

ORIGINAL ARTICLE

## VITAMIN A DEFICIENCY IMPAIRS HOST RESISTANCE TO *LISTERIA MONOCYTOGENES* INFECTION THROUGH EXCESSIVE APOPTOSIS OF MACROPHAGES

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**Abstract** Vitamin A is essential for maintenance of homeostasis, and it also regulates various immune mechanisms. *Listeria monocytogenes* is a gram-positive bacillus that invades the cytoplasm of phagocytes and performs intracellular multiplication, and is often used as a tool for functional analysis of macrophages. In this study, we investigated the changes of macrophages under vitamin A deficiency (VAD) using *L. monocytogenes*. During *L. monocytogenes* infection, apoptosis was increased in CD11b-positive splenocytes of VAD mice compared with vitamin A sufficient (VAS) mice. A similar result was obtained with peritoneal exudate cells (PECs). Significant increase of apoptosis due to the administration of Ro41-5253, an inhibitor of retinoic acid receptor, was also observed in the macrophage cell line RAW264.7. Furthermore, in VAD mice, the number of bacteria in the spleens and livers was significantly increased. These results suggest that excessive apoptosis of macrophages occurs under VAD condition, leading to impaired host resistance to *L. monocytogenes*. Taken together, vitamin A contributes to the maintenance of homeostasis by regulating macrophage functions, indicating the importance of vitamin A supplementation.

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**Key words:** Vitamin A; Vitamin A deficiency; macrophage; *Listeria monocytogenes*; apoptosis.

### Introduction

Vitamin A, one of the fat-soluble vitamins, is the precursor of retinoic acid, which is involved in several biological activities in humans. Vitamin A is present in food stuffs in the form of retinyl ester and provitamin A carotenoids and is stored as a retinyl ester derivative in stellate cells of liver. Vitamin A is transported to target cells in the form of retinol and acts via binding to nuclear receptors called retinoic acid receptor (RAR) and retinoid X receptor (RXR)<sup>1, 2)</sup>. Retinoic acid is found in rhodopsin, which is contained in the retina, and is related not only to vision, but also regeneration of epithelia and innate immunity<sup>2, 3)</sup>.

Thus, vitamin A is essential for maintenance of homeostasis. In populations with low dietary of vitamin A, infection promotes vitamin A deficiency by decreasing absorption, and increasing excretion<sup>4)</sup>.

*Listeria monocytogenes*, a gram-positive bacillus, is an intracellular parasite. Humans and animals can be infected with *L. monocytogenes* through contaminated food and causes sepsis by transmitting via the bloodstream from the intestinal tract. This bacterium is able to develop meningitis in the immunocompromised hosts, particularly in infants, HIV/AIDS patients, and the elderly<sup>5)</sup>. *L. monocytogenes* invades the host cells using the proteins belonging to internalin fami-

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ly<sup>6</sup>) and is passively taken up into macrophages by phagocytosis<sup>7, 8</sup>. Ideally, *L. monocytogenes* is supposed to be killed in mature phagosomes. However, this bacterium lyses the phagosomal membrane by secreting listeriolysin O, internalizes into the cytoplasm of macrophages, and multiplies intracellularly. Moreover, this bacterium is known to be able to escape from the bactericidal effect of macrophages by moving to the adjacent cells through the actin polymerization mediated by ActA protein<sup>9</sup>. Regarding this intracellular replication niche, *L. monocytogenes* has been used to evaluate the function of macrophages<sup>10</sup>.

We have previously reported that the administration of all-trans-retinoic acid (ATRA) suppresses the early production of TNF- $\alpha$  and increases the resistance to *L. monocytogenes* infection in vitamin A deficiency (VAD) mice<sup>11</sup>. Furthermore, excessive lymphocyte apoptosis by type I interferon reduces host resistance to *L. monocytogenes* infection<sup>12</sup>. Based on these results, we aimed to evaluate the effects of retinoic acid on macrophages under vitamin A deficient (VAD) condition using *L. monocytogenes* infection model.

## Materials and Methods

### Preparation of VAD and control mice

Pregnant C57BL/6J mice (Charles River, Yokohama, Kanagawa, Japan) and severe combined immunodeficiency mice (SCID: background strain is C57BL/6J) provided from Riken BioResource Research Center, Tsukuba, Ibaraki, Japan, were fed a chemically defined diet which lacks vitamin A (Oriental Yeast Co., LTD., Tokyo, Japan). Pups were weaned at the age of 4 weeks and continuously maintained on the same diet until an analysis was performed at the age of 10 weeks. Vitamin A Sufficient (VAS) mice were fed control diet containing retinol acetate. They were housed under specific-pathogen-free conditions with a temperature-con-

trolled room at 22°C on a 12-h light-dark cycle at the Institute for Animal Experimentation, Hirosaki University Graduate School of Medicine. All animal experiments in the present study were conducted in accordance with the Animal Research Ethics Committee, Hirosaki University Graduate School of Medicine, and followed the Guidelines for Animal Experimentation, Hirosaki University (Permit number: M15007).

### Infection of mice with *L. monocytogenes*

*L. monocytogenes* Ib 1684 was cultured in tryptic soy broth (BD Diagnosis Systems, Sparks, MD, USA) and the bacterial cells were prepared as previously described<sup>13-15</sup>. The concentration of washed cells was adjusted spectrophotometrically at 550 nm. Mice were infected intravenously with 0.2 ml of a solution containing  $5 \times 10^5$  colony-forming units (CFU) of viable *L. monocytogenes* in 0.01 M phosphate-buffered saline (PBS; pH 7.4). The spleens and livers were removed 24 h and 48 h post infection. The organs were homogenized in PBS with 1% 3-[(3-Cholamidopropyl) dimethylammonio] propanesulfonate (Wako Pure Chemical Industries Ltd., Osaka, Japan). The number of bacteria was counted by plating serial 10-fold dilutions of organ homogenates on tryptic soy agar (BD Diagnosis Systems). Colonies were routinely counted 24 h later<sup>14</sup>.

### TUNEL staining

The spleens were removed before and 24 h post *L. monocytogenes* infection and fixed with 10% formalin neutral buffer solution. After embedding in paraffin, the spleens were sectioned at 5- $\mu$ m in thickness and analyzed with the ApopTAQ kit (Oncor-Appligene, Heidelberg, Germany) according to the manufacturer's instructions. Terminal deoxynucleotidyl transferase-mediated UTP-biotin nick end labeling (TUNEL)-positive images were observed under microscope (Olympus IX73, Tokyo, Japan). Two expert pathologists

evaluated independently. Some paraffin sections were stained with hematoxylin and eosin (H&E).

### Flow cytometry

The spleens of infected C57BL/6J mice were collected 0, 2 h and 6 h post infection and single cell suspensions were prepared. Then, the cells were stained with PE-Cy7 labeling CD11b monoclonal antibody and FITC-Annexin V Apoptosis Detection Kit I (Becton-Dickinson and Company, Tokyo, Japan) according to the manufacturer's instructions<sup>16</sup>. Cells were analyzed by FACS-can<sup>TM</sup> with CELLQuest II<sup>TM</sup> (Becton-Dickinson and Company).

### Macrophage cell line and *L. monocytogenes* infection

Macrophage cell line RAW264.7 cells (ATCC TIB-71, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Tokyo, Japan) and seeded on 24-well culture plates at  $1 \times 10^6$  cells/well. Cells were treated with 10 mM Ro41-5253 (Abcam, Cambridgehire, UK), as RAR inhibitor, or with dimethyl sulfoxide (DMSO, Wako Pure Chemical Industries, Ltd.) as control group for 1 h and infected simultaneously with *L. monocytogenes* at multiplicity of infection (MOI) of 10. After incubation for 30 min, the extracellular bacteria were eliminated with 50 µg/ml gentamicin. The cultured supernatants and cells were collected 4 h post infection. The titers of IL-1β (Quantiine IL-1β ELISA kit, R&D systems, Inc., Minneapolis, MN, USA) and TNF-α (TNF-α Mouse ELISA kit, Thermo Fisher Scientific) in the supernatants were measured and DNA fragmentation in the cells was determined to evaluate cell death as described below.

### Preparation of peritoneal exudate cells (PECs) and *L. monocytogenes* infection

Mice were injected with 3 ml of 4% thiogly-

colate (Eiken Chemical Co., Ltd., Tokyo, Japan) intraperitoneally. After 4 days, mice were injected intraperitoneally with 6 ml cold PBS and peritoneal exudate cells (PECs) were harvested. PECs were seeded on 24-well culture plate at  $1 \times 10^6$  cells/well in RPMI 1640 medium (Thermo Fisher Scientific) supplemented with 0.1 mg/ml recombinant macrophage colony-stimulating factor (PeproTech, Rocky Hill, NJ, USA) and 10% FBS and incubated overnight<sup>17</sup>. Cells were then treated with 10 mM Ro41-5253 or DMSO as control group for 1 h and infected with *L. monocytogenes* at MOI of 10. After incubation for 30 min, the extracellular bacteria were eliminated with 50 µg/ml gentamicin. The infected cells were collected 4 h post infection. DNA fragmentation in the cells was determined to evaluate cell death as described below.

### Evaluation of cell death by ELISA

DNA fragmentation in the cytoplasm was measured by Cell Death Detection ELISA kit (Roche Diagnostics, Rotkreuz, Switzerland) according to the manufacturer's instructions. Apoptosis at a molecular level was determined spectrophotometrically at 405 nm and the ratio of fragmentation versus controls was calculated<sup>18</sup>.

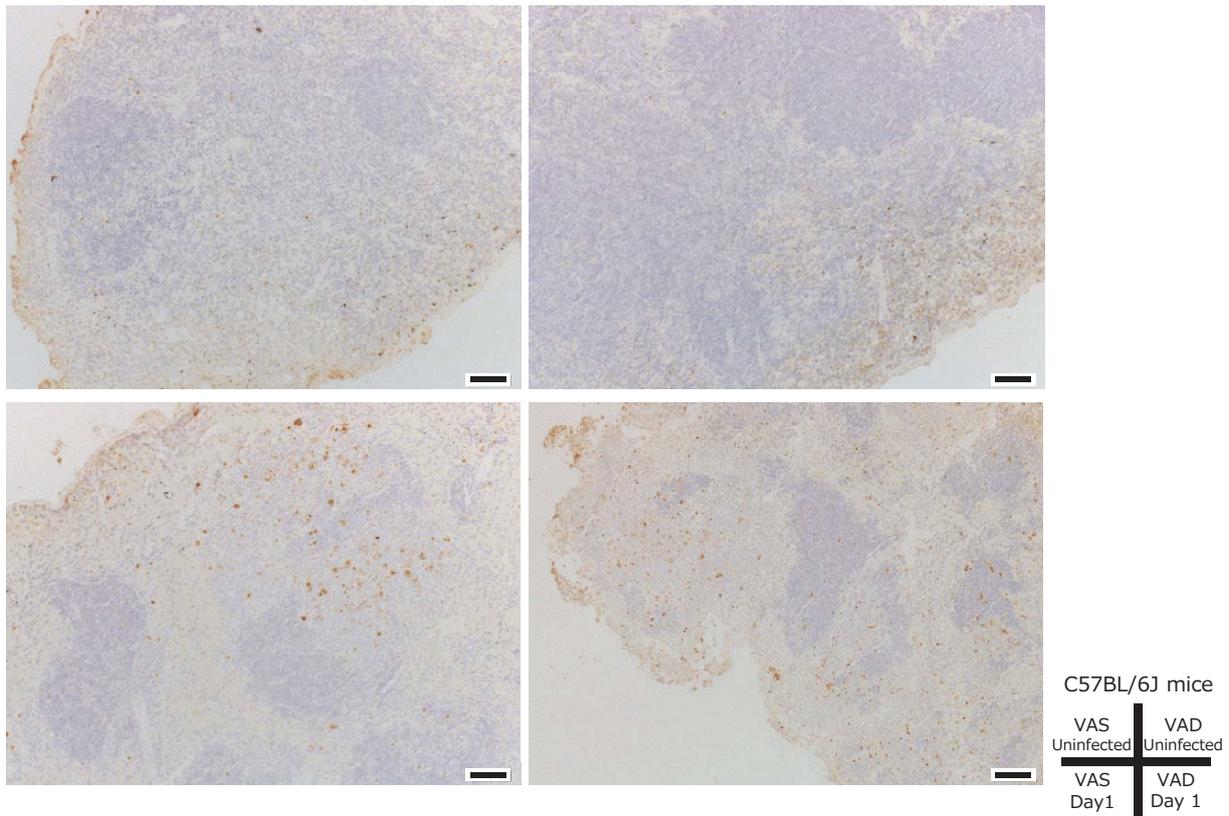
### Statistical analysis

Data were expressed as means ± standard deviations. Unpaired-*t* test (PRISM 7, Graph Pad, San Diego, CA, USA) was used to determine the significance of the differences of numbers. *p* values of <0.05 or 0.01 were considered significant. Each experiment was repeated at least twice.

## Results

### Apoptosis of splenocyte in VAD mice was increased after *L. monocytogenes* infection

C57BL/6J and SCID mice under VAS and VAD conditions were intravenously infected with *L. monocytogenes* and the spleens were

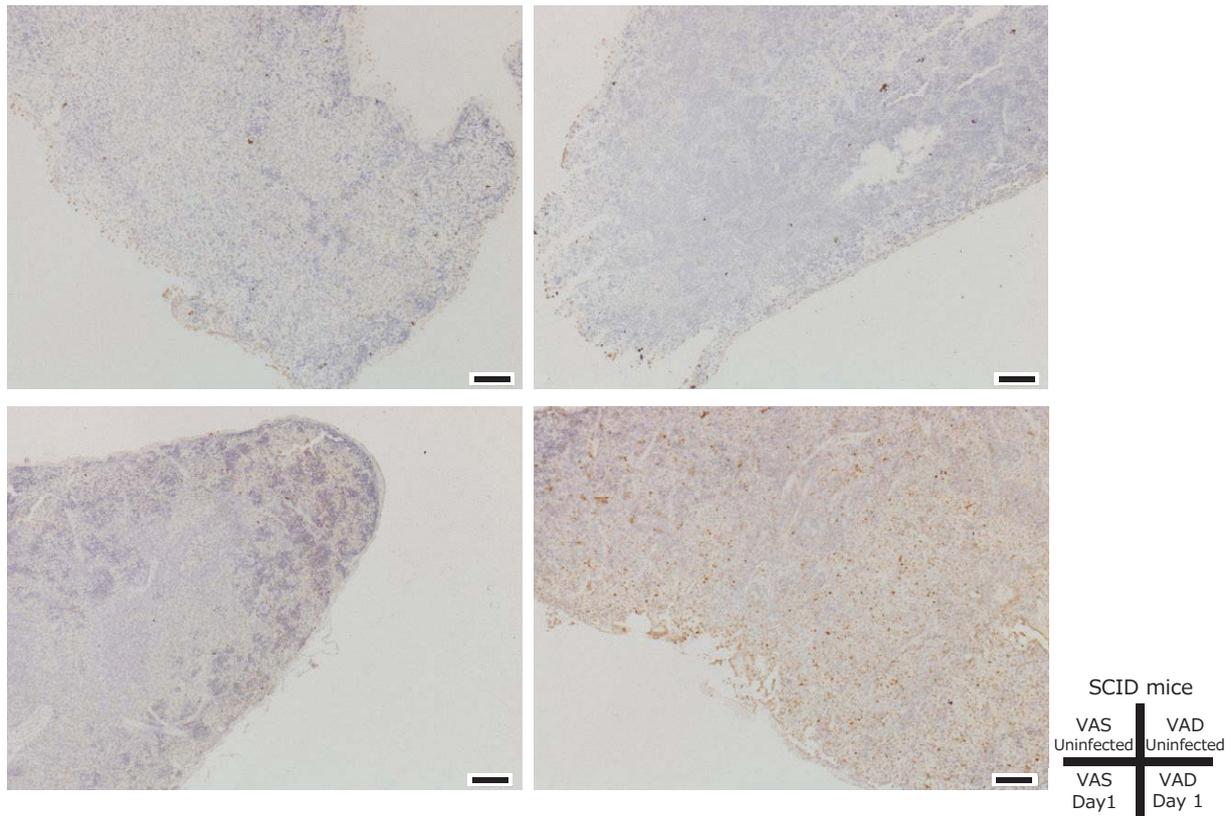


**Figure 1** VAD increases TUNEL-positive cells in C57BL/6J mice and SCID mice infected *L. monocytogenes*.  
 (A) VAS or VAD C57BL/6J mice were infected with  $5 \times 10^5$  CFU of *L. monocytogenes* intravenously. The spleens were collected before (day 0) and 24 h (day 1) post infection. Paraffin sections of splenic tissue were analyzed with TUNEL stain. TUNEL-positive cells were not observed practically in the uninfected organ (day 0). On day 1, TUNEL-positive cells were observed in both VAD and VAS mice, and the number of TUNEL-positive cells in VAD mice was much more than that of VAS mice. Bar = 100 $\mu$ m

subsequently harvested at 24 h after infection. Apoptotic cells in the spleen were then evaluated *in vivo* with TUNEL staining. In the uninfected mice, the number of TUNEL-positive cells did not change under either VAS or VAD conditions (Figure 1A and 1B, upper panel). On the other hand, the number of TUNEL-positive cells increased in both VAD C57BL/6J mice (Figure 1A, lower panel) and SCID mice (Figure 1B, lower panel) at 24 h after infection. Moreover, the apoptotic cells were characterized as macrophages by microscopic findings in H&E stained sections. Therefore, it was assumed that apoptosis increased histologically under VAD condition with *L. monocytogenes* infection, and that the major apoptotic cells were macrophages.

#### Apoptosis of CD11b-positive splenocytes was increased in VAD mice infected with *L. monocytogenes*

Apoptosis of macrophages in VAD mice was analyzed by flow cytometry. The spleens of VAS and VAD mice were collected at 0, 2 h and 6 h post *L. monocytogenes* infection. The number of CD11b-positive cells with Annexin V-positive and 7-AAD-negative which indicate apoptosis were evaluated. Although there was no difference between VAS mice and VAD mice in apoptosis before *L. monocytogenes* infection (0 h), apoptosis of macrophages in VAD mice was increased significantly at 2 h and 6 h after infection (Figure 2).



**Figure 1** VAD increases TUNEL-positive cells in C57BL/6J mice and SCID mice infected *L. monocytogenes*.

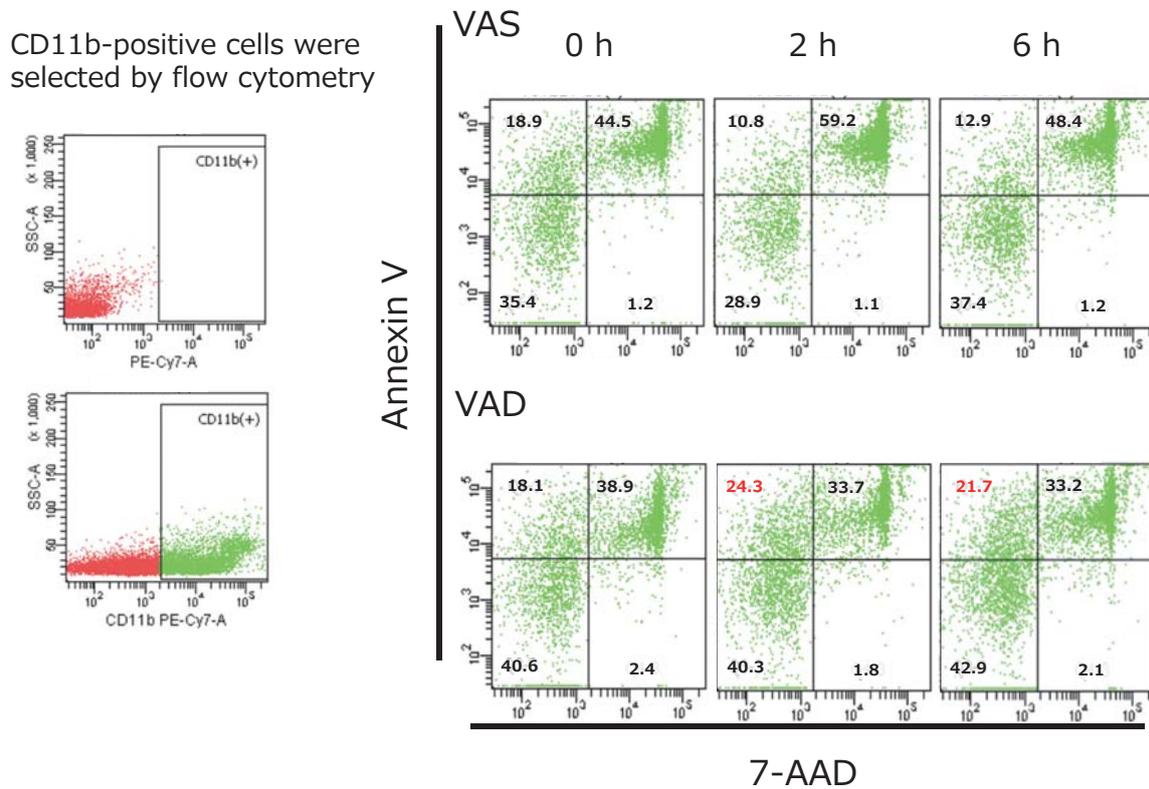
(B) VAS or VAD SCID mice were infected with  $5 \times 10^5$  CFU of *L. monocytogenes* intravenously. The spleens were collected before (uninfected) and 24 h (day1) post infection. Paraffin sections of splenic tissue were analyzed with TUNEL stain. TUNEL-positive cells were not observed practically in the uninfected organ as C57BL/6J mice. On day 1, TUNEL-positive cells were observed in both VAD and VAS mice, and the number of TUNEL-positive cells in VAD mice was much more than that of VAS mice. Bar = 100µm

### Apoptosis was increased in VAD macrophages infected with *L. monocytogenes*

In order to confirm the effects of vitamin A on apoptosis of *L. monocytogenes*-infected macrophages, the macrophage cell line RAW264.7 cells were treated with a RAR inhibitor Ro41-5253. After infection with *L. monocytogenes*, DNA fragmentation was quantitatively analyzed using Cell Death Detection ELISA kit to evaluate the apoptosis of macrophages. At 4 h after *L. monocytogenes* infection, a significant increase in apoptosis of macrophages was observed in the Ro41-5253-treated cells compared with the control group (Figure 3A). Similarly, PECs were harvested from VAS mice and VAD mice and pretreated with Ro41-5253, then the apoptosis of PECs after infection with *L. monocytogenes* was evaluated

using Cell Death Detection ELISA kit. Apoptosis occurred in PECs of VAD mice was significantly increased in comparison with that of VAS mice (Figure 3B, white bars). In addition, Ro41-5253 enhanced a significant increase in apoptosis in only PECs of VAS mice but not in the PECs of VAD mice (Figure 3B). To confirm the effect of vitamin A on apoptosis of macrophages, the titers of IL-1 $\beta$  and TNF- $\alpha$  in the supernatants of RAW264.7 cells at 4 h post infection with *L. monocytogenes* were also measured by ELISA. As shown in Figure 4, production of both IL-1 $\beta$  and TNF- $\alpha$  was significantly decreased in Ro41-5253-treated group.

### Effect of VAD on *L. monocytogenes* infection *in vivo*



**Figure 2** Identification of CD11b-positive cells as apoptotic cells by flow cytometry.

C57BL/6J mice were infected with  $5 \times 10^5$  CFU of *L. monocytogenes* intravenously. The spleens were collected at 0, 2 h and 6 h post infection and single cell suspensions were prepared. Then, the cells were stained with PE-Cy7 labeling CD11b monoclonal antibody and FITC Annexin V Apoptosis Detection Kit I. The CD11b-positive cells were selected by flow cytometry (left panel). Apoptotic cells are indicated by Annexin V-positive and 7-AAD negative staining. The number of apoptotic cells harvested from VAD mice was higher than that from VAD mice at 2 h and 6 h post infection.

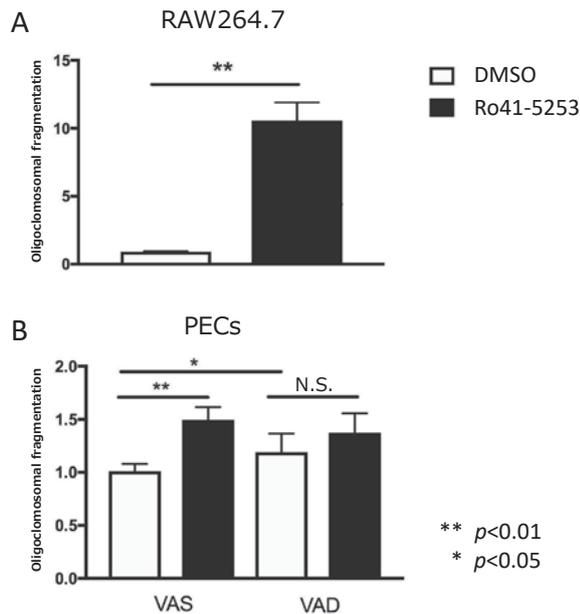
VAS C57BL/6J mice, VAD C57BL/6J mice, VAS SCID mice, and VAD SCID mice were intravenously infected with  $5 \times 10^5$  CFU of *L. monocytogenes*. Mice were dissected after 1 day and 2 days of infection and the spleens and livers were harvested. The number of bacteria in the organs was determined. The number of bacteria in both spleen and liver of C57BL/6J increased significantly under VAD condition from day 1 post infection (Figure 5A, B). A similar result was obtained in SCID mice (Figure 5C, D).

## Discussion

Vitamin A is essential for maintenance of homeostasis and has been known to regulate various immune mechanisms<sup>1-3</sup>). In particular,

transiently reduced blood vitamin A due to infections is known to further deteriorate the infectious state<sup>4,19</sup>). Thus, VAD greatly affects both innate and adaptive immunity. Specifically, VAD attenuates innate immunity by hindering the regeneration of mucosal epithelia and causing the dysfunction of macrophages, neutrophils, and natural killer cells<sup>4,19</sup>). Macrophages are activated by damage-associated molecular patterns and they act as the producing cells of cytokines for phagocytosis and antigen presentation<sup>20</sup>).

VAD increases transcription of IL-12<sup>22</sup>), and corneal abrasions in VAD rats resulted in greater inflammatory damage and IL-1 production<sup>21</sup>). Although these data indicate that VAD increases some macrophage-mediated inflammatory reactions, the phagocytic capacity



**Figure 3** DNA fragmentation in macrophages

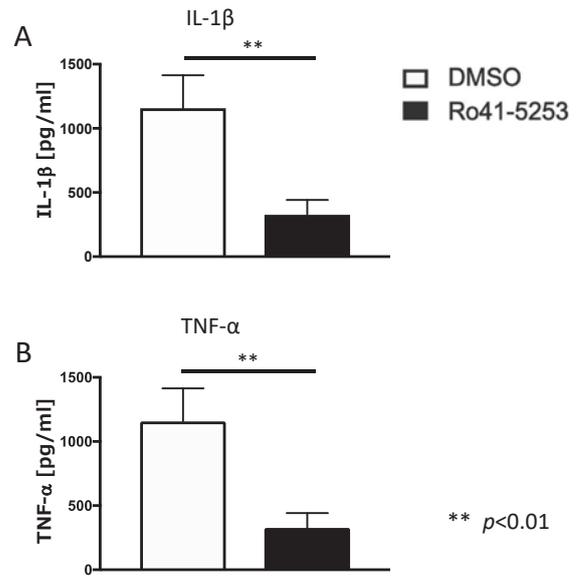
(A) RAW264.7 cells were pretreated with Ro41-5253 and infected with *L. monocytogenes* at MOI of 10. The infected cells were collected 4 h post infection. DNA fragmentation in cells were determined by Cell Death Detection ELISA kit. The apoptosis was enhanced in Ro41-5253-treated cells compared with the untreated cells.

(B) PECs were harvested from VAS or VAD mice and cultured. PECs were pretreated with Ro41-5253 and infected with MOI 10 of *L. monocytogenes*. The infected cells were collected 4 h post infection. DNA fragmentation in cells was determined by Cell Death Detection ELISA kit. The apoptosis of VAD PECs was higher than that of VAS PECs (white bars). In VAS PECs, the apoptosis of cells treated with Ro41-5253 was higher than that of the untreated cells. Apoptosis of VAD PECs showed no significant difference between Ro41-5253-treated and untreated group.

DMSO: dimethyl sulfoxide, Ro41-5253:RAR inhibitor, N.S.: Not significant

of macrophage is impaired by VAD. For example, the phagocytic ability of macrophage is decreased and bactericidal activity of PECs against *Staphylococcus aureus* is reduced in VAD mice<sup>23</sup>).

Therefore, the effect of retinoic acid on macrophage functions was focused in the present study. Using *L. monocytogenes* infection model, host resistance and changes of macrophages under VAD condition were investigated. During

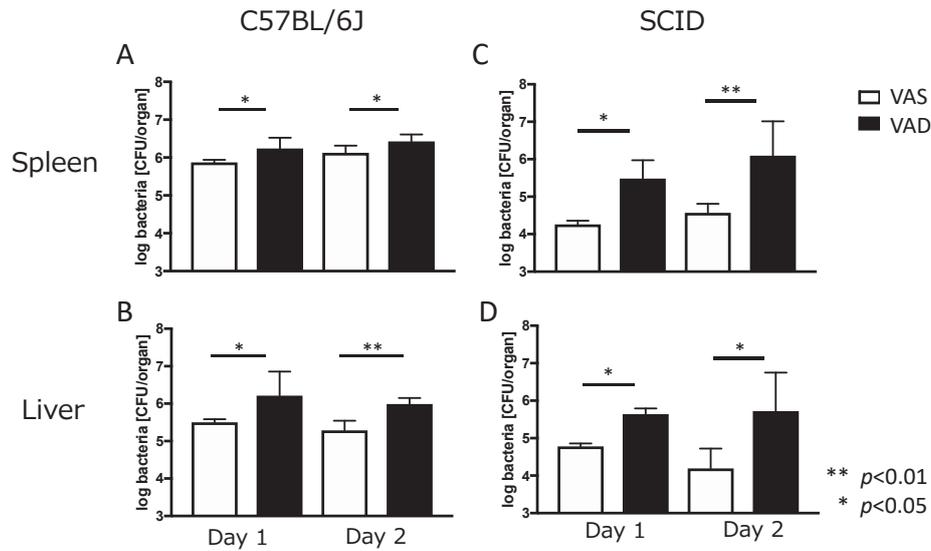


**Figure 4** RAR inhibitor suppressed production of IL-1 $\beta$  and TNF- $\alpha$  from macrophages infected with *L. monocytogenes*.

RAW264.7 cells were treated with or without RAR antagonist (Ro41-5253) for 1 h and infected simultaneously with *L. monocytogenes* at MOI of 10. At 4 h after infection, the production of IL-1 $\beta$  (A) and TNF- $\alpha$  (B) in the cultured supernatant were determined by ELISAs. Cytokine production was suppressed in the cells treated with Ro41-5253.

the early phase of *L. monocytogenes* infection, the number of bacteria in the spleens and livers of C57BL/6J mice increased significantly under VAD (Figure 5A, 5B). In addition, the number of bacteria in VAD SCID mice also increased significantly (Figure 5C, 5D), suggesting the involvement of innate immunity, in particular with macrophages neither T cells nor B cells. In VAD SCID mice infected with *L. monocytogenes*, TUNEL-positive cells increased in the spleen (Figure 1B) and most of these cells were morphologically classified as macrophages. The flow cytometric analysis also revealed that enhancement of apoptosis occurred in CD11b-positive cells. These results suggest that VAD impairs host resistance to *L. monocytogenes* infection through excessive apoptosis of macrophages.

The production of IL-1 $\beta$  and TNF- $\alpha$  in Ro41-5253-treated group decreased significantly in



**Figure 5** Host resistance to *L. monocytogenes* infection was attenuated in VAD.

C57BL/6J and SCID mice were infected with  $5 \times 10^5$  CFU of *L. monocytogenes* intravenously. The spleens and livers were removed 24 h (day 1) and 48 h (day 2) post infection. The number of bacteria in the organs was counted. In C57BL/6J mice (A and B) and SCID mice (C and D), the number of bacteria in the spleen (A and C) and liver (B and D) from VAD mice was increased compared with VAS mice.

RAW264.7 cells after infection with *L. monocytogenes* (Figure 4). Pyroptosis induces the secretion of IL-1 $\beta$  and TNF- $\alpha$  is necessary for necrosis. Thus, these results supported the hypothesis that cell death under VAD condition were not due to pyroptosis or necrosis but associated with apoptosis. Dysfunction of macrophages which is indicated by the decrease of IL-1 $\beta$  and TNF- $\alpha$  production was considered to be caused by increase in apoptosis of these cells, leading to a decrease of cell number.

Apoptosis of PECs after *L. monocytogenes* infection was also evaluated parallelly with RAW264.7 cells (Figure 3A, B). Although Ro41-5253 enhanced the apoptosis in PECs from VAS mice, the apoptosis of PECs from VAD mice was not significantly increased by Ro41-5253 treatment (Figure 3B). During preparation process of PECs, these cells were cultured shortly in the medium supplemented with FBS. Since FBS contains low concentration of vitamin A, the short exposure of a low concentration of vitamin A might recover the dysfunction of PECs obtained from VAD mice.

Our result showed that VAD impairs host resistance to *L. monocytogenes* infection through excessive apoptosis of macrophages. We conclude that vitamin A contributes to the maintenance of homeostasis by regulating macrophage functions, suggesting the importance of vitamin A supplementation for host defense.

## Conflicts of Interest

All authors have no conflicts of interest directly relevant to the content of this article.

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