtained with sorted B cells indicated that live intracellular *L. monocytogenes* could be recovered from splenic lymphocytes following infection of mice. To facilitate a rapid simultaneous analysis of multiple cell types in the spleen, we next chose to use antibody-coated magnetic beads to isolate B cells (CD22.2⁺), T cells (TCR β ⁺), NK cells (DX5⁺), M ϕ (F4/80⁺), and DC (CD11c⁺) from the spleens of BALB/c mice 24 hours after *L. monocytogenes* infection. As shown in Fig. 4A, the number of bacteria recovered from each cell type increased in a dose-dependent manner. For each of the inoculums used, the number of bacteria associated with phagocytes (M ϕ and DC) was 3 to 4-fold greater than the number of bacteria recovered from lymphocytes. At the highest dose given to the mice (~30 LD₅₀), 63 CFU were recovered per 10^4 B cells and 68 CFU were recovered per 10^4 T cells, an apparent infection rate of approximately 0.65% for lymphocytes (Fig. 4A). These results were confirmed by a visual inspection of Diff-Quik stained cells centrifuged onto glass slides which revealed that 5 out of 1,154 B lymphocytes (0.43%) and 4 out of 1050 T lymphocytes (0.38%) were infected with either one or two *L. monocytogenes* per cell. No cells with a morphology typical of either macrophages or dendritic cells were observed on these slides.

Although the infection rate for lymphocytes was very low in mice given 1×10^4 CFU (~1 LD₅₀) of *L. monocytogenes*, we hypothesized that the number of bacteria associated with lymphocytes would increase over time as the bacteria replicated and infected new cells. To test this, we infected BALB/c mice with 1×10^4 CFU of *L. monocytogenes* and isolated either B cells or macrophages from the spleens of these mice at 24, 48, and 72 hours post-infection. As shown in Fig. 4B, there was an exponential increase in the number of CFU recovered from both B cells and macrophages three days post-infection. These data suggest that *L. monocytogenes* are able to survive and multiply within lymphocytes during intravenous infection of mice.

2.4. Lymphocytes represent a substantial portion of the bacterial load in the spleen through out the course of *L. monocytogenes* infection in mice

The experiments described above were all performed with lethal doses of *L. monocytogenes* to maximize the detection of infected lymphocytes at early time points. To test whether lymphocytes also harbored intracellular bacteria during a sublethal infection, we injected BALB/c mice with 1.9×10^3 CFU of *L. monocytogenes* (~0.2 LD₅₀). Groups of mice were sacrificed on days 1, 3, 5, and 7 post-infection, and the number of CFU recovered from splenic B cells, T cells, macrophages and dendritic cells was determined. For all four cell types, the peak bacterial burden was detected at 3 days post-infection (Fig. 5A). The number of CFU recovered per lymphocyte was lower than for macrophages or dendritic cells at most of the time points we examined. However, since lymphocytes typically represent 80-90% of the cells found in the spleen, the total number of intracellular bacteria found in either B cells or T cells actually represented a significant portion of the splenic bacterial load (Fig. 5B). In fact, at each time point, the total number of bacteria found in splenic lymphocytes was greater than the total number of bacteria residing in macrophages plus dendritic cells. Together, these observations confirm that lymphocytes remain infected with *L. monocytogenes* at a low level for the duration of infection in mice, and that intracellular bacteria in lymphocytes account for a large fraction of the bacterial burden in the spleen.

2.6. B cell deficient mice have significantly reduced bacterial loads in the spleen, but not the liver, compared with wildtype mice

To determine if infection of lymphocytes in the spleen altered the dynamics of early *L. monocytogenes* infection in mice, we compared the bacterial load in the spleens and livers of wildtype versus B cell-deficient mice. We chose to use B cell-deficient mice rather than mice that lack all lymphocytes because: (1) B cells represent the major fraction of lymphocytes in the spleen and (2) B cells are not thought to have any significant role in the clearance of primary infection with *L. monocytogenes* [15,16]. Groups of mice were infected intravenously with a sublethal dose of *L. monocytogenes* and the total number of CFU present in the spleen and liver was determined at 24, 28, and 72 hours post-infection. As shown in Fig. 6, there were significantly fewer bacteria present in the spleens of B cell deficient mice at each time point tested. At 72 hours post-infection, the number of CFU in the spleens of wildtype mice remained high, while the bacterial load in the spleens of B cell deficient mice was beginning to be reduced. In contrast, the number of *L. monocytogenes* present in the livers of these two mouse strains was nearly identical over the course of the infection. These data suggest that the presence of B cells as a potential reservoir for *L. monocytogenes* helps to prolong bacterial infection in the spleen.

3. Discussion

In this study, we showed that a small percentage of murine B cells and T cells are infected with *L. monocytogenes* throughout the course of a systemic infection. The efficiency of infection for lymphocytes was significantly lower than has been reported for other non-phagocytic cells. However, lymphocytes are the predominant cell type present in the spleen and we found that *L. monocytogenes* associated with B and T lymphocytes represented at least half of the bacterial burden in the spleen during murine infection. Previous attempts to characterize infected cell types in the spleen using only histochemical methods did not reveal the presence of infected lymphocytes [5,17]. However, it can be difficult to observe bacteria within stained lymphocytes because the nuclei are large and there is often very little cytoplasm present in these cells. In addition, our results indicate that at most time points as few as 1 in a 1000 lymphocytes may be infected with *L. monocytogenes*, therefore, a manual inspection of a few hundred stained cells would not be expected to yield positive results. In this study, we were able to achieve a greater sensitivity by isolating splenocyte subpopulations and then lysing the cells to recover intracellular bacteria. Plitas et al. recently used the same type of approach to determine the number of intracellular *L. monocytogenes* in sorted NK cells and they found approximately 100 CFU per 1×10^6 cells 24 hours after infection of mice [18].

One explanation for the low infection rate for lymphocytes could be that the cells do not express high levels of the appropriate receptors to allow for uptake triggered by bacterial adhesins. *L. monocytogenes* invasion of intestinal epithelial cells is mediated primarily by internalin A following interaction with E-cadherin [19], a transmembrane protein that is expressed in the adherens junctions between epithelial cells. Members of the cadherin superfamily are also expressed on circulating cells, however, this has been best studied for Langerhans cells and specialized subsets of T cells in the skin [20]. Corn et al. recently showed that E-cadherin was expressed in peripheral blood mononuclear cells and lymphoblastoid cell lines derived from normal human donors [21], however, it is not yet clear whether E-cadherin is expressed on the surface of murine lymphocytes.

Internalin B can also mediate *Listeria* uptake via an interaction with c-Met, the hepatocyte growth factor receptor that is expressed on a wide variety of host cells. Several groups have shown that activated B cells, but not T cells, strongly express c-Met [22-24], which suggests that at least a subset of B lymphocytes should be susceptible to internalin B-mediated uptake

of *L. monocytogenes* during infection. B cells are professional antigen presenting cells and as such, also have some level of intrinsic phagocytic capacity. Menon et al. showed that in vitro invasion of murine primary B cells was similar for both *L. monocytogenes* and the non-pathogenic species *Listeria innocua*, an organism that does not express internalin A or internalin B [12]. This suggests that either other bacterial adhesins can promote invasion of lymphocytes, or that the bacteria did not actively invade the B cells and instead were taken up by phagocytosis. In this study, we found that murine B cells in the spleen contained approximately 3-fold more intracellular bacteria than T cells at the peak of the bacterial load three days post-infection (Fig. 6).

L. monocytogenes infection is known to cause a substantial depletion of T cells in the white pulp of the spleen, and this may help the bacteria to temporarily evade an effective cell-mediated immune response. T cell death appears to reach a peak by 48 hours post-infection, and within 6 days the effect is reversed and the architecture of the lymphoid tissue appears normal again [17,25,26]. Carrero et al. have proposed that type I IFN produced by infected macrophages sensitizes T cells to undergo apoptosis following exposure to listeriolysin O, a multifunctional toxin secreted by *L. monocytogenes* [27-29]. In these studies, only activated lymphocytes were susceptible to apoptosis; resting T cells appear to be spared from the effects of LLO-induced apoptosis. During a sublethal infection with *L. monocytogenes*, splenic B cells may also be spared from destruction simply because the low dose of bacteria localizes primarily in the T cell zones of the white pulp. In fact, when mice were infected with higher doses of *L. monocytogenes*, some B cell depletion was also noted in the spleen [17]. Menon et al. further showed that when either mouse primary B cells or the human Ramos RA-1 B cell line were exposed to *L. monocytogenes*, 40-60% of the cells released lactate dehydrogenase within 6 hours [12]. In this study, we did not see a significant loss of viable cells within 8 hours of infection while performing intracellular growth assays on non-adherent transformed cell lines of lymphocytic origin. However, there was no apparent source of type I IFN in the cell culture to sensitize the cells and this could explain why we did not observe any cell death.

We found that B cell deficient (J_H mutant) mice had significantly fewer bacteria in the spleen, but not the liver during the first three days following sublethal infection with *L. monocytogenes*. These results are consistent with previous studies that examined *L. monocytogenes* infection of B cell deficient mice at either earlier or later time points than we used. Ochsenbein et al. found 80-fold fewer bacteria in the spleens of μ MT mice 6 hours post-infection compared with wildtype C57BL/6 mice [30]. Matsuzaki et al. showed that there was no difference in the bacterial titers of the livers of μ MT or wildtype mice 6 days post-infection [31]. Collectively, these observations suggest that the presence of B cells makes mice more susceptible to *L. monocytogenes* infection and we would argue that this is because the B cells provide a reservoir for bacterial growth in the spleen. Since lymphocytes are more susceptible to apoptotic death after exposure to *L. monocytogenes*, it is possible that individual lymphocytes remain infected for a shorter period of time than macrophages in vivo. However, during the earliest stages of the infection, a substantial fraction of the bacteria that are released from dying lymphocytes are likely to re-infect additional lymphocytes. In this way, lymphocytes can serve as a reservoir that promotes prolonged survival of *L. monocytogenes* in the host.

Since B cells can act as professional antigen presenting cells, it seems likely that infected B cells will process and present *L. monocytogenes* antigens to both CD4⁺ and CD8⁺ T cells during infection. This could have a significant impact on our understanding of how the memory T cell response develops during *L. monocytogenes* infection. Shen et al. showed that B cells were required to prevent depletion of antigen-specific CD8⁺ T cells during the contraction phase of the response [16]. Although a specific role for B cells in memory

development has not yet been delineated, one possibility is that antigen presentation by B cells allows for sustained costimulation of effector T cells. Further studies will be needed to determine whether *L. monocytogenes* infection of T cells, B cells, or NK cells is important for the development of an adaptive immune response, or whether infection alters the immune functions of these lymphocytes.

4. Materials and Methods

4.1. Bacterial strains

Wildtype *L. monocytogenes* (*Lm*) strain 10403s was incubated in brain heart infusion (BHI) broth (Difco) at 37°C with agitation until the bacteria reached early stationary phase, and then aliquots were prepared and frozen at -80°C. Prior to infection, frozen aliquots were thawed on ice and grown to early log phase in BHI broth. Dilutions of this culture prepared in PBS were used to infect either tissue culture cells or mice.

4.2. Cell culture

P815 (mouse lymphoblast-like mastocytoma), EL-4 (mouse thymoma), D011.10 (mouse T lymphocyte hybridoma), RMA (mouse T cell lymphoma), J774 (mouse macrophage-like) transformed cell lines were obtained from Michael Starnbach (Harvard Medical School). The A20 cell line (mouse B cell lymphoma) was provided by Subbarao Bondada (University of Kentucky). All cell lines were maintained at 37°C with 7% CO₂ in a medium (RP-10) consisting of RPMI 1640 (GIBCO #21870) supplemented with 10% fetal bovine serum (FBS), L-glutamine, HEPES, 50μM 2-ME and antibiotics.

4.3. Mice

Female BALB/cBy/J mice were purchased from The Jackson Laboratory and housed in specific-pathogen free conditions at the University of Kentucky (UK). B cell-deficient (*J_H* knockout) mice were generously provided by Beth Garvy (UK). Age and gender matched BALB/c/J mice were purchased from The Jackson Laboratory for use as wildtype controls for the experiment shown in Fig. 6. The experimental procedures used in this study were approved by the IACUC at UK. For infections, 8 to 14 week old mice were given an intravenous injection (in the lateral tail vein) of *L. monocytogenes* suspended in 200 μl of PBS.

4.4. Intracellular growth assay (IGA)

Adherent J774 cells were seeded on 12-mm round glass coverslips in a 24-well dish and incubated overnight in RP-10 without antibiotics to reach confluence. *L. monocytogenes* suspended in PBS was added to the cells and the dish was subjected to centrifugation at 700 × g for 5 min. After 25 mins. further incubation at 37°C in 7% CO₂, the cells were washed 3 times with pre-warmed PBS, and then suspended in RP-10 containing 25 μg/ml gentamicin. At indicated time points, the number of CFU associated with each coverslip was determined by placing the coverslip in sterile dH₂O, vortexing vigorously for 30 sec., and plating dilutions on BHI agar. The IGA was adapted for non-adherent cells as follows: (1) the cells were seeded in non-tissue culture treated 60 mm dishes (Falcon #351007) instead of on coverslips; (2) the cells were infected for 60 mins. before washing; (3) for the washes, the cells were transferred to a centrifuge tube and then the cells were re-seeded in a new 60 mm culture dish; (4) at the indicated time points, a portion of the cells was removed, a cell count was obtained, and then the cells were pelleted and resuspended in sterile dH₂O.

4.5. Microscopy

Infected cells were applied to Superfrost glass slides (VWR) using a Cytospin centrifuge (Thermo). The slides were stained with Diff-Quick (Dade Behring) and examined by light microscopy at 100X magnification under oil immersion. Each slide was viewed by two different individuals; each person counted at least 2000 cells per sample. Both the percentage of infected cells and the average number of bacteria present per cell was determined.

4.6. CFU recovery from sorted B cells

Splenocytes were harvested from a BALB/c/By/J mouse infected 24 hours earlier with 1.25×10^5 *Lm* 10403s (12.5 LD₅₀) and stained with anti-CD22.2 monoclonal antibodies (clone Cy34.1; eBioscience). CD22.2 (+) and (-) splenocyte subsets were sorted using a MoFlo sorter (DAKOCytomation) and collected directly into pure FBS. The CD22.2 (+) subset of cells was shown to be 98.74% pure. Both the sort (+) and the sort (-) fractions were washed, lysed in sterile H₂O, and then plated on BHI agar to determine the total number of intracellular bacteria present. As a control, the total number of CFU present in a portion of whole, unfractionated spleen from the same mouse was also determined. Comparison of the total CFU found in whole spleen and the CFU associated with sort-positive plus sort-negative cells showed that 72.85% of the bacterial load present in the spleen was recovered in the cells subjected to the sorting procedure.

4.7. CFU recovery from enriched splenocyte subsets

Splenocytes were harvested from *L. monocytogenes*-infected mice and single cell suspensions were prepared in RP-10 media containing 25 µg/ml gentamicin. The cells were stained with APC-conjugated antibodies (eBioscience) that recognize either F4/80 (clone BM8; macrophage marker), CD11c (clone N418; dendritic cell marker), and DX5 (NK cell marker). APC-conjugated anti-TCR β (clone H57-597) and PE-conjugated anti-CD22.2 antibodies were purchased from BD Biosciences were also used. Anti-PE or anti-APC antibody-coated magnetic beads (BD Biosciences IMag) were then used according to the manufacturer's instructions for positive selection of B cells, T cells, NK cells, macrophages, and dendritic cells. Isolated cells were collected in RP-10/Gent₂₅ and counted. A portion of the cells was lysed in sterile H₂O and serial dilutions were plated on BHI agar. The remainder of the isolated cells were used for Cytospin slide preparations. Preliminary experiments showed that exposing either intracellular (*Lm*-infected J774 cells) or extracellular bacteria to the magnetic beads did not reduce the viability of *L. monocytogenes*.

4.8. Quantification of bacterial load in spleen and liver

Wildtype (BALB/c) and B cell-deficient mice were infected intravenously with 7×10^3 CFU of *L. monocytogenes*. Groups of mice (n=4) were sacrificed at 24, 48, and 72 hours post-infection. Spleens and livers were harvested aseptically and homogenized in 0.2% NP-40. Serial dilutions were plated on tryptic soy agar containing 10 µg/ml streptomycin and the total number of CFU per organ was calculated. A two-way analysis of variance (ANOVA) of the data was performed using Prism software.

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Fig. 1.

Intracellular growth assays performed with either adherent or non-adherent J774 cells showed comparable growth of *L. monocytogenes*. J774 macrophage-like cells were seeded in triplicate either on glass coverslips (adherent) or in non-tissue culture treated dishes (non-adherent) and then infected with *Lm* 10403s (MOI=0.1). Cells were lysed at the indicated time points and the total number of CFU per 10^5 cells (non-adherent) or CFU associated with each coverslip (adherent) was determined. Mean values $\pm$ SD from one of two separate experiments are shown.

Fig. 2. Intracellular growth of *L. monocytogenes* in transformed cell lines of lymphocytic origin. EL-4 thymoma, D011.10 T cell hybridoma, P815 lymphoblast-like, RMA T cell lymphoma, and A20 B cell lymphoma cells were infected at the indicated multiplicities of infection and non-adherent intracellular growth assays were performed. Each cell line was used in at least two separate experiments. Mean values $\pm$ SD for triplicate samples from a representative experiment are shown.

Fig. 3.

L. monocytogenes infection of lymphocytic cell lines was less efficient than infection of macrophage-like cells. (A) Lymphocyte-like cell lines were infected at an MOI of 10. The mean number of intracellular bacteria per 10⁵ cells $\pm$ SD at 90 minutes post-infection is shown for each cell type. (B) Cytopsin preps were stained with Diff-quick and examined under oil immersion. Images are shown at 1000x magnification and arrows indicate bacteria.

Fig. 4.

CFU recovery from the lymphocytes of BALB/c mice infected with a lethal dose of *L. monocytogenes*. (A) BALB/c mice were infected with *Lm* 10403s at the indicated dose and splenocytes were harvested 24 h later. CD22.2⁺ (B), TCR β ⁺ (T), DX5⁺ (NK), F4/80⁺ (M ϕ), and CD11c⁺ (DC) cells were isolated by positive selection using antibody-coated magnetic beads. The cells were washed extensively, lysed in sterile H₂O, and plated on BHI agar to recover viable bacteria. (B) Groups of BALB/c mice (n=3) were infected with 1 × 10⁴ *Lm* 10403s (1 LD₅₀). Splenocytes were harvested at the indicated times post-infection and the total number of intracellular bacteria associated with either B cells (CD22.2⁺) or M ϕ (F4/80⁺) cells was determined. Mean values $\pm$ SD are shown.

Fig. 5.

Lymphocytes harbor a substantial portion of the bacterial load throughout the course of sublethal *L. monocytogenes* infection. BALB/c mice were infected with 1.9×10^3 CFU of *L. monocytogenes*, and groups of mice ($n=3$) were sacrificed at the indicated time points. Splenocyte subsets were isolated and then lysed to determine the total # of intracellular bacteria in each cell type. (A) Total CFU recovered per 10^4 cells is shown. (B) The total number of CFU present in the spleen was calculated using the data shown in (A) and the total splenocyte count for each mouse. Mean values $\pm$ SD for one of two separate experiments are shown.

Fig. 6.

B cell deficient mice harbor fewer *L. monocytogenes* in the spleen, but not the liver compared to wildtype mice. Groups of B cell deficient (JH mutant) or wildtype BALB/c mice (n=4) were infected a sublethal dose of *Lm* 10403s. Spleens and livers were harvested aseptically and homogenized in 0.2% NP-40 at the indicated time points and the total number of CFU per organ was calculated. A two way analysis of variance test indicated that the bacterial burdens in the spleens of wildtype mice compared to B cell-deficient mice were significantly different at all time points tested ($P = 0.0021$). In contrast, no significant differences were observed in the liver ($P = 0.3478$).