

Short-term Fasting Enhances the Protection Against *Listeria Monocytogenes* Infection Through Changes of Intestinal CD103⁺ Dendritic Cells

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Abstract

Gastrointestinal tract is the first organ to be directly affected upon fasting. However, little is known about how the fasting influences intestinal immune system. In the present study, we focused on the changes of intestinal dendritic cells (DCs) in mice upon short-term fasting and how the changes influence protective immunity against *Listeria monocytogenes* (LM) infection. We found that the fasting induces an increased number of CD103⁺CD11b⁻ DCs in both small intestinal lamina propria (SI LP) and mesenteric lymph nodes (mLN) and the SI LP CD103⁺CD11b⁻ DCs undergo an active proliferation and migration by increased levels of GM-CSF and CCR7, respectively. At 24 hours post-infection (hpi) of LM, there was a significant reduction of bacterial burden from the spleen, liver, and mLN of the short-term fasting mice compared to those of *ad libitum* mice. Accordingly, short-term fasting mice showed enhanced survival against LM infection when compared with *ad libitum* mice. Furthermore, significantly high amount of TGF- β 2 and Aldh1a2 expression from CD103⁺CD11b⁻ DCs in mouse infected with LM sequentially caused the following events: the increase of Foxp3⁺ Tregs, preferential change in the composition of CD103⁺ to CD103⁻ DCs, and the induction of IFN- γ producing cells. Collectively, increase of intestinal CD103⁺ DCs by short-term fasting is a key player for protection against LM infection through the changes of functional features from tolerogenic to Th1 immunogenic.

Introduction

Periodic fasting could extend lifespan of bacteria, yeast, worms and mice compared to *ad libitum* diet¹. It has been suggested that intermittent fasting protects mice from various infectious and non-infectious diseases such as diabetes, cancers and neurodegeneration². For instance, the mice which had been fasting for 24–72 h before *L. monocytogenes* infection reduced bacterial burden and prolonged host survival³. Furthermore, fasting appeared to have a positive impact on survival rate after the transplantation of kidney and liver, and the ischemia-reperfusion injury in mice⁴.

The nutritional depletion even for a short time period (*i.e.*, 24 h) reduced total number of cells in the bone marrow and thymus⁵. As gastrointestinal tract is the first organ to be directly affected after the fasting, proteins related to metabolism including glycolysis are decreased, and overall protein synthesis is reduced after the fasting for 24 h. Interestingly, however, proteins involved in cellular protection such as preservation of intestinal integrity were significantly increased at the same time⁶. And, it has been suggested that nutritional depletion could induce the changes in hormones as well as the function of immune cells⁷. For example, leptin can promote expansion of naïve T cells in an IL-2-dependent manner and switch toward more Th1 responses than Th2 responses^{8,9}. Leptin also promotes the maturation of dendritic cells (DCs) through increasing co-stimulatory molecules, producing pro-inflammatory cytokines¹⁰, and the migration to inflamed tissues¹¹.

CD103⁺ DCs in small intestinal lamina propria (SI LP) serve to capture antigens, including apoptotic epithelial cells¹² and bacterial antigens¹³, and migrate into mesenteric lymph node (mLN) in a CCR7-

dependent manner¹⁴. Moreover, they together with TGF- β ^{15,16} and retinoic acid¹⁷ induce regulatory T cell (Treg) differentiation. CD103⁺ DCs can be divided into several subtypes depending on their CD11b or CD8 α expression. CD103⁺CD11b⁻CD8 α ⁺ DCs are known to be specialized for the cross-presentation of cell-associated antigens and priming of CD8⁺ T cells¹⁸. By contrast, CD103⁺CD11b⁺ DCs appear to induce immune tolerance at the steady-state, particularly in intestine; however, paradoxically, these cells were prone to induce Th1 response if exposed to inflammatory condition¹⁹. Likewise, a minor population of intestinal CD103⁻CD11b⁺ DCs appear to efficiently prime naïve CD4⁺ T cells and induce differentiation to either IL-17 or IFN- γ producing effector CD4⁺ T cells²⁰.

The primary function of small intestine is to perform digestion and absorption of incoming nutrients, while upon the fasting, there are structural and functional changes with reduction of metabolic activities²¹. Although effects of fasting on the intestinal epithelial cells have been previously documented, its impact on the intestinal immune cells, DCs in particular, has not yet been fully understood. In this study, changes of CD103⁺ DCs in gut-associated lymphoid organs such as mLN and SI LP by short-term fasting, and immune context by these changes were investigated using a mouse model infected with *L. monocytogenes*.

Results

CD11c^{hi} DCs were increased in mLN and SI LP of short-term fasting mice

Gastrointestinal tract consumes enormous amount of energy and, therefore, could be directly and greatly affected upon the fasting⁶ but little is known about how it influences the gastrointestinal immunity. Thus, we focused on intestinal immune cells under the short-term fasting condition. After 24 h (short-term) of fasting⁵, significantly increased number of CD45⁺ leukocytes in mLN and SI LP was observed from fasting mice when compared to those of *ad libitum* mice (Fig. 1A). Next, to investigate which cell types have been increased among CD45⁺ leukocytes after short-term fasting, various immune cell types including dendritic cells (DCs), neutrophils, macrophages, B cells, natural killer (NK) cells, and T cells were analyzed. Significant increase in a number of CD11c^{hi} DCs in mLN as well as in SI LP were observed when compared to *ad libitum* group (Fig. 1B). On the other hand, neutrophils, macrophages, NK cells, and lymphocyte populations including B cells, CD4, CD8 and $\gamma\delta$ T cells, were not significantly changed in both mLN and SI LP (Supplementary Fig. 1A-C).

Taken together, the results showed that short-term fasting leads to the significant increase of intestinal CD11c^{hi} DCs, not of other major immune cells.

CD103⁺ DCs were dramatically increased in mLN and SI LP from short-term fasting mice

It has been reported that intestinal CD11c^{hi} DCs could be further categorized to several subsets based on the expression of CD103 and CD11b molecules²². The majority of CD11c^{hi} DCs in small intestine expresses the integrin α_E referred as CD103 paired with β_7 ²³. Furthermore, intestinal CD103⁺ DCs play an important role in the maintenance of tolerance against food antigens or commensal bacteria. In addition, this immunological tolerance maintains intestinal homeostasis while suppressing unnecessary intestinal hyper-inflammation that may occur even in the normal individuals²⁴. To investigate which subsets of DCs are increased by short-term fasting, we further examined the change of CD11c^{hi} DCs based on its expression of CD103 and CD11b. Intriguingly, the results showed that the number of CD103⁺CD11b⁻ DCs was significantly increased in mLN from mice with short-term fasting (Fig. 2A and B). Furthermore, CD103⁺ DCs, but not CD103⁻ DCs, in SI LP were significantly increased from mice with short-term fasting when compared with those of *ad libitum* (Fig. 2C and D).

Collectively, these results indicate that increase of CD11c^{hi} DCs in short-term fasting mice mainly arose from the increased number of CD103⁺CD11b⁻ DCs, but not from CD103⁻ DCs, in both mLN and SI LP.

CD103⁺ DCs were actively proliferated in SI LP by GM-CSF that increased after the short-term fasting

We hypothesized that increased number of CD103⁺ DCs in the SI LP was caused by either active cell proliferation at local site or migration. To investigate whether CD103⁺ DCs were proliferating more upon short-term fasting than *ad libitum*, BrdU incorporation assay was performed and the proliferation of intestinal DCs was monitored. Interestingly, there was a significant increase of BrdU uptake in CD103⁺ DCs, both CD103⁺CD11b⁻ and CD103⁺CD11b⁺ subsets, in the SI LP of short-term fasting mice compared to those of *ad libitum* mice, which is consistent with increased cellularity of these subsets in the SI LP (Fig. 3A). However, no significant increase was seen in any subpopulations of CD11c^{hi} DCs in mLN (Supplementary Fig. 2A).

It is well known that GM-CSF is required for the development of DCs at steady-state as well as inflammatory situation²⁵. GM-CSF also induces the development and expansion of conventional DCs²⁶, and facilitates the recruitment of intestinal DCs²⁷. Thus, to investigate whether the increased number of CD103⁺ DCs in short-term fasting mice was affected by GM-CSF increase, mRNA expression of GM-CSF in SI LP was examined. As expected, the expression of GM-CSF was significantly increased in the SI LP after the short-term fasting (Fig. 3B). In order to confirm the possibility of migration mentioned above along with proliferation, expression of the surface proteins on CD103⁺ DCs in SI LP. The results showed that significant increase of CCR7, MHC II, CD205, and PD-L1 on the intestinal CD103⁺ DCs from short-term fasting mice compared to *ad libitum* (Fig. 3C and Supplementary Fig. 2B).

These results suggest that the short-term fasting increases the number of DC subsets, largely CD103⁺CD11b⁻ and CD103⁺CD11b⁺ DCs in the SI LP, through augmenting cell expansion and migration due to the increase of GM-CSF and CCR7 expression, respectively.

Short-term fasting protects mice against *L. monocytogenes* infection

We postulated that increase of CD103⁺ DCs by short-term fasting can affect intestinal immunity against pathogenic infection because CD103⁺ DCs were suggested to act as tolerogenic DCs²⁴. In order to elucidate the role of CD103⁺ DCs increased at short-term fasting in infectious condition, the mice were infected with *L. monocytogenes* that is known to preferentially induce Th1 and Th17 responses²⁸ and bacterial burden was measured. The result showed that colony forming unit (CFU) was significantly decreased in spleen, mLN, and liver at 48 hours post-infection (hpi) in short-term fasting group (Fig. 4A). Together with bacterial burden, to measure the degree of systemic bacteremia, CFU in serum was examined. With consistency, high bacteremia was observed from the sera of mice fed with *ad libitum* but not with a short-term fasting group (Fig. 4B). Furthermore, in order to rule out the possibility that short-term fasting mice consumed the feed much faster after re-feeding during the infection than *ad libitum* mice which could influence severeness of the invasion, CFU in the stomach at 3 hpi was examined and found no differences (Supplementary Fig. 3A).

Next, we questioned whether the reduced bacterial burden in both tissues and serum from short-term fasting mice has a correlation of protection based on host survival. Interestingly, consistent with bacterial burden, the survival of mice which had been short-term fasting was increased compared to that of *ad libitum* mice (Fig. 4C). In the context with survival rate, body weight change of short-term fasting group in Lm-OVA infected group was comparable to that of PBS group (Supplementary Fig. 3B).

Collectively, these data suggest that short-term fasting has beneficial effect on the protection against gastrointestinal *L. monocytogenes* infection.

CD103⁺ DCs and Foxp3⁺ regulatory T cells (Tregs) were increased in mLN during the early phase of *Listeria* infection after short-term fasting

Next, we examined what role the increased intestinal CD103⁺ DCs could play in Lm-OVA infection in short-term fasting mice. Previous studies suggested that CD103⁺CD11b⁻ DCs are usually tolerogenic and involved mainly in the differentiation of Foxp3⁺ Tregs by expressing anti-inflammatory cytokines and inhibitory surface molecules²⁴. So, to investigate whether intestinal CD103⁺CD11b⁻ DCs contribute to the induction of Foxp3⁺ Tregs during *L. monocytogenes* infection, CD103⁺CD11b⁻ DCs and Foxp3⁺ Tregs were examined in mice with/without fasting infected with *L. monocytogenes*. CD103⁺CD11b⁻ DCs were significantly increased in mLN (Fig. 5A) and SI LP (Fig. 5B) of short-term fasting mice compared to those of *ad libitum* mice. The results showed that, in mLN, *L. monocytogenes* infection induced an increase in number of Foxp3⁺ Tregs in both short-term fasting and *ad libitum* mice at 1 dpi (day post infection), but much higher in the fasting than *ad libitum* mice (Fig. top of 5C and D). It has been well reported that CD103 is a marker for identifying *in vivo*-activated Foxp3⁺ Tregs²⁹. Thus, to know whether the increased Foxp3⁺ Tregs in short-term fasting mice are activated functionally, expression of CD103 on Foxp3⁺ Tregs was examined. The results showed that increase of *in vivo*-activated CD103⁺Foxp3⁺ Tregs was observed

within the group of increased Foxp3⁺ Tregs in short-term fasting mice compared to those of *ad libitum* mice (Fig. bottom of 5C and E). In contrast to mLN, in spleen, Foxp3⁺ Tregs were comparable in both groups with no difference in the composition of CD103⁺Foxp3⁺ Tregs (Supplementary Fig. 4). Consistent with the increased Foxp3⁺ Tregs, mRNA level of *foxp3* was 3-fold higher in short-term fasting mice than in that of *ad libitum* mice at 1 dpi (Fig. 5F left). Contrary to *foxp3* level, it was observed that the mRNA levels of *t-bet* were upregulated at 3 days after the infection (Fig. 5F right).

Collectively, it suggests that increased CD103⁺CD11b⁻ DCs in short-term fasting mice induced functional Foxp3⁺ Tregs upon *L. monocytogenes* infection, especially at early stage.

Increased level of TGF-β and RA contributed to CD103⁺ DC with the feature of tolerogenic DCs

We suggested intestinal CD103⁺ DCs to promote increase of Foxp3⁺ Tregs upon *L. monocytogenes* infection in short-term fasting mice. Thus, to elucidate the factors to promote the increase of Foxp3⁺ Tregs, first, changes of the surface molecules on CD103⁺ DCs were examined. It has been reported that increased PD-L1 and decreased CD86 and MHC class II are the phenotypic characteristics of tolerogenic DCs³⁰, and CD205-expressing CD8α⁺ DCs also can induce Foxp3⁺ Tregs through producing TGF-β³¹. Consistent with the previous reports, at 1 dpi, significant increase of PD-L1, CD205 and CCR7 was observed on CD103⁺ DCs from short-term fasting mice compared to *ad libitum*, while there were no changes on CD86 and MHC II (Fig. 6A).

With same context, it has been reported that CD103⁺ DCs producing TGF-β, RA and aldehyde dehydrogenase A2, which is the key enzyme for converting vitamin A to RA, are able to induce Foxp3⁺ Tregs^{15,16}. So, we analyzed the expression of those soluble factors in purified intestinal CD103⁺ DCs of *ad libitum* mice and short-term fasting mice. Interestingly, the mRNA levels of *TGF-β2* and *Aldh1a2* from the purified intestinal CD103⁺ DCs were significantly increased in short-term fasting mice compared to those from *ad libitum* mice (Fig. 6B).

Taken together, the results suggested that increased *TGF-β2* and *Aldh1a2* could contribute to tolerogenic feature of CD103⁺ DCs together with significant increase of PD-L1 and CD205, and those tolerogenic features of CD103⁺ DCs would contribute to increase of Foxp3⁺ Tregs in short-term fasting mice during *L. monocytogenes* infection.

Short-term fasting induced up-regulation of Th1 response in mice infected with *L. monocytogenes*

Even though the results so far suggested increase of CD103⁺ tolerogenic DCs together with Foxp3⁺ Tregs in short-term fasting mice could be involved in the host survival, we went step further to investigate how the bacterial burden was significantly reduced during later time point, after 48 hpi (Fig. 4). It has been well reported that CD103⁻ DCs preferentially induce differentiation of naïve CD4⁺ T cells to IFN-γ-producing Th1 cells²⁰. Thus, we hypothesized that there should be likely the change of CD103⁺ to CD103⁻ DC

subsets after 2 dpi in short-term fasting mice that may reflect to reducing bacterial load. So, we tried to look at changes of DC subsets at 3 dpi. Interestingly, the results showed that the percentage and absolute number of CD103⁻CD11b⁺ DCs are significantly increased in short-term fasting mice compared to *ad libitum* mice at 3 dpi while, reversely, the percentage and absolute number of CD103⁺CD11b⁻ DCs reduced significantly (Fig. 7A). We further investigated whether CD103⁻ DCs have helped forming Th1 environment as reported in the previous study²⁰. Both percentage and absolute number of IFN- γ ⁺ cells among CD4⁺CD3e⁺ (Fig. 7B), CD8⁺CD3e⁺ cells (Fig. 7C), and NK1.1⁺CD3e⁻ (Supplementary Fig. 5A), were increased at 2 and 3 dpi in short-term fasting mice, in agreement with the increased level of *t-bet* expression at 3 dpi (Fig. 5F).

Collectively, in short-term fasting mice, increase of IFN- γ ⁺ cells at the later phase of infection is highly affected by increase of CD103⁻ DCs that contributes to the reduction of bacterial burden by enhancing T cell immune response.

Discussion

In the present study, functional alteration of intestinal immune cells, especially CD11c^{hi} DCs, caused by short-term fasting with/without *L. monocytogenes* infection was investigated. Major findings are as follow: (1) short-term fasting could alter the composition of intestinal innate immune cells, with increase of CD11c^{hi} DCs being most apparent; (2) among CD11c^{hi} cells, CD103⁺CD11b⁻ DCs in both mLN and SI LP from fasted mice proliferated better than those of from *ad libitum* control mice; (3) constitutional changes of DCs were found after the short-term fasting and (4) the expansion and migration of CD11c^{hi} DCs in SI LP after the fasting was closely related with upregulated GM-CSF and CCR7, respectively; and (5) short-term fasting significantly contributed to the protection of the mice from *L. monocytogenes* infection, likely mediated by sharp increase in the induction of Foxp3⁺ Tregs at the early phase and IFN- γ ⁺ cells at the later after the infection.

Several recent studies have suggested that unexpected immune response was provoked by fasting in mice. For instances, fasting during the anorexia enhances the host survival upon experimental autoimmune encephalomyelitis (EAE) model³² or *L. monocytogenes* infection³³. On the other hand, intermittent fasting suppresses antigen-specific antibody production after the immunization with ovalbumin and cholera toxin³⁴. We showed fasting for 24 hours could have enough impact to change the property of intestinal immune cells, particular in DCs to protect against *L. monocytogenes* infection. Nevertheless, appropriate strategy for fasting to most effectively change CD11c^{hi} DCs, and how long the increased CD11c^{hi} DCs can maintain resistance to *L. monocytogenes* infection are in the queue for upcoming studies.

CD103⁺ DCs, in the present study, were the most increased DC subset in mLN and SI LP upon fasting. The increase of CD103⁺CD11b⁻ DCs in SI LP was due to the active proliferation and migration of the cell. It should be noted that the rate of proliferation was much higher in SI LP than mLN, which is consistent

with previous report³⁵ showing that mLN CD103⁺ DCs have slower kinetics of proliferation than SI LP CD103⁺ DCs. On the other hand, increased GM-CSF could influence the rapid recruitment of CD11c^{hi} DCs²⁷. Concordant with this report, we also observed increased GM-CSF in SI LP from short-term fasting mice, suggesting that local increase of GM-CSF induced by fasting might affect the expansion and migration of intestinal CD11c^{hi} CD103⁺ DCs.

In addition to the GM-CSF we have proposed, there are other factors that can affect changing the number of DCs. One of the best known is the flt3 ligand, which plays an important role in the differentiation of hematopoietic stem cells into cDCs³⁶. In addition, flt3 ligand can maintain the normal number of cDCs by directly regulating their proliferation in the periphery³⁷. Therefore, although the results in the present study suggested a role of GM-CSF for the proliferation of CD11c^{hi} cells, it is seemingly necessary to conduct future study for investigating a direct effect of flt3 ligand with/without GM-CSF on proliferation of CD11^{hi} DC subset.

It has been well reported that Foxp3⁺ Tregs not only prevent autoimmune diseases^{38,39}, but also curb vigorous antimicrobial immune responses by restricting excessive inflammation^{40,41}. In the present study, we are proposing a correlation between CD103⁺ DCs and Foxp3⁺ Tregs that short-term fasting is beneficial for the protection of the mice infected with *L. monocytogenes*. During the early stage after the infection, CD103⁺CD11b⁻ DCs and Foxp3⁺ Tregs are significantly increased in short-term fasting mice. In the previous report¹⁵, Foxp3⁺ Tregs induced by CD103⁺ DCs are the safeguard of the host from excessive immune responses after pathogen invasion. We have suggested that the increase of Foxp3⁺ Tregs are occurred by increase of TGF- β 2 expression in short-term fasting mice. It was reported that TGF- β directly promotes expansion of the Foxp3⁺ Treg *in vivo*⁴². Therefore, it is likely that the increase of Foxp3⁺ Treg during the early stage of infection could be a TGF- β -dependent expansion. The possibility for the expansion of Foxp3⁺ Tregs in short-term fasting mice by increase of TGF- β -producing CD103⁺ DCs should be verified further by transferring Foxp3⁺ Tregs into TGF- β deficient or DC-specific IRF8 deficient mice for other possibilities. Furthermore, our result clearly showed the higher expression of *aldh1a2* in CD103⁺ DCs from short-term fasting mice, which might have caused increase of Foxp3⁺ Tregs as well. A study suggested that RA is mainly produced by intestinal DCs and epithelial cells, and inhibition of RA receptor reduced the induction of Foxp3⁺ Tregs⁴³. Thus, these findings suggest that tolerogenic conditions made by short-term fasting might restrain overwhelming immune response and protect host from tissue damage at early time point of infection.

The results in the present study showed that CCR7 and CD205 are also significantly increased from short-term fasting mice infected with *L. monocytogenes*, together with PD-L1. As aforementioned, PD-L1-expressing DCs acted as tolerogenic DCs inducing Foxp3⁺ Tregs^{30,31}. So we thought it will be intriguing to investigate the roles of CCR7 and CD205 in PD-L1-expressing DCs from mice with short-term fasting. It has been well reported CCR7 is one of the major chemokine receptors to regulate migration of DCs from tissues to lymph nodes⁴⁴. And CD205 is directly associated to antigen uptake, then enhances the antigen-

presentation via both MHC class I and MHC class II pathways^{45,46}. Collectively, the results in the present study suggested that CD103⁺ DCs in short-term fasting mice may have the roles as, by highly expressing CCR7, CD205, and PD-L1, the most efficiently migrating DCs to lymph node to provoke systemic immune responses as well as maintain immune tolerance.

Based on the previous works, CD103⁻ DCs have more immunogenic nature in both steady state and infectious conditions where they induce naïve T cells to differentiate IFN- γ producing Th1 cells²⁰ and preferentially produce proinflammatory cytokines¹⁵. Despite the immunostimulatory characteristic of CD103⁻ DCs in the steady state, number of these cells was much lower than CD103⁺ DCs, so that intestinal immune system stays preferentially on the tolerance condition. At 1 dpi shown in the present study, CD103⁺CD11b⁻ DCs and Foxp3⁺ Tregs were increased in their number in short-term fasting mice compared to those of *ad libitum* mice. On the other hand, increase in Th1 response at 3 dpi in short-term fasting mice infected with *L. monocytogenes*. The idea that the increase in number of CD103⁻ DCs were directly involved in the induction of Th1 response of mice infected with *L. monocytogenes* remains to be proven for its mode of mechanism. Nevertheless, these results have suggested that the changes of intestinal CD11c^{hi} DCs in short-term fasting mice are critical for both maintaining the immunological tolerance by increase of CD103⁺ DCs at the early phase of *L. monocytogenes* infection and forming Th1 biased environment by increase of CD103⁻ DCs at late phase.

Collectively, the present study suggested that the short-term fasting influenced the characteristic of intestinal CD11c^{hi} DCs to balance between maintaining tolerance and excessive immune response caused by *L. monocytogenes* infection. The balance was regulated through the induction of intestinal CD103⁺CD11b⁻ DCs coincident with increased number of Foxp3⁺ Tregs during the early phase of infection, then following induction of CD103⁻CD11b⁺ DCs with IFN- γ ⁺ cells at the later phase of infection. These results provide an insight on how fasting influences innate immune system and an implication in designing efficient strategies for oral prophylactic vaccination and chemo-treatment.

Materials And Methods

Animals and short-term fasting

Female BALB/c mice, 6 weeks old, were purchased from Orient Bio Inc., Korea. Mice were divided into two groups, one fed *ad libitum* and the other fasted for 24 h with water provided. In order for the mice to avoid eating their own feces during starvation, the mice were transferred into new bedding cages when the fasting started. All experimental procedures were approved by Institutional Animal Care and Use Committee at Seoul National University (Approval number: SNU-130510-4-1), Korea. We confirmed that all animal experiments were carried out by adhering ARRIVE guidelines. All animal experiments were performed and carried out in accordance with relevant guidelines and regulations.

Bacteria preparation and infection

Recombinant *Listeria monocytogenes* expressing ovalbumin (Lm-OVA) and its parental 10403s strain were kindly provided by Dr. Hao Shen (University of Pennsylvania, Philadelphia, PA, USA). Bacteria, cultured with brain heart infusion media for 8 h at 140 rpm on a shaking incubator at 30 °C, were harvested by centrifugation and thoroughly washed twice with PBS. Bacteria count was estimated by measuring optical density at 600 nm as previously described⁴⁷. The number of bacteria administered to the mice was validated by CFU counting through a serial dilution and plating. For infection studies *in vivo*, 1×10^8 CFU of Lm-OVA in 200 μ l of PBS were administered by intragastric (i.g.) route at 24 h after the fasting⁴⁸.

Determination of bacterial colony

To determine bacterial burden, spleen, liver and mLNs were taken after the perfusion using PBS. Each organ was homogenized with PBS with 0.1% Triton x-100. To examine the number of *Listeria* in the blood, at least 200 μ l of blood was collected by eye-bleeding and centrifuged at 6300 x g for 10 min to separate serum and cells. Serial diluents were plated on brain heart infusion agar plate for 12 to 16 h at 37 °C incubator and CFU was examined.

SI LP cell isolation

Small intestine, fats, connective tissues and Peyer's patches removed, was cut longitudinally and washed in cold PBS. Subsequently, it was cut into 1 cm pieces and transferred to the flask containing 20 ml of digestion solution composed with 1 x Hank's balanced salt solution without Ca^{2+} and Mg^{2+} (Sigma-Aldrich, St. Louis, MO, USA), 5% FBS (Gendepot, Barker, TX, USA), 1 mM DL-Dithiothreitol (DTT) (Sigma-Aldrich), and 2 mM EDTA (Sigma-Aldrich). Tissues were dissociated by gentle stirring for 20 minutes (min) at 37 °C and the supernatant discarded. The remaining fractions of SI LP were chopped with scissors thoroughly and digested by stirring with RPMI-1640 media containing 2% FBS, 0.5 mg/ml of collagenase type VIII (Sigma-Aldrich) and 40 μ g/ml of DNase I (Roche, Indianapolis, IN, USA) for 30 min at 37 °C. LP suspensions were filtered through a 70- μ m filter and washed with RPMI-1640.

In vivo proliferation assay

During short-term fasting, mice were injected intraperitoneally (i.p.) with 1 mg of BrdU dissolved in distilled PBS. After 12 h, single cells were prepared from SI LP and mLNs. After surface staining with fluorochrome-conjugated antibodies, the cells were washed thoroughly. To fix and permeabilization, the stained cells were suspended in 100 μ l of cytofix/cytoperm buffer (BD Biosciences, San Jose, CA, USA), incubated for 20 min at room temperature (RT) and washed with 1 x BD perm/wash buffer. Suspending cells in 100 μ l of BD perm/wash buffer plus (BD Biosciences) and incubated for 10 min at 4 °C in the dark. Incubated cells were washed with 1 x BD perm/wash buffer and centrifuged. After re-fixing the cells with 100 μ l of the buffer, suspended 1×10^6 of cells were incubated with DNase reaction solution for 1 h at 37 °C. The cells were suspended in 50 μ l of 1 x BD perm/wash buffer containing fluorescent anti-BrdU-FITC. The cells were incubated for 20 min at RT, washed with 1 x BD perm/wash buffer and analyzed by using flow cytometry (BD Biosciences).

Flow cytometry, intracellular cytokines, and Foxp3 staining

Fluorochrome-conjugated monoclonal antibodies to 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), -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) antibodies were purchased from BD Biosciences. Monoclonal antibodies to mouse anti-CD69 PerCP-Cy5.5 (H1.2F3), -F4/80 APC (BM8), -Ly6G BV421 (1A8), -CD11b BV605 (M1/70), -CD4 BV605 (RM 4–5), -CCR7 Alexa647 (4B12) were purchased from Biolegend (San Diego, CA, USA). For surface molecule staining, the cells were stained with proper combination of antibodies as described above 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 (PMA, 20 ng/ml) and ionomycin (200 ng/ml) in the presence of 3 µl/ml of Brefeldin A (BD Biosciences) and incubated for 5 h at 37 °C. After surface staining, the cells were stained with anti-IFN γ -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, the cells were incubated with anti-Foxp3 Alexa647 antibody (MF23) (BD Biosciences) for 20 min at RT in the dark. After the staining, flow cytometric analysis was performed using FACS Canto II (BD Biosciences) and analyzed using FlowJo software (Tree Star, CA, USA). Cell sorting was performed using FACS Aria (BD Biosciences).

Real-time quantitative polymerase chain reaction (PCR)

cDNA was examined for the frequency of different transcripts by real time quantitative PCR using Power SYBR Green PCR master mix (Applied Biosystems, Warrington, UK). Primers used 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', *tgfb2*: forward 5'- GCC CCT GCT GTA CCT TCG T -3', reverse 5'- TGC CAT CAA TAC CTG CAA ATC T -3'. Thirty cycles of PCR were performed in duplicate for each primer. Relative quantification was determined using the $\Delta\Delta C_t$ 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 \pm standard deviation was determined for each group. For comparison of means between two groups, the data were analyzed using two-tailed paired student's *t*-test and considered statistically significant when *p*-value was less than 0.05. Otherwise, it was mentioning in the figure legend.

Declarations

Acknowledgements

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Author contributions

C-HY conceived and designed the experiments. K-ML, GK, and Y-JJ performed the experiments and analyzed the data. Y-JJ and K-ML wrote the draft of the manuscript. C-HY, Y-JJ, Y-CK, HWK, HC, B-CP, J-HC, and S-HH contributed to analyses and discuss the experimental work together with a critical revision of the manuscript. All authors discussed and finalized the manuscript.

Conflict of interest

The authors declare no financial or commercial conflict of interests.

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Figures

Figure 1.

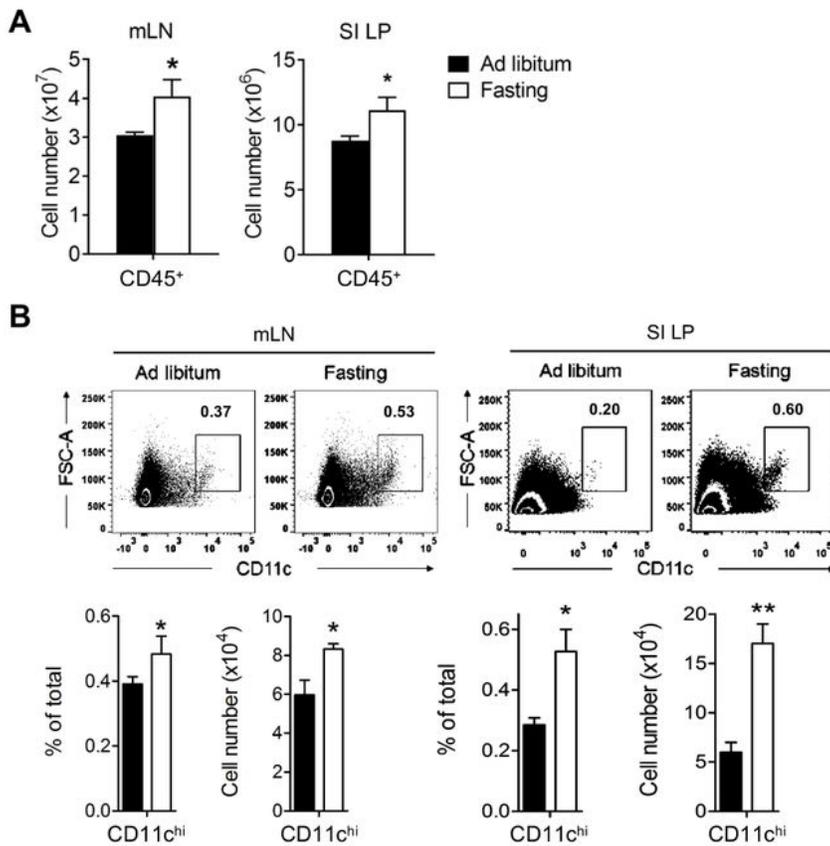


Figure 1

Change of CD11c^{hi} cells from SI LP and mLN in mice after short-term fasting. Mice were fasted for 24 h. (A) The absolute number of CD45⁺ leukocytes in mesenteric lymph node (mLN) and small intestinal lamina propria (SI LP) was measured. (B) The percentage and absolute number of CD11c^{hi} dendritic cells (DCs) were examined in the mLN and SI LP. Statistical significance by unpaired Student's t-test. Data representative of 2-3 independent experiments, n=3-6 mice

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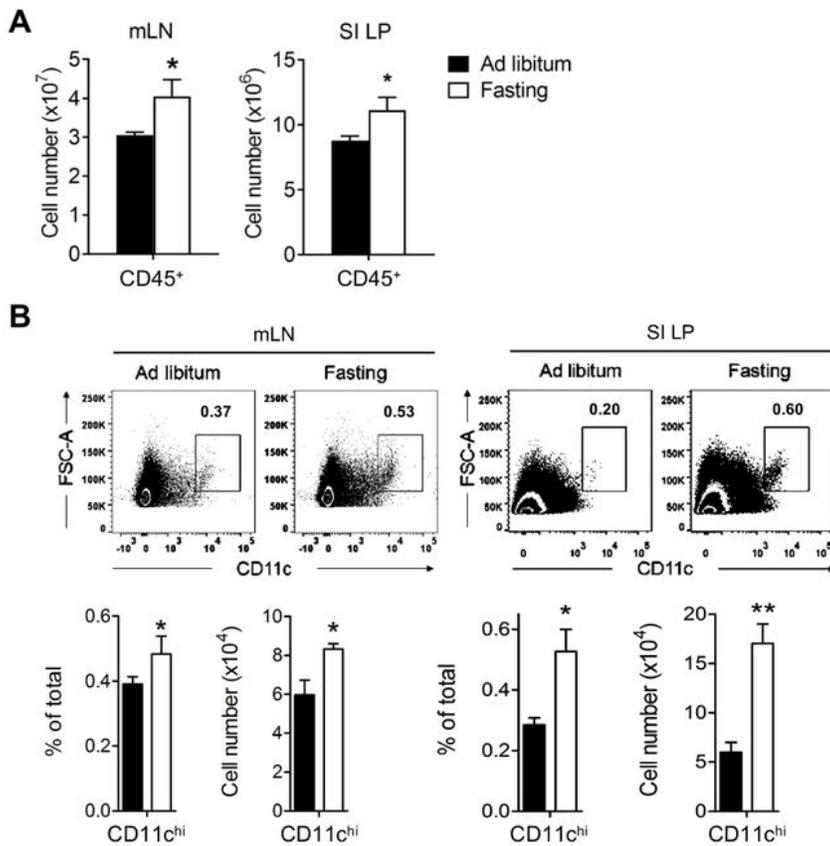


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Figure 2.

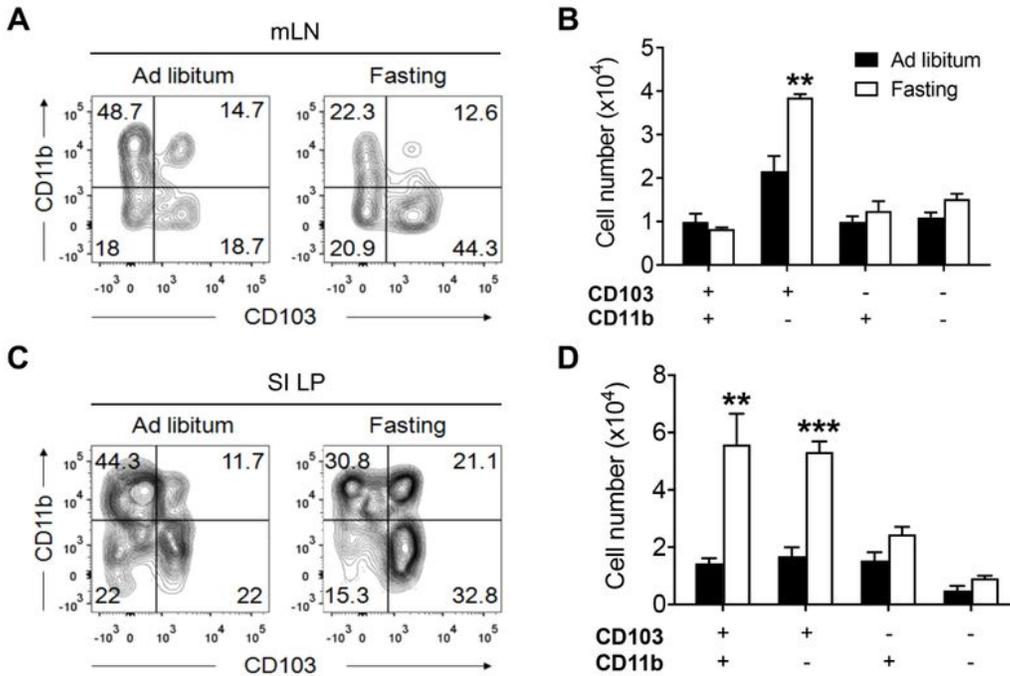


Figure 2

Subtypes of CD11chi DCs based on the expression of CD103 and CD11b in mLN and SI LP in mice after short-term fasting. Mice were fasted for 24 h. (A and C) Dot plot displaying CD11chi DCs based on the expression of CD103 and CD11b in (A) mLN and (C) SI LP. (B and D) The absolute number of CD11chi DC subsets in (B) mLN and (D) SI LP. Statistical significance by unpaired Student's t-test. Data representative of 2-3 independent experiments, n=3-5 mice.

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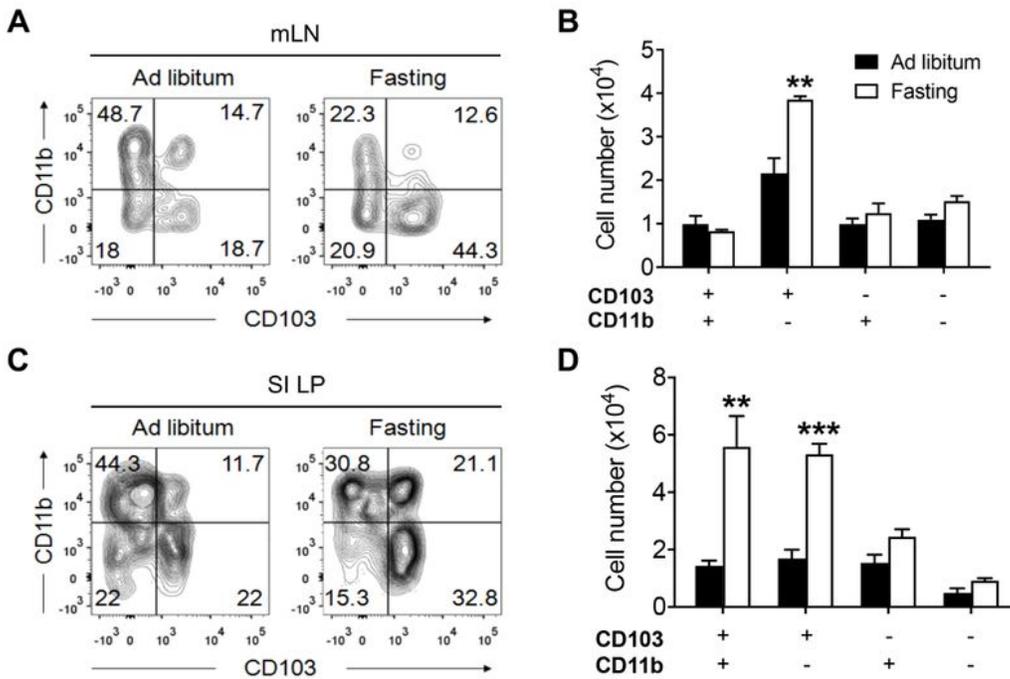


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Figure 3.

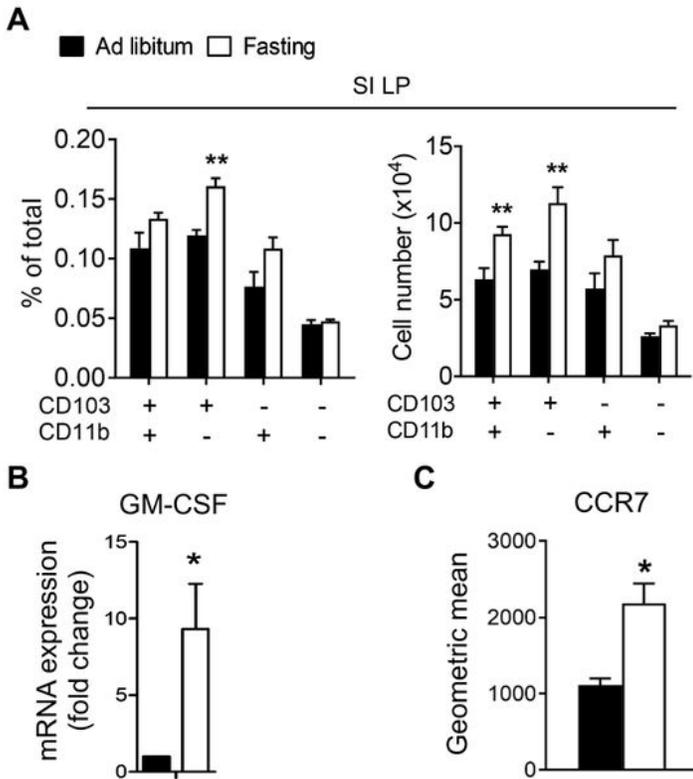


Figure 3

Increase of CD11chi DC subsets in SI LP in mice after short-term fasting. Mice were fasted for 24 h. (A) The percentage and absolute numbers of BrdU+ cells among CD11chi DCs subsets were examined. (B) The mRNA expression of GM-CSF was measured in total SI LP cells. (C) Expression of CCR7 was examined in CD103+ DCs. Statistical significance by unpaired Student's t-test. The representative results from 2-3 independent experiments, n=3-4 mice.

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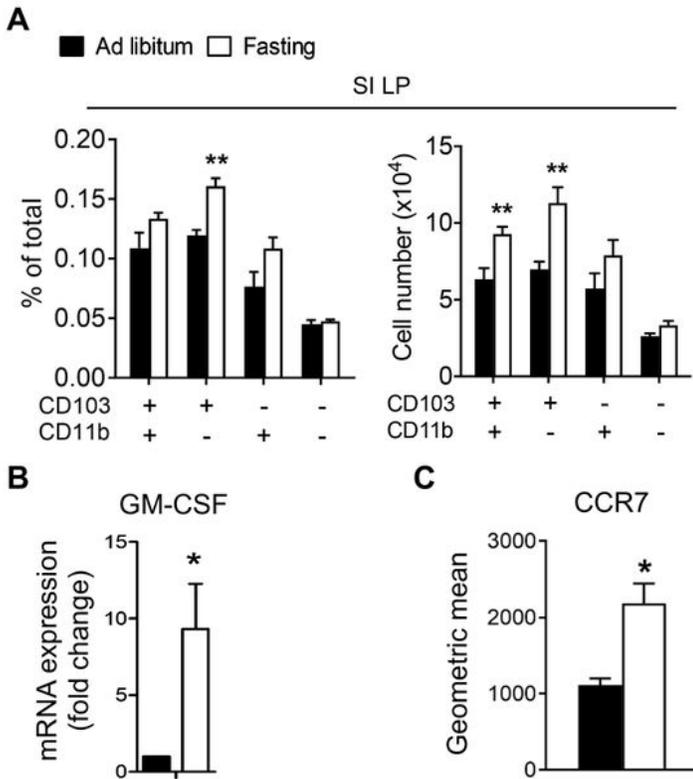


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Figure 4.

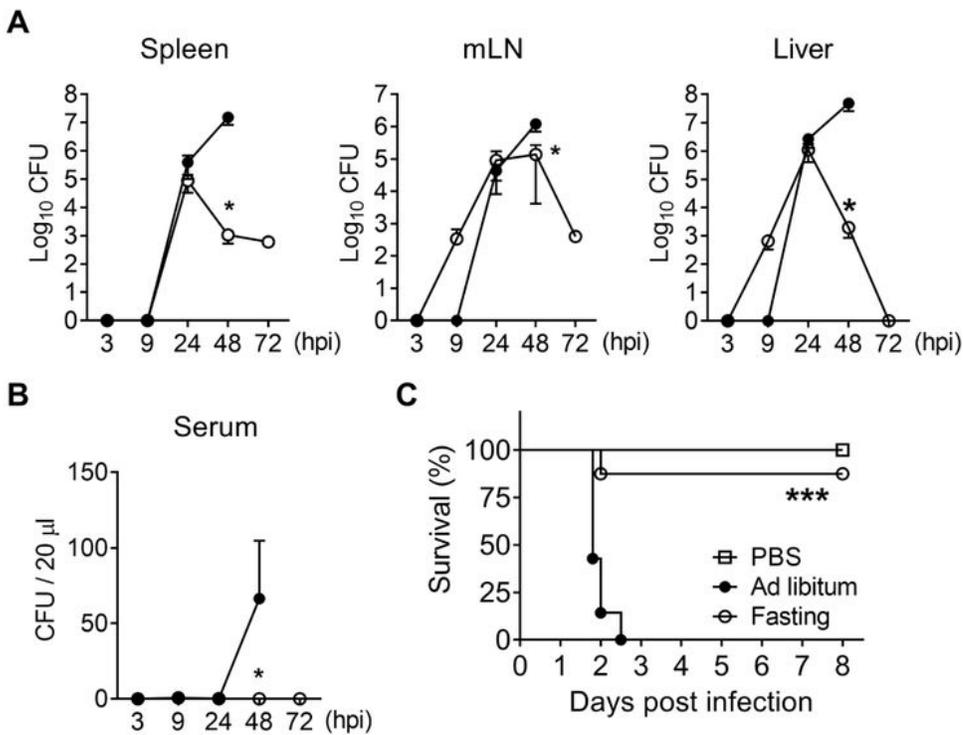


Figure 4

Bacterial burden and survival rate in short-term fasting mice infected with *L. monocytogenes*. Mice were fasted for 24 h and then infected with *L. monocytogenes*. (A-B) Colony forming unit (CFU) was measured in (A) peripheral organs (spleen, mLN, and liver) and (B) serum at Lm-OVA 0, 3, 9, 24, 48 and 72 hours post infection (hpi). (C) Survival rate was monitored for 8 days. The statistics for survival test was

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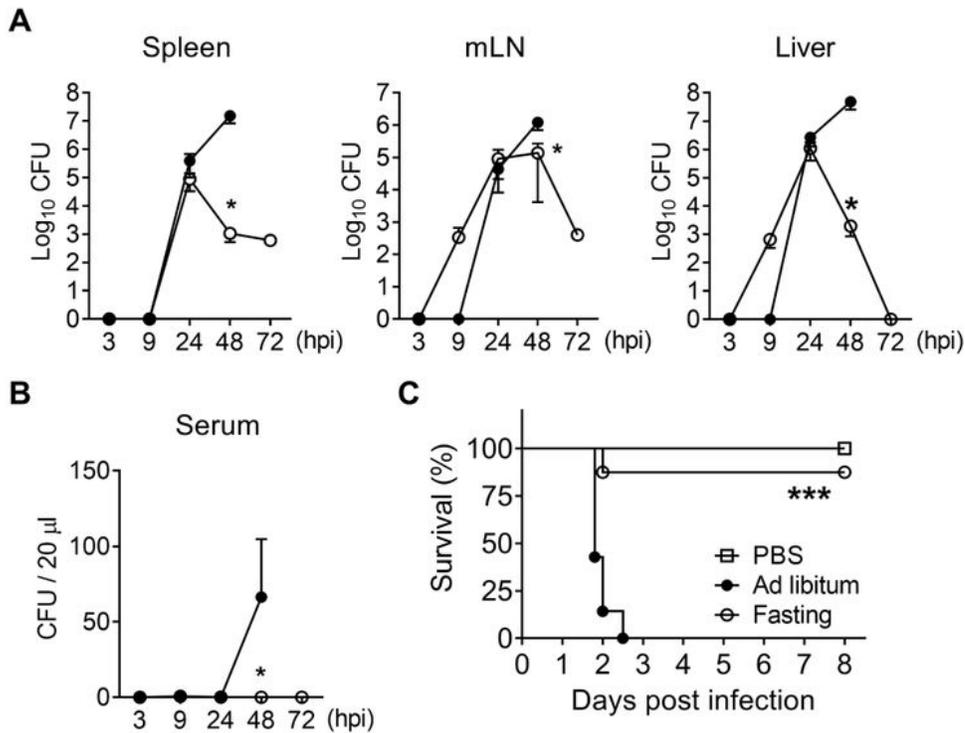


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Figure 5.

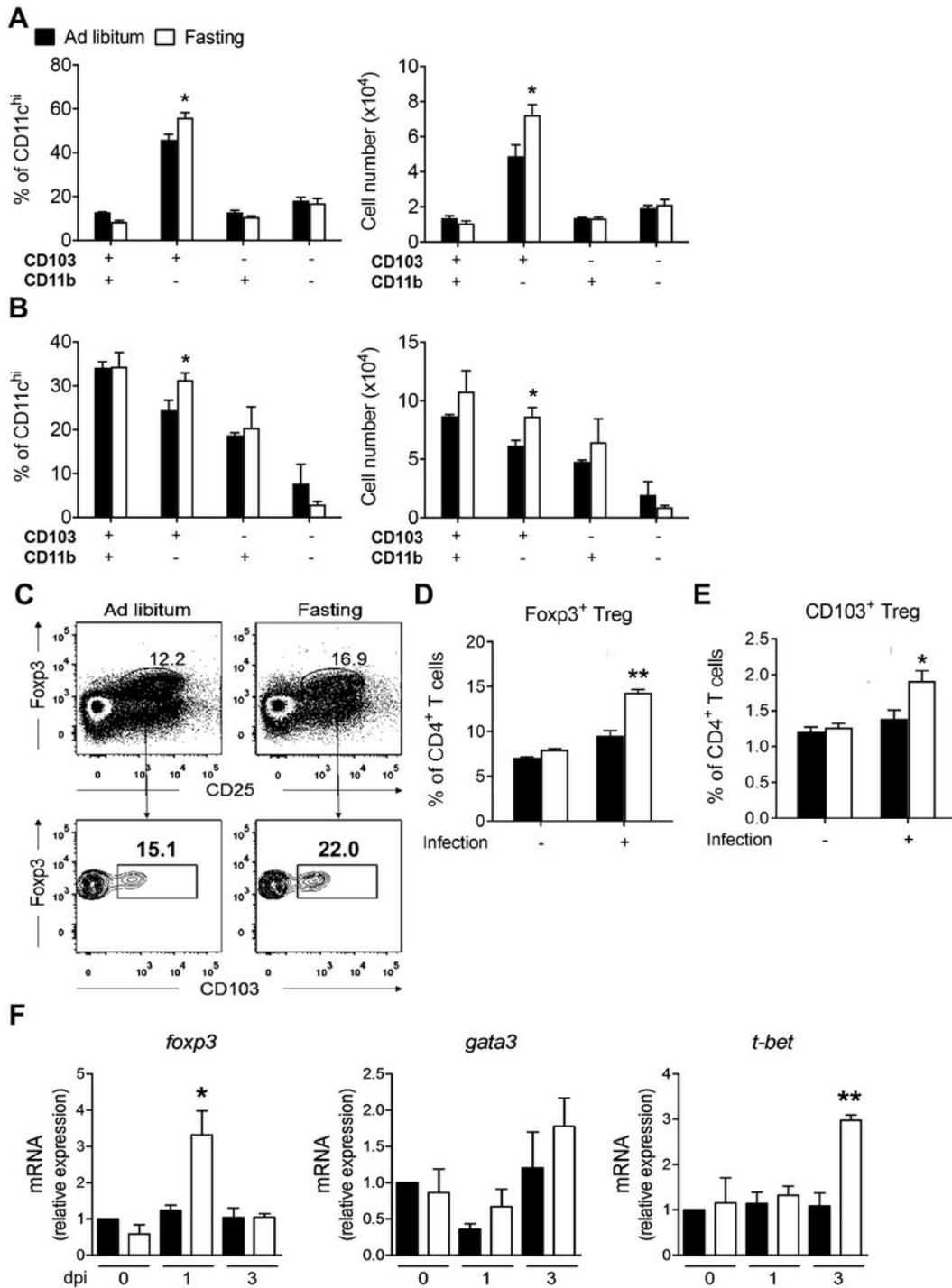


Figure 5

Induction of CD103⁺CD11b⁻ DCs and Foxp3⁺ Tregs in mice with short-term fasting followed by *L. monocytogenes* infection. Mice were fasted for 24 h and then infected with *L. monocytogenes*. (A-B) The

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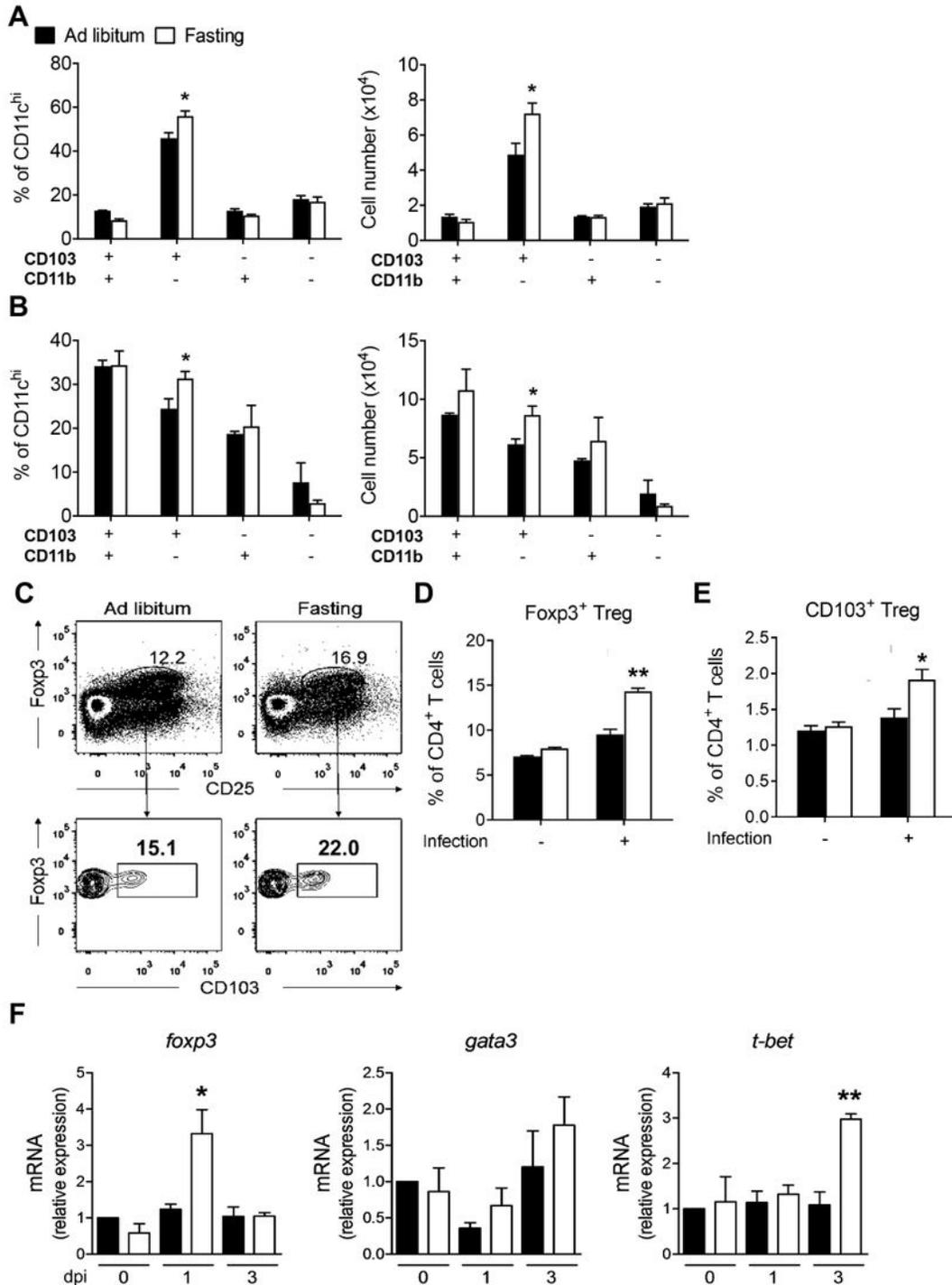


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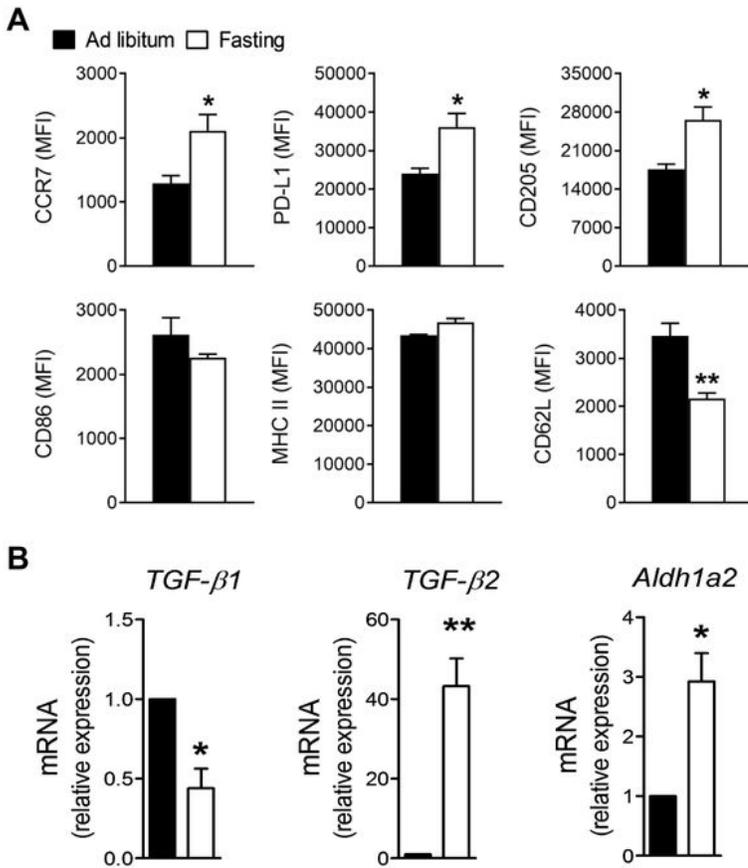


Figure 6

Tolerogenic characteristics of CD103⁺ DCs in mice with short-term fasting followed by *L. monocytogenes* infection. Mice were fasted for 24 h and then infected with *L. monocytogenes*. (A) Expression of CCR7, PD-L1, CD205, CD86, MHC II, and CD62L was examined in CD103⁺ DCs. (B) The mRNA expression of TGF-β1, TGF-β2 and aldehyde dehydrogenase 2 (*aldh1a2*) was measured in CD103⁺ DCs. Statistical

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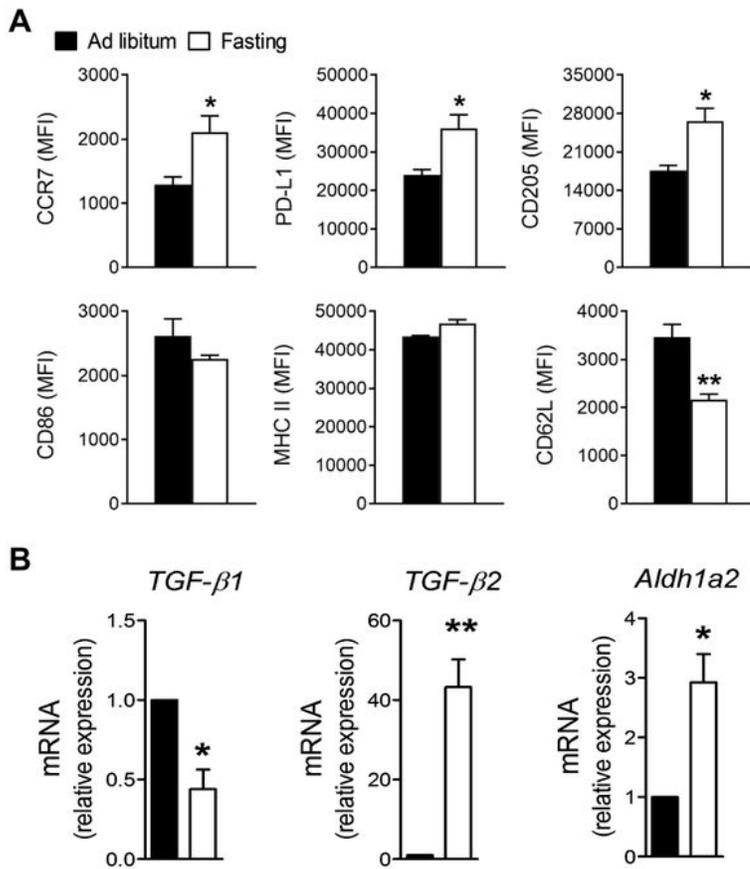


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Figure 7.

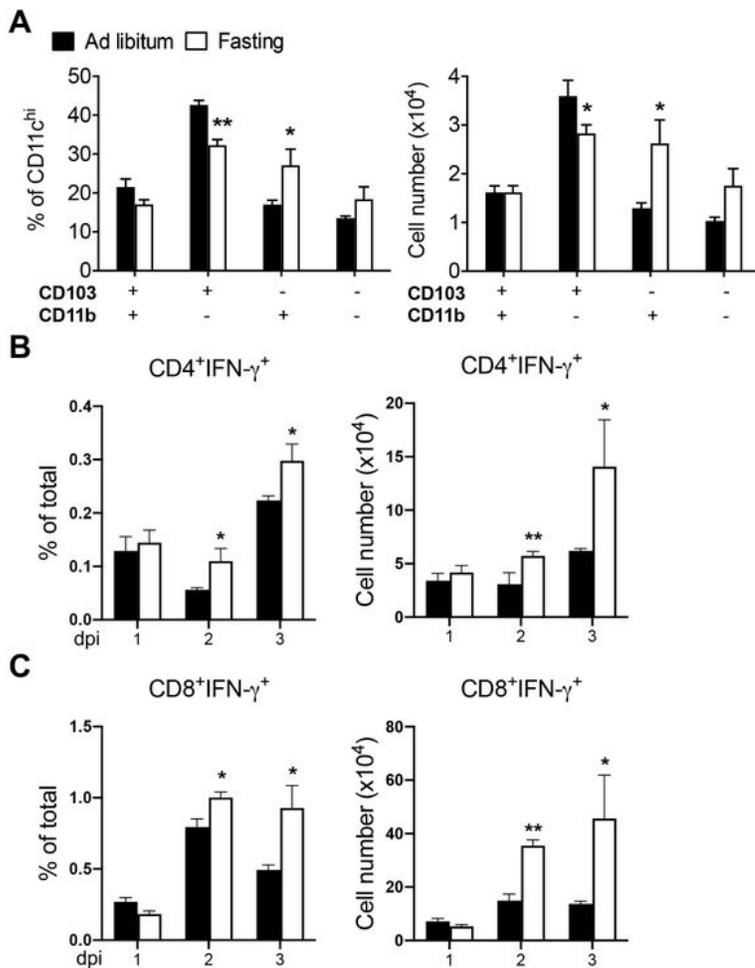


Figure 7

Composition of IFN- γ ⁺ cells among CD4⁺ T cells and CD8⁺ T cells in short-term fasting mice infected with *L. monocytogenes*. Mice were fasted for 24 h and then infected with *L. monocytogenes*. (A) The

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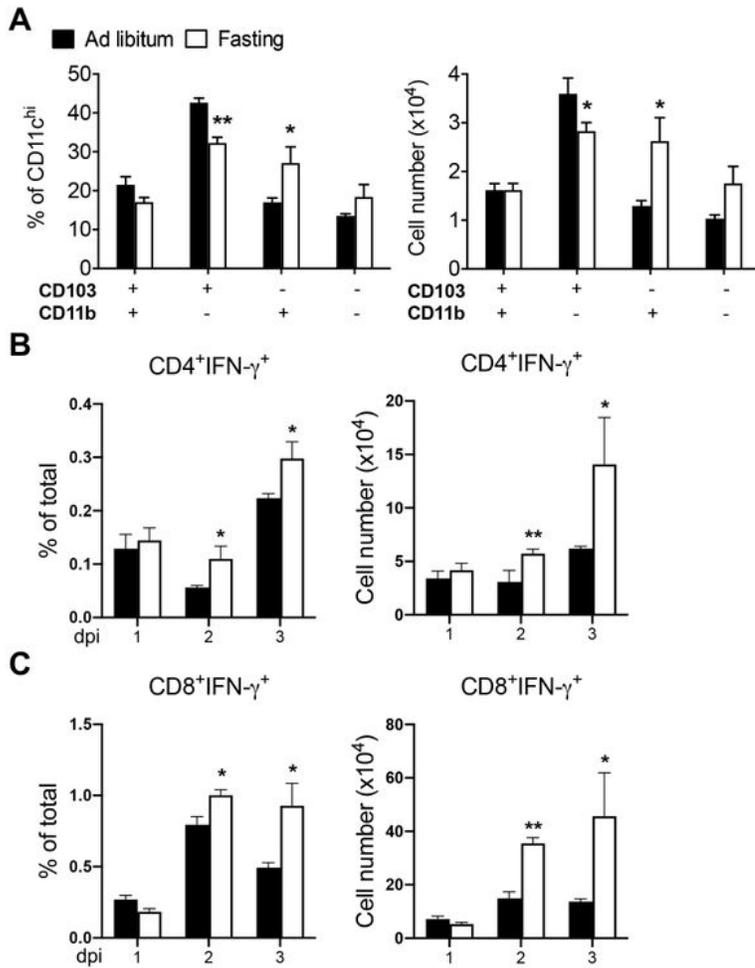


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