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Original Article

Change of Dendritic Cell Subsets Involved in Protection Against *Listeria monocytogenes* Infection in Short-Term-Fasted Mice

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ABSTRACT

The gastrointestinal tract is the first organ directly affected by fasting. However, little is known about how fasting influences the intestinal immune system. Intestinal dendritic cells (DCs) capture antigens, migrate to secondary lymphoid organs, and provoke adaptive immune responses. We evaluated the changes of intestinal DCs in mice with short-term fasting and their effects on protective immunity against *Listeria monocytogenes* (LM). Fasting induced an increased number of $CD103^{+}CD11b^{-}$ DCs in both small intestinal lamina propria (SILP) and mesenteric lymph nodes (mLN). The SILP $CD103^{+}CD11b^{-}$ DCs showed proliferation and migration, coincident with increased levels of GM-CSF and C-C chemokine receptor type 7, respectively. At 24 h post-infection with LM, there was a significant reduction in the bacterial burden in the spleen, liver, and mLN of the short-term-fasted mice compared to those fed *ad libitum*. Also, short-term-fasted mice showed increased survival after LM infection compared with *ad libitum*-fed mice. It could be that significantly high TGF-β2 and Aldh1a2 expression in $CD103^{+}CD11b^{-}$ DCs in mice infected with LM might affect to increase of $Foxp3^{+}$ regulatory T cells. Changes of major subset of DCs from $CD103^{+}$ to $CD103^{-}$ may induce the increase of IFN-γ–producing cells with forming Th1-biased environment. Therefore, the short-term fasting affects protection against LM infection by changing major subset of intestinal DCs from tolerogenic to Th1 immunogenic.

Keywords: Fasting; Dendritic cells; *Listeria monocytogenes*

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Conflicts of interest

The authors declare no potential conflicts of interest.

Abbreviations

APC, antigen-presenting cell; CCR7, C-C chemokine receptor type 7; DC, dendritic cell; hpi, hours post-infection; LM, Listeria monocytogenes; mLN, mesenteric lymph nodes; RT, room temperature; SILP, small intestinal lamina propria.

Author Contributions

Conceptualization: Lee KM, Yun CH; Data curation: Ju YJ, Lee KM, Kim G, Yun CH; Funding acquisition: Yun CH; Investigation: Lee KM, Yun CH; Project administration: Ju YJ, Lee KM, Yun CH; Supervision: Yun CH; Validation: Ju YJ, Yun CH; Writing - original draft: Ju YJ, Lee KM, Yun CH; Writing - review & editing: Ju YJ, Lee KM, Kim G, Kye YC, Kim HW, Chu H, Park BC, Cho JH, Chang PS, Han SH, Yun CH.

INTRODUCTION

Periodic fasting extends the lifespan of bacteria, yeast, worms, and mice compared to an *ad libitum* diet (1). Intermittent fasting protects mice from infectious and non-infectious diseases such as diabetes, cancer, and neurodegeneration (2). For instance, mice fasting for 24–72 h before *Listeria monocytogenes* (LM) infection showed a reduced bacterial burden and prolonged survival (3). Furthermore, fasting increased the survival rate after kidney and liver transplantation and ischemia-reperfusion injury in mice (4).

Even short-term nutritional depletion (*i.e.*, 24 h) reduces the total number of cells in the bone marrow and thymus (5). Because the gastrointestinal tract is the first organ directly affected by fasting, proteins related to metabolism are decreased, and protein synthesis is reduced after fasting for 24 h. Interestingly, however, proteins involved in cellular protection such as preservation of intestinal integrity were significantly increased (6). Also, nutritional depletion alters hormone levels and immune cell function (7). For example, leptin promoted expansion of naïve T cells in an IL-2–dependent manner and switched from Th2 to Th1 responses (8,9). Leptin also promoted dendritic cell (DC) maturation by inducing co-stimulatory molecules, proinflammatory cytokines (10), and migration to inflamed tissues (11).

To provoke an adaptive immune response, activation of and antigen presentation by professional antigen-presenting cells (APCs) is required. DCs survey and capture antigen at the local site and deliver it to the draining secondary lymphoid organ for naïve T-cell priming (12). DCs have heterogenic subsets depending on their state of activation, tissue, and differentiation lineage (13). In the gastrointestinal tract, especially in the small intestinal lamina propria (SILP), DCs can be classified based on the expression of CD103 (14). $CD103^+$ DCs in SILP capture antigens, including apoptotic epithelial cells (15) and bacterial antigens (16), and migrate to the mesenteric lymph node (mLN) in a C-C chemokine receptor type 7 (CCR7)-dependent manner (17). Moreover, together with TGF-β (18,19) and retinoic acid (RA) (20), they induce Treg differentiation. $CD103^+$ DCs can be divided into several subtypes depending on their CD11b or CD8α expression. $CD103^+CD11b^-CD8\alpha^+$ DCs are specialized for cross-presentation of cell-associated antigens and priming of $CD8^+$ T cells (21). Also, in $Batf3^{-/-}$ mice which lack intestinal $CD103^+CD11b^-$ DCs, there was no evidence of spontaneous gastrointestinal inflammation (22). So, $CD103^+CD11b^+$ DCs have been postulated to play a role for immune tolerance in the intestine; however, paradoxically, it has been reported that those cells induce a Th1 and Th17 response under inflammatory conditions (23,24). Likewise, a minor population of intestinal $CD103^-CD11b^+$ DCs prime naïve $CD4^+$ T cells and induce differentiation to IL-17– or IFN-γ–producing effector $CD4^+$ T cells (25).

The primary function of the small intestine is digestion and absorption of nutrients. Upon fasting, there are structural and functional changes and reduced metabolic activities (26). Although the effect of fasting on intestinal epithelial cells has been documented, its impact on intestinal immune cells, DCs in particular, is unclear. We evaluated the changes in $CD103^+$ DCs in gut-associated lymphoid organs (such as mLN and SILP) caused by short-term fasting and investigated the immune context induced by these changes in mice infected with LM.

MATERIALS AND METHODS

Animals and short-term fasting

Female BALB/c mice, 6 weeks old, were purchased from Orient Bio Inc., South Korea. Mice were divided into two groups, one fed *ad libitum* and the other fasted for 24 h with water provided. To prevent the mice from eating their own feces during starvation, they were transferred to new bedding cages when fasting started. The experimental procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU-130510-4-1). Animal experiments were carried out in compliance with the ARRIVE guidelines.

Bacteria preparation and infection

Recombinant LM expressing ovalbumin (LM-OVA) and the parental 1043S strain were provided by Dr. Hao Shen (University of Pennsylvania, Philadelphia, PA, USA). Bacteria cultured in brain heart infusion medium for 8 h at 140 rpm on a shaking incubator at 30°C were harvested by centrifugation and thoroughly washed twice with PBS. Bacteria were enumerated by measuring the optical density at 600 nm (27). The number of bacteria administered to the mice was validated by colony forming units (CFUs) counting by serial dilution and plating. For infection *in vivo*, 1×10^8 CFU of LM-OVA in 200 µL of PBS were administered intragastrically after fasting for 24 h (28).

Enumeration of bacteria

The spleen, liver, and mLNs were removed after perfusion with PBS. Each organ was homogenized in PBS with 0.1% Triton x-100. To enumerate *Listeria*, at least 200 µl of blood were collected by eye-bleeding and centrifuged at 6,300×g for 10 min to separate serum. Serial dilutions were plated on brain heart infusion agar for 12 to 16 h at 37°C and CFUs were counted.

SILP cell isolation

The small intestine, fat, connective tissues, and Peyer's patches were removed, cut longitudinally, and washed in cold PBS. The organs were cut into 1-cm pieces and transferred to flasks containing 20 ml of digestion solution comprising 1× Hank's balanced salt solution without Ca^{2+} or Mg^{2+} (Sigma-Aldrich, St. Louis, MO, USA), 5% fetal bovine serum (FBS; Gendepot, Barker, TX, USA), 1 mM DL-dithiothreitol (Sigma-Aldrich), and 2 mM EDTA (Sigma-Aldrich). Tissues were dissociated by gentle stirring for 20 min at 37°C and the supernatant was discarded. The SILP fractions were chopped using scissors and digested by stirring in RPMI-1640 medium containing 2% FBS, 0.5 mg/mL collagenase VIII (Sigma-Aldrich), and 40 µg/ml DNase I (Roche, Indianapolis, IN, USA) for 30 min at 37°C. Lamina propria suspensions were passed through a 70-µm filter and washed with RPMI-1640.

In vivo proliferation assay

During short-term fasting, mice were injected intraperitoneally with 1 mg of BrdU in distilled PBS. After 12 h, single cells were prepared from the SILP and mLN. After surface staining with fluorochrome-conjugated antibodies, the cells were washed thoroughly. Then, the cells were fixed and permeabilized in 100 µl of Cytofix/Cytoperm buffer (BD Biosciences, San Jose, CA, USA) for 20 min at room temperature (RT) and washed with BD perm/wash buffer (BD Biosciences). Cells were suspended in 100 µl of BD perm/wash buffer plus (BD Biosciences) and incubated for 10 min at 4°C in the dark. Next, the cells were washed with BD perm/wash buffer and centrifuged. After fixation in 100 µl of buffer, 1×10^6 cells were incubated in DNase reaction solution for 1 h at 37°C. The cells were suspended in 50 µl of BD perm/wash buffer

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containing fluorescent anti-BrdU-FITC. The cells were incubated for 20 min at RT, washed with BD perm/wash buffer, and subjected to flow cytometry analysis.

Flow cytometry, intracellular staining, and Foxp3 staining

For cell surface staining, anti-CD11c FITC (HL3), -CD11b PE-Cy7 (M1/70), -CD103 BV421 (M290) or APC (2E7), -CD8a BV421 (53-6.7) or V450 (53-6.7), -I-Ad APC (AM5-32.1), -CD25 PE-Cy7 (PC61), -CD62L BV605 or APC-Cy7 (MEL-14), -CD3e FITC (145-2C11), -PD-L1 PE (J43), -CD80 PE (16-10A1), -CD86 PE (GL1), -NK1.1 (PK136) -CD44 APC-Cy7 (1M7), and -CD45 APC (30-F11) fluorochrome-conjugated antibodies were purchased from BD Biosciences. Mouse anti-CD69 PerCP-Cy5.5 (H1.2F3), -F4/80 APC (BM8), -Ly6G BV421 (1A8), -CD11b BV605 (M1/70), -CD4 BV605 (RM 4-5), and -CCR7 Alexa647 (4B12) monoclonal antibodies were purchased from BioLegend (San Diego, CA, USA). The cells were stained with the appropriate antibodies and incubated for 20 min at 4°C in the dark.

For intracellular staining, isolated single cells were stimulated with phorbol 12-myristate 13-acetate (20 ng/ml) and ionomycin (200 ng/ml) in the presence of 3 μl/ml Brefeldin A (BD Biosciences) and incubated for 5 h at 37°C. Next, cells were stained with anti-IFNγ-PE (XMG1.2) and -IL-17A-PerCP-Cy5.5 (TC11-18H10) antibodies (BD Biosciences) for 20 min at 4°C in the dark.

For Foxp3 intracellular staining, cells were incubated with anti-Foxp3 Alexa647 antibody (MF23) (BD Biosciences) for 20 min at RT in the dark. Next, flow cytometry was performed using a FACS Canto II (BD Biosciences) and analyzed by FlowJo software (Ashland, OR, USA). Cell sorting was performed using a FACS Aria (BD Biosciences). For all staining protocols, cells were analyzed by staining with live/dead discriminating dye (Tonbo Biosciences, CA, USA); dead cells were excluded.

Real-time quantitative PCR

cDNA was subjected to real-time quantitative PCR using Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK). The primers were *gm-csf*: forward 5′-CTG CCT TAA AGG GAC CAA GAG A-3′, reverse 5′-TTC CGC TGT CCA AGC TGA GT-3′; *foxp3*: forward 5′-GGA TGA GCT GAC TGC AAT TCT G-3′, reverse 5′-GTA CCT AGC TGC CCT GCA TGA-3′; *gata3*: forward 5′-GCC TCG GCC ATT CGT ACA T-3′, reverse 5′-GTA GCC CTG ACG GAG TTT C-3′; *t-bet*: forward 5′-TCG TGG AGG TGA ATG ATG GA-3′, reverse 5′-GA GTG ATC TCT GCG TTC TGG TA-3′; *aldh1a2*: forward 5′-TTG GCT TAC GGG AGT ATT CAG AA-3′, reverse 5′-GCC TCG GCC TCT TAG GAG TT-3′; *tgfb1*: forward 5′-TCG ACA TGG AGC TGG TGA AA-3′, reverse 5′-GAG CCT TAG TTT GGA CAG GAT CTG-3′; and *tgfb2*: forward 5′-GCC CCT GCT GTA CCT TCG T-3′, reverse 5′-TGC CAT CAA TAC CTG CAA ATC T-3′. Thirty PCR cycles were performed in duplicate for each primer. Relative quantification was performed using the *ΔΔCt* method and normalized to expression of the housekeeping gene *gapdh*: forward 5′-CTC CAC TCA CGG CAA ATT CA-3′, reverse 5′-GCC TCA CCC CAT TTG ATG TT-3′.

Statistical analysis

The mean value ± standard deviation was determined for each group. For comparison of means, the two-tailed unpaired Student's *t*-test was used. Differences were considered significant at $p<0.05$ unless otherwise specified.

RESULTS

CD11c^{hi} DCs were increased in the mLN and SILP of short-term-fasted mice

The gastrointestinal tract consumes much energy and so is directly affected by fasting (6), but little is known about the influence of fasting on gastrointestinal immunity. Therefore, we focused on changes in the intestinal immune system caused by short-term fasting. After 24 h of fasting, a significantly increased number of CD45$^+$ leukocytes was observed in the mLN and SILP of fasted mice compared to *ad libitum*-fed mice (**Fig. 1A**). Next, we investigated the numbers of CD45$^+$ DCs, neutrophils, macrophages, B cells, natural killer (NK) cells, and T cells. A significant increase in the number of CD11c^{hi} DCs was observed in the mLN and SILP compared to *ad libitum*-fed mice (**Fig. 1B** and **Supplementary Fig. 1**). In contrast, neutrophils, macrophages, NK cells, and lymphocyte populations (including B cells, CD4, CD8, and γδ T cells) in the mLN and SILP were not significantly changed (**Supplementary Fig. 1** and **Supplementary Fig. 2**).

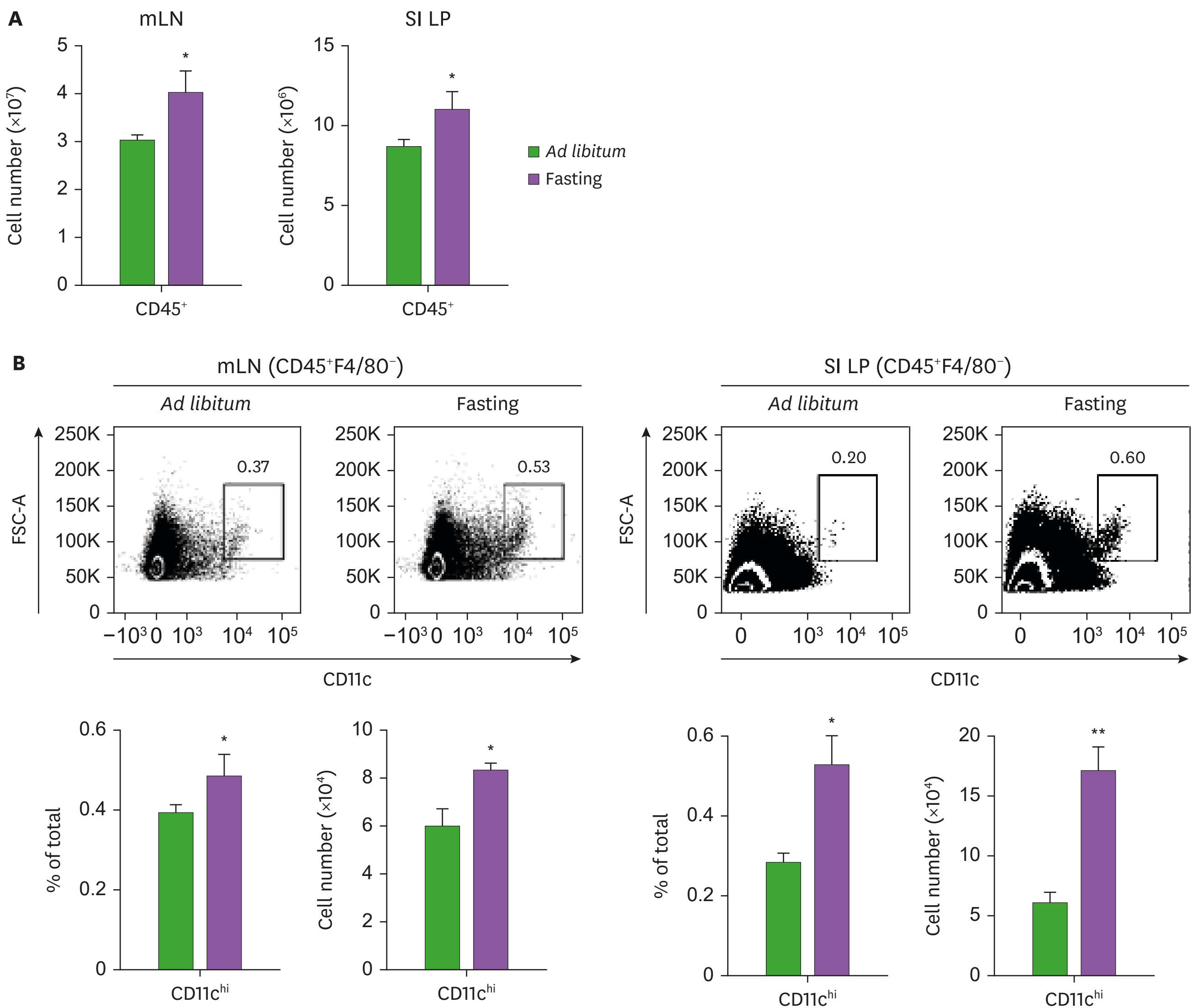

Figure 1. Change of CD11c^{hi} cells in the SILP and mLN after short-term fasting. Mice were fasted for 24 h. (A) Absolute number of CD45$^+$ leukocytes in the mLN and SILP. (B) Percentage and absolute number of CD45$^+$F4/80$^-$CD11c^{hi} DCs in the mLN and SILP. Unpaired Student's *t*-test. Data are representative of two or three independent experiments, n=3–6 mice.
*p<0.05; **p<0.01.

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Taken together, the results showed that short-term fasting leads to a significant increase in the population of intestinal CD11c^{hi} DCs, but not of other immune cell types.

CD103$^+$ DCs were dramatically increased in mLN and SILP from short-term-fasted mice

Intestinal CD11c^{hi} DCs can be categorized into several subsets based on their CD103 and CD11b expression (29). The majority of CD11c^{hi} DCs in the small intestine expresses the integrin α_E referred to as CD103 paired with β_7 (30). Furthermore, intestinal CD103$^+$ DCs play an important role in maintaining tolerance to food antigens and commensal bacteria. In addition, immunological tolerance maintains intestinal homeostasis and suppresses unnecessary intestinal hyper-inflammation, which can occur even in normal individuals (14). To investigate the DC subsets increased by short-term fasting, we examined CD11c^{hi} DCs based on CD103 and CD11b expression. Intriguingly, the number of CD103$^+$CD11b$^-$ DCs was significantly increased in the mLN of short-term-fasted mice (**Fig. 2A and B**). Furthermore, CD103$^+$ DCs, but not CD103$^-$ DCs, in the SILP were significantly increased in short-term-fasted mice compared to *ad libitum*-fed mice (**Fig. 2C and D**).

Collectively, these results indicate that the increased CD11c^{hi} DCs in short-term-fasted mice were mainly CD103$^+$CD11b$^-$ DCs, but not CD103$^-$ DCs, in the mLN and SILP.

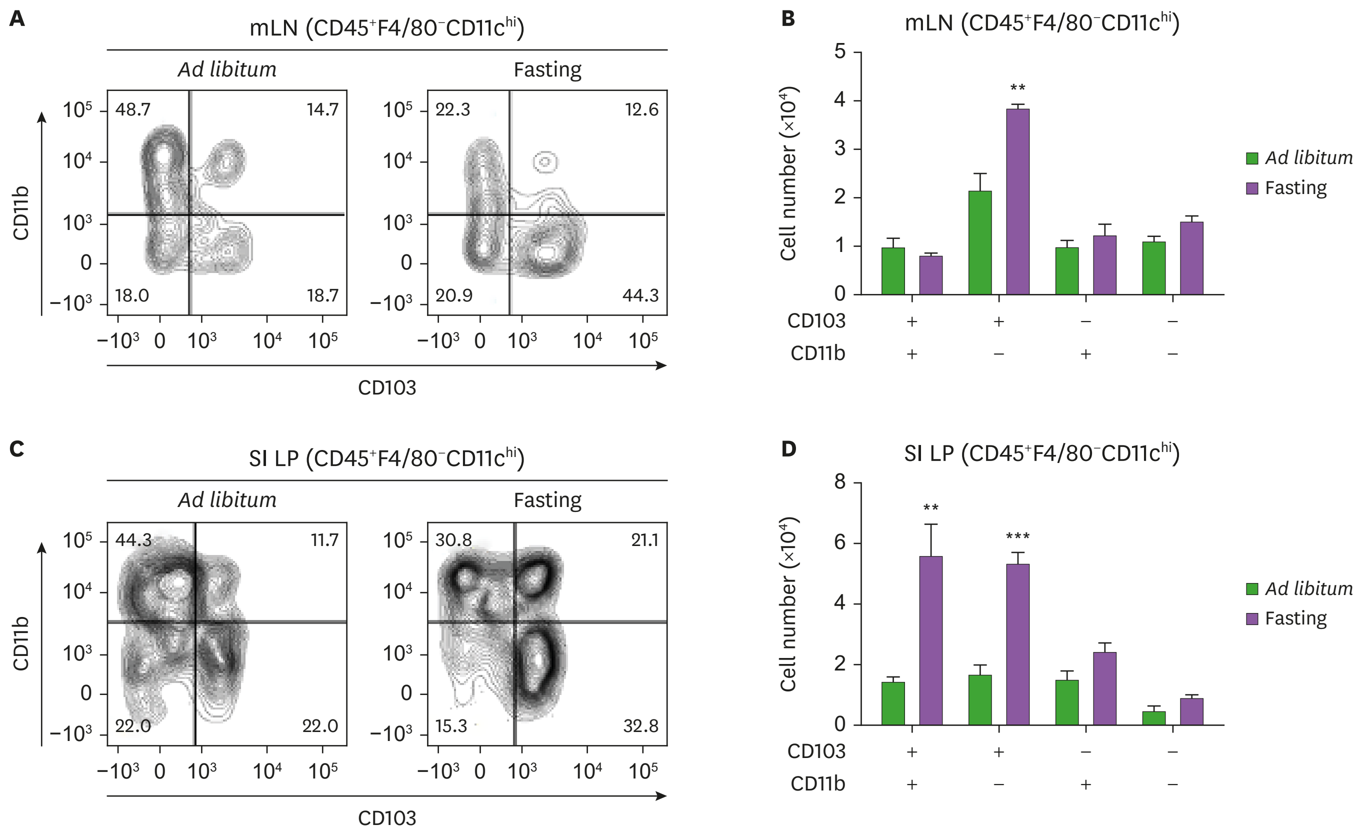

Figure 2. Subtypes of CD11c^{hi} DCs based on CD103 and CD11b expression in mLN and SILP after short-term fasting. Mice were fasted for 24 h. (A, C) Contour plots of CD45$^+$F4/80$^-$CD11c^{hi} DCs based on CD103 and CD11b expression in the (A) mLN and (C) SILP. (B and D) Absolute number of CD45$^+$F4/80$^-$CD11c^{hi} DC subsets in the (B) mLN and (D) SILP. Unpaired Student's *t*-test. Data are representative of two or three independent experiments, n=3–5 mice.
**p<0.01; ***p<0.001.

CD103$^+$ DCs proliferate in the SILP by GM-CSF

We hypothesized that the increased number of CD103$^+$ DCs in the SILP was caused by local cell proliferation or migration or both. To investigate CD103$^+$ DC proliferation, we performed a bromodeoxyuridine (BrdU) incorporation assay. Interestingly, BrdU uptake significantly increased in CD103$^+$CD11b$^-$ and CD103$^+$CD11b$^+$ DCs in the SILP of short-term-fasted mice compared to *ad libitum*-fed mice (**Fig. 3A**). Furthermore, BrdU uptake increased in CD103$^+$CD11b$^-$ DCs in the mLN of short-term-fasted mice (**Supplementary Fig. 3A**). These results were correlated with the increased numbers of cell subsets in the SILP and mLN (**Fig. 2**).

GM-CSF is required for the development of DCs under steady-state and inflammatory conditions (31). GM-CSF also induces the development and expansion of conventional DCs (32) and facilitates the recruitment of intestinal DCs (33). Therefore, we evaluated the mRNA level of GM-CSF in the SILP. The expression of GM-CSF was significantly increased in the SILP after short-term fasting (**Fig. 3B**). To assess their migration capacity, expression of CCR7 on CD103$^+$ DCs in the SILP was examined (34). CCR7 expression was significantly increased in intestinal CD103$^+$ DCs after short-term fasting compared to *ad libitum*-fed mice (**Fig. 3C**). In addition, other functional markers of DCs, such as major histocompatibility complex class II (MHC II), CD205, and PD-L1, were increased (**Supplementary Fig. 3B**).

Therefore, short-term fasting increased the number of DC subsets, largely CD103$^+$CD11b$^-$ and CD103$^+$CD11b$^+$ DCs, in the SILP in correlation with increase of GM-CSF and CCR7 expression, respectively.

Short-term fasting protects mice against LM infection

Next, we postulated that the increase in CD103$^+$ DCs caused by short-term fasting modulates intestinal immunity because CD103$^+$ DCs may be tolerogenic (14). To elucidate the role of CD103$^+$ DCs in infection, mice were infected with LM, which induces Th1 and Th17 responses (35) and the bacterial burden was measured. The number of CFUs was significantly decreased in the spleen, mLN, and liver at 48 h post-infection (hpi) in short-term-fasted mice (**Fig. 4A**). In addition, high bacteremia was detected in mice fed *ad libitum* but not those on short-term fasting (**Fig. 4B**). Furthermore, and ruling out the possibility that short-term-fasted mice consumed food more rapidly after re-feeding, the number of CFUs in the stomach at 3 hpi was not different (**Supplementary Fig. 4A**).

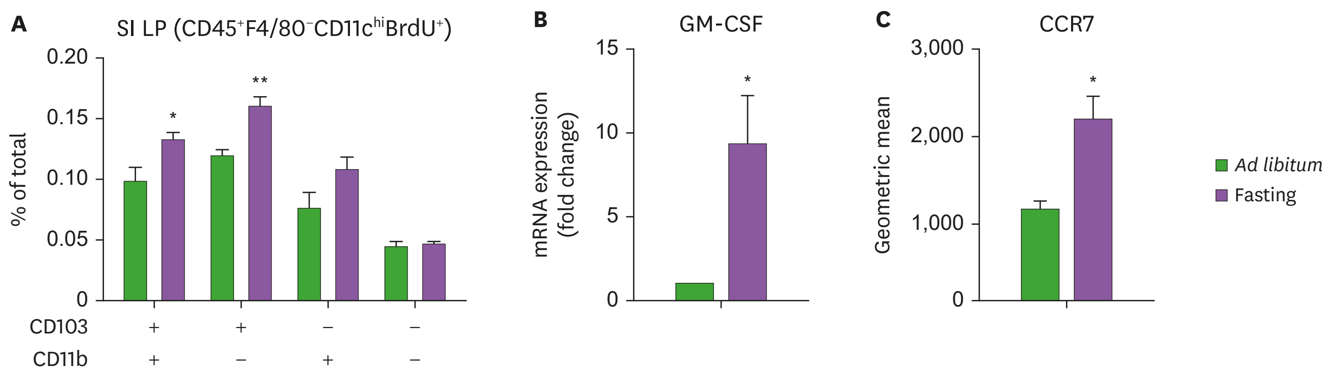

Figure 3. Increase of CD11c^{hi} DC subsets in the SILP after short-term fasting. Mice were fasted for 24 h. (A) BrdU uptake by CD45$^+$F4/80$^-$CD11c^{hi} DCs subsets. (B) mRNA level of GM-CSF in total SILP cells. (C) CCR7 expression in CD45$^+$F4/80$^-$CD11c^{hi}CD103$^+$ DCs. Unpaired Student's *t*-test. Results are representative of two or three independent experiments, n=3–4 mice.
*p<0.05; **p<0.01.

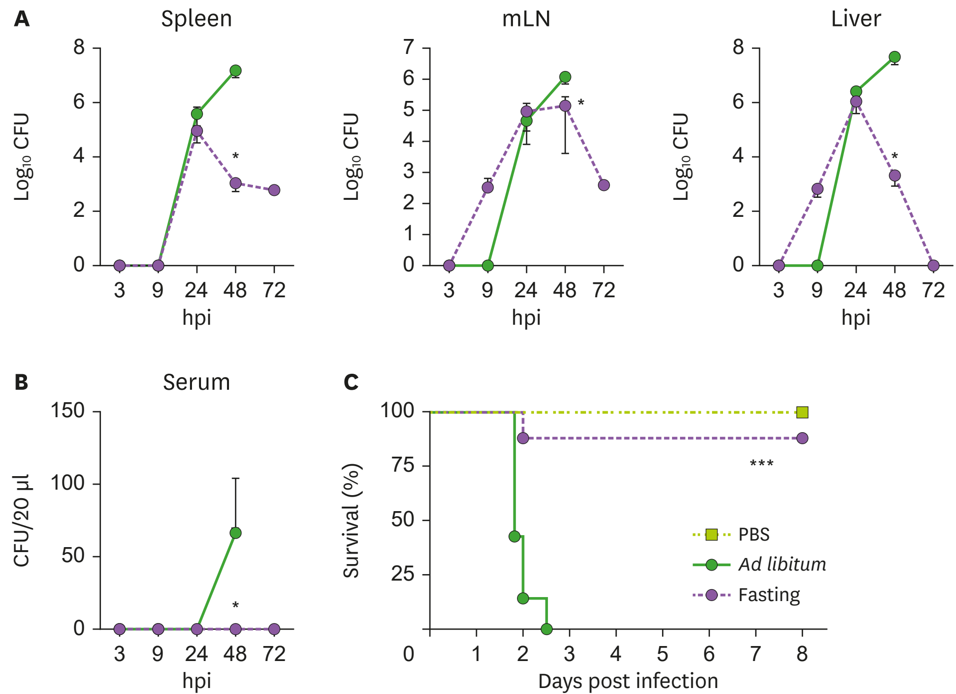

Figure 4. Bacterial burden and survival rate in short-term-fasted mice infected with LM. Mice were fasted for 24 h and infected with LM. (A, B) Number of CFUs in (A) peripheral organs (spleen, mLN, and liver) and (B) serum at 0, 3, 9, 24, 48, and 72 hpi with Lm-OVA. (C) Survival rate over 8 days; log-rank (Mantel–Cox) test. Other analyses by unpaired Student's *t*-test. Results are representative of two or three independent experiments, n=5–6 mice. $^{*}p<0.05$; $^{***}p<0.001$.

Next, we evaluated the effect of short-term fasting on survival. Consistent with the bacterial burden, the survival of short-term-fasted mice was increased compared to *ad libitum*-fed mice (**Fig. 4C**). Also, the body weight change of short-term-fasted mice infected with LM-OVA was comparable to that of the PBS group (**Supplementary Fig. 4B**).

Therefore, short-term fasting protects against gastrointestinal LM infection.

$CD103^+$ DCs and $Foxp3^+$ Tregs were increased in the mLN during early *Listeria* infection after short-term fasting

Next, we examined the role of the increased intestinal $CD103^+$ DCs in LM-OVA infection in short-term fasting mice. $CD103^+CD11b^-$ DCs are tolerogenic and mediate the differentiation of $Foxp3^+$ Tregs by expressing anti-inflammatory cytokines and inhibitory surface molecules (14). Therefore, to investigate whether intestinal $CD103^+CD11b^-$ DCs contribute to the induction of $Foxp3^+$ Tregs during LM infection, $CD103^+CD11b^-$ DCs and $Foxp3^+$ Tregs were examined in LM-infected mice with/without fasting. $CD103^+CD11b^-$ DCs were significantly increased in the mLN (**Fig. 5A**) and SILP (**Fig. 5B**) of short-term-fasted mice compared to *ad libitum*-fed mice. In the mLN, LM infection induced an increase in number of $Foxp3^+$ Tregs in short-term-fasted and *ad libitum*-fed mice at 1 dpi; the magnitude of the increase was greater in the short-term-fasted mice (**Fig. 5C top and D**). CD103 is a marker of *in vivo*-activated $Foxp3^+$ Tregs (36-38). Therefore, to determine whether the increased $Foxp3^+$ Tregs in short-term-fasted mice were functionally active, we examined their CD103 expression. The number of *in vivo*-activated $CD103^+Foxp3^+$ Tregs among $Foxp3^+$ Tregs was higher in short-term-fasted mice compared to *ad libitum*-fed mice (**Fig. 5C bottom and E**). By contrast, in the spleen, $Foxp3^+$ Tregs were comparable in the two groups and their composition did not differ

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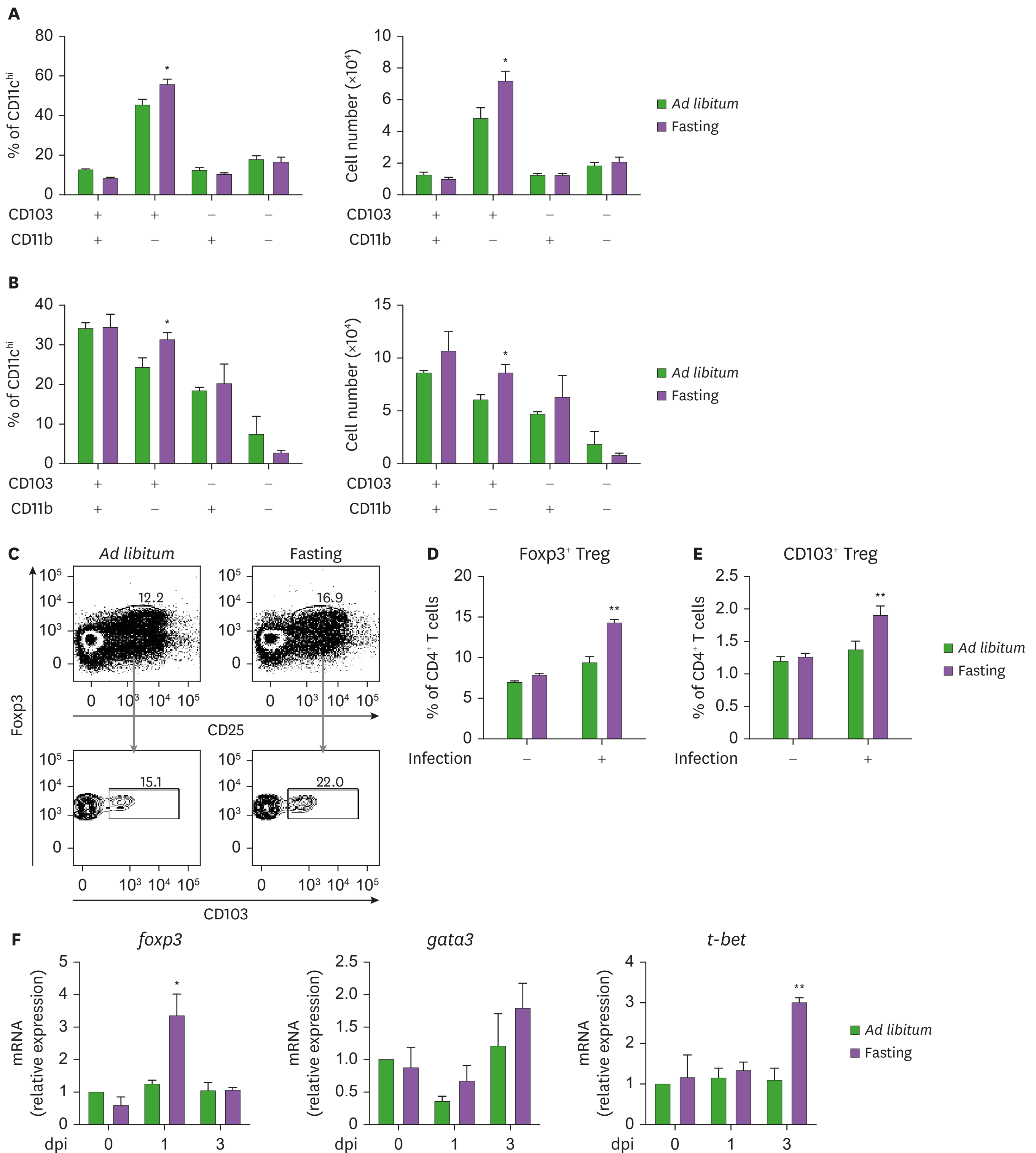

Figure 5. Induction of CD103$^+$CD11b$^-$ DCs and Foxp3$^+$ Tregs in short-term-fasted mice infected with LM. Mice were fasted for 24 h and infected with LM. (A, B) Percentage (left panel) and absolute number (right panel) of CD45$^+$F4/80$^-$CD11c^{hi} DC subsets in (A) mLN and (B) SILP of mice infected with LM. (C, D) Foxp3$^+$ Tregs in the mLN at 1 dpi: (C) dot plot and (D) percentage of CD3$^+$CD4$^+$ cells. (E) Percentage of Foxp3$^+$CD103$^+$ Tregs among CD3$^+$CD4$^+$ cells. (F) mRNA levels of *foxp3*, *gata3*, and *t-bet* in CD3$^+$CD4$^+$ cells in the mLN. Unpaired Student's *t*-test. Results are representative of two or three independent experiments, n=4–6 mice.
*p<0.05; **p<0.01.

(**Supplementary Fig. 5**). Also, the *foxp3* mRNA level was threefold higher in short-term-fasted mice than in *ad libitum*-fed mice at 1 dpi (**Fig. 5F left**). The *t-bet* mRNA level was significantly upregulated in short-term-fasted mice compared to *ad libitum*-fed mice at 3 dpi (**Fig. 5F right**).

Collectively, these results suggest that the increased $CD103^+CD11b^-$ DCs in short-term-fasted mice induced functional $Foxp3^+$ Tregs upon LM infection, especially at the early stage.

Increased TGF-β and RA levels contributed to the tolerogenicity of $CD103^+$ DCs

Next, to elucidate the factors responsible for the increase of $Foxp3^+$ Tregs, we examined the cell surface molecules of $CD103^+$ DCs. Increased PD-L1 and decreased CD86 and MHC II expression are phenotypic characteristics of tolerogenic DCs (39), and $CD205^+CD8\alpha^+$ DCs producing TGF-β increase $Foxp3^+$ Tregs (40). At 1 dpi, PD-L1, CD205, and CCR7 expression was significantly increased in $CD103^+$ DCs of short-term-fasted mice compared to *ad libitum*-fed mice, but CD86 and MHC II expression was unchanged (**Fig. 6A**).

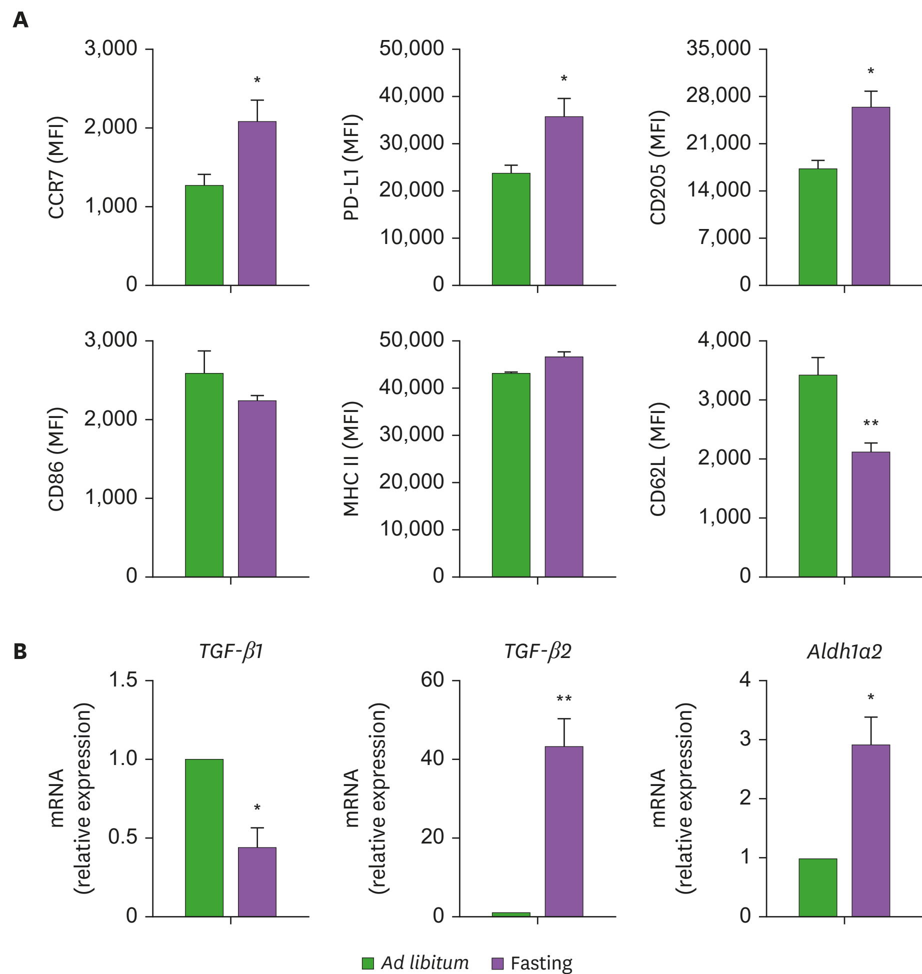

Figure 6. Tolerogenic characteristics of $CD103^+$ DCs in short-term-fasted mice infected with LM. Mice were fasted for 24 h and infected with LM. (A) Expression of CCR7, PD-L1, CD205, CD86, MHC II, and CD62L in $CD45^+F4/80^-CD11c^{hi}CD103^+$ DCs. (B) mRNA levels of *TGF-β1*, *TGF-β2*, and *aldehyde dehydrogenase 2 (aldh1a2)* in $CD45^+F4/80^-CD11c^{hi}CD103^+$ DCs. Unpaired Student's *t*-test. Results are representative of two or three independent experiments, n=4–5 mice.
$^*p<0.05$; $^{**}p<0.01$.

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CD103+ DCs producing TGF-β, RA, and aldehyde dehydrogenase A2 (Aldh1a2) induce Foxp3+ Tregs (18,19). Therefore, we examined their expression in purified intestinal CD103+ DCs. Interestingly, the *TGF-β2* and *Aldh1a2* mRNA levels in intestinal CD103+ DCs were significantly increased in short-term-fasted mice compared to *ad libitum*-fed mice (**Fig. 6B**).

Taken together, the results suggested that increased *TGF-β2*, *Aldh1a2*, PD-L1, and CD205 expression may contribute to the tolerogenicity of CD103+ DCs. Such tolerogenic CD103+ DCs may have a correlation with an increase of Foxp3+ Tregs in short-term-fasted mice infected with LM.

Short-term fasting upregulated the Th1 response in mice infected with LM

Next, we investigated the LM burden after 48 hpi. CD103− DCs preferentially induce the differentiation of naïve CD4+ T cells to IFN-γ–producing Th1 cells (25). Therefore, we expected that the number of CD103− DCs would increase after 2 dpi in short-term-fasted mice. At 3 dpi, the percentage and absolute number of CD103−CD11b+ DCs were significantly increased in short-term-fasted mice compared to *ad libitum*-fed mice; by contrast, the percentage and absolute number of CD103+CD11b− DCs were reduced significantly (**Fig. 7A**). We further

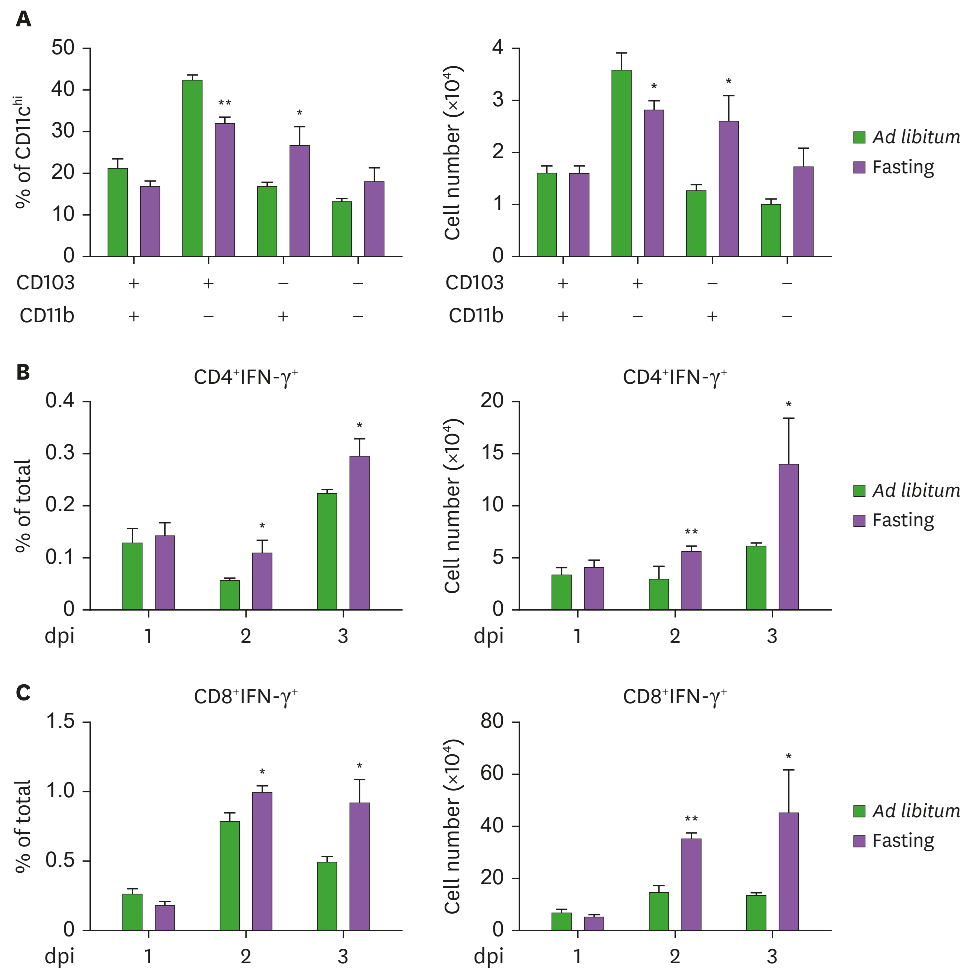

Figure 7. Composition of IFN-γ+ cells among CD4+ T cells and CD8+ T cells in short-term-fasted mice infected with LM. Mice were fasted for 24 h and infected with LM. (A) Percentage and absolute number of CD45+F4/80− CD11chi DC subsets in the mLN at 3 dpi. (B, C) Percentage and absolute number of IFN-γ+ cells among (B) CD4+ T lymphocytes (CD3+CD4+), and (C) CD8+ T lymphocytes (CD3+CD8+) in the mLN at 1, 2, and 3 dpi. Unpaired Student's *t*-test. Results are representative of two or three independent experiments, n=4–6 mice. *p<0.05; **p<0.01.

investigated whether CD103^{-} DCs promote a Th1 environment (25). The percentage and absolute number of IFN-γ^{+} cells among CD4^{+}CD3^{+} (**Fig. 7B**), CD8^{+}CD3^{+} (**Fig. 7C**), and NK1.1^{+}CD3^{-} (**Supplementary Fig. 6**) cells were increased at 2 and 3 dpi in short-term-fasted mice, in agreement with the increased *t-bet* expression at 3 dpi (**Fig. 5F**).

In summary, increased IFN-γ^{+} cells in short-term-fasted mice at the later phase of infection may be correlated with an increase of CD103^{-} DCs in accordance with reduction of the bacterial burden by enhancing T cell–mediated immune responses.

DISCUSSION

We investigated the functional alterations of intestinal immune cells, especially CD11c^{hi} DCs, caused by short-term fasting with/without LM infection. The findings were as follows: short-term fasting altered the composition of intestinal innate immune cells, including an increase in CD11c^{hi} DCs; among CD11c^{hi} cells, CD103^{+} DCs in the mLN and SILP from short-term-fasted mice proliferated more than those from *ad libitum*-fed mice; the expansion and migration of CD103^{+} DCs in the SILP after fasting was linked to upregulation of GM-CSF and CCR7, respectively; and short-term fasting significantly contributed for the protection in mice infected with LM through induction of Foxp3^{+} Tregs for regulating excessive immunopathology at the early phase and IFN-γ^{+} cells to deal with the infected cells at the later phase.

Unexpected immune responses may be provoked by fasting. For instance, fasting during anorexia enhances survival in mice with experimental autoimmune encephalomyelitis (41) or LM infection (42). By contrast, intermittent fasting suppresses antigen-specific antibody production after immunization with ovalbumin and cholera toxin (43). Fasting for 24 h altered the properties of intestinal immune cells, particularly DCs, protecting against LM infection. A fasting strategy to induce CD11c^{hi} DC subset alterations and the duration of protection against LM infection warrant further studies.

CD103^{+} DCs were the most increased DC subset in the mLN and SILP upon short-term fasting. The increase of CD103^{+}CD11b^{-} DCs in the SILP was a result of cell proliferation and migration. Also, the rate of proliferation was higher in the SILP than the mLN, as reported previously (44), showing that mLN CD103^{+} DCs proliferate more slowly than SILP CD103^{+} DCs. In addition, GM-CSF is essential for the development of CD103^{+} DCs, but not CD103^{-} DCs, in non-lymphoid tissues, including the SILP (45). Indeed, the GM-CSF in the SILP was increased in short-term-fasted mice compared to *ad libitum*-fed mice, which may be linked to the increased number of intestinal CD103^{+} DCs. Flt3 ligand also plays an important role in the differentiation of hematopoietic stem cells into conventional DCs (cDCs) (46). In fact, Flt3 ligand maintains a normal number of cDCs by directly regulating their proliferation in the periphery (47). It was reported that absence of GM-CSFR in Csf2r$^{-/-}$ mice affects mostly the development of CD103^{+}CD11b^{+} DCs in SILP, but not of CD103^{-}CD11b^{+} DCs. Also, in Flt3$^{-/-}$ mice, the development of CD103^{+}CD11b^{+} DCs in SILP was impaired, and CD103^{+}CD11b^{-} and CD103^{-}CD11b^{+} DCs were significantly diminished in SILP (48). In addition, in the same context, it was reported that CX3CR1^{hi} (CD103^{-}CD11b^{+}) DCs in SILP have poor responsiveness to Flt3 and GM-CSF (49), whereas CD103^{+} DCs in SILP responded stronger to Flt3, coincident with strong antigen-presentation ability (16). Therefore, although our results suggested a role for GM-CSF in the increased proliferation of CD103^{+} DC subsets in short-term-fasted

mice compared to *ad libitum*-fed mice, an investigation of the direct effect of Flt3 ligand with/without GM-CSF on proliferation is needed.

It has been well reported that CCR7 plays an important role for migration of DCs (34). We showed $CD103^+$ DCs in fasted mice express increased CCR7 compared to *ad libitum*-fed mice (**Fig. 3C**). So, we could have inferred that $CD103^+$ DCs in fasted mice could have migrated better into mLN than those of *ad libitum*-fed mice, thus likely proliferated by increased GM-CSF.

$Foxp3^+$ Tregs not only prevent autoimmune diseases (50,51), but also curb vigorous antimicrobial immune responses by restricting inflammation (52,53). We report a correlation between $CD103^+$ DCs and $Foxp3^+$ Tregs, and that short-term fasting protects mice against LM. During the early stage of infection, $CD103^+CD11b^-$ DCs and $Foxp3^+$ Tregs were significantly increased in short-term-fasted mice. $Foxp3^+$ Tregs induced by $CD103^+$ DCs prevent excessive immune responses to pathogens (18). It is important to note that $CD103^+$ DCs can be classified into two distinct subsets based on the expression of CD11b. It has been demonstrated that lack of intestinal $CD103^+CD11b^-$ DCs in $Batf3^{-/-}$ mice have no symptoms of spontaneous inflammation in the intestine. The authors postulated that $CD103^+CD11b^+$ DCs would play a role for maintaining intestinal homeostasis via regulating Tregs induction (22). Although this is a report contrary to what we propose in the present study, it is not yet known how short-term fasting affects the relationship between intestinal $CD103^+CD11b^+$ DC and induction of Tregs. It appears to be necessary, in future, to examine the cause and consequence of changes in the intestinal DC subsets when $Batf3^{-/-}$ mice are applied to the short-term fasting model.

We have suggested that the increase of $Foxp3^+$ Tregs was a result of increased TGF-β2 expression in short-term-fasted mice. TGF-β promotes expansion of $Foxp3^+$ Tregs *in vivo* (54). Therefore, the increase of $Foxp3^+$ Tregs during early infection may be mediated by TGF-β. This should be verified by transferring $Foxp3^+$ Tregs into TGF-β-deficient or DC-specific IRF8-deficient mice. Furthermore, *Aldh1a2* expression was higher in $CD103^+$ DCs from short-term-fasted mice, which might have caused the increase in $Foxp3^+$ Tregs. RA is mainly produced by intestinal DCs and epithelial cells, and inhibition of RA receptor reduced the induction of $Foxp3^+$ Tregs (55). Therefore, tolerogenic conditions in short-term-fasted mice might restrain the immune response and prevent tissue damage during the early phase of LM infection.

PD-L1 expression, together with CCR7 and CD205, was also significantly increased in short-term-fasted mice infected with LM. PD-L1-expressing DCs function as tolerogenic DCs by inducing $Foxp3^+$ Tregs (39,40). Therefore, we investigated the roles of CCR7 and CD205 in PD-L1-expressing DCs from short-term-fasted mice. CCR7 regulates migration of DCs from tissues to draining lymph nodes (34). CD205 is directly associated with antigen uptake and enhances antigen presentation by the MHC I and II pathways (56,57). It has been suggested that intestinal $CD103^+$ DCs have better potential and ability to uptake non-invasive bacteria efficiently using intraepithelial dendrites (16). In addition, $CD103^+CD11b^+$ DCs have been reported to be the first DC subset to transport bacteria, *Salmonella Typhimurium*, to the mLN after oral infection (48). Collectively, our results suggested that the increased $CD103^+$ DCs in short-term-fasted mice are migratory DCs maintaining intestinal tolerance, and can transport bacterial antigen the most firstly when the host infected.

$CD103^-$ DCs are more immunogenic than $CD103^+$ DCs under steady state and infectious conditions and induce differentiation of naïve T cells into IFN-γ–producing Th1 cells (25)

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and production of proinflammatory cytokines (18). However, the number of $CD103^-$ DCs was lower than that of $CD103^+$ DCs, indicating maintenance of tolerance. Indeed, at the early stage (1 or 2 days) after bacterial infection, $Foxp3^+$ Tregs are important for inducing a protective and non-pathogenic Th17 response and later maximizing the gut Th1 response (58). At 1 dpi, $CD103^+CD11b^-$ DCs and $Foxp3^+$ Tregs were increased in number in short-term-fasted mice compared to *ad libitum*-fed mice. By contrast, the Th1 response at 3 dpi was increased in short-term-fasted mice infected with LM. Although it is still a controversy, $CD103^-CD11b^+$ DCs in mLN are known to be a subset derived from blood, not from intestine (25). So, we have thought reason that increase of $CD103^-CD11b^+$ DCs at 3 dpi is causing by the migration, not by subset switching from $CD103^+$ into $CD103^-$. However, the exact mechanisms on how short-term-fasted mice alter the major subset of DCs in intestine during bacterial infection should be followed up by further investigation.

Our results suggest that the changes of intestinal $CD11c^{hi}$ DC subsets in short-term-fasted mice are critical for maintaining intestinal tolerance during early LM infection and later for forming a Th1-biased environment by increasing the number of $CD103^-$ DCs. It has been suggested that intestinal $CD103^-CD11b^+$ DCs induce differentiation of naïve $CD4^+$ T cells into IFN-γ–producing Th1 cells (25). On the other hand, there is another intestinal DC subset, $CD103^+CD11b^+XCR1^+$ DCs of which to differentiation of naive T cells into Th1 cells (59). The authors demonstrated that there was a significant decrease of intestinal T cell population, especially potential to produce IFN-γ, in mice when $XCR1^+$ DCs were depleted. In addition, the mice lacking $XCR1^+$ DCs showed more susceptible to DSS-induced colitis. Therefore, further investigation on the contribution of $CD103^+CD11b^-XCR1^+$ DCs to the increase of IFN-γ producing cells would be meaningful.

In the context with induction of IFN-γ^+ cells at 3 dpi, the present study showed that neutrophils were increased in short-term fasting mice infected with LM when compared to the *ad libitum*-fed mice (**Supplementary Fig. 7**). It could be that the increase of neutrophils may enhance bacterial clearance in LM infection. This is in agreement with the previous report that the infiltration of $Ly6G^+$ neutrophils is critical for bacterial clearance and host survival (60). Based on these findings, we could suggest that establishment of Th1 environment in mice infected with LM by increased $CD11b^+$ DCs is important for the protective immunity.

To examine the antigen-specific $CD4^+$ and $CD8^+$ T cells upon LM infection, it is usual to check IFN-γ production in intestinal $CD4^+$ or $CD8^+$ T cells at 7-8 dpi by re-stimulating with cognate epitope *in vitro* (61-63). However, unfortunately, in experimental condition of current study, we could not have checked antigen-specific IFN-γ producing $CD4^+$ or $CD8^+$ T cells because infected mice with *ad libitum*-fed (as the control group) were severely affected by infection and many are almost dead (**Fig. 4B**). Therefore, further works would be required to overcome the limitation of current study through finding even more perfect infection condition verifiable both unique effect of short-term fasting to intestinal DCs and surviving the mice for investigating antigen-specific T cell response.

Although we have suggested the effect of fasting to intestinal DC subsets only in the present study, it has been demonstrated that effect of mild or transient restriction of dietary intake is not limited to DCs, but can affect various immune cells, such as T cells, B cells, neutrophils, macrophages, and monocytes, to modulate immune responses (64). Furthermore, fasting could increase the resistance to colonization of *Salmonella Typhimurium*, thus reduce host inflammatory responses through suppressing NF-κB expression and downstream inflammatory mediators in whole cecal tissue lysates (65). Conversely, it has been reported

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that fasting suppresses antigen-specific antibody production in the Ova-vaccination model and Ova-induced diarrhea model by inducing the migration of naïve B cells to the bone-marrow in addition to the immune-enhancing effect (43). Therefore, further study that examines the comprehensive effect of fasting to modulate host immune responses from the perspective of changes in immune cells together with microbiome would be required.

In summary, short-term fasting influenced the characteristics of intestinal $CD11c^{hi}$ DCs to balance tolerance and the immune response to LM infection. The balance was regulated by induction of intestinal $CD103^{+}CD11b^{-}$ DCs and $Foxp3^{+}$ Tregs during the early phase of infection, followed by induction of $CD103^{-}CD11b^{+}$ DCs with IFN-γ^{+} cells. These results provide insight into the influence of fasting on the innate immune system and could inform the development of strategies for oral prophylactic vaccination and treatment.

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SUPPLEMENTARY MATERIALS

Supplementary Figure 1

Gating strategy for flow cytometry analysis of immune cells. Single cells were prepared and pre-gated for single live $CD45^{+}$ cells, and then specifically further gated for each cell type as displayed, respectively.

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Supplementary Figure 2

Changes on the composition of $CD45^{+}$ cells in mLN and SI LP from mice with short-term fasting. Mice were fasted for 24 h. Changes of neutrophils and macrophages (A), B cells and NK cells (B), and T cells (C) were examined. Statistical significance was examined by using unpaired Student's *t*-test. The representative results from 2–3 independent experiments, n=3–6 mice.

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Supplementary Figure 3

Increase of the $CD11c^{hi}$ DC subsets in mice with short-term fasting. Mice were fasted for 24 h. (A) BrdU uptake among $CD45^{+}F4/80^{-}CD11c^{hi}$ DCs subsets were examined in mLN. (B) Expression of MHC II, CD86, CD205, and PD-L1 was examined in $CD45^{+}F4/80^{-}CD11c^{hi}CD103^{+}$ DCs of SILP. Statistical significance was examined by using unpaired Student's *t*-test. The representative results from 2–3 independent experiments, n=3–4 mice.

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Supplementary Figure 4

Bacterial burden in stomach and body weight change after short-term fasting in mice infected with LM. Mice were fasted for 24 h and then infected with LM. (A) CFU was measured in stomach at 3 hpi. (B) Body weight was monitored for 8 days. To note that the body weight of short-term fasting group was measured from day-1 because of the fasting. The statistics for body weight result was analyzed by log-rank (Mantel-Cox) test and all other statistical significance by unpaired Student's *t*-test. The representative results from 2–3 independent experiments, n=5–6 mice.

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Supplementary Figure 5

Induction of splenic Foxp3$^+$ Tregs in mice with short-term fasting followed by LM infection. Mice were fasted for 24 h and then infected with LM. (A, B) Foxp3$^+$ Tregs were analyzed in spleen at 1 dpi and shown in (A) dot plot and (B) percentage among CD3$^+$CD4$^+$ cells. Statistical significance was examined by using unpaired Student's t-test. The representative results from 2–3 independent experiments, n=3-4 mice.

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Supplementary Figure 6

Composition of IFN-γ$^+$ cells among NK cells in short-term fasting mice infected with LM. Mice were fasted for 24 h and then infected with LM. The percentage and absolute number of IFN-γ$^+$ cells among NK cells (NK1.1$^+$CD3$^-$NKp46$^+$) in mLN at 1, 2 and 3 dpi. Statistical significance was examined by using unpaired Student's *t*-test. The representative results from 2–3 independent experiments, n=4–6 mice.

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Supplementary Figure 7

Changes of neutrophils and macrophages in mice infected with LM. Mice were fasted for 24 h and then infected with LM. Percentage and absolute number of neutrophils and macrophages from SILP was examined at 3 dpi. Statistical significance was examined by using unpaired Student's *t*-test. Results are representative of two or three independent experiments, n=4–6 mice.

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