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Essential role of CCL2 in clustering of splenic ERTR-9^{+} macrophages during infection of BALB/c mice by *Listeria monocytogenes*

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Abstract

Early interactions between pathogens and host cells are often decisive for the subsequent course of infection. Here we investigated early events during infection by Listeria monocytogenes, an ubiquitously occurring facultative intracellular microorganism that exhibits severe pathogenicity mainly in immunocompromised individuals. We show that the inflammatory chemokine CCL2 is highly up- regulated early after Listeria infection in spleen of BALB/c mice. ERTR-9^+ macrophages of the marginal zone were identified as the only infected cells and exclusive producers of CCL2 at the early time point. Consequently, clusters of different cell types were formed around infected ERTR-9^+ cells. Metalophillic MOMA-1^+ marginal zone macrophages were however excluded from the clusters and migrated into the B cell follicles. Depletion of CCL2 during infection resulted in a different composition of cell clusters in the spleen and increased mortality rate of treated mice. Interestingly, ERTR-9^+ macrophages no longer were part of clusters in such mice, but remained at their original location in the marginal zone.

Introduction

Listeria monocytogenes, a Gram-positive facultative intracellular bacterium, is associated with serious infections in immunocompromised individuals, newborn children, the elderly and pregnant women (10,11,25,27). The murine experimental equivalent of listeriosis represents one of the best-understood bacterial infection models to date (30). In addition, various extensively studied tissue culture systems have revealed the Listeria infection cycle and the key components essential for it (for review see: Vazquez -Boland et al (36).

During this work it became clear that Listeria, when interacting with a particular host cell, induces strong reactions within this cell (see for example (23,29)). This is, on the one hand, due to host cell receptors, like toll like receptors, scavenger receptors or to uncharacterized intracytosolic receptors that interact with bacterial components (12,23). On the other hand, virulence factors have been shown to also induce specific signal cascades upon encountering the host cell surface. The spectrum of such events ranges from rearrangement of the cytoskeleton induced by the internalins (3,6,15) to induction of apoptosis of dendritic cells (DCs) and T cells by listeriolysin (17,18). In addition, it is well established that Listeria provokes the secretion of several cytokines in macrophages (10,21,34) and the up-regulation of cell adhesion molecules on endothelial cells (9).

Despite of these extensive studies, little is known about the immediate *in vivo* effects of Listeria on cells that are encountered first during infection i.e the macrophages that are responsible for the removal of the bacteria from circulation. Induction of proliferation and migration within the spleen has been described (5,26). Similarly, the early production of pro-inflammatory cytokines has also been ascribed to them (7,35).

In vitro it was observed that CCL2 is amongst the inflammatory cytokines and chemokines instantaneously produced by primary macrophages and macrophage lines upon Listeria infection (13,29,30) (unpublished data). In agreement, a protective role of CCL2 in murine listeriosis has been observed recently (31). Therefore, we wanted to investigate whether macrophages are responsible for the production of this mediator in the early phase of listeriosis *in vivo*. Results showed that expression of CCL2 at 4h post infection (p.i.) was restricted exclusively to ERTR-9^{+} macrophages of the marginal zone (MZ), which also was the population exclusively infected at this time point. Interestingly, experiments with mice in which CCL2 was depleted by administration of specific antibodies demonstrated that CCL2 is specifically involved in the activation of the ERTR-9^{+} macrophage population to migrate into the infectious foci.

Materials and methods

Bacterial strains and culture: Wild type strain *L. monocytogenes* EGD-e serotype 1/2a was used in all experiments. Bacteria were grown in BHI (brain heart infusion) broth at 37^{o}C overnight, then suspensions were diluted 1/5 in fresh medium and incubated for two hours at 37^{o}C, until they reached mid-log phase. Depending on the experiment, bacteria were washed and resuspended in PBS.

Mice: Female BALB/c mice were purchased from Harlan (Borchem, Germany) and used at the age of 10-12 weeks.

Antibodies: rat-anti-mouse ERTR-9 biotin (BMA Biomedicals), rat-anti-mouse MOMA-1 biotin (BMA Biomedicals), MOMA-1 FITC (Pharmingen), rabbit-anti-*L. monocytogenes* (Dunn Labortechnik), goat-anti-rabbit AMCA (Sigma), goat-anti-rabbit Alexa 488 (Molecular Probes), rat-anti-mouse Gr-1 PECy7 (eBioscience), rat-anti-B220 CyChrome (Pharmingen), hamster-anti-murine-CD3 (Pharmingen), goat-anti-hamster Cy5, rat-anti-mouse DX5 (Pharmingen), CD11b biotinylated (anti-Mac-1, clone: M1/70.15.11.5), CD11b PE (eBioscience), CD11c biotinylated (N418, clone: CHB 229), CD11c APC (eBioscience), rabbit-anti-mouse Laminin1γ chain (ImmunDiagnostic). Goat-anti-mouse CCL2 affinity purified polyclonal Abs (R&D Systems) were used to deplete mice of CCL2.

PCR. Primers for Real-Time RT PCR were selected by DNASTAR, Primer Select software (Lasergene) and purchased from MWG (forward; reverse) CCL2: (GCCCCACTCACCTGCTGCTA ; TTTACGGGTCAACTTCACATTCAA

Real-Time RT PCR to quantitate of chemokines transcripts: After infection of mice subpopulation of splenic cells were isolated by cell sorting. Total RNA from cells was prepared with the RNeasy MiniKit (Qiagen) according to the manufacturer's protocol. DNA contamination in the total RNA was eliminated by incubation with DNaseI (Amersham Pharmacia Biotech, Freiburg, Germany). cDNA was prepared using Superscript II RNaseH$^-$ (Invitrogen) according to the manufacturer's instructions and used in either RT-PCR or Real-Time RT PCR. The latter reaction was conducted using a SYBR Green PCR Master Mix kit (Applied Biosystem) in the GeneAmp 5700 Sequence Detection System (Applied Biosystem). Results were normalized using the housekeeping gene RPS9

In vivo infection: BALB/c female mice were infected intravenously (iv) with 100xLD_{50} ($5x10^5$) Listeria. After 0, 2, 4, 6, 24 h spleens were removed, part of the organs were used for RNA extraction, aliquots were frozen in liquid nitrogen for histology and from part of the spleen cell suspensions were prepared. The rest of the organ was homogenized in PBS supplemented with 0.2% NP-40 and plated

Isolation of cells from the spleens of infected mice: Spleen cell suspensions from infected animals were prepared by washing out the spleens with IMDM supplemented with antibiotics (penicillin 100µg/ml, streptavidin 100µg/ml, gentamycin 20 µg/ml), erythrocytes were lysed for 2 min in ACK buffer, and then washed three times in PBS containing EDTA. Cell suspensions were then used for a FACS analysis or cell sorting.

Polyclonal Abs treatment: To neutralize mouse CCL2, affinity purified polyclonal goat antibodies against murine CCL2 (R&D Systems) were used. 10µg were injected iv in 100 µl of sterile PBS 1 hr before bacterial infection. Animals were infected iv with $5x10^5$ CFU of Listeria,

then 4, 6, 24 h pi, respectively, mice were sacrificed, spleens removed and prepared for histology and flow cytometry.

Quantification of L. monocytogenes load in organs of infected mice: Mice were sacrificed at indicated time points, fragments of spleens were homogenized in 1 ml of 0,2% NP-40 in PBS. 50 µl of these homogenates were plated on BHI agar plates in duplicates and CFU (Colony Forming Units) estimated after overnight incubation at 37°C.

Quantification of L. monocytogenes load in sorted spleen cell populations: Cells from infected mice were sorted based on surface markers as described for FACS staining incubated with TritonX/PBS and plated on BHI agar plates in duplicates. CFU (Colony Forming Units) were estimated after overnight incubation at 37°C.

Flow cytometry and cell sorting: Single cell suspensions were prepared in FACS buffer at a density $5x10^5$ cell per well. Cells were treated with anti-mouse FcR Abs, followed by staining with appropriate Abs. Flow cytometry was performed using a FACSCalibur or FACSCanto (Becton Dickinson). Data were analyzed with CellQuestPro software or FACSDiva Softwear (Becton Dickinson).

Described populations of spleen cells were sorted after Abs staining using MOFLO (Cytomation), or FACSVantage DiVa and subsequently reanalyzed to confirm a sort quality, which reached 80-90%, depending on cell population.

Immunohistochemistry: Spleens were embedded in Tissue-Tek O.C.T. compound (Sakura), snap-frozen in liquid nitrogen and stored at –20°C. Cryostat sections of 7µm were prepared, air dried for 2h at room temperature and fixed in acetone (2 min at –20°C). After thawing, slides were

blocked with 0.05% BSA in PBS, stained with appropriate antibodies. After staining and washing, slides were dried, mounted with Neo-Mount (Merck) and analyzed using a laser scanning confocal microscope.

Confocal microscopy: Four-color confocal microscopy (AMCA, FITC/Alexa 488, Cy3/Alexa568, APC/Cy5/CyChrome) of cryosections was performed using LSM 510 META (Zeiss). To avoid overlapping emissions, fluorescent dyes were selectively excited in two series and fluorescence of single channels was measured by photon counting. Images were processed with Confocal Assistant 4.02, ImagePro 4.5 (Media Cybernetics) and Adobe Photoshop 7.

Results

Clustering of splenic macrophages after infection with L. monocytogenes

The established regulation of the pro-inflammatory cytokines and chemokines after Listeria infection should result in a severe repositioning of cells in the spleen due to migration and influx of leukocytes and lymphocytes. Indeed, when immune histology was employed a severe restructuring of the spleen was observed. At 4h after intravenous injection of (i.v.) $5x10^5$ bacteria, the architecture of the spleen with white and red pulp, separated by the marginal zone (MZ) was still very little affected (Fig. 1A), but these structures were completely altered at the later stage of infection - 24h pi (Fig. 1A). At this time, B cell follicles in the white pulp remained intact and MOMA-1^+ metalophillic marginal zone macrophages, usually located at the inner rim of the marginal zone, had migrated into these follicles. ERTR-9^+ macrophages that usually are located at the outer rim of the marginal zone had formed clusters around infection foci in close vicinity of MZ. To such clusters, most of the CD11b$^+$ cells were attracted, consisting of neutrophils and macrophage populations (Fig. 1B). Additionally, many T cells were found in such clusters (data not shown).

Staining of laminin, which detects the endothelial cells of inner membranes and blood vessels, revealed that generally the splenic marginal sinus is in the middle of the cluster (Fig. 1B). Thus, the clusters surrounding infectious foci are formed in the area of marginal zone.

Importantly, identical clusters were observed when mice were infected with $2x10^3$ bacteria. However, their formation required three days to be completed (data not shown).

Exclusive infection of ERTR-9$^+$ macrophages by L. monocytogenes

The marginal zone of the spleen is the area where the closed blood circulation opens into the splenic sinus. Therefore, both ERTR-9$^+$ as well as MOMA-1$^+$ macrophages should be exposed to blood borne pathogens after i.v. infection. However, in the case of Listeria, apparently only one type of macrophage is infected at 4h pi. Co-staining of splenic cryosections for macrophages and Listeria revealed that bacteria were only found associated with ERTR-9$^+$ macrophages (Fig. 2A), not CD11b$^+$ cells. Even when other cell types appeared to co-localize with Listeria, larger magnifications revealed that the bacteria are in long cellular extensions of ERTR-9$^+$ cells that might be in contact with many other cells in the spleen. This was confirmed by cell sorting and subsequent plating. At 4h pi Listeria were only recovered at significant numbers from the ERTR-9$^+$ macrophages (Table 1).

By 24h pi all bacteria were located within the clusters and are partially associated with ERTR-9$^+$ cells. However, plating of sorted splenic cell populations revealed that now also other phagocytic cells were infected with Listeria (Table 1).

Interestingly, MOMA-1$^+$ macrophages that are at the beginning of the infection also located in the marginal zone were poorly infected, although they were able to phagocyte Listeria efficiently *ex vivo* (data not shown). Nevertheless, despite the lack of direct contact with the bacteria at the early phase of infection, MOMA-1$^+$ macrophages become activated and migrated away from the large clusters into the B cell areas of the white pulp (Fig. 1A and 2C).

ERTR-9$^+$ macrophages are responsible for early production of CCL2

CCL2 was found to be the first chemokine up-regulated in adherent splenocytes isolated from Listeria infected mice under our conditions (data not shown). Since CCL2 is a chemokine

194 specific for macrophage attraction and the first visible effect of Listeria infection in mice is the
195 migration of macrophages, one might hypothesize that CCL2 is involved in the cluster formation.
196 Consequently, one would expect that cells infected first, in this case ERTR-9^+ macrophages, are
197 the main producers of CCL2 at the early stage of infection. To test this hypothesis, we sorted
198 macrophages and dendritic cells from mice infected with $5x10^5$ bacteria at 4 and 24h pi on the
199 basis of the cell markers: ERTR-9^+, MOMA-1^+, F4/80^+ (macrophages of the red pulp), CD$11c^+$
200 (total DC) and CD$11c^{int}$/B220^+ (plasmacytoid dendritic cells, pDCs). Analysis of these sorted
201 cells by RT-PCR revealed that ERTR-9^+ macrophages indeed were the major producers of CCL2
202 at 4h pi (Fig. 3A). Only pDCs showed a slight expression of this chemokine at that time point. At
203 later time points, consistent with the presence of bacteria in several phagocytic cell types, all the
204 cell populations tested are able to produce CCL2. Real-Time RT-PCR confirmed these results
205 with isolated ERTR-9^+ and MOMA-1^+ macrophages from infected mice. Only ERTR-9^+ cells
206 exhibited a significant up-regulation of CCL2 mRNA at 4h pi (Fig 3B).

207 Importantly, when the same cells were isolated from mice that were infected with $2x10^3$ Listeria
208 expression of CCL2 mRNA at early time points could be detected exclusively in ERTR-9^+ cells
209 although the RT-PCR signal was low compared to the signal obtained with cells from mice
210 infected with a high dose (data not shown).

211 ***Higher susceptibility of CCL2 depleted mice to infection by L. monocytogenes***

212 Since the chemokine CCL2 that attracts macrophages is apparently up regulated already during
213 the early phase of Listeria infection in the spleen of BALB/c mice, we decided to investigate the
214 importance of CCL2 in the host defense against Listeria. To this end, CCL2 was depleted by
215 injecting specific antibodies 1h before infection. Subsequently, mice were infected with $5x10^5$

bacteria. An antibody dose of 10µg/mouse was chosen although already lower amounts were shown to neutralize completely the *in vivo* activity of CCL2 (22). When 10^4 Listeria were injected into CCL2 depleted mice a higher susceptibility compared to controls was evident although the numbers of CFU were not dramatically different in both types of animals (Figure 4 and data not shown). This is consistent with findings of Serbina et al (31,33). Unexpected discrepancy between bacterial CFU in spleen and mortality of infected mice was similarly found by Depaolo (8) in case of Salmonella infection. The reason for this phenomenon is not clear so far.

Altered splenic cell composition in CCL2 depleted mice infected with L. monocytogenes

In view of the fact that depletion of the CCL2 chemokine alters defense against Listeria, it was important to verify the influence of anti-CCL2 treatment on cell composition kinetics in the infected spleen. Therefore, single cell suspensions were stained for cell surface markers of the major splenic cell populations. Consistent with a role of CCL2 at the early phase of infection, the most significant differences in cell composition were visible at 4h pi (Fig. 5). As expected, mainly macrophages and dendritic cells were affected by CCL2 depletion. Antibodies treated animals showed no increase in the frequency of ERTR-9^+ and MOMA-1^+ macrophages and CD11c^{int} B220^+ plasmacytoid dendritic cells at 4h pi. In contrast, in untreated mice the percentage of MOMA-1^+ and ERTR-9^+ initially increased (Fig. 5). Numbers of T cells, NK cells and B cells, as well as granulocytes, appeared to be not affected by the depletion. The same frequencies were observed in both types of mice with the fraction of T and NK cells dramatically decreasing during this period. This is most likely due to the apoptosis induced by Listeria described before (18). CD11b^+ and Gr-1^+ cells increased significantly in frequency during the

observation time and this increase was unaffected by CCL2 depletion. Thus, CCL2 appears to be involved in the recruitment and possibly the survival of splenic subpopulations of macrophages and to some extend dendritic cells after Listeria infection.

Effect of CCL2 depletion on the clustering of macrophages

Based on the differences described above, we decided to compare the architecture of the spleen after infection of control versus CCL2 depleted mice. Interestingly, in CCL2 deficient mice, ERTR-9^+ macrophages neither migrate nor form clusters during Listeria infection (Fig. 6A). Less than 8% of ERTR-9^+ cells are associated with clusters (Fig. 6C) and even at 24h pi the original marginal zone is still apparent. Nevertheless, cell clusters were formed independently of ERTR-9^+ cells in the marginal zone area and contained, like in untreated mice, CD11b$^+$ macrophages, Gr-1^+ neutrophils and some T cells (data not shown) as well as bacteria. We observed no difference in migration of MOMA-1^+ macrophages in both types of mice. These cells migrate to the B cell follicles in anti-CCL2 Abs treated mice as well as in untreated mice (Fig. 6B). These results suggest a strong influence of CCL2 chemokine on ERTR-9^+ marginal zone macrophage activation and migratory capacities.

Discussion

The marginal zone of the spleen is an unique region, where the closed blood circulation opens into splenic sinus. It is a transit area for the cells leaving the bloodstream and entering the white pulp (24). Due to its strategic function, marginal zone contains large number of resident macrophages, B cells, dendritic cells and fibroblasts. All these populations interact and are dependent upon each other. An especially important role in this location play macrophages, since they sample blood borne antigens and pathogens. These cells could be divided into at least two populations: Marginal Zone Macrophages (MZM) and Marginal Zone Metallophilic Macrophages (MMM). MMZ are recognized by ERTR-9 and MMM by MOMA-1 antibodies, respectively. Both populations have virtually no CD11b, CD11c, F4/80 but subpopulations of them are positive for B220 (data not shown).

It was previously shown that macrophages of the marginal zone are able to ingest Listeria (1,5), but it was postulated that both populations are equally contributing to this process. We have demonstrated that only one macrophage type, ERTR-9^{+}, co-localize with bacteria at the early stage of infection. Already at 2h pi bacteria can be observed inside ERTR-9^{+} cells (>90%, data not shown) while some still resided in marginal sinus (<10%). At 4h pi all detectable bacteria were found in the ERTR-9^{+} population.

The reason for such an exquisite cell specificity of Listeria is not clear, but the anatomical location certainly contributes to this, since cells of the marginal zone are preferentially exposed to blood borne pathogens. In addition, *ex vivo* experiments have shown that MOMA-1^{+} cells are able to internalize bacteria almost as well as ERTR-9^{+} macrophages (data not shown). This raises

274 the question why MOMA1$^+$ macrophages, occupying the same location are not infected and do
275 not take part in cluster formation. One explanation could be that ERTR-9$^+$ macrophages express
276 specific receptors like the C-type lectin characterized by the ERTR-9 antibody (SIGNR1) or
277 additional adhesion molecules (16,20).

278 Migration of cells in and out of lymphoid organs during infection with bacteria is an intensively
279 studied phenomenon presently. Chemokines and chemokine receptors are most important in this
280 process. In particular, the pro-inflammatory chemokine CCL2, previously called monocyte
281 chemotactic protein 1 (MCP-1), is immediately up regulated upon infections with many bacterial
282 pathogens. In general, it attracts monocytes and macrophages towards infectious foci.

283 Infection with *Listeria monocytogenes* results in up-regulation of many proinflammatory
284 cytokines and chemokines in the spleen (33,13). Here we show that CCL2 is one of the first
285 chemokines up regulated in ERTR-9$^+$ macrophages, which are the only population infected by
286 Listeria during the early phase of infection. Up-regulation of CCL2 in macrophages infected with
287 Listeria was also observed *in vitro* (unpublished data), (13,31) and *ex vivo* using splenic adherent
288 cell population isolated from infected BALB/c mice (unpublished data). Immediate up-regulation
289 of CCL2 was independent of whether a lethal or sublethal dose was employed for infection.
290 Thus, our data also show that ERTR-9$^+$ macrophages of the marginal zone are the major
291 producers of CCL2 at the early stage of infection. This implies that direct infection with Listeria
292 is required for initiation of CCL2 production.

293 The early expression of the pro-inflammatory chemokine CCL2 by infected ERTR-9$^+$ marginal
294 zone macrophages suggests it's important role in innate host defense. Indeed, antibody depletion
295 of CCL2 strongly enhanced the mortality of BALB/c mice infected with Listeria. Consistently, a

strong effect was observed by others in recombinant C57Bl/6 mice lacking CCL2 expression (31,33).

However, the finding that upon CCL2 depletion ERTR-9$^+$ macrophages do not migrate towards infectious foci of Listeria was unexpected. Normally, at 24h after infection inflammatory cell clusters were formed by ERTR-9$^+$ cells together with CD11b$^+$ macrophages, Gr-1$^+$ granulocytes and CD3$^+$ T cells around Listeria infected cells. In contrast, in CCL2 depleted mice no migration of ERTR-9$^+$ cells was observed, but the migration of all other cell subsets was unaltered. Similarly, colonization of spleen by Listeria was very little influenced (data not shown). This appears contradictory to data of Sebrina et al. (31) that demonstrated a strong increase of Listeria in the spleen of mice with a deleted gene for CCL2. However, the same authors now demonstrated that lack of CCL2 responsiveness did not influence the immigration of CD11b$^+$ into the spleen after Listeria infection (32). Rather it results in a block of emigration of such cells from the bone marrow and their absence in the circulation.

The reason for the different behavior of ERTR-9$^+$ cells is unclear. Additional chemokines might be produced to which cells other then ERTR-9 might have receptors. The same reasoning might explain the differential survival of monocyte/granulocyte populations with or without CCL2 depletion. On the other hand, CCL2 might signal in ERTR-9$^+$ macrophages via additional receptors as described for astrocytes (4,37). Thus, depletion of CCL2 might influence ERTR-9$^+$ macrophages more severe than other cells.

Taken together, our data allow the following interpretation of the sequence of events during Listeria infection. Infection of ERTR-9$^+$ macrophages, the primary target cells for Listeria, induces CCL2 production in these cells resulting in the attraction of other phagocytic cells

318 including non-infected ERTR-9$^+$ macrophages, CD11b$^+$ macrophages, Gr-1$^+$ granulocytes as
319 well as some CD3$^+$ T cells. Thus, infected ERTR-9$^+$ marginal zone macrophages represent the
320 condensation nucleus of the cell clusters formed in the spleen. When CCL2 is depleted, clusters
321 are formed nevertheless due to additional mediators, but uninfected ERTR-9$^+$ macrophages no
322 longer take part in cell migration. Obviously, migration of this population depends exclusively
323 on an early CCL2 signal. Moreover, it is possible that due to the CCL2 dysfunction, infected
324 ERTR-9$^+$ macrophages are killed by the bacteria and therefore are no longer detectable in the
325 clusters at 24h pi. Such scenario is suggested by the severe decrease the number of such cells at
326 24 hrs pi.

327 Our interpretation of the sequence of events is in apparent contrast to the suggestion by Muraille
328 et al.(26,5). Cluster formation was observed by them, but was interpreted as a migration of
329 infected macrophages into the T cell areas of the spleen. Laminin staining of cryosections
330 allowed us to clarify this controversy. T cells and most other cells are in fact migrating towards
331 the infectious foci formed in the marginal zone. The complex clusters of cells sometimes reach
332 from the marginal zone into the white and/or the red pulp. However, the majority of such clusters
333 do not surround the central arteriole, a hallmark of the T cell zone, although occasionally clusters
334 reach up to this vessel.

335 Intriguingly, the second population of macrophages found in the marginal zone, recognized by
336 MOMA-1 antibodies is not infected by Listeria. It is unexpected, considering the anatomical
337 localization of these macrophages in the marginal zone and their verified ability to efficiently
338 phagocyte Listeria *ex vivo*. Evidently, these macrophages are not attracted to the clusters of
339 phagocytic cells formed around the infectious foci. Rather, they migrate to the B cell follicles

independently of CCL2. It is tempting to speculate that these macrophages up-regulated the chemokine receptor CXCR5 that is responsible for homeostatic recruitment of B cells to the follicles (2,14,19,25,28). Clustering of MOMA-1^{+} macrophages in the B cell follicles might then be due to the location of a cellular source of the chemokine CCL13, the ligand of CXCR5.

Taken together, our results imply an important function for CCL2 during the initial phase of the innate immune response against *L. monocytogenes*. On the other hand, our data also extend the complexity of host reactions that are encountered during Listeria infection.

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Table 1 Number of bacteria in isolated splenocyte populations after Listeria infection in BALB/c mice

Cells	Markers	CFU[1.] 4h	CFU[1.] 24h
MZM[2.]	ERTR-9^{+}	1201 (13)	396 (4)
mDCs[3.]	ERTR-9^{-}CD11c^{+}CD11b^{int}	0	72 (1)
TipDCs[4.]	ERTR-9^{-}CD11c^{int}CD11b^{+}	0	2093 (93)
MMZM[5.]	MOMA-1^{+}	0	269 (3)
Granulocytes	MOMA-1^{-}CD11b^{+}CD11c^{-}Gr-1^{+}	0	70329 (85)
Macrophages	MOMA-1^{-}CD11b^{+}CD11c^{-}Gr-1^{-}	4 (0,04)	28426 (94)

[1.] per population in one spleen* (per 10^4 sorted cells)

[2.] Marginal zone macrophages

[3.] Myeloid dendritic cells

[4.] TNF/iNOS producing dendritic cells

[5.] Metalophillic marginal zone macrophages

* The cells from five sorted spleens were counted, and approximate value for one spleen estimated

Figure legends

Figure 1. Migration of macrophage subsets in the spleen after infection with *L. monocytogenes*. Cryosections from the spleens of infected mice at indicated time points were prepared, stained with appropriate fluorescent Abs and analyzed using confocal microscopy. **A.** Clustering of ERTR-9$^+$ Marginal Zone Macrophages (green) around infection foci and migration of MOMA-1$^+$ Metallophilic Marginal Zone Macrophages (red) into B cell follicles (blue). **B.** Clustering of CD11b$^+$ cells during infection with *L. monocytogenes*. CD11b$^+$ cells are stained in red and laminin in green. At 4h pi CD11b$^+$ cells are scattered through the red pulp. At 24h pi these cells form clusters around infection foci in the MZ area. No clusters were observed in the white pulp or surrounding the central arteriole. Arrows indicate central arterioles. Size bar 100μm. Pictures represents data from at least three independent experiments, with at least three mice. More than 20 fields of view were analyzed in each experiment.

Figure 2: ERTR-9$^+$ macrophages are the sole cell population involved in uptake of *L. monocytogenes* from blood. Splenic cryosections of infected mice were prepared as mentioned in Figure 1. **A.** 4h pi. ERTR-9$^+$ macrophages are exclusively associated with bacteria (green) at the early infection stage – 4h pi. Listeria green, CD11b blue, ERTR-9 red. Size bar 20μm. **B.** 24h pi. At 24h ERTR-9$^+$ macrophages and CD11b macrophages and neutrophils form clusters around infection foci. Listeria green, CD11b blue, ERTR-9 red. Size bar 20μm. **C.** MOMA-1$^+$ macrophages do not participate in

cluster formation and migrate into B cell area of the white pulp. MOMA-1 red, CD11b blue, Listeria green. Size bar 20µm. Pictures represents data from at least three independent experiments, with at least three mice. More than 20 fields of view were analyzed in each experiment.

Figure 3: CCL2 expression in macrophage and DC populations of the spleen after infection by *L. monocytogenes*. Mice were infected with $5x10^5$ CFU of *L. monocytogenes*. Spleens of mice 4 and 24h pi or of uninfected controls were removed, macrophage and DC populations sorted using the indicated markers and RNA isolated. RT-PCR or Real-Time RT-PCR from cDNA were used to study gene expression of CCL2. **A.** Induction of CCL2 in different macrophage and DC populations revealed by RT-PCR. **B.** Quantitation of CCL2 mRNA induction in ERTR-9^+ and MOMA-1^+ macrophages using Real Time RT-PCR. Cells were sorted from five pooled spleens, all experiments were repeated at least three times.

Figure 4: Increased mortality of BALB/c mice depleted of CCL2 after *L. monocytogenes* infection. BALB/c mice (treated previously with anti-CCL2 Abs or control Abs) were infected with 10^4 CFU of bacteria. The mortality rate of infected animals was examined. Solid line represents controls, dotted line represents CCL2 depleted animals. Experiment was done twice with at least five animals per group.

519 **Figure 5: Alteration in cellular composition of the spleen in anti-CCL2 Abs treated**
520 **mice after *L. monocytogenes* infection.** BALB/c mice treated with anti-CCL2 or goat
521 IgG (control mice) were infected with Listeria, at indicated time points single cell
522 suspensions were prepared from spleens of infected animals. Cells were stained using
523 Abs recognizing surface markers characteristic for the major splenic cell populations
524 and analyzed using a FACSCalibur. Control mice (solid line) and anti-CCL2 treated
525 mice (dotted line) are shown. All stainigs were repeated twice, with at least three
526 animals per group, results are expressed as means ± standard deviations. * $p \leq 0.01$.
527

528 **Figure 6: Clustering of phagocytic cells by *L. monocytogenes* is changed after**
529 **CCL2 depletion.** Spleens were isolated from infected animals treated or untreated with
530 anti-CCL2 24h after infection. Cryosections were prepared and stained with appropriate
531 fluorescent Abs. **A.** Migration of ERTR-9^+ macrophages is no longer observed in CCL2
532 depleted mice and bacteria are no longer associated with these macrophages CD11b
533 cells are stained in blue, ERTR-9 in red and Listeria in green. **B.** Migration of MOMA-1^+
534 cells into the B cell follicle is unaltered in CCL2 depleted mice comparing to the normal
535 BALB/c mice. MOMA-1 cells stained in red, B220 in blue and Listeria in green. Size bars
536 represent 50µm. These experiments were done twice using three mice. More than 20
537 fields of view were analyzed in each experiment. Control staining of spleen from
538 infected but untreated mice is depicted in Figure 2 since these experiments were done
539 in parallel. **C.** The percentage of ERTR-9^+ cells associated with infectious foci at 24h pi
540 in BALB/c mice treated with anti-CCL2 compared to untreated control mice. ERTR-9^+
541 cells were counted in fields of view of immune histological sections from spleens of at

least three treated and untreated mice, to calculate means and standard errors. More than 10 fields of view were analyzed for each condition.