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Intestinal CD103$^+$ dendritic cells induced by short-term fasting are essential for the protection against *Listeria monocytogenes* infection

단기 절식으로 유도된 장내 CD103$^+$ 수지상 세포의 리스테리아 감염 방어

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농 학 석 사 학 위 논 문

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지도교수 윤철희
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2015년 02월

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Summary

Despite the fact that gastrointestinal tract is the largest organ producing and consuming a great amount of energy and the first organ to be directly affected after the fasting, very little is known about how fasting influences intestinal immune cells. Innate immune cells in gastrointestinal tract play a critical role as an initial sensor of antigens and inducer of proper immune response. In the current study, I focused on the changes of intestinal innate cells, especially $CD103^+$ dendritic cells (DCs), in mice upon 24 hr short-term fasting and how the fasting influences the protection against intragastric *Listeria monocytogenes (L. monocytogenes)* infection. The results showed that the mice with short-term fasting increased the number of $CD103^+CD11b^-$ DCs in both small intestinal lamina propria (SI LP) and mesenteric lymph nodes (mLN) after either fasting or fasting followed by infection. Induction of SI LP $CD103^+CD11b^-$ DCs during short-term fasting was caused by active proliferation, but this phenomenon was confined only in SI LP. Furthermore, the expression of CCR7, PD-L1 and CD205 was up-regulated on $CD103^+CD11b^-$ DCs in mice which had been short-term fasting and infected with *L. monocytogenes*. Surprisingly, short-term fasting increased the survival rate compared to control (*ad libitum*) mice when infected with *L. monocytogenes*. At early time points post infection (pi; 3, 9 and 24 hr), there was no significant difference in bacterial clearance; strikingly, at 48 hr pi, however, bacterial clearance was significantly increased in spleen, liver and mLN from starved mice compared to control mice, as measured by colony forming units (CFU) of *L. monocytogenes*. Mechanistically, at the early time points pi, the increase of $CD103^+CD11b^-$ DCs after fasting induced significantly high $Foxp3^+$ Tregs

in mLN, which was in line with increased mRNA level of *TGF-β2* and aldehyde dehydrogenase A2 (*aldh1a2*). Curiously, at 3 days pi, the composition of $CD11c^{hi}$ DCs was entirely altered toward the expansion of $CD103^-$ DCs, with induction of IFN-γ^+ NK cells and $CD4^+$ and $CD8^+$ T cells in mLN.

The present study suggests that short-term fasting might induce the tolerogenic condition in the small intestine through increased $CD103^+CD11b^-$ DCs. Accordingly, at day 1 pi, $Foxp3^+$ Tregs were significantly increased. However, at day 2 pi, the *L. monocytogenes* bacterial burden was significantly reduced and at the same time, $CD103^-$ DCs and IFN-γ^+ cells were prominently increased in mLN. Collectively, the results showed that the constitution of intestinal $CD11c^{hi}$ DCs would be a key player for the maintenance of gut homeostasis and/or induction of immunity.

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List of Abbreviations

Aldh1a2	Aldehyde dehydrogenase 1 family, member A2
BM	Bone marrow
BrdU	5-bromo-2'-deoxyuridine
CCR7	C-C chemokine receptor type 7
CCR9	C-C chemokine receptor type 9
CD	Cluster of differentiation
CFU	Colony forming units
DC	Dendritic cells
Foxp3	Forkhead box P3
GALT	Gut-associated lymphoid tissues
Gata3	Trans-acting T-cell-specific transcription factor GATA-3
GM-CSF	Granulocyte macrophage colony-stimulating factor
Hpi	Hours post infection
IEC	Intestinal epithelial cells
IEL	Intraepithelial lymphocytes
IFN- γ	Interferon-gamma
I.g.	Intragastric
I.p.	Intraperitoneal
IL-17	Interleukin-17
L.monocytogenes	*Listeria monocytogenes*
Lm-OVA	*Listeria monocytogenes* expressing OVA
MFI	Mean of fluorescence intensity
MHC class II	Major histocompatibility complex class II
mLN	Mesenteric lymph nodes
NK cell	Natural killer cell
OVA	Ovalbumin
PD-L1	Programmed death-ligand 1
Pi	Post infection
SI IEL	Small intestinal intraepithelial lymphocyte
SI LP	Small intestinal lamina propria
T-bet	T-box transcription factor TBX21
TGF-β	Transforming growth factor-β
Treg	Regulatory T cells
RA	Retinoic acid

I. Introduction

Periodic fasting could extend lifespan of bacteria, yeast, worms and mice compared to their *ad libitum* counterpart group (Fontana et al., 2010). Moreover, it has been suggested that intermittent fasting protects mice from diabetes, cancers and neurodegeneration (Longo and Mattson, 2014) and that fasting induced resistance to cellular stresses after chemotherapy by up-regulation of the growth of cancer cells due to reduction of insulin-like growth factor 1 (Fontana et al., 2010). This is caused by decreased growth hormone (Al-Regaiey et al., 2005, Brown-Borg and Bartke, 2012) followed by reduced or no protein intake (Meynet and Ricci, 2014, Fontana et al., 2008). Furthermore, fasting increased survival rate after the transplantation of kidney and liver, and the ischemia-reperfusion injury (Mitchell et al., 2010). Mice which had been fasting for 24 ~ 72 hrs before *L. monocytogenes* infection reduced bacteria burden and prolonged host survival time (Wing and Young, 1980). In this study, peritoneal macrophages appeared to play a role in the inhibition of DNA synthesis of tumor cells, though the exact mechanism was not elucidated.

It has previously been postulated that at nutritional depletion during the fasting even for short time period would reduce total number of cells in the lymphoid organs such as bone marrow and thymus (Shushimita et al., 2014). Furthermore, it is obvious that gastric and intestinal tract is the first organ to be directly affected after the fasting. In fact, after 12 hr fasting, proteins related to metabolism including glycolysis were decreased in intestine and at 24 hr of fasting, overall protein synthesis were reduced. Interestingly, however, proteins which are involved in cellular protection such as preservation of intestinal integrity were significantly increased at the same time (Lenaerts et al., 2006). It has been suggested that nutritional depletion that can induce the changes in hormones (Ahima et al., 1996) modulates the function of immune cells. For example, leptin can metabolically license T cells (Saucillo

et al., 2014, La Cava and Matarese, 2004) and regulate the maturation of dendritic cells (DCs) (Mattioli et al., 2009, Moraes-Vieira et al., 2014).

Innate immune cells in gastrointestinal tract are critical components playing an important role as initial sensor and inducer of proper immune responses. In intestinal lamina propria (LP), the majority of DCs express $CD103^+$ (Persson et al., 2013), which regulate homeostasis between inflammation and immune tolerance (Varol et al., 2009). In particular, $CD103^+$ DCs in LP serve to capture antigens, including apoptotic epithelial cells (Huang et al., 2000) or bacterial antigens (Farache et al., 2013), and migrate into mLN in a CCR7-dependent manner (Jang et al., 2006, Forster et al., 2008). Moreover, LP $CD103^+$ DCs present the antigens to specific naïve $CD4^+$ T cells and, together with transforming growth factor β (TGF-β) (Coombes et al., 2007, Sun et al., 2007) and retinoic acid (RA) (Hill et al., 2008), induce T regulatory (Treg) cell differentiation. $CD103^+$ DCs are divided into mainly two subtypes depending on the expression of CD11b and CD8α. $CD103^+CD11b^-CD8\alpha^+$ DCs are specialized for the cross-presentation of cell-associated antigens and prime $CD8^+$ T cells, with superior to other subtypes (Cerovic et al., 2014). By contrast, $CD103^+CD11b^+$ DCs (mostly $CD8\alpha^-$) in the intestine could induce immune tolerance in steady state; curiously, however, these cells are prone to induce Th1 responses under inflammatory condition. Likewise, a minor population of intestinal $CD103^-$ DCs is known to efficiently prime naïve T cells and preferentially induce differentiation to IL-17 producing effector T cells (Cerovic et al., 2013). In case of infection, $CD103^-CD11b^+$ DCs stimulate naïve $CD4^+$ T cells to differentiate into IFN-γ^+ cells.

In the present study we showed that $CD103^+CD11b^-$ DCs in mLN and SI LP were increased by active cellular proliferation after the fasting. Importantly, mice with a short-term fasting increased a survival rate compared to *ad libitum* group upon *L. monocytogenes* infection.

II. Materials and methods

Animals

Female Balb/c mice, 6 weeks old, were purchased from Orient Bio Inc., Korea. All experimental procedures in relation to mouse were approved by Institutional Animal Care and Use Committee (IACUC) at Seoul National University (Approval number: SNU-130510-4-1), Korea.

Short-term fasting

Mice were divided into two groups; Mice were fed *ad libitum* and fasted, when needed, for 24 hrs with water provided. In order for the mice to avoid eating their own feces during fasting, the mice were transferred into new bedding cages when the fasting was started.

Bacteria preparation

Recombinant *Listeria monocytogenes* expressing ovalbumin (Lm-OVA) and its parental 10403s strain (Shen et al., 1995, Foulds et al., 2002) were kindly provided by Dr. Hao Shen (University of Pennsylvania, Philadelphia, PA, USA). Bacteria were cultured with brain heart infusion media for 8 hrs at 140 rpm on a shaking incubator at 30°C. They were harvested by centrifugation and thoroughly washed twice with PBS. Bacteria count was estimated by measuring optical density at 600 nm as previously described (Koch, 1970). The number of bacteria administered to the mice was validated by colony forming unit (CFU) counting through a serial dilution and plating.

Infection study

For survival test, 1 x 10^8 or 1 x 10^9 CFU of Lm-OVA or wild type *L. monocytogenes* (10403s)

in 200 μl of PBS were intragastrically (i.g.) administered. Otherwise stated, all experiments were i.g. administrated with 1 x 10^8 Lm-OVA at 24 hrs after the fasting.

Determination of bacteria burden

To determine bacteria 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 *Listeria* in the blood, at least 200 μl of blood was collected by eye-bleeding and centrifuged at 8,000 rpm for 10 min to separate serum and cells. Serial diluents were plated on brain heart infusion agar plate for 12 to 16 hrs 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. Then, 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) containing 5% fetal bovine serum (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 mins at 37°C and filtered through a sieve. The supernatant, containing mucus, intestinal epithelial cells and intraepithelial lymphocytes (IEL), was discarded and vigorously flushed twice with pre-warmed PBS. Tissues were collected and incubated with 20 ml of the digestion solution for discarding mucus layer and intestinal epithelial cells. The remained cells were washed with pre-warmed PBS one more time. The remaining fractions of small intestinal lamina propria (SI LP) were chopped with scissors thoroughly and digested by stirring with RPMI media containing 2% FBS, 0.5 mg/ml of collagenase type VIII (Sigma-Aldrich) and 40 μg/ml of DNase I (Roche, Indianapolis, IN, USA) for 30 mins at 37°C. LP suspensions were filtered

through a 70-μm filter and washed with RPMI.

BrdU incorporation

During short-term fasting, mice were injected intraperitoneally (i.p.) with 1 mg of BrdU dissolved in distilled PBS. After 12 hrs, single cells were produced from SI LP and mLN. After cell surface staining, cells were washed thoroughly. To fix and permeabilization, the stained cells were suspended in 100 μl of cytofix/cytoperm buffer (BD Biosciences, San Jose, CA, USA), incubated for 20 mins at room temperature (RT) and washed with 1 x BD perm/wash buffer. Suspending cells in 100 μl of BD perm/wash buffer plus (BD Biosciences) and incubated for 10 mins 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 μl of the buffer, suspended 1 x 10^6 of cells with 30 μg of DNase solution were diluted in distilled PBS and incubated for 1 hr at 37°C. The cells were suspended in 50 μl of 1 x BD perm/wash buffer containing fluorescent anti-BrdU-FITC. The cells were incubated for 20 mins at RT, washed with 1 x BD perm/wash buffer and analyzed by using flow cytometry.

Antibodies

Fluochrome-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), -I-Ad APC (AM5-32.1), -CD25 PE-Cy7 (PC61), -CD62L BV605 or APC-Cy7, -CD3e FITC (145-2C11), -PD-1 PE (J43), -CD80 PE (16-10A1), -CD86 PE (GL1) and -CD44 APC-Cy7 (1M7) 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)

Flow cytometry

For surface molecule staining, the cells were stained with antibodies as described above and diluted in 50 µl of PBS for 20 mins at 4°C in the dark. The cells were washed 2 times with cold PBS and transferred into the tubes with 250 µl of 2% FBS in PBS. For Foxp3 intracellular staining, the cells were fixed with 100 µl of Foxp3 fixation buffer (BD Biosciences). Then, the cells were incubated for 30 mins at 4°C in the dark and washed with 150 µl of freshly prepared pre-warmed Foxp3 permeabilization buffer (BD Biosciences). To permeabilize the cells, the pellet re-suspended in 100 µl of pre-warmed Foxp3 fixation buffer was incubated for 30 mins at 37°C in the dark. The cells were washed and incubated with anti-Foxp3 Alexa647 antibody (MF23) (BD Biosciences) for 20 mins at RT in the dark. After the staining, the cells were washed with 200 µl of PBS twice. Flow cytometric analysis was performed using FACS Canto II (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR, CA, USA). Cell sorting was performed using FACS Aria (BD Biosciences).

Intracellular staining

Isolated single cells were seeded at 2 x 10^5 / 200 µl in 96 well U-bottom culture plates 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 hrs at 37°C. After the cell surface staining, cells suspended with 100 µl of BD perm/wash buffer were stained with anti-IFNγ-PE and -IL-17A-PerCP-Cy5.5 antibodies (BD Biosciences) for 20 mins at 4°C in the dark. Then, the cells were washed with 1 x BD perm/wash buffer and analyzed by using flow cytometry.

Antigen uptake assay

After the separation of mLN $CD11c^+$ cells by using MACS bead isolation kit (MACS miltenyi Biotec, Germany) followed by sorting out $CD11c^{hi}CD24^+$ cells through flow cytometry, 1 x 10^6 cells were stimulated with 200 ng/ml of OVA-alexa fluor 488 (Life technologies, Carlsbad, CA, USA) in 1 ml of RPMI media containing 10% FBS, 1% antibiotics, 1% sodium pyruvate, 2% HEPES, 50 µM 2-Mercaptoethanol and 0.2% Gentamycin (all from Gibco, Grand Island, NY, USA) for 60 mins at 37°C. For normalization, the cells were stimulated with OVA-Alexa488 at 4°C with the same setting.

cDNA synthesis

One day post Lm-OVA infection followed by fasting, single cells produced from mLN were counted and treated with 1 ml of TRIZOL (Invitrogen, Carlsbad, CA, USA) per 1 x 10^6 cells. To compare the expression level of transcription factors in mLN, total RNA was produced by the addition of 200 µl of chloroform followed by centrifugation at 4°C, 12,000 x *g* for 17 mins. Then, 500 µl of isopropanol was added for 10 mins at room temperature for RNA precipitation and centrifuged at 4°C, 12,000 x *g* for 12 mins. RNA pellet was obtained after washing with 75% ethanol and air dried for 10-15 mins. Pellets were suspended with diethyl pyrocarbonate (DEPC) water (Sigma-Aldrich) and quantified with NanoDrop (Amersham Biosciences, USA) at A_{260}/A_{280}.

Real time quantitative 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 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 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. For multiple group comparison, one-way ANOVA was used. All statistical analysis was performed using Prism (version 5.01).

III. Results

1) Mouse with short-term fasting shows increased leukocyte numbers in mLN and SI LP

Gastrointestinal tract consumes enormous amount of energy and therefore could be directly affected during the fasting but little is known about how fasting influences the intestinal immune system. In the present study, we focused how short-term fasting affects the gut-associated lymphoid tissues (GALT). After 24 hr of fasting in mice, total number of splenocytes was decreased (data not shown) as expected. Surprisingly, however, the fasting significantly increased total number of $CD45^+$ leukocytes in mLN and SI LP while no difference was observed in SI IEL when compared to those of *ad libitum* group (Fig. 1).

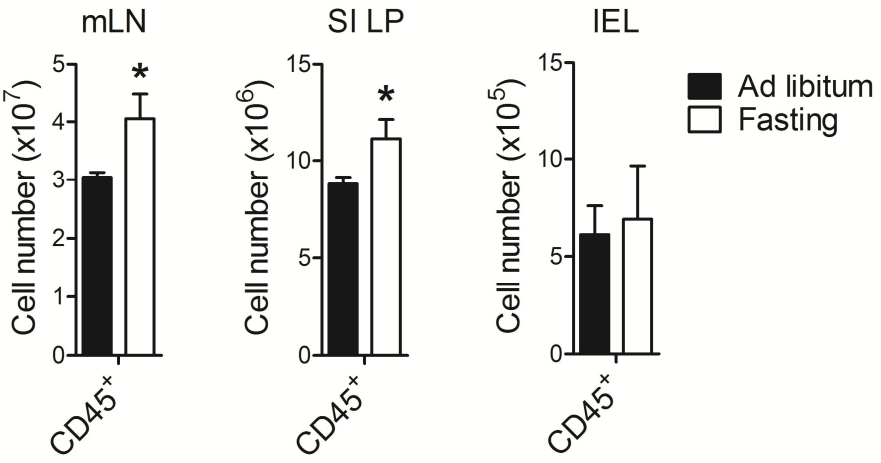


Figure 1. Changes of leukocyte numbers in mLN, SI LP and SI IEL from the mouse fasted for short-term. Balb/c mice were starved for 24 hrs and single cells produced in each organ were stained with anti-CD45 antibodies to examine the number of leukocytes in mLN, SI LP and small intestinal intraepithelial lymphocytes (SI IEL). Statistics were performed by using Student's t test. All values are mean ± S.D. of at least four replicates. * indicates a significant difference at $p<0.05$.

2) $CD11c^{hi}$ DCs are increased in SI LP in mouse after short-term fasting

In order to clarify further which cell types are increased after the short-term fasting, phenotypic analysis of immune cells was performed in mLN and SI LP. After fasting, the mice showed a significant increase in a number of $CD11c^{hi}$ cells both in mLN (Fig. 2A) and to a much larger extent in SI LP (Fig. 2B) compared to those of *ad libitum* control mice. No differences in the percentage and number of $CD8^+$, $CD4^+$ and γδ T cells (Fig. 2C) and B cells (data not shown) in mLN were found. Curiously, neutrophils and macrophages were reduced in the fasted mice compared to control mice (supplementary Fig. 1). Interestingly, in the SI LP, however, the number of $CD4^+$ T cells increased (Fig. 2D). Taken together, the result showed that short-term fasting leads to the significant changes in the intestinal immune cell compartment, largely with $CD11c^{hi}$ cells coincident with a little $CD4^+$ T cells being increased.

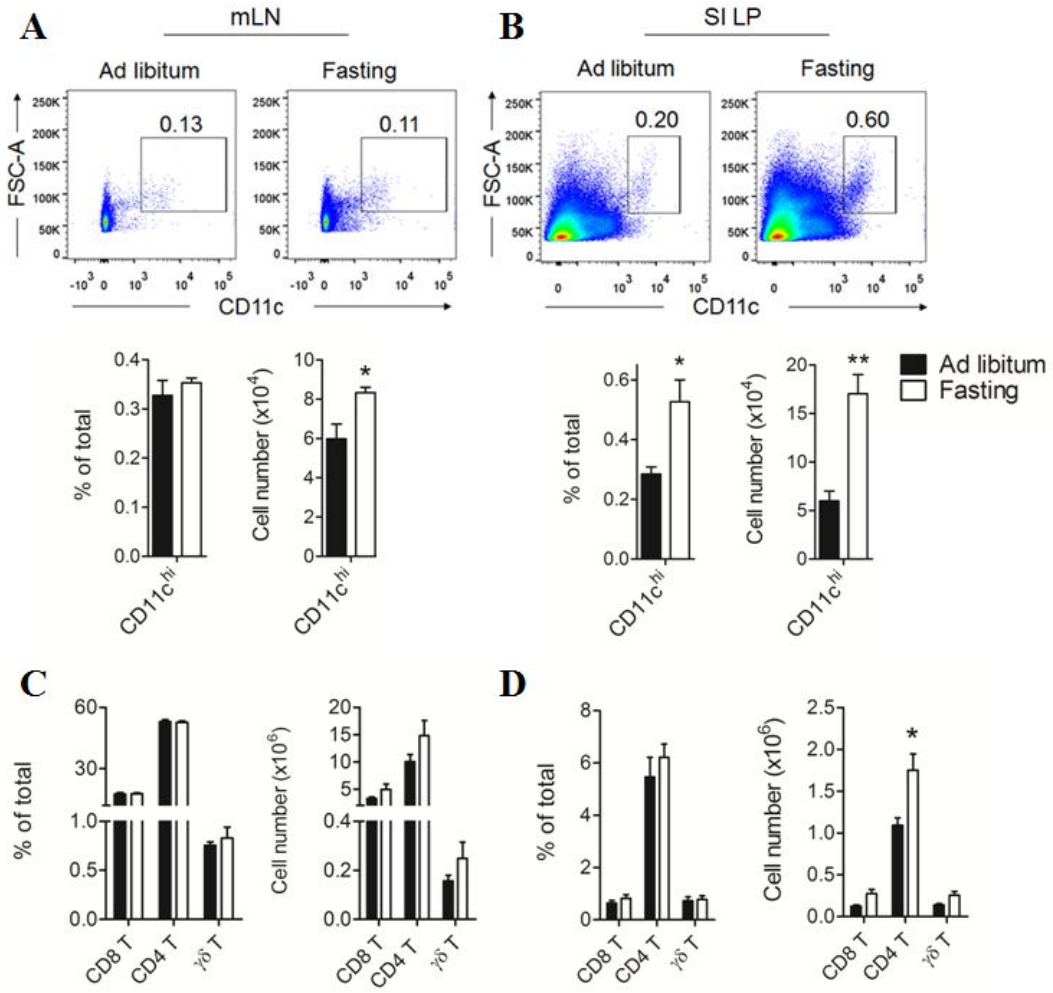


Figure 2. Composition of CD11c^{hi} cells in SI LP and mLN after short-term fasting. Balb/c mice were starved for 24 hrs, and single cells produced from mLN and SI LP were stained with anti-CD11c, -CD3e, -CD4, -CD8 and -γδ TCR antibodies and analysed by flow cytometry. CD11c^{hi} dendritic cells were examined in the (A) mLN and (B) SI LP based on the expression of CD11c and size of cells (upper panel). The percentage of total cells and absolute number of cells (lower panel) was calculated in both (A) mLN and (B) SI LP. The cells were pre-gated on CD3e^{+} cells for CD8^{+}, CD4^{+} and γδT lymphocytes

from (C) mLN and (D) SI LP, and analyzed for the percentage of total cells and absolute number of each cell type. The one representative result out of three separate experiments is shown. All values are mean ± S.D. of five replicates and statistics were calculated by using Student's t test. Significant difference at $p<0.05$ and $p<0.01$ was indicated as * and **, respectively.

3) $CD103^{+}CD11c^{hi}$ DCs are dramatically increased in mLN and SI LP in mice after short-term fasting

Among the intestinal $CD11c^{hi}$ cells, phenotypic subset was analyzed based on the expression of CD103 and CD11b molecules. It is known that the majority of $CD11c^{hi}$ cells in small intestine expresses the integrin αEβ7 referred as CD103 (Cepek et al., 1994). Furthermore, intestinal $CD103^{+}$ DCs play an important role in the maintenance of tolerance against incoming food antigens and commensal bacteria and prevent unnecessary hyper-inflammation by regulating a delicate balance preferentially toward tolerogenic condition (Scott et al., 2011). Intriguingly, the percentage of $CD103^{+}$ DCs in mLN and SI LP was significantly increased in mice after short-term fasting. Especially in mLN, even though there was no difference in the percentage of $CD11c^{hi}$ cells (Fig. 2A), number of $CD103^{+}CD11b^{-}$ DCs was approximately 2 times higher in fasted group than in *ad libitum* group (Fig. 3A). Likewise, in the SI LP, $CD103^{+}CD11b^{-}$ DCs and $CD103^{+}CD11b^{+}$ DCs were all considerably increased in the fasted mice (Fig. 3C).

Further analysis of the increased DC subsets in the fasted mice revealed that $CD103^{+}CD11b^{-}$ DCs in mLN were exclusively $CD8\alpha^{-}$ but were composed both of $CD8\alpha^{-}$ and $CD8\alpha^{+}$ in SI LP (Fig. 3C and D). It was noting that the increased $CD103^{+}CD11b^{+}$ DCs observed in SI LP were also $CD8\alpha^{-}$ (Fig. 3D). Taken together, these results indicate that short-term fasting caused the alteration of $CD11c^{hi}$ cells, with $CD103^{+}CD11b^{-}$ or $CD103^{+}CD11b^{+}$ DCs being sharply increased in mLN and SI LP.

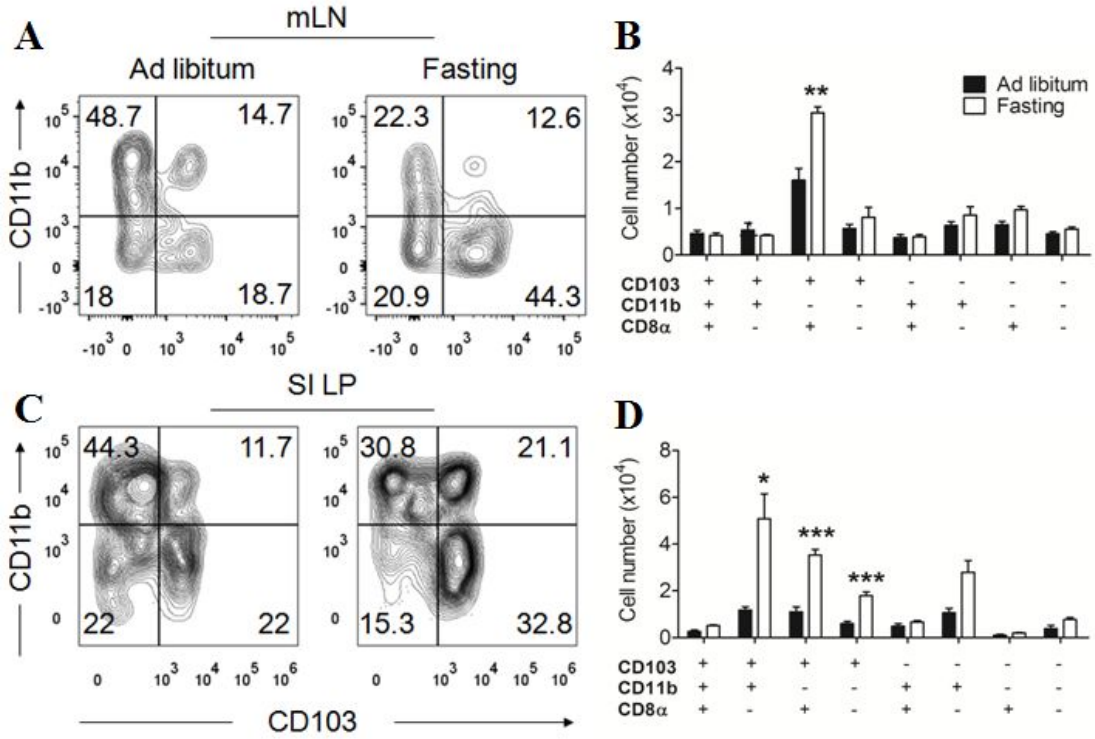


Figure 3. Composition of four subtypes of CD11c^{hi} DCs based on the expression of CD103, CD11b and CD8α in SI LP and mLN after short-term fasting. Balb/c mice were starved for 24 hrs, and single cells produced from mLN and SI LP were stained with anti-CD11c, -CD103, -CD11b and -CD8α and analyzed by flow cytometry. CD11c^{hi} DCs were divided into different subtypes in the (A) mLN and (C) SI LP based on the expression of CD103 and CD11b. (B and D) The CD11c^{hi} cells stained with anti-CD103, -CD11b and -CD8α antibodies were analyzed for the absolute numbers. The one representative result out of three is shown. Values are mean ± S.D. of at least five replicates and statistics was calculated by using Student's t test and all. *, ** and *** indicate a significant differences at $p<0.05$, $p<0.01$ and $p<0.001$, respectively.

4) DCs are actively proliferated in SI LP after short-term fasting.

To address the question of whether increased cellularity in mLN and SI LP was caused by either active cell proliferation at local site or enhanced migration, BrdU labeling assay was performed by injecting mice i.p. with BrdU and monitoring the proliferation of intestinal immune cells for 12 hrs after the fasting. There was no difference in the percentage of $BrdU^+$ cells between fasted and control mice in both mLN (Fig. 4A) and SI LP (Fig. 4C), with T cell compartments being largely unchanged, albeit slight decrease in the percentage, but not absolute number, of $CD8^+$ T cells in SI LP (Fig. 4B, D and E). Interestingly, however, there was a significant increase of BrdU uptake in both $CD103^+CD11b^-$ DCs and $CD103^+CD11b^+$ DCs in the SI LP of fasted mice compared to those of control mice (Fig. 4D, E), which was consistent with increased cellularity of these subsets in the SI LP (Fig. 3C, D).

It is well known that the development of DCs are regulated by GM-CSF (van de Laar et al., 2012), which facilitates the recruitment of intestinal DCs (Hirata et al., 2010). The mRNA level of GM-CSF was significantly increased in the SI LP after the short-term fasting while decreased in BM (Fig. 4F). These results suggested that the short-term fasting increases the number of DC subsets, largely $CD103^+CD11b^-$ DCs and $CD103^+CD11b^+$ DCs in the SI LP, through augmenting cell proliferation due to the increased expression of GM-CSF rather than the migration.

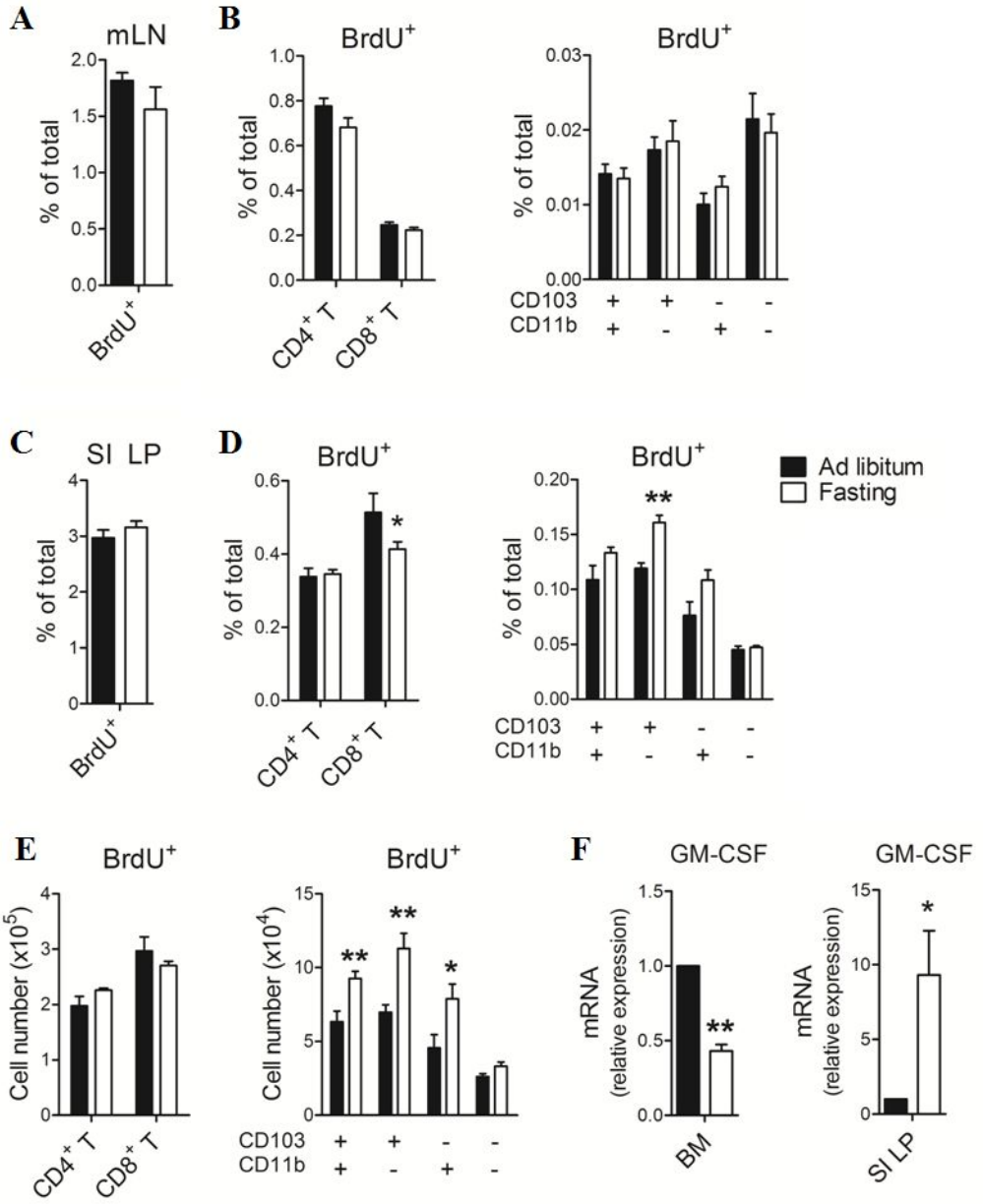


Figure 4. Proliferation of the cells in mLN and SI LP after short-term fasting. Balb/c mice fasted for 12 hrs were received 1 mg of 5-bromo-2'-deoxyuridine (BrdU) solution dissolved in 100 μl of PBS through i.p. route. After 12 hrs, single cells were produced from mLN and SI LP, and stained with anti-CD11c, -CD103, -CD11b, -CD3e, -CD4, and -CD8

antibodies. The composition of $BrdU^+$ cells in $CD45^+$ leukocytes are shown in (A) mLN and (B) SI LP. The percentage of $BrdU^+CD3e^+CD4^+$, $BrdU^+CD3e^+CD8^+$, $BrdU^+CD3e^-CD11c^+CD103^+CD11b^+$, $BrdU^+CD3e^-CD11c^+CD103^+CD11b^-$ cells, $BrdU^+CD3e^-CD11c^+CD103^-CD11b^+$ and $BrdU^+CD3e^-CD11c^+CD103^-$ $CD11b^-$ cells among total $CD45^+$ leukocytes in (B) mLN and (D) SI LP was observed. (E) Absolute numbers of $BrdU^+CD3e^+CD4^+$, $BrdU^+CD3e^+CD8^+$, $BrdU^+CD3e^-CD11c^+CD103^+CD11b^+$, $BrdU^+CD3e^-CD11c^+CD103^+CD11b^-$ cells, $BrdU^+CD3e^-CD11c^+CD103^-CD11b^+$ and $BrdU^+CD3e^-CD11c^+CD103^-CD11b^-$ cells in SI LP were examined. (F) The mRNA expression level of GM-CSF in BM and SI LP taken at 24 hrs fasting. The values were normalized by house-keeping gene *gapdh*. Total mRNA was synthesized into cDNA and real-time PCR was carried out. All statistics were performed by using Student's t test and all values are mean ± S.D. of at least three replicates. * and ** denote a significant differences at $p<0.05$ and $p<0.01$, respectively.

5) Up-regulation of CCR7, PD-L1, CD205 and MHC class II on DCs in mLN after short-term fasting

To examine the characteristics of $CD11c^{hi}$ cells increased after the fasting, expression of various cell surface markers was monitored through flow cytometry. Thus, mean fluorescence intensity (MFI) of CCR7, PD-L1, CD205 (Shrimpton et al., 2009), CD86, MHC class II and CD62L on $CD11c^{hi}$ cells in the mLN between the fasted and control mice was compared. The expression of CCR7, PD-L1, CD205 and MHC class II, but not CD86 and CD62L, was significantly increased on $CD11c^{hi}$ cells in the mLN of fasted mice compared to those in control mice (Fig. 5A)

Next, to examine the ability of soluble antigen-uptake, $CD24^{+}CD11c^{hi}$ cells were sorted from mLN after the short-term fasting and treated OVA-conjugated Alexa 488 *in vitro* for 60 min at 37°C. $CD103^{+}$ $CD11b^{-}$ and $CD103^{-}CD11b^{+}$, but not $CD103^{+}CD11b^{-}$, DCs showed the most prominent antigen-uptake capacity (Fig. 5B); however, this ability was almost identical in the cells from fasted mice as compared to those from control mice, suggesting that the fasting dose not have any impact on antigen-uptake function of DC subsets. Notably, there was a moderate but significant increase in the expression of CD205 on $CD103^{+}CD11b^{+}$ and $CD103^{+}CD11b^{-}$ mLN DCs from fasted mice (Fig. 5C). Collectively, these data suggest that the fasting would affect migratory capacity of DC subsets by upregulating tissue homing receptors and enforce either tolerogenic or immunogenic potential of $CD103^{+}$ DCs in the gut.

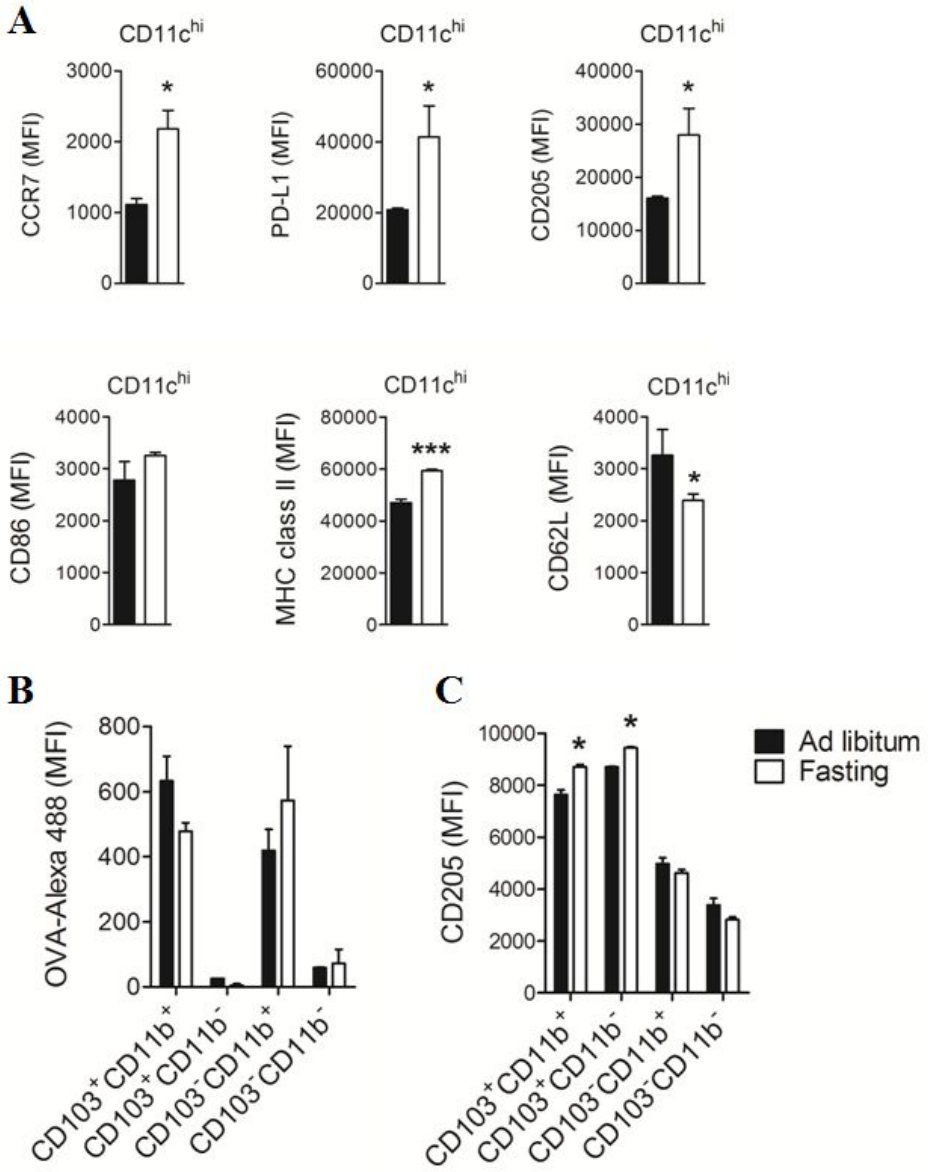


Figure 5. The expression of CD86, CCR7, PD-L1 and CD205 on mLN CD11c^{hi} cells and antigen uptake ability of their subtypes. Balb/c mice were starved for 24 hrs, and single cells produced from mLN were stained with anti-CD11c, -CD103, -CD11b, -CD86, -CCR7, -PD-L1, -CD205, -I-Ad and -CD62L. The cells were analyzed for the expression of

cell surface markers by flow cytometery. (A) MFI values of CCR7, PD-L1, CD205, CD86, MHC class II and CD62L expression on $CD11c^{hi}$ cells from mLN were examined. (B) $CD11c^{+}$ cells from mLNs were separated and stained with anti-CD24, -7AAD and -CD11c antibodies. $CD24^{+}CD11c^{hi}$ cells were sorted by using FACS Aria II. Antigen uptake ability of the cells was examined after the treatment of Alexa 488-ovalbumin (OVA; 200 ng/ml) for 60 min at 37°C. To compensate the value, the cells were incubated at 4°C. Mean of fluorescence intensity (MFI) of OVA was measured by flow cytometry and differences at 37°C and 4°C were regarded as criterion of the antigen uptake. (C) MFI of CD205 on each subtype of $CD11c^{hi}$ cells from mLN after OVA stimulation. All values are mean ± S.D. of three replicates. Significant differences at $p<0.05$ and $p<0.001$ was denoted as * and ***, respectively.

6) Short-term fasting protects mice against intragastric infection with *L. monocytogenes* at the early time point

In order to elucidate the role of DCs increased in mLN on intestinal immunity, mice were infected with *L. monocytogenes* which is known to induce strong systemic Th1 response (Orgun et al., 2008). After short-term fasting, mice were infected with pre-determined lethal dose of 1 x 10^8 *L. monocytogenes* expressing OVA (Lm-OVA) through intragastric route (data not shown). The survival of mice which had been short-term fasting was unexpectedly increased compared to control mice fed *ad libitum* (Fig. 6A, *p value*=0.0003) with no change of body weight (Fig. 6B) after the infection. Similar results were also obtained (supplementary Fig. 2) with wild type strain of *L. monocytogenes* (strain 10403s).

To examine the bacterial clearance, colony forming unit (CFU) of *L. monocytogenes* in spleen, liver and mLNs at 3, 9, 24, 48 and 72 hrs post-infection (hpi). The results showed that CFU in short-term fasted mice was significantly lower in spleen, liver and mLN at 48 hrs than in control mice (Fig. 6C). To measure a symptom of systemic bacteremia caused by *Listeria* infection, CFU in serum was examined. The bacteremia was apparent in mice fed *ad libitum* but not in fasted mice (Fig. 6D). Furthermore, in order to rule out the possibility that fasted mice eat consumed much faster after re-feeding than control mice which may influence the speed of invasion of the pathogen CFU in stomach at 3 hpi was examined and found no difference (Fig. 6E). These data clearly showed that short-term fasting protects mice against gastrointestinal *Listeria* infection.

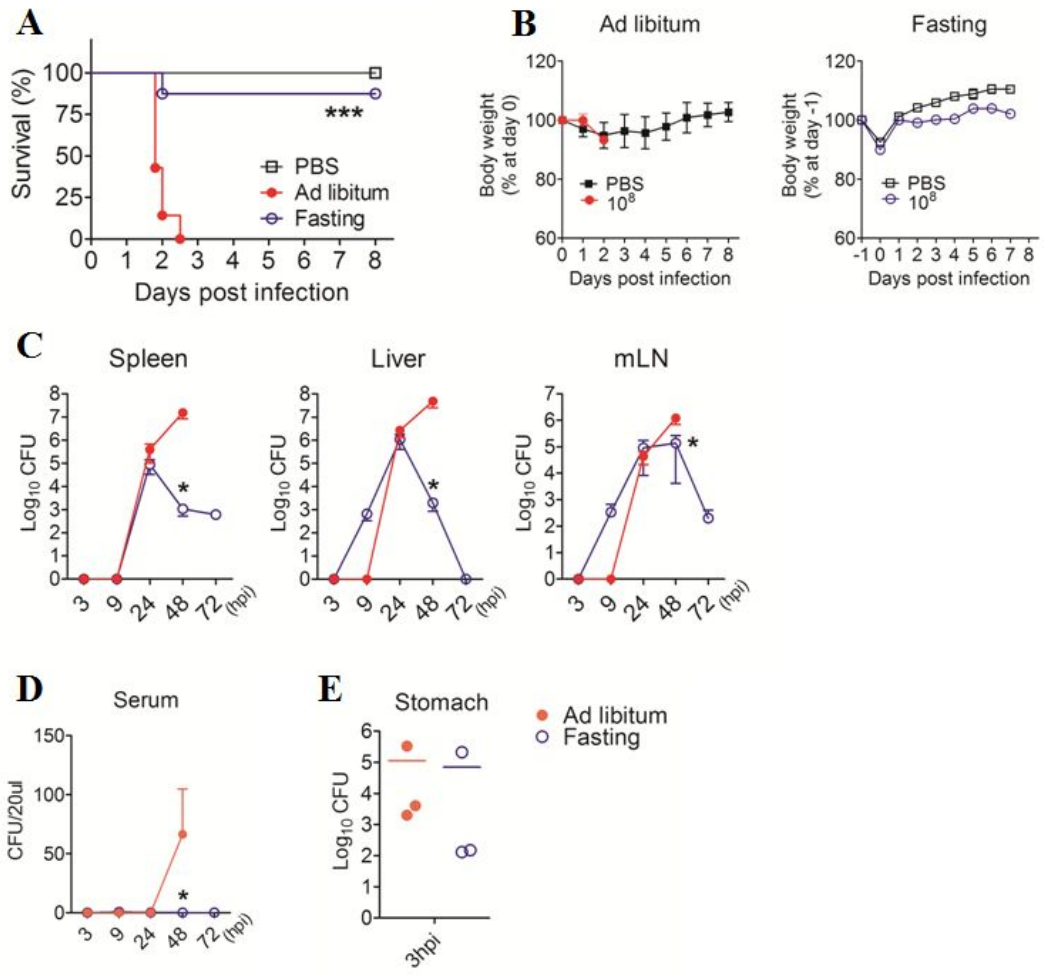


Figure 6. Survival rate, body weight gain and CFU in organs after short-term fasting in mice infected with *Listeria* through intragastric route. Balb/c mice starved for 24 hrs were infected with 1 x 10^8 of Lm-OVA through intragastric administration. (A) Survival and (B) body weight gain were monitored for 8 days. (C, D) Colony forming units (CFU) of Lm-OVA were measured in spleen, liver, mLN and serum at 3, 9, 24, 48 and 72 hours post infection (hpi). (E) Lm-OVA in stomach was measured at 3 hpi. The statistics for survival test was analyzed by log-rank (Mantel-Cox) test and all other experiments by student's t-test.

7) $CD103^+$ DCs and $Foxp3^+$ Tregs are increased in mLN during the early phase of *Listeria* infection after short-term fasting

To examine the potential impact of intestinal $CD103^+CD11b^-$ DCs which was increased after fasting (Fig. 3) on *Listeria* infection, differentiation of $Foxp3^+$ regulatory T cells (Tregs) were examined since it was preferentially induced by $CD103^+CD11b^-$ DCs (Scott et al., 2011). The results in the present study showed that mLN $CD103^+CD11b^-$ DCs were increased after the short-term fasting with or without *Listeria* infection (Fig. 7A). In fact, $CD103^+CD11b^-$ DCs were increased whilst $CD103^+CD11b^+$ DCs decreased in mLN (Fig. 7B). It was intriguing that *Listeria* infection increased $Foxp3^+$ Tregs in both groups but much higher in the fasted group (Fig. 7C), with corresponding increase of activated $CD103^+Foxp3^+$ Tregs (Fig. 7D). This phenomenon was detectable only at day 1 pi but then disappeared at day 2 and 3 pi (supplementary Fig. 3). Consistent with the increased Treg cells, mRNA level of transcription factor *foxp3* at day 1 pi was 3-fold higher in mLN of fasted mice than in that of control mice (Fig. 7E). Notably, mRNA level of *t-bet* was also upregulated but only at day 3 pi. These data suggest that there is a link between the increased $CD103^+CD11b^-$ DCs in mLN after fasting and the induction of $Foxp3^+$ Tregs.

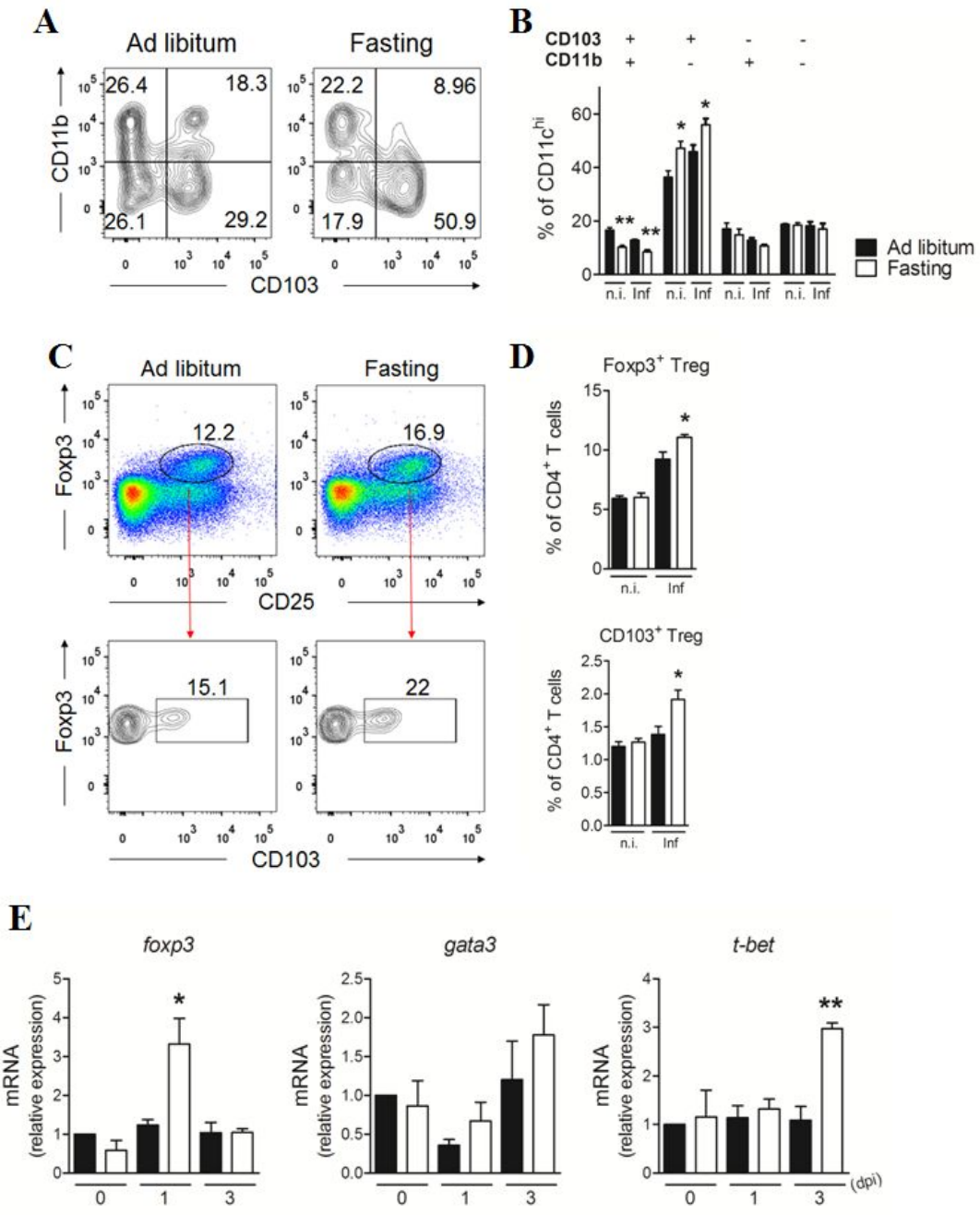


Figure 7. Induction of lymph-borne CD103$^+$CD11b$^-$ DCs and Foxp3$^+$ Tregs in mice with short-term fasting followed by *Listeria* infection. Balb/c mice starved for 24 hrs were

infected with *L. monocytogenes*. (A) The mLN was taken at day 1 pi, suspended single cell was stained with anti-CD11c, -CD11b and -CD103 antibodies, and analyzed by using flow cytometry. The cells gated on $CD11c^{hi}$ were examined based on the expression of CD103 and CD11b. (B) Bar graph indicated proportion of each subgroup among the $CD11c^{hi}$ cells. 'n.i.' and 'Inf' referred as non-infected and infected, respectively. (C, D) $Foxp3^+$ Tregs were analyzed in mLN after day 1 pi showing (C) a dot plot and (D) bar graph on the percentage of $Foxp3^+$ cells among $CD3^+CD4^+$ cells. The percentage of activated $CD103^+$ Tregs among $CD3^+CD4^+$ cells is shown in lower panel. (E) The quantification of the mRNA level of *foxp3, gata3* and *t-bet* in mLN after day 1 and 3 pi. All data were normalized with the expression of house-keeping gene, *gapdh* and the group of non-infected *ad libitum* served as a control.. All values are mean ± S.D. of three replicates Student's t test was used for the statistical analysis. The representative result out of three is shown. * and ** indicate a significant difference at $p<0.05$ and $p<0.01$, respectively.

8) Foxp3$^+$ Tregs are not induced in the peripheral lymphoid organ

To examine the induction of Foxp3$^+$ Tregs in the peripheral lymphoid organ, spleen was taken after the short-term fasting followed by *Listeria* infection. They were not increased in spleen, unlike in mLN, at day 1 (Fig. 8A and B) and 3 pi (data not shown). In addition, there was no difference in the composition of activated CD103$^+$Foxp3$^+$ Tregs (Fig. 8A). Collectively, this result suggest that the increased Foxp3$^+$ Tregs observed in mice after fasting followed by *Listeria* infection was caused by preferential accumulation of inducible Tregs at a local draining site such as mLN, not by a modulation of peripheral lymphoid organ.

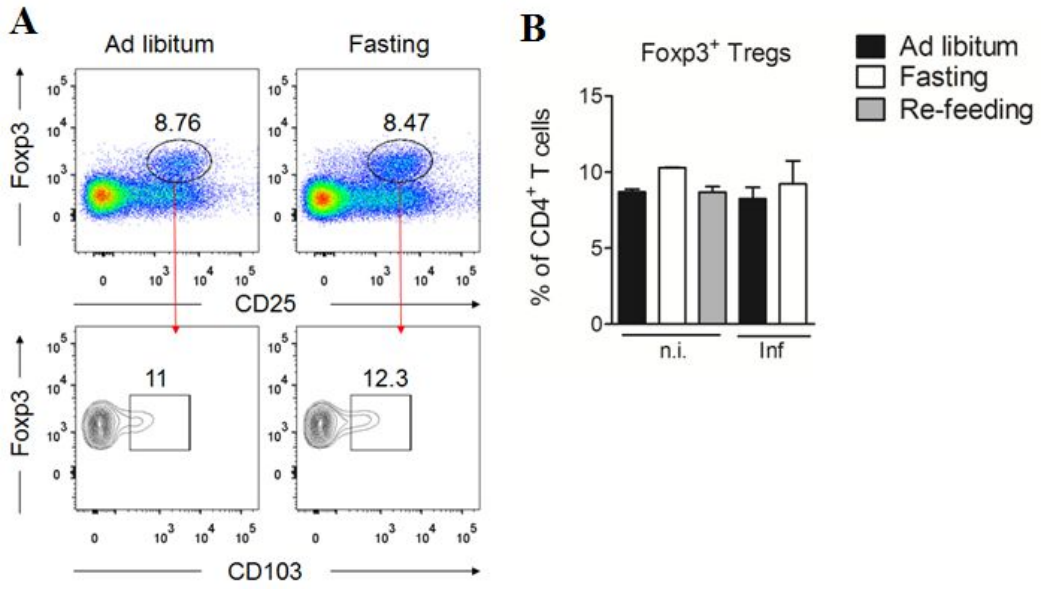


Figure 8. Splenic Foxp3$^+$ Tregs after short-term fasting followed by *Listeria* infection. Balb/c mice were starved for 24 hrs, and single cells derived from spleen were stained with anti-CD3e, -CD4, -CD25, -CD103 antibodies followed by intracellular staining with anti-Foxp3 antibody. (A) The percentage of splenic Foxp3$^+$ Tregs and activated CD103$^+$ Foxp3$^+$ Tregs are shown as a dot plot. (B) The composition of Foxp3$^+$ Tregs among the CD3$^+$CD4$^+$ cells is analyzed by using flow cytometry. 'n.i' and 'Inf' referred as non-infected and infected, respectively. Re-feeding represented the mice had been short-term fasting and followed 1 day re-feeding. Statistics were performed by using Student's t test. All values are mean ± S.D. of three replicates.

9) Down-regulation of CD86 and MHC class II was correlated with increased level of TGF-β and retinoic acids on lymph-borne CD103$^+$ DCs from mice infected *with Listeria* had been fasted for short-term

To see the characteristics of lymph-borne DCs after *Listeria* infection, CCR7 on CD11c^{hi} DCs and each subgroup of DCs from mLN were examined. At day 1 pi, expression of CCR7, PD-L1 and CD205 were increased on CD11c^{hi} cells (Fig. 9A). However, the expression of CD86 and MHC class II were significantly decreased on CD11c^{hi} DCs. Next, major factors in relation to CD103$^+$ DCs that contribute to the induction of Foxp3$^+$ Tregs at day 1 pi (Fig. 7D) were examined. Several reports showed that CD103$^+$ DCs producing TGF-β and RA (Coombes et al., 2007, Sun et al., 2007) are able to induce Foxp3$^+$ Tregs. Thus, mRNA levels of *TGF-β1, TGF-β2* and *aldehyde dehydrogenase A2 (Aldh1a2)*, which is the key enzyme for converting vitamin A to RA, were examined. In agreement with the previous work, CD103$^+$ DCs in mLN from *ad libitum* group produced much higher *TGF-β2* and *Aldh1a2* than CD103$^-$ DCs from same group (supplementary Fig. 4). More importantly, the result showed a significant increase of mRNA transcripts on *TGF-β2* and *Aldh1a2* in mLN CD103$^+$ DCs from fasted mice compared to those from control mice (Fig. 9B). Taken together, in early phase of *Listeria* infection, the activation level of CD11c^{hi} DCs was decreased as the reduction of CD86 and MHC class II level, on the other hand, the tolerogenic characteristics of DCs were increased as induction of transcripts of *TGF-β2* and *Aldh1a2*.

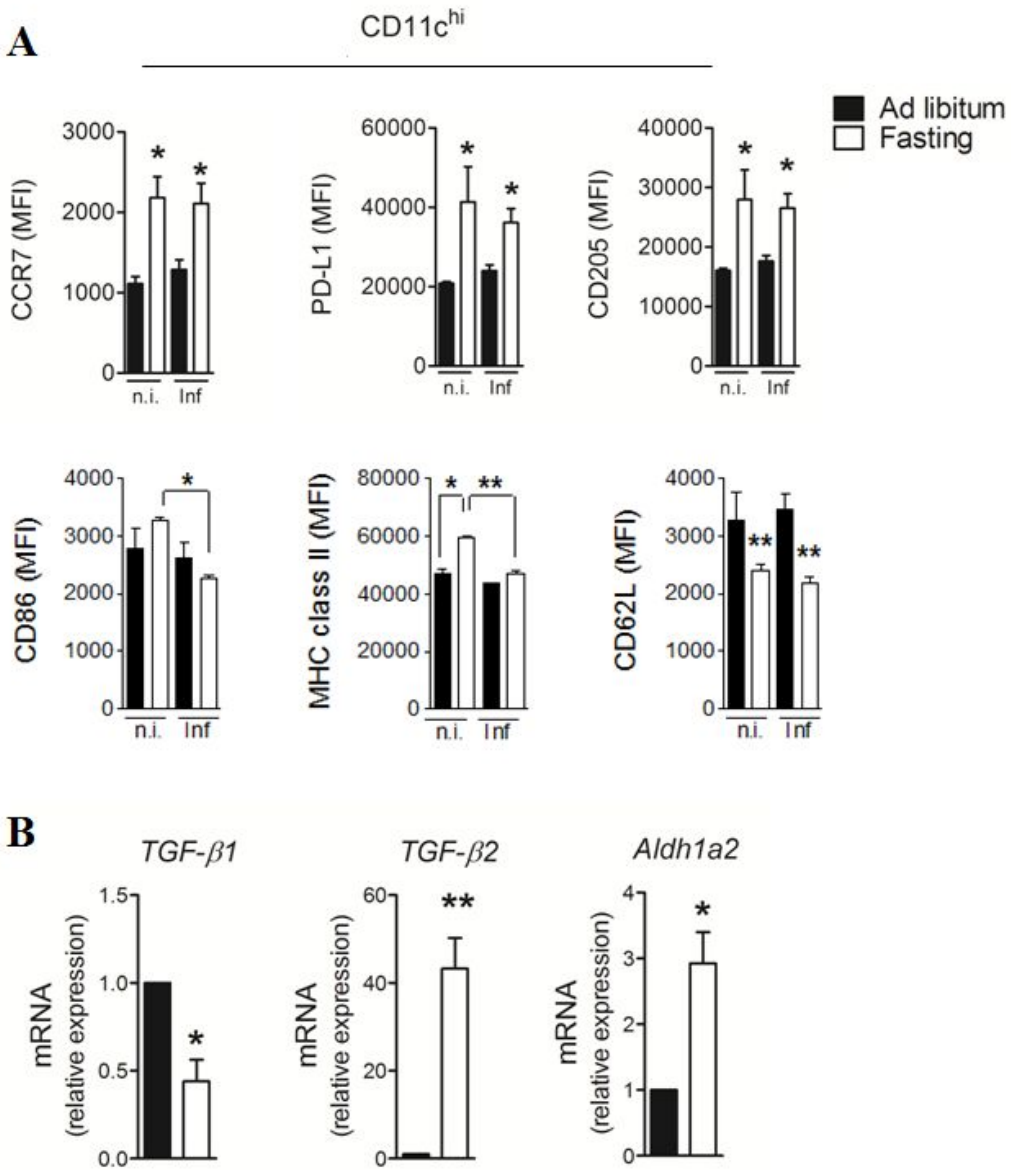


Figure 9. Characteristics of lymph-borne CD103$^+$ DCs in mice with short-term fasting followed by *Listeria* infection. Balb/c mice were starved for 24 hrs followed by *Listeria* infection and single cells were produced from mLN at each indicated time point. Single cells were stained with anti-CD11c, -CD103, -CD11b, -CCR7, -PD-L1, -I-Ad, -CD62L and

–CD205 antibodies and analyzed by flow cytometry. (A) 'n.i.' and 'Inf' referred as non-infected and infected, respectively. At day 1 pi, mean fluorescence intensity (MFI) of CCR7, PD-L1, CD205, CD86, I-Ad and CD62L on $CD11c^{hi}$ DC in mLN. (B) mLNs from 6 mice of each groups were pooled and purified for $CD11c^{+}$ cells through bead separation and $CD103^{+}$ DCs were sorted by using cell sorter. By using real-time PCR mRNA levels of *TGF-β1, TGF-β2* and *aldehyde dehydrogenase 2 (aldh1a2)* were measured. Statistics were performed by using Student's t test. All values are mean ± S.D. of three replicates. * and ** indicate a significant difference at $p<0.05$ and $p<0.01$, respectively.

10) Short-term fasting induced up-regulation of Th1 response in mice infected with *Listeria*

We have found that mLN $CD103^-$ DCs were increased while the $CD103^+$ DCs reduced in fasted mice (Fig. 10A). Based on the reports, in the state of inflammation, $CD103^-$ DCs prefer to induce the differentiation of naïve $CD4^+$ T cells to IFN-γ-producing Th1 cells. Furthermore, IFN-γ is an essential factor to promote a Th1 response for the efficient clearance of the pathogen (Pamer, 2004, Humann and Lenz, 2010). The results showed that both percentage and absolute number of IFN-γ^+ cells among $NK1.1^+CD3e^-$ (Fig. 10B), $CD4^+CD3e^+$ (Fig. 10C) and $CD8^+CD3e^+$ cells (Fig. 10D) were dramatically increased at day 2 and 3 pi in mLN of fasted mice, which was in strong agreement with the increased level of T-bet expression (Fig. 7E). In spleen, there was no difference on the percentage of IFN-γ^+CD4^+ and IFN-γ^+CD8^+, but the IFN-$\gamma^+NK1.1^+$ cells were increased (data not shown). Moreover, the infilteration of splenic neutrophils were observed at day 3 pi in starved mice (supplementary Fig. 5). Taken together, in contrast with the early time point, at the later phase of infection, IFN-γ^+ cells were highly induced.

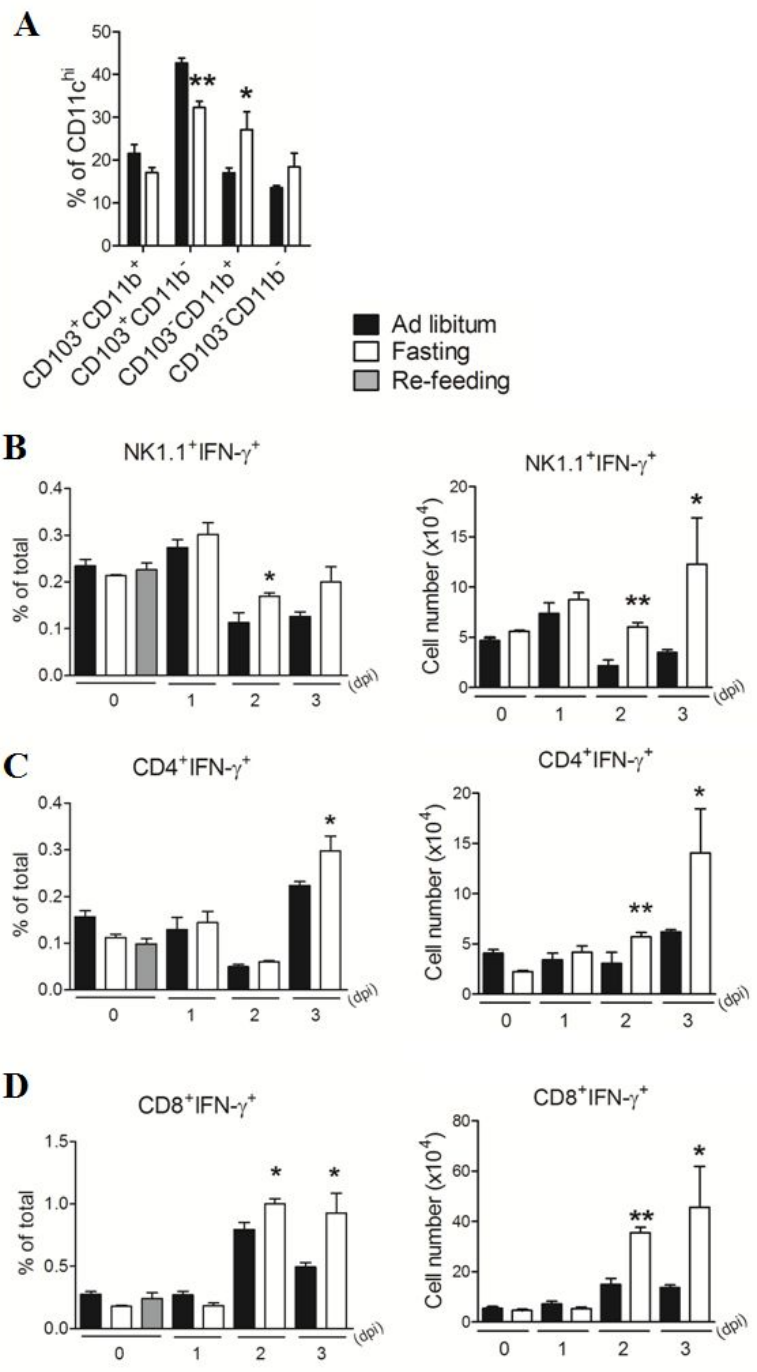
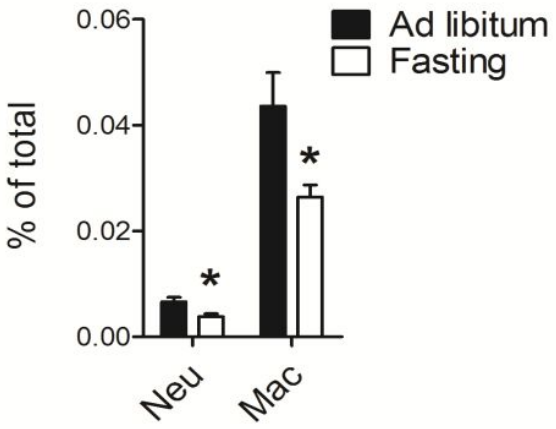


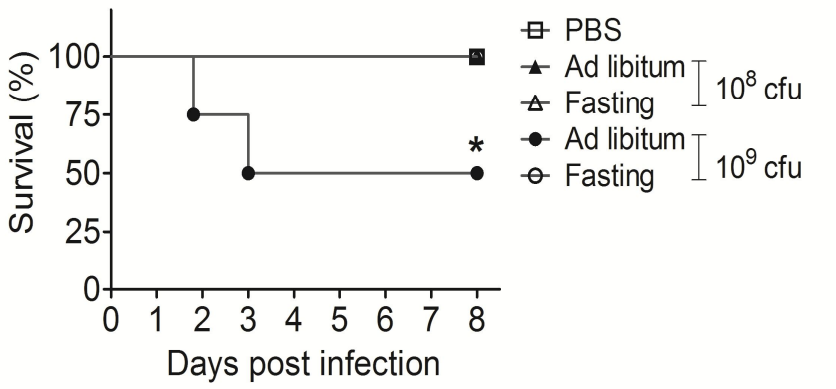
Figure 10. Composition of IFN-γ$^+$ cells among NK cells, CD4$^+$ T cells and CD8$^+$ T cells

in mLN in fasted mice infected with *Listeria*. Mice were divided into short-term fasting, *ad libitum*, and fasting and then re-feeding for 24 hrs and infected with *L. monocytogenes*. Mice were sacrificed and harvested mLNs at the indicated time points. Single cells were stained with anti-CD4, -CD8, -CD3e, -NK1.1, -NKp46, -IFN-γ and -IL-17A antibodies and analyzed by flow cytometry. (A) The constitution of subset of mLN $CD11c^{hi}$ cells at day 3 pi was examined based on the expression of CD103 and CD11b. (B-D) The percentage total cells (left) and absolute number (right) of IFN-γ^+ cells among the (B) NK cells ($NK1.1^+CD3e^-NKp46^+$) (C) $CD4^+$ T lymphocytes ($CD3e^+CD4^+$) and (D) $CD8^+$ T lymphocytes ($CD3e^+CD8^+$) in mLN at day 1, 2 and 3 pi. All statistics were performed by using Student's t test. All values are mean ± S.D. of at least three replicates. * and ** indicate a significant difference at $p<0.05$ and $p<0.01$, respectively.

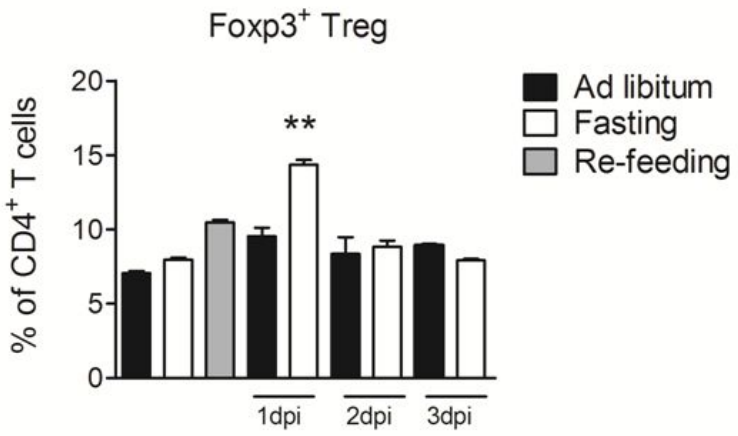
IV. Supplementary results



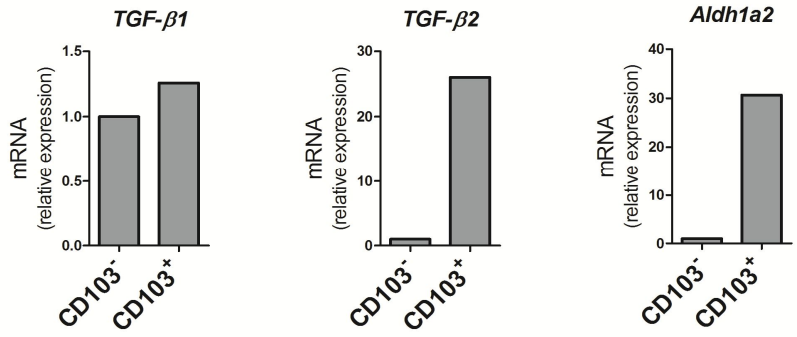
Supplementary Fig. 1. The percentage of neutrophils and macrophages in mLN from mice after short-term fasting. Single cells were produced from mLN of mice after short-term fasting. Neutrophils ($Ly6G^{+}CD11b^{hi}F4/80^{-}CD11c^{-}$) and macrophages ($F4/80^{+}CD11b^{hi}Ly6G^{-}$) were examined by using flow cytometry. Statistics were calculated by using Student's t test. All values are mean ± S.D. of at least three replicates. * indicates a significant difference at $p<0.05$.



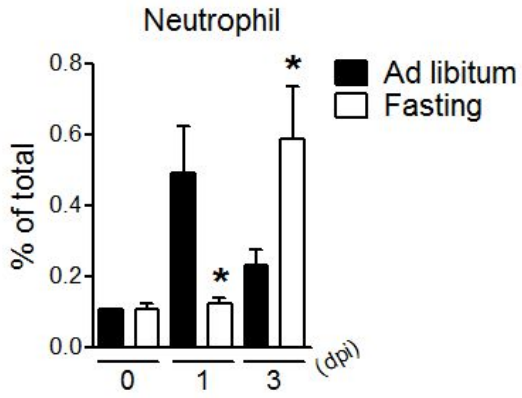
Supplementary Fig. 2. Survival of mice after short-term fasting followed by wild type *L. monocytogenes* (10403s) infection. Female Balb/c mice, 6 weeks old, were fasted for 24 hrs and administrated with 1 x 10^8 or 1 x 10^9 of wild-type *L. monocytogenes* through i.g. route. Then, the survival was monitored for 8 days. At least 5 mice were allotted in each group. The statistics for survival test were analyzed by log-rank (Mantel-Cox) test. * indicates a significant difference at $p<0.05$.



Supplementary Fig. 3. The percentage of Foxp3$^+$ Tregs in mLN. Balb/c mice starved, fed *ad libitum*, or refed (fasting for 24 hrs and then fed for 24 hrs) were infected with *L. monocytogenes* through i.g. route. Single cells were prepared and the composition of Foxp3$^+$ Tregs in mLN at day 1, 2 and 3 pi was examined. All values are mean ± S.D. of at least three replicates. ** indicates a significant difference at $p<0.01$.



Supplementary Fig. 4. The mRNA level of TGF-β1, TGF-β2 and aldh1a2 in mLN CD103$^+$ and CD103$^-$ DCs. The mLNs from 6 Balb/c mice fed *ad libitum* were pooled and purified for CD11c$^+$ cells using bead separation followed by CD103$^+$ and CD103$^-$ DCs sorting by using cell sorter. Total mRNA was synthesized into cDNA and real-time PCR was performed. The mRNA level of *TGF-β1, TGF-β2* and *retinaldehyde dehydrogenase 2 (aldh1a2)* was examined. Statistics were performed by using Student's t test. All values are mean ± S.D. of three replicates.



Supplementary Fig. 5. The percentage of neutrophils in spleen from mice after short-term fasting followed by *Listeria* infection. Balb/c mice starved for 24 hrs or not were infected with *Listeria*. Single cells from spleen were analyzed using anti-CD11c, -Ly6G, -CD11b and -F4/80. Neutrophils ($Ly6G^{+}CD11b^{hi}F4/80^{-}CD11c^{-}$) were monitored by using flow cytometry. Statistics were calculated by using Student's t test. All values are mean ± S.D. of at least three replicates. * indicates a significant difference at $p<0.05$.

V. Discussion

In the present study, we focused on the alteration of intestinal immune cells, especially $CD11c^{hi}$ DCs, caused by short-term fasting or *Listeria* infection after fasting. Major findings are as follows: (1) Short-term fasting (24 hr) could alter the composition of intestinal innate immune cells, with increase of $CD11c^{hi}$ cells being most apparent; (2) Among $CD11c^{hi}$ cells, $CD103^{+}$ $CD11b^{-}$ DCs in both mLN and SI LP from starved mice proliferated better than from *ad libitum* control mice; (3) phenotypic changes of DCs were found in mLN after the short-term fasting and (4) the recruitment and proliferation of $CD11c^{hi}$ DCs in SI LP after the fasting was closely related with upregulated GM-CSF; and (5) short-term fasting significantly contributed on the protection of the mice from *Listeria* infection, likely mediated by sharp increase in the induction of $Foxp3^{+}$ Tregs at the early phase after the infection via TGF-β2 coincident with the augmented Th1 responses at later time point.

The primary function of small intestine is to perform digestion and absorption of incoming nutrients, while upon the fasting it goes through structural and functional changes with reduction of metabolic activity (Wang et al., 2006). Although effects of fasting on the IECs were previously reported, its impact on intestinal immune cells remains largely unknown. Thus, in the present study, we focused on the effect of fasting in terms of changes of cellularity in GALT, especially in mLN and SI LP. The results showed that SI LP $CD11c^{hi}$ cells are the most increased population of innate cells after fasting, which was caused by cell proliferation (Fig. 11). Of the $CD11c^{hi}$ cells, $CD103^{+}CD11b^{-}$ DCs were the most increased DC subset in mLN and SI LP upon fasting. Surprisingly, the induction of SI LP, but not mLN, $CD103^{+}CD11b^{-}$ DCs was due to the active cell proliferation even during fasting. It should be noted that the rate of proliferation was much higher in SI LP than mLN, which is consistent

with previous report showing that mLN $CD103^+$ DCs have slower kinetics than SI LP $CD103^+$ DCs (Jaensson et al., 2008).

In dermis and epithelium, rapid recruitment of $CD11c^{hi}$ cells was caused by prior recruitment of circulating $Gr1^+$ monocytes and shown to participate in cross-priming $CD8^+$ T cells *in vivo* (Le Borgne et al., 2006). In addition, increased GM-CSF production influenced the recruitment of $CD11c^{hi}$ DCs (Hirata et al., 2010). In agreement with this, we also observed increased mRNA level of GM-CSF in SI LP from starved mice, suggesting that local increase of GM-CSF induced by fasting might affect the migration and function of intestinal $CD11c^{hi}$ cells.

In steady states, DCs reside lamina propria, efficiently uptake self-antigens and induce peripheral self-tolerance. In support of this notion, OVA uptake in all DC subsets was apparent but much higher in $CD11b^+$ DCs than $CD11b^-$ DCs. Commonly, $CD11b^+$ cells are thought to be macrophages, except a few reports suggested that $CD103^-CD11b^+CD11c^+$cells are a subset of DCs specifically dependent on Flt3 ligand (Scott et al., 2014). In the present study, there was no difference on the antigen uptake ability of mLN $CD103^+CD11b^-$ $CD11c^{hi}CD24^+$ DCs from between starved and control mice. It is probable that soluble antigen may not be a suitable for studying uptake of intestinal DCs, since it has been reported that soluble antigens do not represent a physiological source of antigens involved in cross-presentation of intestinal $CD103^+CD11b^-$ DCs (Cerovic et al., 2014) and, more importantly, that $CD103^+$ DCs inefficiently uptake soluble antigens (Farache et al., 2013). It was noting in the present study that the expression of CD205 which is related to the uptake of apoptotic cells was significantly increased in mLN $CD11c^{hi}$ cells from starved mice.

Compared with other subtypes of lymph-borne DCs, the $CD103^+CD11b^-$ DCs have a stronger tolerogenic activity in a way of skewing differentiation of naïve T cells toward $Foxp3^+$ Tregs but not IFN-γ^+ cells (Huang et al., 2013). However, in this study, even though

the significant induction of $CD103^+CD11b^-$ DCs in mLN was found, change of $Foxp3^+$ Tregs was not observed. This discrepancy might be explained at least in part by previous report showing that increased number of $Foxp3^+$ Tregs with high proliferative capacity was detectable in starved mice (Procaccini et al., 2010), suggesting that the duration of starvation is seemingly important.

TGF-β and RA produced by $CD103^+$ DCs could induce $Foxp3^+$ Tregs (Coombes et al., 2007) for retaining immune tolerance. For this reason, they are expressed at high level in the gastrointestinal regions when compared to other peripheral tissues. Expression of TGF-β2 on the intestinal $CD103^+$ DCs was much higher than on $CD103^-$ DCs, but there was no difference on the level of TGF-β1, which was consistent with the previous report (Huang et al., 2013). In case of RA, the result clearly showed the higher expression of *aldh1a2* in $CD103^+$ DCs from fasting group, which might increase number of $Foxp3^+$ Tregs. RA is mainly produced by intestinal DCs and IECs. It has been shown that inhibition of RA receptor rectricted the induction of $Foxp3^+$ Tregs (Mucida et al., 2007).

The present study reported an unprecedented phenomenon that short-term fasting is beneficial for the protection of the mice infected with *Listeria.* It was particularly noticed that, during the early time after the infection, $CD103^+CD11b^-$ DCs and $Foxp3^+$ Tregs were significantly induced in mLN. Based on the previous reports, $Foxp3^+$ Tregs induced by $CD103^+CD11b^-$ DCs are the safeguard of the host from excessive immune responses after the invasion by pathogens. $Foxp3^+$ Tregs not only prevent autoimmune diseases (Sakaguchi et al., 2008, Levings et al., 2006), but also curb vigorous antimicrobial immune responses by restricting excessive inflammation (Belkaid and Tarbell, 2009, Belkaid and Rouse, 2005). In supporting this, there was a significant infiltration of neutrophils in spleen in the *ad libitum* group at the early phase of the infection. On the contrary, at day 3pi, splenic neutrophils were increased in starved mice when compared to the control, suggesting better chance of bacterial

clearance in the later phase of infection. This is in agreement with the previous report that the infiltration of $Ly6G^+$ neutrophils in spleen at day 3 pi is critical for bacterial clearance and host survival (Shi et al., 2011). Thus, these findings suggest that tolerogenic conditions made by short-term fasting might restrain overwhelming immune response and protect host from tissue damage.

In steady states, $CD103^+CD11b^+$ DCs are also involved in the maintenance of tolerogenic conditions similar to $CD103^+CD11b^-$ DCs. However, upon the antigenic stimulation, they play an importance role in Th17-mediated immune responses (Persson et al., 2013, Kinnebrew et al., 2012, Lewis et al., 2011). Upon inflammatory response, intestinal $CD103^+$ DCs lose their tolerogenic nature and become an inflammatory antigen-presenting cell, but the mechanism related to the transition of nature has not been demonstrated.

By contrast, lymph-borne $CD103^-$ DCs have more immunogenic nature in both steady and infection states where they induce naïve T cells to IFN-γ producing Th1 cells (Cerovic et al., 2013) and produce more proinflammatory cytokines (Coombes et al., 2007). Despite the immunostimulatory character of $CD103^-$ DCs in the steady state, number of these cells are much lower than $CD103^+$ DCs, so that intestinal immune system stays preferentially on the tolerance condition. At 1 day after the infection shown in the present study, $CD103^+CD11b^-$ DCs and $Foxp3^+$ Tregs were increased in their numbers with high TGF-β and RA compared to those of *ad libitum* group (Fig. 12). On the other hand, the induction of IFN-γ^+ cells at day 3 pi after the fasting might be due to the upregulation of $CD103^-$ DCs (Fig. 13). These results suggest that the function of intestinal $CD11c^{hi}$ cells, based on the expression of CD103 and CD11b, is critical for the differentiation of specific T cell either to main the tolerance or to induce the immunity.

Collectively, the present study demonstrated that short-term fasting altered the characteristic of intestinal DCs to benefit the host from excessive immune response caused by *Listeria* infection through induction of intestinal $CD103^{+}CD11b^{-}$ DCs coincident with increased number of $Foxp3^{+}$ Tregs during the early phase of infection. These results provide an insight on the mechanism how fasting influnces innate immune system and implication in designing efficient strategies for oral prophylactic and chemo treatment including vaccination.

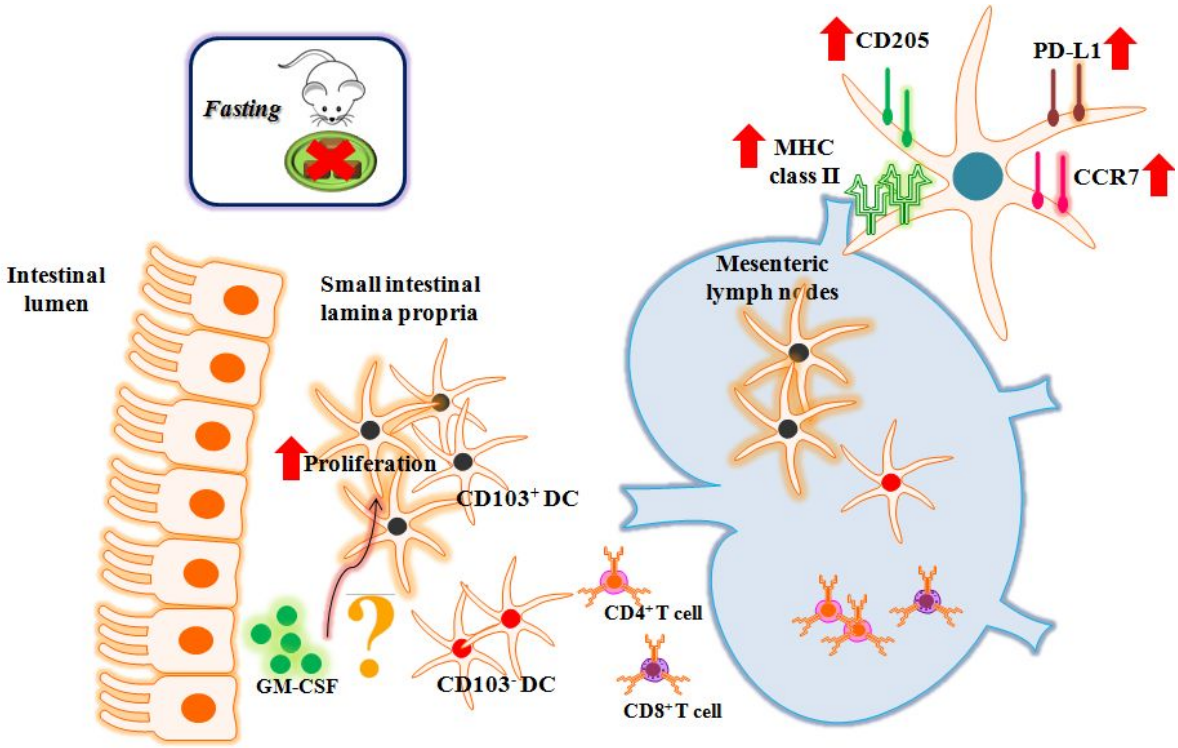


Figure 11. Schematic diagram of intestinal changes in mouse after short-term fasting.

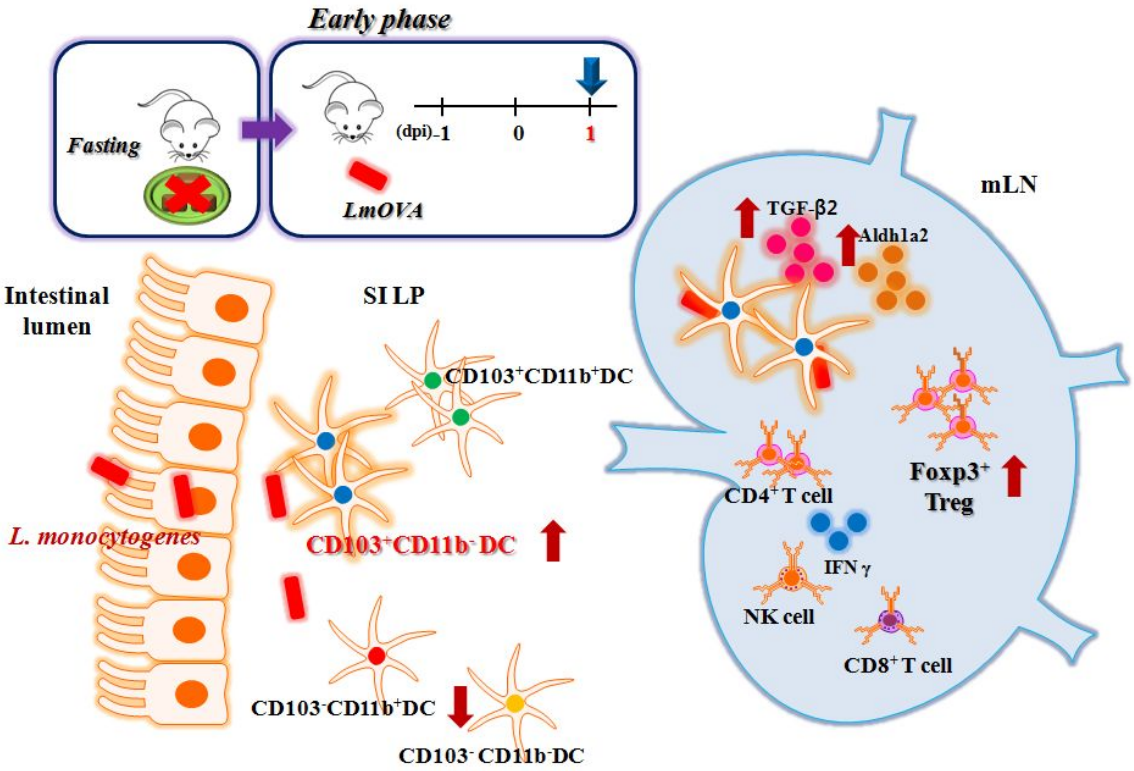


Figure 12. Schematic diagram of intestinal changes at the early phase of *L. monocytogenes* infection in mouse after short-term fasting.

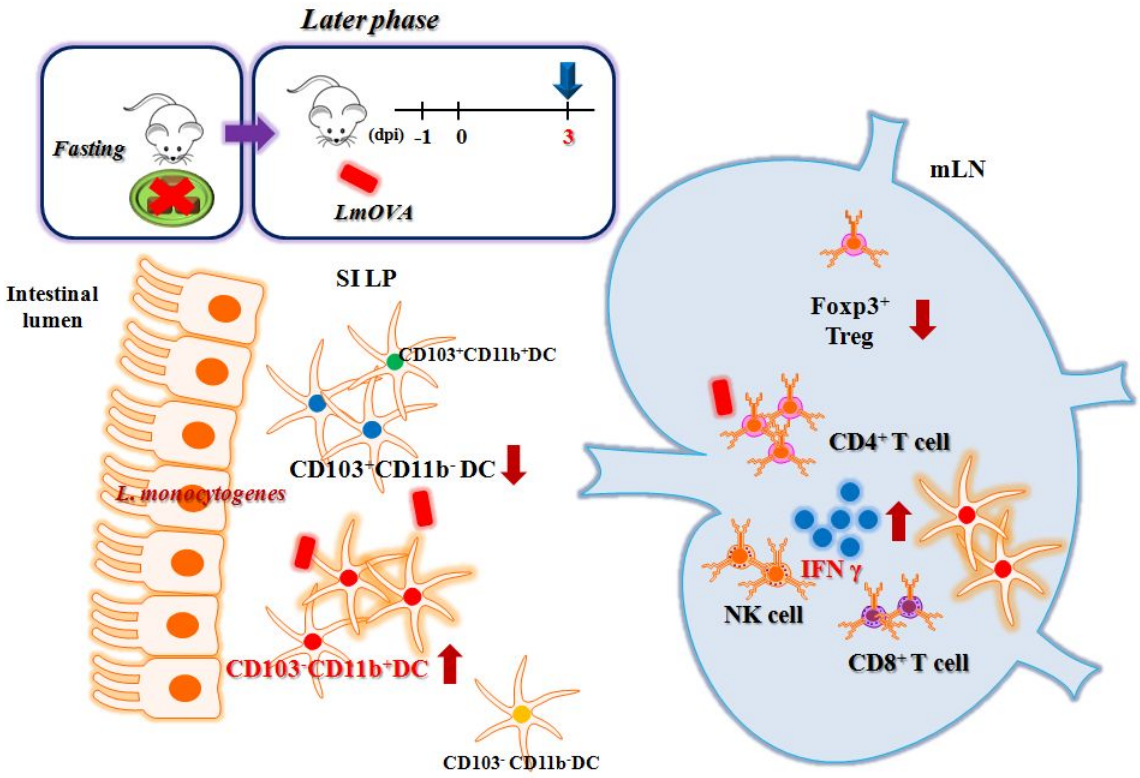


Figure 13. Schematic diagram of intestinal changes at the later phase of *L. monocytogenes* infection in mouse after short-term fasting.

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VII. Summary in Korean

소장은 입을 통해 들어오는 음식물을 소화 및 흡수를 하는 동시에 많은 에너지를 소모하는 주요 기관으로써, 단식이 소장에 주는 영향에 대한 연구가 활발하다. 그러나, 짧은 단식이 소장의 상피세포에 주는 영향 및 단식으로 인해 변화되는 장내 미생물의 변화 및 단백질 발현의 변화에 대한 연구는 활발함에도 불구하고, 단식 자체가 장 면역세포에 끼치는 효과에 대한 연구는 이뤄지지 않고 있다. 장관에 존재하는 선천성 면역 세포는 음식물뿐만 아니라, 장내 미생물의 잔해물 그리고 죽어가는 장 상피세포의 잔해를 흡수하여 장관 막 림프절로 가져간 뒤, T 림프구에 자신이 가져온 항원을 제시한다. 이 때, T 림프구는 자가 항원 및 무해한 항원으로 판단하게 되며, 조절 T 세포 (Regulatory T cell, Treg)를 이용하여 면역 관용을 일으킨다.

따라서, 본인은 본 연구를 통해 짧은 단식이 장 면역 세포의 이동 및 기능 변화에 초점을 맞추면서, 짧은 단식(24시간)으로 인해 변화된 장 내 환경이 후에 침입 하게 될 리스테리아 균에 대한 마우스의 면역 반응에 대해 연구하였다. 특히, 장내에서 조절 T 세포를 유도하는데 가장 큰 역할을 하는 CD103 마커를 발현하는 ($CD103^+$) 수지상 세포에 초점을 맞췄다. 짧은 단식을 한 마우스의 소장 점막 고유층 (Lamina propria) 및 장관 막 림프절에서의 $CD103^+CD11b^-$ 수지상 세포가 유의적으로 증가하는 것을 확인하였다. $CD103^+CD11b^-$ 수지상 세포의 증가는 활발한 급증과, 장 면역 기관으로의 이동이 원인이다. 이때, 소장 점막 고유층에서 과립구 대식세포 콜로니 자극 인자 (GM-CSF) 의 유의적 증가가 세포의 급증 및 이동에 영향을 끼칠 것이라 사료된다. 하지만, 자세한 메커니즘은 본 연구에서 밝히지 못했다. 이렇게 증가한 수지상 세포에서 세포 이동에 관련된 인자 (CCR7), 죽어가는 세포를 흡입하는 데 필요한 표면 마커 (CD205), 마지막으로 T 세포의 활성을 제지하는 마커 (PD-L1)가 단식을 한 마우스 그룹에서 유의적으로 증가함을 확인했다. 하지만, 외부 항원 모델인 OVA 를 흡입하는 능력 실험에서는

차이가 없었다.

짧은 단식 후, 강한 Th1 반응을 일으키는 리스테리아 균을 구강으로 감염을 한 결과, 단식을 한 마우스 그룹에서의 생존율이 유의적으로 높았다. 감염 24시간 째 까지는 비장, 간, 장관 막 림프절에 존재하는 리스테리아 수에 큰 차이가 없었으나, 48시간이 지나면서 단식을 해준 마우스에서 리스테리아 수가 급감하는 것을 확인했다. 심지어 단식을 하지 않은 마우스의 경우에는 48시간째 혈액에서 박테리아가 발견되는 균혈증 증세를 보였다. 박테리아의 감소가 일어나는 시점을 기준인 감염 1일 째와 그 이후 시간에서의 CD103 수지상 세포의 변화를 나눠서 관찰하였다. 그 결과, 감염 1일에는 $CD103^{+}CD11b^{-}$ 수지상 세포가 단식만을 했을 때와 같이 여전히 증가하는 것을 확인하였다. $CD103^{+}CD11b^{-}$ 수지상 세포는 Foxp3를 발현하는 Treg을 유도하는 것으로 잘 알려진 것 과 같이, 확인을 한 결과, 1일 째에서 $Foxp3^{+}$ Treg 세포가 유의적으로 증가했으며, 이 시점에서 메신저 리보 핵산 (mRNA) 수준에의 *foxp3* 전사인자 발현량도 증가하였다. 이러한 현상은 $CD103^{+}$ 수지상 세포에서 만들어지는 형질전환생장인자 β (TGF-β) 와 레티노산 (Retinoic acid) 로 인해 유도된 것으로 mRNA 수준에서 관찰했다.

반면, 감염 3일째에서는 $CD103^{+}CD11b^{-}$ 수지상 세포가 감소하는 동시에, $CD103^{-}$ 수지상 세포가 증가하는 현상을 발견했다. $CD103^{-}$ 수지상 세포는 $CD103^{+}$ 수지상 세포와는 다르게 Th1 반응을 유도하여 IFN-γ 를 생성하는 것으로 잘 알려져 있다. 본 연구자는 감염 3일 째, 장관 막 림프절에서 $CD103^{-}$ 수지상 세포가 증가와 IFN-γ 를 생성하는 CD4, CD8 그리고 NK 세포의 유의적 증가를 확인하였다. 또한, 이 시점에서 *t-bet* 전사인자의 발현량 증가를 mRNA 수준에서 관찰하였다.

본 연구를 통하여, 짧은 단식이 장 내 $CD103^{+}$ 수지상 세포의 기능 및 구성의 변화에 끼치는 영향을 처음으로 발견했다. 이는 향후 경구 백신 개발, 질병의 예방 혹은 치료에 있어 방향성을 제시해줄 것이라 예상한다.