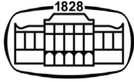

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Acute neonatal *Listeria monocytogenes* infection causes long-term, organ-specific changes in immune cell subset composition

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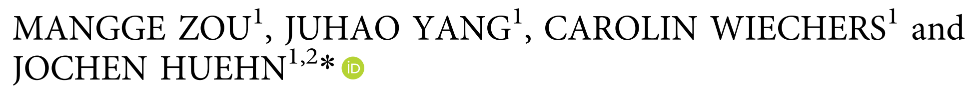
ORIGINAL RESEARCH PAPER



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ABSTRACT

Listeria monocytogenes (*Lm*) is a food-borne pathogen with a high chance of infecting neonates, pregnant women, elderly and immunocompromised individuals. *Lm* infection in neonates can cause neonatal meningitis and sepsis with a high risk of severe neurological and developmental sequelae and high mortality rates. However, whether an acute neonatal *Lm* infection causes long-term effects on the immune system persisting until adulthood has not been fully elucidated. Here, we established a neonatal *Lm* infection model and monitored the composition of major immune cell subsets at defined time points post infection (p.i.) in secondary lymphoid organs and the intestine. Twelve weeks p.i., the $CD8^+$ T cell population was decreased in colon and mesenteric lymph nodes (mLNs) with an opposing increase in the spleen. In the colon, we observed an accumulation of $CD4^+$ and $CD8^+$ effector/memory T cells with an increase of T-bet$^+$ T helper 1 (Th1) cells. In addition, 12 weeks p.i. an altered composition of innate lymphoid cell (ILC) and dendritic cell (DC) subsets was still observed in colon and mLNs, respectively. Together, these findings highlight organ-specific long-term consequences of an acute neonatal *Lm* infection on both the adaptive and innate immune system.

KEYWORDS

listeria monocytogenes, neonatal infection, long-term consequences, immune system, organ-specific

INTRODUCTION

Listeria monocytogenes (*Lm*) is a Gram-positive facultative intracellular bacterium, which ubiquitously exists in the environment and can be spread by consumption of contaminated food [1]. For immunocompetent adults, the intestinal microbiota could markedly reduce the colonization of ingested *Lm* and prevent *Lm* dissemination [2]. Ingested *Lm* is thus usually trapped and cleared from the intestinal tract [3, 4]. However, neonates, pregnant women, elderly and immunocompromised individuals are at a high risk of *Lm* infection with systemic dissemination [5, 6]. Upon ingestion of *Lm* in individuals from these risk groups, *Lm* penetrates through the intestinal epithelial barrier. Subsequent to the internalization into epithelial cells, *Lm* rapidly replicates, and invades into mesenteric lymph nodes (mLNs), spleen and liver, finally disseminating into the brain and placenta via systemic circulation [7–10].

Lm infection in pregnant mothers normally leads to nonspecific and mild maternal illness, but causes a stillbirth or acute neonatal *Lm* infection in offspring [11–13]. *Lm* infection in neonates is the third leading cause of neonatal bacterial meningitis [14] and is a major source of neonatal sepsis [11]. Neonatal listeriosis is a severe disease caused by neonatal *Lm* infection with a high fatality rate [15]. Based on the manifestation of the symptoms, neonatal listeriosis is subdivided into early onset disease (EOD) and late onset

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disease (LOD) [16]. EOD manifests within the first 6 days of life with precedent mild maternal symptoms, while neonates usually present clinical features including respiratory distress or pneumonia, septicemia and meningitis [17]. The mortality rate of EOD is about 20%, and the follow-up study revealed that 40% of surviving neonates suffered with severe neurological and developmental sequelae [18]. LOD manifests between day 7–28, and is typically diagnosed in term neonates with asymptomatic mothers [17, 19]. LOD patients usually suffer from septicemia and meningitis with an approximate mortality rate of 10% [16, 20]. After recovery from LOD, severe complications were frequently observed, including physical growth and mental retardation [17]. Although the neurological and developmental sequelae of neonatal listeriosis were already recognized in the 10-year follow-up study in the 1980s, the potential immunological sequelae of neonatal listeriosis is still incompletely understood.

Upon acute *Lm* infection, neutrophils, macrophages, natural killer cells and $\gamma\delta$ T cells are mobilized to control the bacterial growth at the initial stage [21–24]. Meanwhile, *Lm* infection induces an adaptive immune response through both $CD4^+$ and $CD8^+$ T cells, leading to a full clearance of *Lm* and the generation of a memory T cell pool [25–28].

Neonatal mice show a markedly increased susceptibility to *Lm* infection when compared to adult mice due to an insuffcient induction of cytotoxic T cell (CTL) activity accompanied with poor IFN-γ production [29]. Likewise, *Lm*-infected neonatal mice mount an impaired T helper 1 (Th1) response, likely due to the lower expression of critical Th1 response mediators, e.g., mannose-binding lectins and toll-like receptors [29].

Although the neonatal immune response against *Lm* was studied in detail [29], it has not been systematically investigated if neonatal *Lm* infection can cause long-term effects on the immune system that persist until adulthood. In the present study, we established a neonatal *Lm* infection model with 1-day-old neonates and analyzed infection-induced alterations in the composition of major T cell, dendritic cell (DC) and innate lymphoid cell (ILC) subsets at defined time points post infection (p.i.) in secondary lymphoid organs and the intestine. Interestingly, we could demonstrate organ-specific long-term consequences of neonatal *Lm* infection on both the adaptive and innate immune system that persisted as late as 12 weeks post infection, suggesting that acute neonatal infections can cause sustained changes within the immune system.

MATERIALS AND METHODS

Mouse strains

Foxp3^{hCD2}xThy1.1 mice (BALB/c background) were bred and housed under specific pathogen-free conditions in isolated ventilated cages at the Helmholtz Centre for Infection Research (Braunschweig, Germany).

Bacterial strains

Listeria monocytogenes (*Lm*) strain 10403S was used throughout the study. *Lm* was inoculated and plated on Brain Heart Infusion (BHI, Roth) agar plates. After 18-h overnight culture at 37 °C, single colonies were picked and transferred to 5 mL BHI medium in 15 mL Falcon tube. Subsequently, the bacteria were cultured overnight at 200 rpm at 37 °C. The next day, bacteria were harvested for infections.

Infection

0-day- or 1-day-old newborn Foxp3^{hCD2}xThy1.1 mice were intragastrically injected with different doses of *Lm*. Subsequently, the infected mice were monitored regularly and gain of body weight was documented. At different time points p.i., mice were euthanized by CO_2 asphyxiation, and organs were removed for analysis of bacterial burden and immune cell subset composition.

Bacterial burden analysis

At indicated time points p.i., mLNs, spleen, liver and stomach were isolated and placed into 15 mL Falcon tubes with 2 mL sterile PBS containing 0.2% (v/v) IGEPAL-CA 630 (Sigma-Aldrich). Organs were homogenized at 30,000 rpm for 30 s. To assess the bacterial burden in organs, serial dilutions of the homogenates were plated on BHI agar plates. After 20 h, the colony forming units (CFU) were counted as CFU per organ.

Cell isolation from organs

Single cell suspensions of splenocytes were prepared by meshing spleens through 30 μM cell strainers, followed by erythrocytes lysis. Single cell suspensions of lymph nodes (LNs) were prepared by enzyme-mediated digestion in RPMI 1640 medium (Gibco) containing 0.2 mg/mL DNase I (Roche), 0.2 mg/mL collagenase D (Roche) and 0.15 U/mL dispase (Roche) as described previously [30]. Colons were opened longitudinally and washed with PBS to remove feces. After two times incubation with PBS containing 5 mM EDTA buffer for 20 min at 37 °C, colons were cut into small pieces and digested in RPMI 1640 medium (Gibco) containing 0.1 mg/mL DNase I (Roche), 1 mg/mL collagenase D (Roche) and 0.1 U/mL dispase (Roche). After 1 h digestion at 37 °C, cells were centrifuged and subjected Percoll (GE Healthcare) gradient centrifugation (40%/80%, 780 g, 20 min, at room temperature with acceleration and brake off). Cells in the interphase layer were harvested, washed, and resuspended in PBS containing 0.2% bovine serum albumin (BSA, Sigma-Aldrich) buffer.

Antibodies and flow cytometry

Flow cytometric analysis was performed as described recently [31]. In brief, single-cell suspensions were labeled directly with the following fluorochrome-conjugated antibodies purchased from either BioLegend, BD Biosciences or

eBioscience: anti-human CD2 (RPA-2.10 and TS118), anti-mouse CD3 (17A2), anti-mouse CD4 (RM4-5), anti-mouse CD8α (53-6.7), anti-mouse CD11c (N418), anti-mouse CD19 (6D5), anti-mouse CD44 (IM7), anti-mouse CD45 (30-F11), anti-mouse CD45R (RA3-6B2), anti-mouse CD62L (MEL-14), anti-mouse CD127 (A7R34), anti-mouse Gata3 (L50-823 and TWAJ), anti-mouse Gr1 (RB6-8C5), anti-mouse Ly6G (1A8), anti-mouse MHCII (M5/114.15.2), anti-mouse RORγt (Q31-378), anti-mouse T-bet (4B10) and anti-mouse Ter119 (TER-119). Live/Dead discrimination was carried out using LIVE/DEAD Fixable Blue Dead Cell Stain (Thermo Fisher Scientific). Surface staining was performed for 15 min on ice in PBS (Gibco) containing 0.2% BSA. Intracellular stainings for T-bet, Gata3 and RORγt were performed with the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's instructions. Flow cytometry samples were acquired at the LSR Fortessa flow cytometer with Diva software (BD Biosciences) and data were analyzed using FlowJo software (Tree Star). Absolute cell numbers were counted with the Accurri C6 Cytometer (Beckton Dickinson) (Supplementary Table 1).

Statistical analysis

Group sizes were estimated according to a presumed standard deviation (SD) and an expected type I error of <0.05. The sample size was adjusted, if required, based on initial results. For all figures, each data point represents a single mouse if not stated otherwise. Prism software (GraphPad) was utilized for statistical analysis and graphs. For comparison of unmatched groups, two-tailed Mann–Whitney test was applied. If comparing more than three groups one-way ANOVA followed by Bonferroni's post-test was used. All data are presented as mean or mean ± SD and $P < 0.05$ are considered as significant. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$.

Ethics

All animal experiments were conducted in compliance with the German animal protection law (TierSchG BGBl. I S. 1105; 25.05.1998). Animals were housed and handled in accordance with good animal practice as defined by FELASA and the national animal welfare body GV-SOLAS. All animal experiments were approved by the Lower Saxony Committee on the Ethics of Animal Experiments as well as the Lower Saxony State Office of Consumer Protection and Food Safety under the permit number 33.19-42502-04-17/2489. All experiments were performed in accordance with the institutional, state, and federal guidelines.

RESULTS

Establishment of a neonatal *Lm* infection model

To establish a neonatal *Lm* infection model, 0-day- or 1-day-old newborn mice were intragastrically infected with either

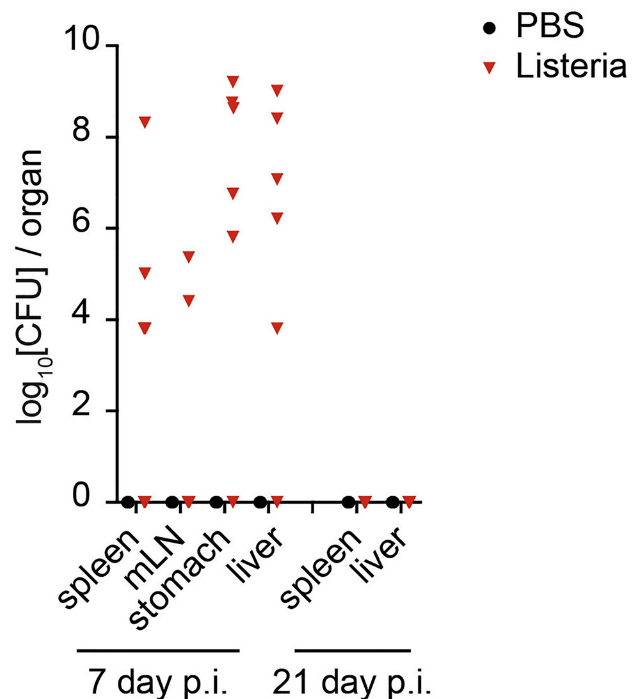

Fig. 1. Lm colonization and dissemination following intragastric administration. 1-day-old newborn Foxp3^{hCD2}xThy1.1 (BALB/c) mice were intragastrically injected with 20 CFU of *Lm*. Seven days and 21 days p.i., mice were sacrificed for analysis of bacterial colonization and dissemination. Spleen, mLNs, stomach and liver were collected, homogenized and plated on BHI agar plates. CFU were counted and given as CFU per organ (n = 4–6/group from at least three litters). mLN, mesenteric lymph node; CFU, colony forming unit

10, 20 or 100 CFU of *Lm*, and their survival and body weight were monitored regularly. 0-day-old newborns were highly susceptible to *Lm* infection, and already at the lowest infection dose succumbed within the first 4 days (Supplementary Figure 1A). 1-day-old mice were more resistant to *Lm* infection. They showed an approximately 80% survival rate at the infection dose of 20 CFU and 85% survival rate at 10 CFU, accompanied with a steady increase of body weight (Supplementary Figure 1A, B). However, infected newborns showed less body weight increase per day when compared to PBS-treated control mice within the first 4 days p.i., with the most dramatic reduction in relative gain of body weight occurring at day 3 (Supplementary Figure 1C). For all subsequent studies, 1-day-old mice were infected with 20 CFU of *Lm* since these conditions resulted in a clear manifestation of the pathological phenotype without severely compromising the survival rate. Firstly, we determined the bacterial colonization and dissemination in various organs during the course of the infection in the newly established neonatal *Lm* infection model. For quantification of the bacterial burden, liver, spleen, mLNs and stomach were collected from neonatally infected mice at 7 and 21 days p.i. 7 days p.i., *Lm* could be detected in spleen, liver, mLNs and stomach, demonstrating a systemic dissemination of *Lm*. Yet, already 21 days p.i. *Lm* was undetectable in spleen and

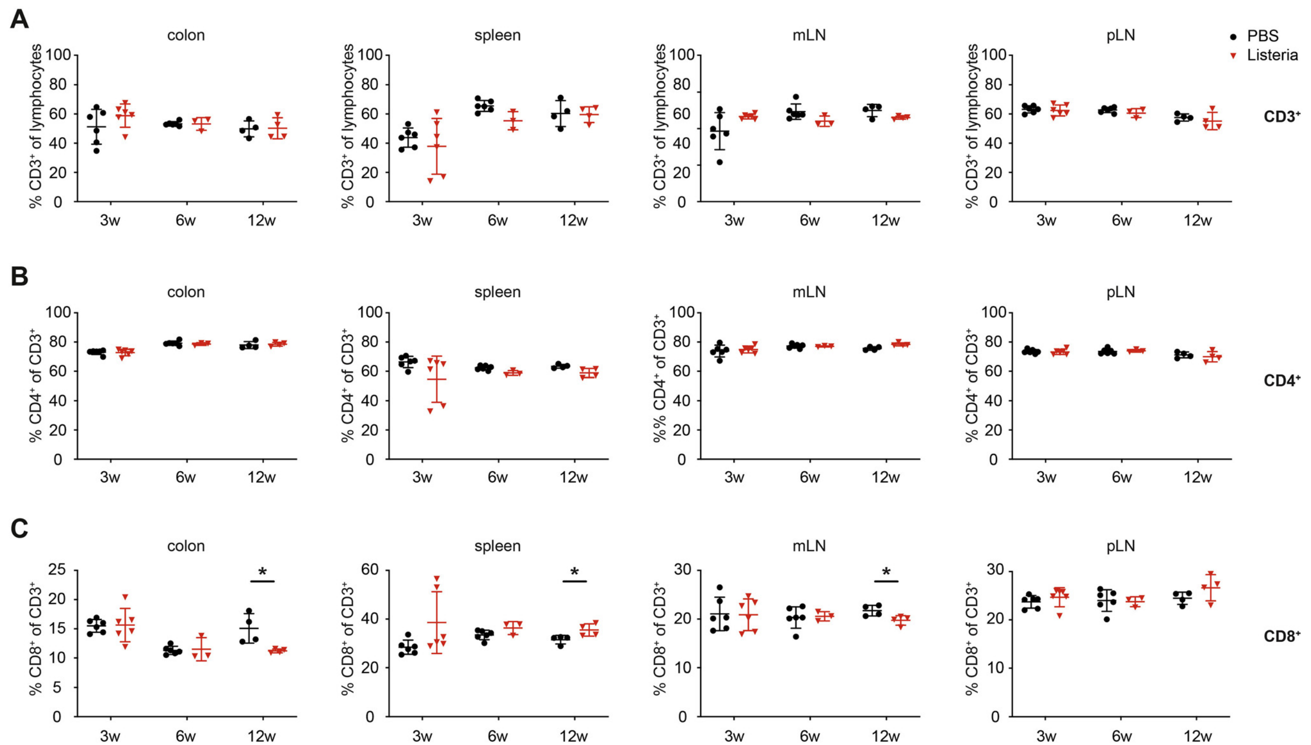

Fig. 2. Long-lasting impact of neonatal *Lm* infection on the T cell compartment. 1-day-old newborn Foxp3^{hCD2}xThy1.1 (BALB/c) mice were intragastrically injected with 20 CFU of *Lm*. Colon, spleen, mLN and pLN were collected and analyzed at indicated time points p.i. The frequencies of total CD3^{+} T cells among whole lymphocytes (A), CD4^{+} T cells among total CD3^{+} T cells (B) and CD8^{+} T cells among total CD3^{+} T cells (C) were plotted. Each dot represents a single mouse. Data were pooled from two to four independent experiments. *$P < 0.05$. mLN, mesenteric lymph node; pLN, peripheral lymph node. CFU, colony forming unit

liver (Fig. 1), indicating efficient clearance of *Lm* before weaning in this neonatal infection model.

Long-term impact of neonatal *Lm* infection on major T cell populations

The immune response against neonatal *Lm* infection has been studied in detail [29]. Yet, the long-term impact of this neonatal perturbation on the immune status in adulthood has not been systematically analyzed, so far. Here, we utilized the newly established neonatal *Lm* infection model and firstly analyzed the composition of major T cell subsets 3, 6 and 12 weeks p.i. in colon, spleen, mLNs and skin-draining peripheral lymph nodes (pLNs) by flow cytometric analysis (Supplementary Figure 2A). No significant changes were observed between neonatally infected mice and PBS-treated controls regarding the frequency of total CD3^{+} T cells among whole lymphocytes as well as the frequency of CD4^{+} T cells among CD3^{+} T cells in all organs at all indicated time points (Fig. 2A, B). Likewise, the frequency of CD8^{+} T cells among CD3^{+} T cells was comparable in all organs between infected groups and PBS-treated controls at 3 and 6 weeks p.i., suggesting that the T cell compartment is rather stable at these time points. Interestingly, when neonatally infected mice reached the age of 12 weeks, a significantly reduced frequency of CD8^{+} T cells was observed in colon and mLNs when compared to PBS-treated controls, while at the same time the frequency of CD8^{+} T cells was increased significantly in the spleen and showing a trend in pLNs (Fig. 2C). This finding suggests an age- and organ-specific impact of neonatal *Lm* infection on the CD8^{+} T cell population.

Having demonstrated that neonatal *Lm* infection has a long-term impact on the CD8^{+} T cell population, we next investigated whether the pool of effector/memory CD8^{+} T cells, characterized as CD44^{high}CD62L^{-} cells (Supplementary Figure 2B), was long-lastingly altered upon neonatal *Lm* infection. While no gross changes were observed in the frequencies of CD8^{+} effector/memory T cells in spleen, mLNs and pLNs of neonatally infected mice when compared to PBS-treated controls at any time point p.i., we observed an increased frequency of CD44^{high}CD62L^{-} CD8^{+} T cells in the colon of neonatally infected mice reaching statistical significance at the time points 6 and 12 weeks p.i. (Fig. 3A), suggesting long-lasting alterations within the pool of CD8^{+} effector/memory T cells in the intestine. The situation was slightly different for CD4^{+} effector/memory T cells, which were also characterized as CD44^{high}CD62L^{-} cells (Supplementary Figure 2B). Here, we observed an age-dependent increase in the frequency of CD4^{+} effector/memory T cells in the colon and spleen of PBS-treated controls, which was further enhanced in the colon of neonatally infected mice at 12 weeks p.i. (Fig. 3B). This increase could not solely be explained by an accumulation of regulatory T cells (Tregs), characterized by the expression of the transcription factor

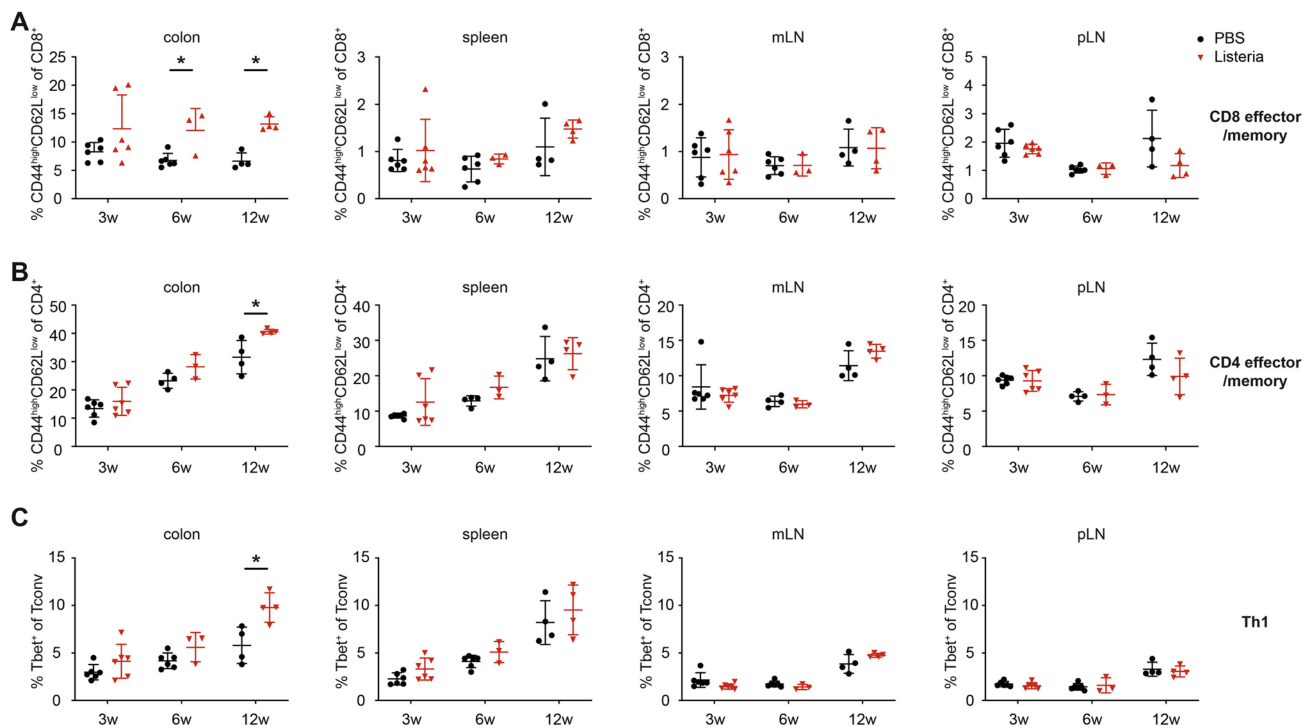

Fig. 3. Long-lasting impact of neonatal *Lm* infection on effector/memory T cells. 1-day-old newborn Foxp3^{hCD2}xThy1.1 (BALB/c) mice were intragastrically injected with 20 CFU of *Lm*. Colon, spleen, mLN and pLN were collected and analyzed at indicated time points p.i. The frequencies of CD44^{high}CD62L^{-} cells among CD8^{+} T cells (CD3^{+}CD4^{-}CD8^{+}) (A), CD44^{high}CD62L^{-} cells among CD4^{+} T cells (CD3^{+}CD4^{+}CD8^{-}) (B) and T-bet^{+} (Th1) cells among CD4^{+} Tconv cells (CD3^{+}CD4^{+}CD8^{-}hCD2^{-}) (C) were plotted. Each dot represents a single mouse. Data were pooled from two to four independent experiments. $^{*}P < 0.05$. mLN, mesenteric lymph node; pLN, peripheral lymph node. CFU, colony forming unit

Foxp3 (Supplementary Figure 2C), as we did not observe any significant alterations in the frequency of Foxp3^{+} Tregs between neonatally infected mice and PBS-treated controls in any organ at any time point p.i. (Supplementary Figure 3A). Yet, 12-week-old neonatally infected mice displayed a significantly higher proportion of inflammatory Th1 cells, characterized as CD3^{+}CD4^{+}CD8^{-}T-bet^{+} cells (Supplementary Figure 2D), in the colon when compared to PBS-treated controls (Fig. 3C), while only subtle alterations were observed among CD4^{+} effector/memory T cells harboring a Th2 phenotype (characterized as CD3^{+}CD4^{+}CD8^{-}Gata3^{+}) in mLNs (Supplementary Figures 2D and 3B). Together, these data suggest that neonatal *Lm* infection has a long-term impact on the T cell compartment with the strongest alterations appearing in the colon.

Long-lasting impact of neonatal *Lm* infection on the ILC compartment

Recent studies suggest that ILCs are essentially required to control early replication of *Lm* in the intestine and restrict dissemination of bacteria to peripheral tissues after oral infection in adult mice. In particular, ILC1 and ILC3 subsets respond to infection with proliferation and production of IFN-γ and IL-22, respectively [32, 33]. Here, we asked whether neonatal *Lm* infections can long-lastingly affect the ILC compartment within the colon and mLNs. When we first analyzed the frequency of total ILCs, characterized as CD45^{+}CD3^{-}CD19^{-}CD11c^{-}Gr1^{-}Ter119^{-}CD127^{+} cells (Supplementary Figure 4), among whole lymphocytes, no significant changes were observed between neonatally infected mice and PBS-treated controls in the colon at 3 and 6 weeks p.i. and in the mLNs at all time points (Fig. 4A). Interestingly, when neonatally infected mice reached the age of 12 weeks, the frequency of total ILCs within the colon increased significantly when compared to PBS-treated controls. This increase of total ILCs in neonatally infected mice at the late time point p.i. was accompanied by a significant reduction in the frequency of colonic ILC3s, characterized as CD45^{+}CD3^{-}CD19^{-}CD11c^{-}Gr1^{-}Ter119^{-}CD127^{+}RORγt^{+} cells (Supplementary Figure 4), while no gross differences were observed within mLNs between neonatally infected mice and PBS-treated controls (Fig. 4B). Yet, we observed a slight, but significant increase in the frequency of ILC1s, characterized as CD45^{+}CD3^{-}CD19^{-}CD11c^{-}Gr1^{-}Ter119^{-}CD127^{+}T^{-}bet^{+} cells (Supplementary Figure 4), at 3 weeks p.i. in both colon and mLNs when neonatally infected mice were compared to PBS-treated controls, which likely still represents the acute response against the neonatal *Lm* infection as the difference disappeared during aging (Fig. 4C). Viewed as a whole, our finding suggests a long-term impact of neonatal *Lm* infection on the ILC compartment, resulting in an altered ILC subsets composition.

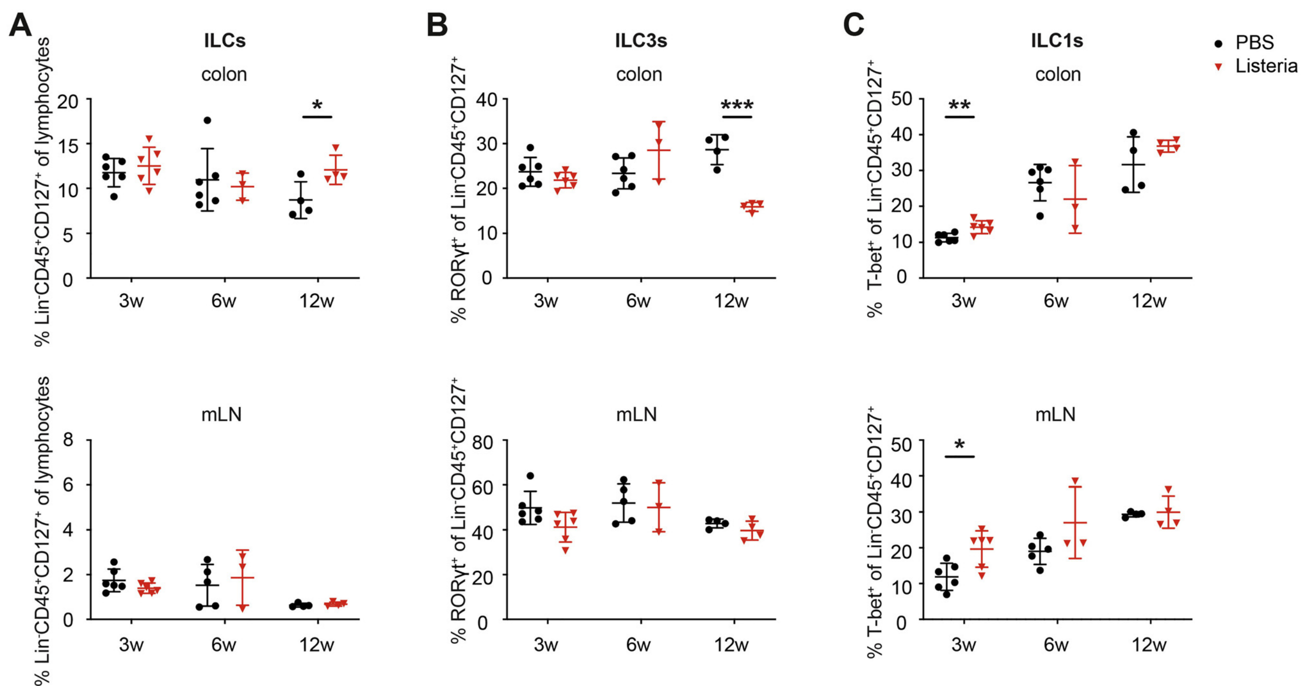

Fig. 4. Long-lasting impact of neonatal *Lm* infection on the ILC compartment. 1-day-old newborn Foxp3^{hCD2}xThy1.1 (BALB/c) mice were intragastrically injected with 20 CFU of *Lm*. Colon and mLN were collected and analyzed at indicated time points p.i. The frequencies of total ILCs ($CD45^+CD3^-CD19^-CD11c^-Gr1^-Ter119^-CD127^+$) among whole lymphocytes (A), RORγt$^+$ cells (ILC3s) among total ILCs (B), and T-bet$^+$ cells (ILC1s) among total ILCs (C) were plotted. Each dot represents a single mouse. Data were pooled from two to four independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. mLN, mesenteric lymph node. CFU, colony forming unit

Long-lasting impact of neonatal *Lm* infection on the DC compartment

DCs are known to play dual roles during *Lm* infection. On the one hand, they act as antigen-presenting cells, integrate signals derived from tissues and initiate T cell priming in secondary lymphoid organs in order to control the infections [34]. On the other hand, they may facilitate the establishment and dissemination of *Lm* due to their antigen-capturing and migratory properties [35]. Here, we aimed to dissect the potential long-term impact of an acute neonatal *Lm* infection on the composition of DC subsets, namely migratory DCs (MigDC, $CD3^-CD45R^-Ly6G^-MHCII^{high}CD11c^+$) and resident DCs (ResDC, $CD3^-CD45R^-Ly6G^-MHCII^+CD11c^{high}$) (Supplementary Figure 5), in the gut-draining mLNs. Interestingly, while no differences in the frequencies of MigDCs and ResDCs were observed between neonatally infected mice and PBS-treated controls at 3 and 6 weeks p.i., an altered DC subset composition was observed at 12 weeks p.i. with an increase in the frequency of MigDCs and a decrease in the frequency of ResDCs when neonatally infected mice were compared to PBS-treated controls (Fig. 5). Together, our finding suggests that neonatal *Lm* infection has a long-term impact on the DC compartment, which is emerging only at late time points p.i.

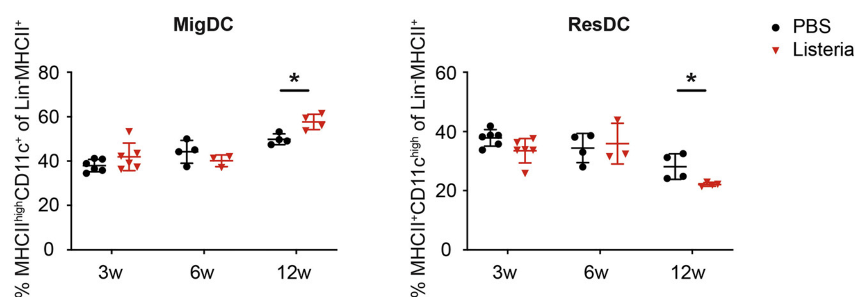

Fig. 5. Long-lasting impact of neonatal *Lm* infection on the DC compartment. 1-day-old newborn Foxp3^{hCD2}xThy1.1 (BALB/c) mice were intragastrically injected with 20 CFU of *Lm*. mLNs were collected and analyzed at indicated time points p.i. The frequencies of $MHCII^{high}CD11c^+$ cells (MigDCs) (A) and $MHCII^+CD11c^{high}$ cells (ResDCs) (B) among $MHCII^+Lin^-$ cells were plotted. Each dot represents a single mouse. Data were pooled from two to four independent experiments. *$P < 0.05$. Lin: $CD3^+CD45R^+Ly6G^+$. CFU, colony forming unit

DISCUSSION

Lm, a Gram-positive and food-borne bacterium, has a predilection to infect elderly and immunocompromised individuals, pregnant women and neonates. It can cause listeriosis especially in these vulnerable populations [1, 11] and clinical data demonstrated that *Lm* infection in neonates can drive sepsis or meningitis with high fatality rates [11]. Neonatal *Lm* infection models have been used previously to study the neonatal immune response against an acute bacterial challenge. Byun et al. have demonstrated that neonatal mice display an impaired Th1 response and an insufficient induction of CTL activity, which might contribute to the increased susceptibility of neonates to *Lm* infection [29]. Yet, in this model 5-day-old mice were infected with *Lm* which might omit the phenotype and consequences of EOD. In another model, 1-day-old mice were infected intranasally with *Lm*, but here the effects of the neonatal infection on the central nervous system were in the focus of this study [8]. Thus, the long-term consequences of neonatal *Lm* infection on the immune status in adulthood have not been systematically analyzed so far to the best of our knowledge.

In the present study, we established an acute neonatal *Lm* murine infection model with 1-day-old newborns by direct injection of *Lm* into the stomach to mimic the natural food-derived infection route, which results in a systemic dissemination of *Lm* to the mLNs, spleen and liver. Subsequently, we dissected the long-lasting consequences of neonatal *Lm* infection on major cell types of the immune system that were reported to be involved during *Lm* infection, namely T cells, ILCs and DCs [25–29, 32, 33, 35]. In line with the previously reported organ-specific $CD4^+$ and $CD8^+$ T cell responses upon *Lm* infection [36, 37], we observed long-term and organ-specific alterations in the $CD4^+$ and $CD8^+$ T cell compartment upon neonatal *Lm* infection. However, these alterations were not restricted to the adaptive immune system as long-term changes were also observed within the ILC and DC compartment, indicating that likewise the innate immune system was long-lastingly affected after neonatal *Lm* infection. It is important to note that most of the long-term changes, particularly those within the ILC and DC compartment, occurred at the site of the infection, the colon and mLNs. These observations are in accordance with findings from a recently published study, in which an acute infection of adult mice with the gastrointestinal pathogen *Yersinia pseudotuberculosis* resulted in an altered intestinal immune status that persisted several months after pathogen clearance [38]. The authors demonstrated that sustained inflammation driven by signals derived from the microbiota caused a permanent remodeling of the mesentery and an increased lymphatic leakage in the mesenteric adipose tissue, finally deviating MigDCs to the adipose tissue and preventing them from entering the mLNs. As a consequence, these sequelae of a cured infection resulted in a long-lastingly impaired intestinal immune status, including tolerance and protective immunity [38]. It is tempting to speculate that similar mechanisms contribute to the formation of the “immunological scars” that were observed in the present study. However, one interesting difference between these two studies is apparent as in the neonatal *Lm* infection model several of the long-term changes, namely the reduced frequency of colonic $CD8^+$ T cells, the increased frequency of splenic $CD8^+$ T cells, the higher proportion of inflammatory Th1 cells and total ILCs in the colon that was accompanied by a decreased frequency of ILC3s, and the altered DC subset composition in the mLNs, solely appeared at the late time point p.i., in 12-week-old neonatally infected mice, and were not simply long-lastingly sustained after pathogen clearance. The molecular mechanisms underlying this late appearance of these alterations are unknown, but it is obvious that the neonatal period is not only highly vulnerable to the infection itself, but also to the acquisition of the long-term alterations. It is well known that during the very short so-called “neonatal window of opportunity” our immune system rapidly matures, the microbiome is stably established and the susceptibility to immune-mediated diseases in adulthood is fine-tuned [30, 39–41]. The *Lm* infection occurring within this neonatal period might directly interfere with these maturation and fine-tuning processes, thus finally leading to long-term consequences that appear later in life.

Our data highlight that acute neonatal *Lm* infections can induce long-term alterations in the immune system. However, whether these sustained immunological alterations will result in pathological outcomes or not needs to be elucidated in future studies. Yet, it is already known that some food-borne bacteria can lead to severe immunological sequelae. *Campylobacter*, the most common cause of gastroenteritis, can cause campylobacteriosis in humans, with a high incidence in children [42, 43]. The majority of patients recovers spontaneously within 7–10 days without any medical intervention, whereas a subgroup of patients can develop severe neurological, orthopedical and gastrointestinal sequelae with a latency of weeks to months p.i. [44]. Approximately 0.1% of *Campylobacter*-infected individuals develop Guillain-Barré syndrome, a serious autoimmune-mediated neurological disorder that occurs as a consequence of a cross-reaction of antibodies induced during the infection with the sugar moieties on nerve gangliosides in affected individuals [45, 46]. Meanwhile, *Campylobacter* infections were also associated with reactive arthritis, Reiter’s Syndrome, irritable bowel syndrome, Crohn’s disease and ulcerative colitis [42, 44]. In addition to *Campylobacter*, non-typhoidal *Salmonella enterica* and *Shigella* can also drive the sequelae of reactive arthritis after recovery from acute infections [47, 48]. In the present study, we have observed elevated Th1 cells in colon 12 weeks p.i., however the antigen-specificity of these cells is unknown. Thus, it is tempting to speculate that these Th1 cells might contribute to the initiation of Crohn’s disease, which is known to be mainly driven by Th1 cells, when mice get older. Viewed as a whole, the frequent long-term immunological alterations at the site of infection in the neonatal *Lm* infection model might pose a latent threat of immunological sequelae during aging, when

the tightly balanced gastrointestinal immune system gets slightly dysregulated.

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Authors' contribution: MZ and JH designed study and wrote the manuscript. MZ, JY and CW performed experiments and analyzed data. All authors had full access to all data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1556/1886.2020.00007.

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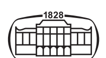

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