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Listeria monocytogenes replicate in bone marrow-derived CD11c+ cells, but not in dendritic cells isolated from the murine gastrointestinal tract

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Abstract

Recent fate mapping studies and gene expression profiles suggest that commonly used protocols to generate bone marrow-derived cultured dendritic cells yield a heterogeneous mixture, including some CD11c^{hi} cells that may not have a bona fide counterpart in vivo. In this study, we provide further evidence of the discordance between ex vivo-isolated and in vitro-cultured CD11c^{+} cells by analyzing an additional phenotype, the ability to support cytosolic growth of the facultative intracellular bacterial pathogen *Listeria monocytogenes*. Two days after foodborne infection of mice with GFP-expressing *L. monocytogenes*, a small percentage of both CD103^{neg} and CD103^{+} conventional DC (cDC) in the intestinal lamina propria and MLN were GFP^{+}. However, in vitro infection of the same subsets of cells harvested from naïve mice resulted in inefficient invasion by the bacteria (< 0.1 % of the inoculum). The few intracellular bacteria detected survived for only a few hours. In contrast, cultured CD103^{neg}CD11c^{+} cells induced by GM-CSF readily supported exponential growth of *L. monocytogenes*. Flt3L-induced cultures yielded CD103^{+}CD11c^{+} cells that more closely resembled cDC with only a modest level of *L. monocytogenes* replication. For both culture protocols, the longer the cells were maintained in vitro, the more readily they supported intracellular growth. The results of this study suggest that cDC are not a niche for intracellular growth of *L. monocytogenes* during intestinal infection of mice.

Introduction

Conventional dendritic cells (cDC) respond to infection by secreting cytokines, migrating to draining lymph nodes, and presenting antigen to T cells. Despite the critical role of cDC in mediating both innate and adaptive immunity, direct interactions between the foodborne intracellular bacterial pathogen *Listeria monocytogenes* and the various subsets of DC found *in vivo* remain largely undefined. Most of what is known about the growth of *L. monocytogenes* inside dendritic cells is derived from either in vitro-generated bone marrow-derived cultured cells, or inferred from systemic infection of mice after global depletion of CD11c^{+} cells (1,2).

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Early studies showed that GM-CSF was an important media component for generating both human and mouse DC *in vitro* (3–6). Bone marrow-derived cells cultured in the presence of GM-CSF are useful for *in vitro* studies to efficiently internalize and present antigen to $CD4^+$ T cells or to cross-present antigen to $CD8^+$ T cells. As a result of their frequent use in such assays, $CD11c^+$ GM-CSF-derived cells are often thought of as prototypic DC. However, it is now appreciated that different subtypes of DC can arise from either circulating monocytes or common dendritic progenitors in the bone marrow (7–9). For example, activation of the Flt3 receptor by Flt3-L promotes the differentiation of committed DC progenitors into cDC that express $CD8\alpha^+$ and $CD103^+$ in both lymphoid and non-lymphoid tissues, including the gut lamina propria (10,11). Flt3-L can also be used as a media supplement to generate $CD103^+$ $CD11c^{hi}$ cells from bone marrow progenitors (12,13).

cDC subsets in the intestinal lamina propria and the draining mesenteric lymph nodes (MLN) have unique characteristics compared to splenic cDC, which are likely caused by continual exposure to gut-derived microbial products and dietary antigens (14). CD103 (integrin α_E chain) is expressed on the majority of DC in the intestinal lamina propria including both BATF3-dependent cDC1 and IRF4-dependent cDC2, two recently described subsets that are derived from circulating pre-DC (15). Expression of CD103 by DC in the MLN is correlated with the induction of $Foxp3^+$ regulatory T cells and gut-homing T cells (16,17). In contrast, $CD103^{neg}$ DC are considered more inflammatory due to a greater ability to secrete TNF-α and IL-6 after in vitro stimulation (16), as well as efficiently inducing IFN-γ- and IL-17-producing effector T cells (11). In the intestinal LP, both $CD103^{neg}$ and $CD103^+$ DC express CCR7 and migrate in lymphatic fluid (11).

L. monocytogenes are facultative intracellular pathogens, and much research effort has focused on understanding the ability of these organisms to invade a wide variety of mammalian cells, survive and replicate in the cytosol, and spread to neighboring cells. However, we recently showed that following oral transmission, a large proportion of *L. monocytogenes* in the gut are actually extracellular (18). Intracellular growth was not required to establish infection in either the ileum or the colon, but replication in an as yet unidentified cell type was critical for colonization of the MLN (18). $Ly6C^{hi}$ monocytes were the predominant cell type that associated with *L. monocytogenes* in the MLN, but invasion of these cells is inefficient, and they did not serve as an intracellular growth niche for *L. monocytogenes* (19). In this study, we examined the ability of *L. monocytogenes* to invade, survive, and replicate in dendritic cells. We found that cDC harvested from the MLN of naïve mice and infected directly *ex vivo* did not support intracellular growth of the bacteria. In contrast, *L. monocytogenes* replicated exponentially inside in vitro-generated, bone marrow-derived $CD11c^+$ cells, which suggests that these cultured cells do not closely resemble intestinal cDC.

Materials and Methods

Bacteria

Mouse-adapted ($InlA^m$-expressing) *L. monocytogenes* EGDe derivatives SD2000, SD2710 (constitutive GFP^+) and SD2001 (GFP^{neg} vector control) were described previously (18). All

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strains were grown in Brain Heart Infusion (BHI) broth shaking at 30°C to early stationary phase, aliquoted, and stored at 80°C.

Mice

Female BALBc/By/J (BALB) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 4 weeks of age and housed in a specific-pathogen free facility (9 AM – 7 PM dark cycle). Mice were 6–9 weeks old when used in experiments. All procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Foodborne infection

Aliquots of *L. monocytogenes* were thawed, incubated at 30°C in BHI broth without shaking for 1.5 h, washed once with PBS, and then suspended in a 2:3 mixture of PBS and salted sweet cream butter (Kroger). A 2–3 cm piece of white bread (Kroger) saturated with *L. monocytogenes* was fed to mice near the onset of their dark cycle as described previously (20,21). The concentration of *L. monocytogenes* was estimated from the known titer of the bacterial aliquot and the actual inoculum was determined by plating serial dilutions of homogenized contaminated bread pieces.

Isolation of MLN and intestinal lamina propria cells

Lymph nodes were cut into 4 pieces each, and the total MLN were placed in 4 ml of RP-5 media (RPMI 1640 (Invitrogen 21870)/20 mM HEPES/5% FBS). Collagenase type IV (300 U/ml; Worthington) and DNase I (120 U/ml; Worthington) were added and the nodes were digested for 30 min at 37°C shaking at 250 rpm in a 50 ml conical tube containing a sterile stir bar (2 cm). Large intestines (cecum and colon) were flushed with 8 ml cold CMF buffer (Ca^{2+}/Mg^{2+}-free HBSS/10 mM HEPES/25 mM sodium bicarbonate/2% FBS) and then everted using a sterile weaving needle with button thread (22). Mucus was removed by shaking in a 50 ml conical tube with 25 ml CMF for 1 min. Epithelial cells were removed and the LP cells were isolated from the interface of a 44%/70% Percoll gradient as described previously (20).

Flow cytometry

Antibodies specific for CD16/CD32 (93), CD45 (30-F11), CD11c (N418), CD11b (M1/70), Ly6G (1A8-Ly6g), B220 (RA3-6B2), MHC-II (M5/114.15.2), CD3 (145-2C11), F4/80 (BM8), CD19 (eBio1D3) from eBioscience; Ly6C (HK1.4) and CD103 (2E7) from BioLegend were used. Data were acquired using an iCyt Synergy and analyzed with FlowJo (Tree Star). The percentage of *Listeria*-associated (GFP^{+}) cells in each population was determined by using cells from mice infected with *L. monocytogenes* SD2001 as a negative gating control in each experiment as described previously (18,19).

Ex vivo infection of cDC

Sorted $CD103^{+}$ or $CD103^{neg}$ cDC isolated from the MLN were seeded in 96-well round-bottom ultra-low attachment plates (Corning) and incubated in media with 20% FBS (100 μl) for at least 30 min at 37°C in 7% CO_2 prior to infection. In some experiments, cells were plated in half-area 96-well dishes to increase cell density; however, no differences were

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noted based on the type of dish used. Due to the relatively low yield of sorted cDC (~0.4 to 3 $\times 10^4$ per mouse), cells were divided equally into two wells and infected at a normalized MOI. The cells isolated from each mouse were used to analyze infection at two different time points; no technical replicates were plated. Thus, sorted cells from individual mice served as biological replicates for each experiment. Cells were used for intracellular growth assays as indicated below except that only one wash was used to remove extracellular bacteria.

Intracellular growth assay

Gentamicin protection assays were used to identify the number of intracellular CFU. Cultured cells were seeded in either 96-well round bottom (1×10^5/well) or 24-well (2.5×10^5/well) flat-bottom ultra-low attachment dishes (Corning). Where indicated, 12 mm diameter glass coverslips were placed in the wells prior to cell seeding. Aliquots of *L. monocytogenes* were thawed, incubated at shaking at 37°C in BHI broth for 1.5 h, washed once with PBS, and then suspended at the appropriate concentration. The concentration of *L. monocytogenes* was estimated from the known titer of the bacterial aliquot and the actual MOI was determined by plating serial dilutions of the inoculum. Extracellular bacteria were removed 30–60 min after infection by washing 3× with pre-warmed HBSS and then suspended in RP-10 media containing 10 μg/ml gentamicin. The percentage of inoculum internalized was calculated by dividing the number of CFU recovered after at least 20 min. exposure to gentamicin by the total number of CFU added to each well. At each time point, cells were harvested, washed once, lysed in sterile H_2O and plated on BHI agar.

In vitro generation of bone marrow-derived CD11c$^+$ cells

Bone marrow was flushed from the femurs and tibias of uninfected mice and suspended in 10 ml RPMI 1640 (Invitrogen # 21870), L-glutamine, HEPES, 2-ME, and 10% FBS (RP-10 media) in 100 mm non-TC petri plates. For GM-CSF-induced cultures, the protocol of Lutz et al. (23) was followed using 3% J558 supernatant as a source of GM-CSF. For Flt3-L-induced cultures, CHO FLT3-L-secreting cells (provided by Thomas Mitchell, University of Louisville with permission from Nick Nicola, The Walter and Eliza Hall Institute of Medical Research) were used as a source of Flt3-L as described (24). For day 16 cultures, 1.5×10^6 bone marrow cells/ml were cultured in RP-10 with 12.5% Flt-3L supernatant plus 0.75% J558 supernatant as described (25). 5 ml of fresh media was added on day 5 and non-adherent cells were removed on day 9 and transferred to new plates in fresh media (3×10^5 cells/ml). For day 6 cultures, 1.5×10^6 bone marrow cells/ml were cultured in RP-10 supplemented with 20% Flt3-L supernatant and no J558 supernatant. 10 ml of fresh media was added on day 3.

Microscopy

For Diff-Quik® (Dade-Behring) staining, cells were spun onto Superfrost slides (VWR) for 6 min. at 600 rpm using a Cytospin. Cells were then fixed in methanol 5 sec, stained in solution I for 10 sec, and stained in solution II 5 sec. Cells were dried and mounted with Permount® under glass coverslips. For F-actin staining, cells were spun onto slides, air-dried, fixed with formalin for 10 min, washed 3 times with PBS, and then permeabilized in TBS-T (TBS/0.1% Triton X-100/1% BSA, pH=8.8) for 15 min at room temp. Texas Red®-X

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Phalloidin (ThermoFisher) was added for 20 min at room temp. Slides were then washed 8 times in TBS-T, and 8 times with TBS alone. For differential "in/out" staining of *L. monocytogenes*, cells were washed 3 times with cold buffer (Ca^{2+}/Mg^{2+}-free HBSS/1% FBS/1 mM EDTA) and then incubated with Difco *Listeria* O Antiserum Poly (BD Biosciences cat. # 223021) (1:10) in PBS with 3% BSA for 20 min on ice. Washed cells were then incubated with goat anti-rabbit IgG-Texas Red® (ThermoFisher) for 20 min on ice. Stained cells were spun onto poly-L-lysine-coated Superfrost slides (VWR), formalin fixed at 4°C for 10 min, washed with PBS, and mounted under coverslips with ProLong® Diamond antifade (Molecular Probes). All cells were visualized using a Zeiss Axio Imager.Z1 with a 100×/1.4NA PlanApo oil immersion objective and analyzed with AxioVision software.

Statistics

Statistical analysis was performed using Prism for Macintosh (version 6; Graph Pad). *P* values of <0.05 were considered significant and are indicated as follows: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$.

Results

L. monocytogenes associate with both CD103$^+$ and CD103^{neg} DC in the gut

Ly6C^{hi} monocytes were previously identified as the primary cell type associated with GFP-expressing *L. monocytogenes* in the gut-draining MLN two days after foodborne infection with either wild type or murinized (InlA^m-expressing) bacteria (19). CD11c$^+$ cells represented only a minor fraction of the total GFP$^+$ cells at that early time point, but the number of *L. monocytogenes*-associated CD11c$^+$ cells did increase slightly by 72 hpi. In this study, we used a similar flow cytometric approach to confirm that oral infection also resulted in an association of the bacteria with CD11c$^+$ cells in the intestinal lamina propria (Fig. S1). Only 1.8% of all GFP$^+$ cells were Ly6G$^-$Ly6C$^{-/+}$CD11c^{hi} (Fig. S1D), similar to what was observed previously in the MLN (19). The key difference noted in the lamina propria was that the majority of *L. monocytogenes*-associated cells were Ly6G^{hi} neutrophils, rather than Ly6C^{hi} monocytes.

CD103$^+$ cDC can express E-cadherin (26), and binding of the *L. monocytogenes* surface protein InlA to E-cadherin is known to induce a "zipper-like" mechanism of bacterial uptake (27). Therefore, we postulated that *L. monocytogenes* would associate primarily with CD103$^+$ subsets of DC in the gut. To test this, female BALB/c/By/J mice were fed 10^8 CFU of a mouse-adapted *L. monocytogenes* strain that constitutively expressed GFP. Two days post-infection (dpi), mononuclear cells from the large intestine lamina propria were analyzed by flow cytometry (Fig. 1A) and the percentage of *L. monocytogenes*-associated (GFP$^+$) cells in each cDC subset was determined using the approach outlined in Fig. S1C. Approximately equal numbers of CD103^{neg} and CD103$^+$ cDC were isolated from the large intestine lamina propria of each infected mouse (Fig. 1B). As expected, only a minor fraction of the cDC was GFP$^+$. However, there was no preferential association of *L. monocytogenes* with the CD103$^+$ cells. Thus, within 48 hours of foodborne transmission, *L.*

monocytogenes were directly interacting with both CD103^{+} and CD103^{neg} cDC in the intestinal lamina propria.

CD103^{+} cDC are migratory cells that traffic from the gut to the MLN (28). To find out if *L. monocytogenes* preferentially associated with the CD103^{+} cells that disseminated to the draining lymph nodes, we also analyzed total cells from the MLN at 72 hpi (Fig. 1C). Similar numbers of CD103^{+} and CD103^{neg} cDC were found in the MLN and there was still no preferential association of *L. monocytogenes* with either cDC subset (Fig. 1D). Similar numbers of GFP^{+} cells were found in the CD11b^{+} and CD11b^{neg} subsets (data not shown), suggesting that *L. monocytogenes* did not preferentially associate with any of the four subsets of intestinal DC. To find out if the cells contained live, replicating intracellular bacteria, sorted cells were lysed and plated on BHI agar. In the majority of cases, no CFU were detected; the cells from one mouse yielded one CFU (data not shown). This was not a surprising result given the small number of cDC that were isolated and the low percentage of cells that were GFP^{+} two days post-infection; the expected number of CFU would be just at or below the limit of detection using this approach. However, it was also possible that 48 hours after infection, most of the cDC present in gut had been exposed to pro-inflammatory cytokines that activated the cells to restrict intracellular growth of *L. monocytogenes*.

L. monocytogenes inefficiently invade cDC and cannot sustain intracellular survival

To find out if either CD103^{neg} or CD103^{+} cDC were capable of supporting intracellular growth of *L. monocytogenes*, the cells were isolated from the MLN of uninfected mice and then infected *ex vivo* (Fig. 2A). *L. monocytogenes* was added to approximately 1×10^{4} sorted cells per well in low attachment plates at an MOI of 10. One hour after infection, only 0.1% of the inoculum added to either CD103^{neg} or CD103^{+} DC was protected from gentamicin (Fig. 2B). By comparison, adherent phagocytic cells such as bone marrow-derived macrophages can take up nearly all of an inoculum within 1 hour, and phagocytose about 1–5 % of the bacteria if the cells are cultured in low adherence dishes (19). Non-phagocytic cells such as Caco-2 intestinal epithelial cells also typically internalize 1–3% of *L. monocytogenes* when infected at high MOI (19,29). No gentamicin resistant bacteria were recovered when the cells were infected at low dose (MOI=0.1–0.5) or at an intermediate dose (MOI=1–5) (data not shown). Therefore, the cDC infected directly *ex vivo* did not efficiently internalize mouse-adapted *L. monocytogenes*.

After incubating for three additional hours (4 hpi), the number of gentamicin-resistant CFU recovered from the cDC remained the same (Fig. 2C). This suggested that intracellular *L. monocytogenes* survived, but did not replicate inside the cDC. To find out if the intracellular bacteria had escaped from the phagocytic vacuole into the cytosol, we stained F-actin and examined the cells microscopically for the presence of actin tails (Fig. 3A). Of 137 infected cells recovered from three different mice, only three cells harbored bacteria with actin tails (Fig. 3B). The vast majority of GFP^{+} cells 4 hpi did not co-localize with actin, and most cells were associated with only one to three bacilli per cell (Fig. 3C). By eight hours post-infection, the number of intracellular *L. monocytogenes* had decreased significantly in both CD103^{neg} and CD103^{+} cDC, and in some cases, no gentamicin-resistant bacteria were recovered (Fig. 2C). Together, these results indicate that *L. monocytogenes* are inefficiently

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taken up by cDC, and those few intracellular bacteria that survive have a truncated life cycle compared with L. monocytogenes intracellular growth previously observed in macrophages.

In vitro-generated, bone marrow-derived CD103^{neg}CD11c^{+} cells readily support intracellular growth of L. monocytogenes—Westcott et al. previously reported that bone marrow-derived cultured CD11c^{hi} cells were a suboptimal niche for intracellular growth of *L. monocytogenes* when compared to bone marrow-derived macrophages (30). However, in contrast to our results with ex vivo infections, in that study, *L. monocytogenes* survived inside the CD11c^{+} cells for at least 8 hours and the number of gentamicin-resistant bacteria grew 30-fold during that time period. To explain this discrepancy and to better understand the culture conditions that lead to an intracellular growth phenotype, we re-examined the ability of *L. monocytogenes* to replicate in cultured CD11c^{+} cells.

In the study by Westcott et al., bone marrow cells were cultured with rGM-CSF for six days before the loosely adherent CD11c^{+} cells were magnetically enriched, allowed to adhere to glass coverslips, and then infected. In contrast, the *ex vivo* cDC infections shown in Fig. 2 were conducted in round-bottom low-attachment plates. Since in vitro culture of bone marrow cells with GM-CSF also generates adherent cells with more macrophage-like features (3), it was possible that allowing the cells to attach was promoting efficient invasion and replication. To test this, bone marrow cells were cultured for eight days in the presence of GM-CSF containing cell supernatants and then the loosely adherent cells were transferred to low-attachment 24-well plates with or without glass coverslips and cultured for an additional 24 hours. At this time point, approximately 80% of the cells expressed intermediate to high levels of CD11c and lacked CD103 expression regardless of whether or not the cells were transferred to glass coverslips during the last day of culture (Fig. 4A and data not shown). Attachment to glass also did not alter the expression levels of MHC-II or B220 (Fig. 4B). One hour after infection with *L. monocytogenes*, similar numbers of gentamicin-resistant bacteria were recovered, which suggested that adherence to glass did not significantly alter initial invasion of *L. monocytogenes* (Fig. 4C). The number of intracellular bacteria increased 32-fold over eight hours for the cells cultured on glass coverslips (Fig. 4D). However, only a 6-fold increase in CFU was observed for cells infected in suspension in low attachment plates. Thus, adherence to glass accounted for at least some of the ability of *L. monocytogenes* to replicate inside in vitro-generated CD103^{neg}CD11c^{+} cells.

GM-CSF can promote the development of macrophages as well as neutrophils (31), and ~15–20% of the cells in our cultures were typically CD11c^{neg} (Fig. 4A). Thus, it was possible that a minor proportion of heavily infected CD11c^{neg} cells in the bulk cultures could account for the intracellular growth we observed. To test this, CD11c^{+} and CD11c^{neg} cells were sorted on day eight and then cultured overnight in low-attachment plates prior to infection. As shown in Fig. S2A, the CD11c^{+} cells had a round mononuclear morphology with a relatively large cytoplasmic to nucleus ratio. The CD11c^{neg} cells had characteristic monocytic kidney-shaped nuclei, and a minor proportion of the cells had lobular nuclei characteristic of granulocytes. Gentamicin-resistant *L. monocytogenes* were recovered from the CD11c^{+}, but not the CD11c^{neg} cells (Fig S2B). The sorted CD11c^{+} cells had a slight

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reduction in total CFU compared to unsorted bulk cultures, but exponential growth of intracellular bacteria was still observed. Thus, the enhanced ability of in vitro-generated CD11c^{+} cells to support intracellular growth of *L. monocytogenes* was not explained by the presence of other CD11c^{neg} cells in the culture.

L. monocytogenes survive, but do not replicate exponentially, inside bone marrow-derived CD103^{neg}CD11c^{+}MHC-II^{hi} cells that are cultured for only six days

Helft *et al.* recently demonstrated that CD11c^{+} cells generated in vitro with GM-CSF contain two populations of cells: MHC-II^{hi} cells that expressed DC surface markers such as CCR7 and CD135 and MHC-II^{int} cells that express markers of the monocyte/macrophage lineage such as CD115, F4/80 and CD64 (32). The MHC-II^{hi} cells were derived from both common dendritic precursors (CDP) and monocytes. In an attempt to isolate CD11c^{+} cells that more closely resembled cDC that recently differentiated from CDP, we harvested cells from an earlier time point in the culture (Fig. 4E) and sorted the MHC-II^{hi} cells (Fig. 4F). After 6 days, the CD11c^{+}MHC-II^{lo} cells had a macrophage-like morphology with a large cytoplasm and phagocytic vesicles, whereas, the CD11c^{+}MHC-II^{hi} cells had relatively larger nuclei with a more dendritic-like morphology (Fig. 4G).

L. monocytogenes were efficiently taken up in a dose-dependent manner by the sorted MHC-II^{hi} cells within one hour of infection (Fig. 4H). However, there was only a 3 to 7-fold increase in gentamicin-resistant bacteria in these cells over eight hours (Fig. 4I). Many of the intracellular bacteria were localized to the cytosol and associated with actin tails 8 hpi (Fig. 3D). The average number of bacilli associated with each infected cell increased over time with about 8% of the cells containing 15 or more *L. monocytogenes* by 8 hpi (Fig. 3E). Together, these results indicated that the efficiency of *L. monocytogenes* growth inside in vitro-generated CD103^{neg}CD11c^{+} cells was as least partially dependent on length of time in culture, and that GM-CSF-derived cells did not closely resemble cDC isolated from the MLN in regards to intracellular growth phenotype.

CD103^{+}CD11c^{+} cells generated using FLT3-L do not efficiently support intracellular replication of *L. monocytogenes*—Activation of CD135 (FLT-3) is critical for differentiation of DC progenitors into cDC that express CD103, and a recent report described a longer culture method to selectively generate CD103^{+} DC *in vitro* using FLT3-L (25). Nearly all of the cells generated using this approach were CD11c^{+}MHC-II^{+} after 16 days in culture, and approximately two-thirds of the cells were CD103^{+} (Fig. 5A). Microscopic examination of these cells revealed a unique morphology in regards to overall diameter and nuclear shape relative to the GM-CSF-cultured CD11c^{+} cells (Fig. 5B).

One hour after infection of CD103^{+}CD11c^{+} cells in suspension at low MOI, ~ 6 % of the inoculum was gentamicin-resistant (Fig. 5C). Thus, the FLT3L-induced cultured CD103^{+} cells had an invasion rate that was less than the GM-CSF cultured CD103^{neg} cells (Fig. 4C), but more efficient than the cDC harvested from MLN and infected directly *ex vivo* (Fig. 2C). As shown in Fig. 5D, there was no change in intracellular CFU recovered at 3 hpi, but by 8 hours, the number of gentamicin-resistant CFU increased 4-fold. Reducing the FLT3-L culture period to just six days resulted in a mixture of CD103^{+}CD11c^{hi} cells as well as

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$CD11c^{int}B220^{+}$ plasmacytoid DC (Fig. 5E). The majority of the cells that expressed a cDC phenotype ($CD11c^{hi}B220^{-}$) co-expressed both MHC-II and CD103. To determine if any of these cells could support intracellular growth of *L. monocytogenes*, the cells were infected in bulk at various MOI. As shown in Fig. 5F, *L. monocytogenes* readily invaded these cells within 30 minutes in a dose-independent manner however, the number of gentamicin-resistant CFU did not increase over time (Fig. 5G).

To confirm that *L. monocytogenes* were able to invade these cells, we performed differential "in/out" staining one hour after infection with GFP^{+} *L. monocytogenes.* As shown in the representative images in Fig. 3F, approximately 70% of the cells had at least one internalized (green) bacterium. Furthermore, actin staining of cells infected for 8 h indicated that about 10% of the cells counted harbored *L. monocytogenes* associated with actin tails (Fig. 3G). Consistent with the lack of exponential growth observed in the gentamicin protection assay, only 2 of the infected cells were associated with more than ten bacilli per cell (Fig. 3H). Together, these data indicate that *L. monocytogenes* can efficiently invade in vitro-generated $CD103^{+}CD11c^{+}$ cells, and survive in the cytosol, but intracellular growth is either inhibited or delayed compared to either macrophages or GM-CSF-induced cultured cells.

Discussion

In this study, we demonstrated that *L. monocytogenes* associated with a minor fraction of cDC in the intestinal LP and MLN during foodborne infection. However, *L. monocytogenes* inefficiently invaded these cells, and did not survive for more than a few hours. In striking contrast, in vitro-generated, bone marrow-derived-derived $CD11c^{+}$ cells that phenotypically resembled cDC readily supported intracellular growth of *L. monocytogenes.* Interestingly, the length of time cells were maintained in monoculture with growth factors such as GM-CSF and Flt-3L correlated with increased replication of intracellular bacteria (Fig. 6). Our data suggest that Flt3-L induced cultured cells most closely resemble cDC in the gut, but still differ significantly in their ability to restrict long term survival of intracellular *L. monocytogenes.*

Pron *et al.* previously used a microscopy approach to show that cells that expressed OX-62 (CD103) were the first cell type to associate with *L. monocytogenes* near the Peyer's Patches and in the draining MLN of rats infected via a ligated intestinal loop (33). In the mouse, interactions between *L. monocytogenes* and DC have been primarily studied in the spleen following intravenous infection. The migratory $CD8\alpha^{+}$ DC found in the spleen are thought to be equivalent to the $CD103^{+}$ DC found in non-lymphoid tissues, based on transcriptional network analysis and the sharing of key transcriptional factors required for development, (34,35). Although some investigators have reported that *L. monocytogenes* preferentially interacted with $CD8\alpha^{+}$ DC in the spleen (1,2), another study found more $CD8\alpha^{-}$ DC associated with *L. monocytogenes* (36). Similar to our results, these earlier studies found only a small number of live, replicating *L. monocytogenes* associated with DC in the spleen (1–13 CFU per 1000 cells) (1,2,37,38). Despite this association of live CFU with splenic DC, it is still unclear if *L. monocytogenes* actively replicated inside those cells over time. In fact, it has been suggested that the key role for $CD8\alpha^{+}$ DC in the spleen may actually be to translocate bacteria from the red pulp into the periarteriolar lymphoid sheath

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of the white pulp, allowing for subsequent replication in other permissive cell types such as macrophages (1,36,39). In that case, there would be no requirement for intracellular survival or replication in DC, and transport of extracellular, adherent bacteria would be most efficient.

The exact mechanism by which DC limit intracellular growth of *L. monocytogenes* remains unclear. As we previously proposed in monocytes (19), it is possible that a reduced acidification of phagosomes in cDC or monocyte-derived DC can delay bacterial escape by inhibiting optimal activity of the pore-forming toxin listeriolysin O. Once in the cytosol of DC, *L. monocytogenes* may be killed by autophagy. Matsumura *et al.* recently suggested that the actin-bundling protein Fascin-1 may promote an association between the autophagosomal marker LC3 and *L. monocytogenes* in GM-CSF-induced cultured CD11c^{+} cells (40). *L. monocytogenes* normally avoid being trapped in autophagosomal membranes by expressing ActA at one pole to mediate actin-based motility in the cell cytosol (41). Expression of ActA is typically induced at least 200-fold after *L. monocytogenes* gain to the host cell cytosol (42), and this has been attributed to allosteric binding of glutathione to the transcriptional regular PrfA (43). Soon after entry into the host cell cytoplasm, actin surrounds *L. monocytogenes* forming a “cloud” (44), and actin tails form only after polar expression of ActA is initiated (45,46). We found little evidence of ActA-mediated localization of actin into polar tails in cDC infected ex vivo; however, many of the intracellular bacteria were surrounded by actin (data not shown). Thus, it is possible ActA expression is not induced in the cytosol of cDC. If this were true, it would suggest that the cytosol of cDC may have a lower glutathione concentration than normally found in cells that are permissive for intracellular growth, such as macrophages.

Although it has long been understood that bone marrow-derived GM-CSF cultured cells contained a heterogeneous mixture of cells, the abundant yield of cells and the reproducibility of results when used for in vitro assays has led to widespread acceptance of these cells as prototypic dendritic cells. The results presented here further confirm the assertion made by Helft et al. that most GM-CSF cultures contain a large percentage of cells with attributes more closely resembling macrophages, which they referred to as GM-Macs (32). Indeed, *L. monocytogenes* replicate exponentially in the cytosol of bone marrow-derived CD64^{hi}F4/80^{+} macrophages, but not in monocytes (19), or as demonstrated here, in cDC analyzed directly ex vivo. Prolonged maintenance of cultured cells in vitro may alter the properties of DC-like cells due to extended feedback regulation from cytokines secreted into the media (47). Alternatively, the function of the cultured cells may be altered over time due to the absence of regulatory factors normally expressed by other immune cells or stromal cells in vivo. This study further highlights the need to define cells within the myeloid lineage based on functional attributes such as susceptibility to infection, rather than by surface marker phenotype, and to use primary cells analyzed directly ex vivo whenever possible.

Supplementary Material

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Acknowledgments

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Abbreviations

BALB	BALB/c/By/J
cDC	conventional dendritic cell
gent^R	resistant to 10 μg/ml gentamicin
hpi	hours post-infection
MLN	mesenteric lymph nodes

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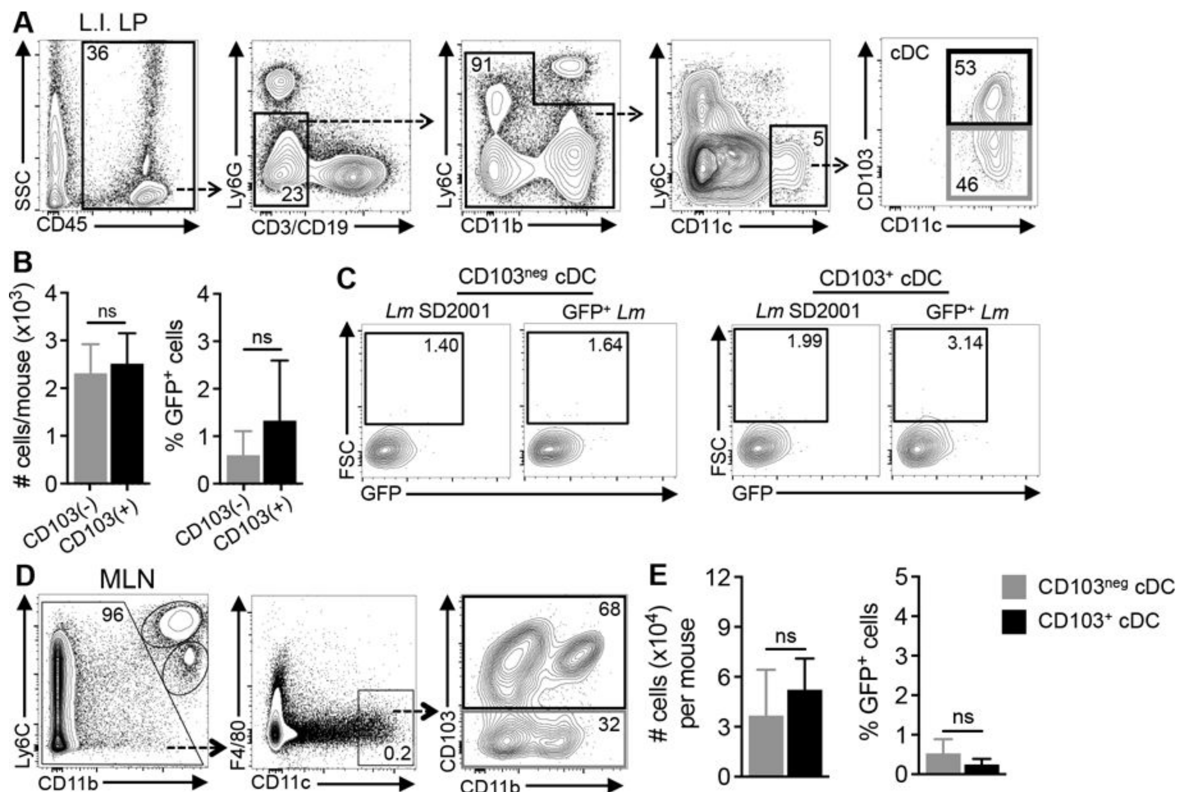

FIGURE 1.

L. monocytogenes do not preferentially associate with CD103^{+} cDC in the gut. BALB mice were fed 1–6 × 10^{8} CFU of either *Lm* SD2710 (GFP^{+}) or *Lm* SD2001 (vector control). **(A and C)** Gating schemes used to identify CD103^{neg} and CD103^{+} cDC isolated from the large intestine lamina propria (LI-LP) 48 hpi or MLN 72 hpi. **(B and D)** Mean (±SD) total number of cDC (left graph) and mean (±SD) percentage of GFP^{+} (*Lm*-associated) cDC in each subset (right graph). Pooled data for n=6 mice (LI-LP) or n=4 mice (MLN) from at least two separate experiments are shown. Statistical significance was determined using Mann-Whitney analysis.

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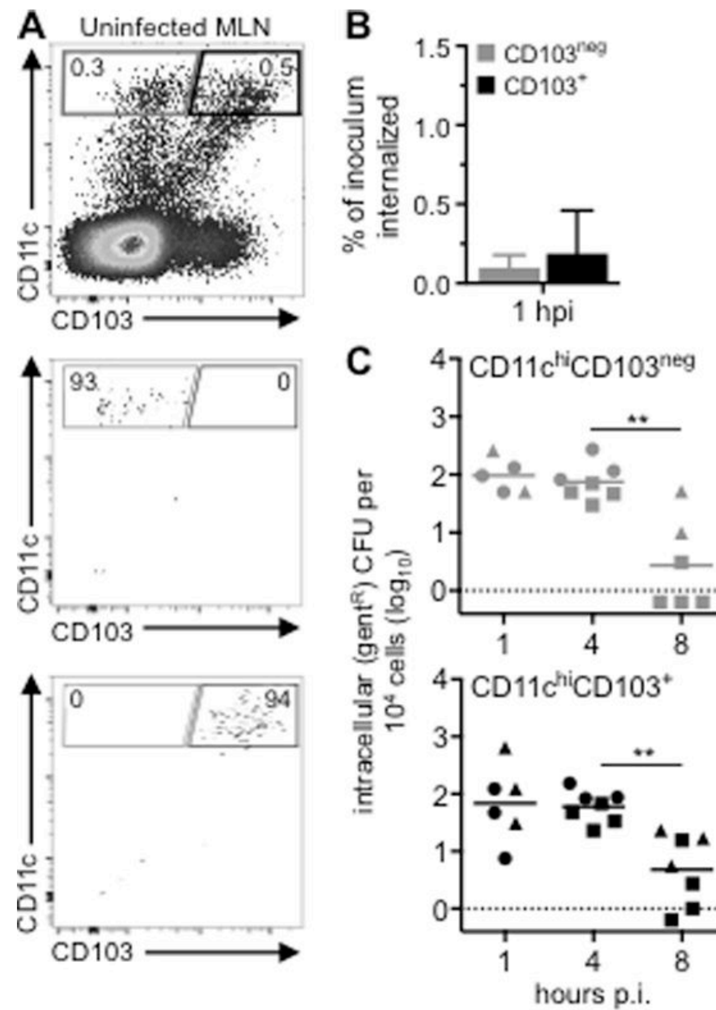

FIGURE 2.

cDC isolated from the MLN of naïve mice do not support intracellular growth of *L. monocytogenes.* **(A)** CD11c^{hi}CD103^{neg} or CD11c^{hi}CD103^{+} cells were sorted from the MLN of uninfected BALB mice. Sorted cells were infected with *Lm* SD2000 (MOI=10–14) directly ex vivo. **(B)** Mean percentage (±SD) of the *Lm* inoculum that was resistant to 10 μg/ml gentamicin (gent^{R}) after 1 h. **(C)** Intracellular growth assay for CD103^{neg} (grey) and CD103^{+} (black) cDC infected directly ex vivo. Pooled data from three separate experiments (designated by circles, squares, & triangles) are shown. Cells sorted from a single mouse were used at two time points. Statistical significance was determined using Mann-Whitney analysis.

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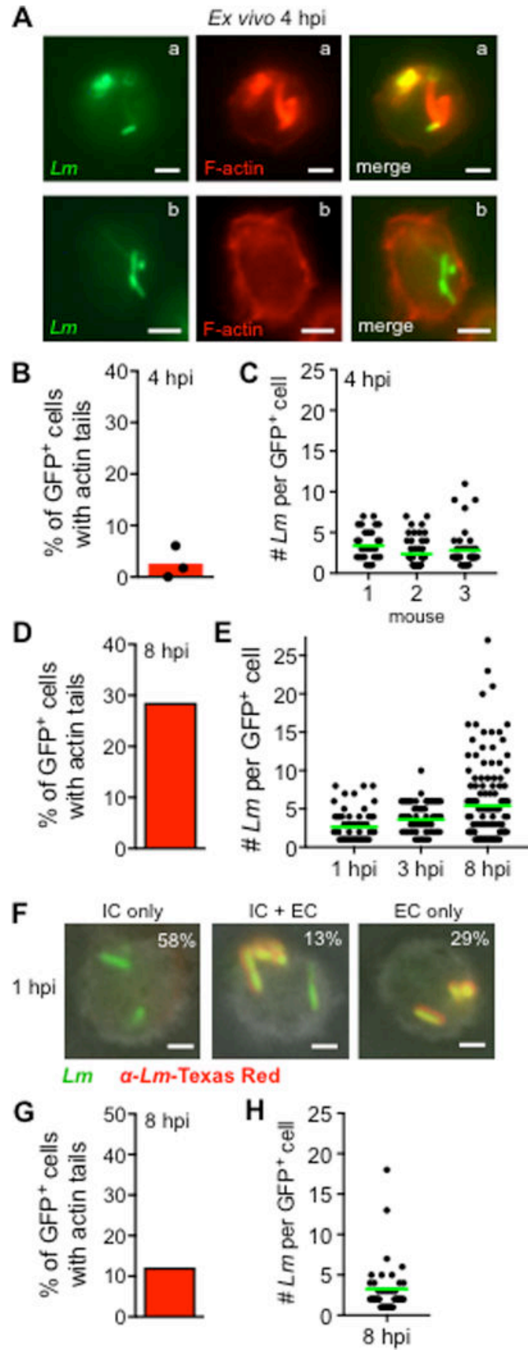

FIGURE 3.

Microscopic analysis of *L. monocytogenes*-infected CD11c^{+} cells. **(A-C)** CD11c^{hi} cells were sorted from the MLN of uninfected BALB mice and infected with *Lm* SD2710 (MOI=8) directly ex vivo for 1 h. Cells were spun onto slides and stained with phalloidin-Texas Red 4 hpi. **(A)** Representative images: cell in "a" contains *Lm* associated with actin tails and cell in "b" contains *Lm* not associated with actin. **(B)** Bar indicates mean percentage of GFP^{+} cells that had *Lm* associated with actin tails from each mouse (n=3). **(C)** Dots represent the number of *Lm* associated with each infected cell; horizontal green lines

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indicate mean values. **(D-E)** Bone marrow-derived CD11c^{+}MHC-II^{hi} cells sorted after 6 d of culture in GM-CSF were infected for 30 min with *Lm* SD2710 (MOI = 0.2). A total of 300 cells were examined for each time point. **(D)** Percentage of *Lm*-infected cells that contained bacteria with actin tails. **(E)** Dots indicate the number of bacilli associated with each infected cell. **(F-H)** Bone marrow-derived cells cultured in FLT3-L for 6 d were infected with *Lm* SD2710 (MOI=1). **(F)** Representative images for differential "in/out" staining of unpermebilized cells spun onto slides and stained with *Lm*-specific antibodies 1 hpi. The percentage of cells with intracellular bacteria (IC only), both intracellular and extracellular (IC + EC), or only extracellular (EC only) are given in the upper right corner of each image. A total of 400 cells were counted. **(G)** Percentage of *Lm*-infected cells that contained bacteria with actin tails. **(H)** Dots indicate the number of *Lm* associated with each infected cell 8 hpi. Scale bars, 2 μm.

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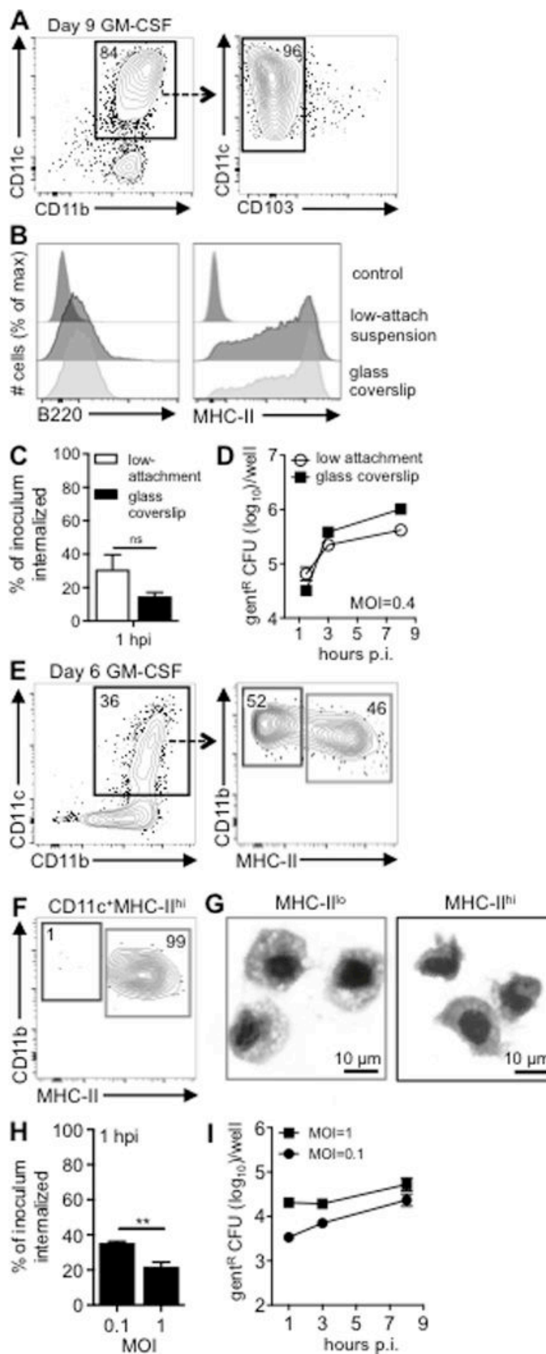

FIGURE 4.

In vitro generated, bone marrow-derived CD103^{neg}CD11c$^+$ cells readily support exponential growth of *L. monocytogenes*. **(A-D)** Bone marrow cells were cultured in GM-CSF for 8 days seeded in low-attachment dishes with or without glass coverslips and analyzed on day 9. **(A)** The majority of cultured CD11c$^+$ cells lacked CD103. (**B**) Overnight incubation on glass did not alter expression of MHC-II or B220. **(C)** Mean percentage (±SD) of the *Lm* SD2000 inoculum that was gent^R after 30 min. infection (MOI=0.4). **(D)** Intracellular growth assay indicates mean values (±SD) for triplicate samples. **(E)** Gating scheme used to

identify $CD11c^{+}MHC\text{-}II^{lo}$ and $CD11c^{+}MHC\text{-}II^{hi}$ cells after 6 d of culture in GM-CSF. **(F)** Post sort analysis of sorted $CD11c^{+}MHC\text{-}II^{hi}$ cells. **(G)** Diff-Quik staining of sorted $CD11c^{+}MHC\text{-}II^{lo}$ and $CD11c^{+}MHC\text{-}II^{hi}$ cells. **(H)** Mean percentage (±SD) of the *Lm* SD2000 inoculum that was $gent^{R}$ after 30 min. infection at the indicated MOI. **(I)** Intracellular growth assay showing mean values (±SD) for triplicate samples. For all panels, data from one of two separate experiments is shown.

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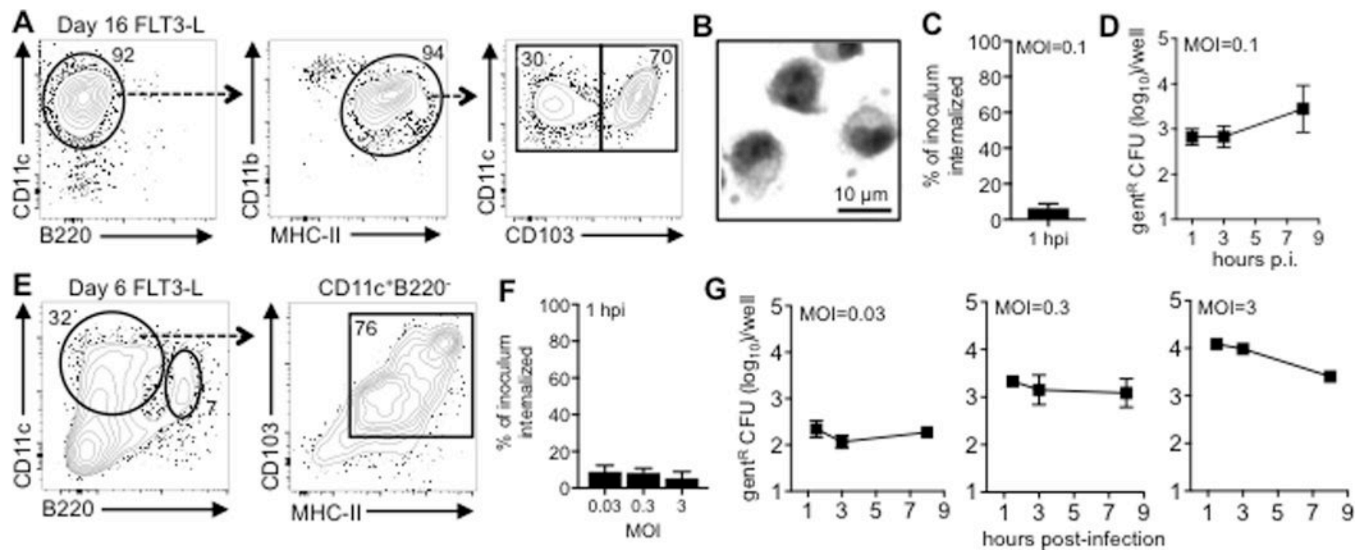

FIGURE 5.

Flt3-L-cultured cells do not efficiently support the intracellular replication of *L. monocytogenes.* Gating scheme **(A)** and Diff-Quik staining **(B)** of bone marrow cells cultured for 16 days in 12.5% Flt3-L sup plus 0.75% GM-CSF sup. **(C)** Mean percentage (±SD) of *Lm* SD2000 inoculum that was gent^R 1 h after infection of day 16 cells. **(D)** Intracellular growth assay using day 16 cells showing mean values (±SD) for triplicate samples. **(E)** Surface phenotype of bone marrow cells cultured for 6 days in 20% FLT3-L supernatant alone. **(F)** Mean percentage (±SD) of *Lm* SD2000 inoculum that was gent^R 1 h after infection of day 6 cells at various MOI. **(G)** Intracellular growth assay using day 6 cells showing mean values (±SD) for triplicate samples. In panels C, D, F, and G data are representative of at least two separate experiments.

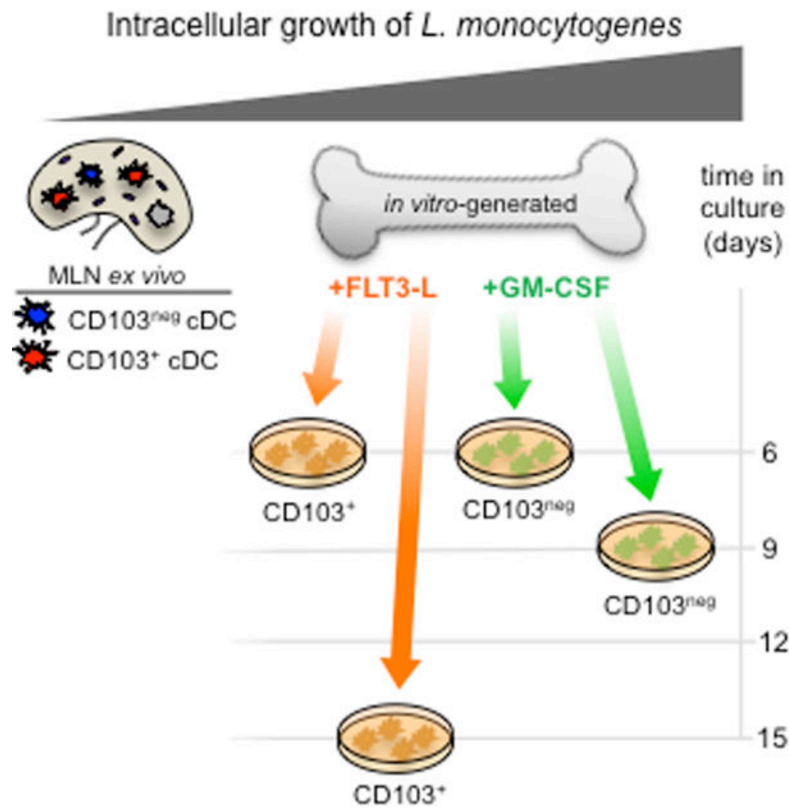

FIGURE 6.

Intracellular growth of *L. monocytogenes* in CD11c+ cells is ontogeny-dependent. Cartoon depicts the relative ability of the cells used in this assay to support intracellular growth of *L. monocytogenes*. Cells on the left are most restrictive and cells on the right are most permissive for exponential replication.

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