ALL-TRANS-RETINOIC ACID (ATRA) INCREASES HOST RESISTANCE TO LISTERIA MONOCYTOGENES INFECTION

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Abstract  Dietary vitamin A is an essential precursor of tissue retinol, which participates in a variety of biological processes including innate immunity. Functions of vitamin A mainly depend on retinoic acid (RA), principally all-trans-RA (atRA) and 9-cis-RA. We assessed whether atRA is beneficial in host resistance against bacterial infections or not. Vitamin A-deficient (VAD) mice were highly susceptible to infection with *Listeria monocytogenes*. Pre-treatment with atRA enhanced host resistance against *L. monocytogenes* infection in both VAD and VAS mice. Interferon (IFN) -γ production in atRA pre-treated VAS mice was not higher compared with the control VAD mice. The effect of atRA was independent of T cells and B cells. The bactericidal activity in macrophages obtained from atRA pre-treated VAS mice was almost the same level compared with the control VAS mice. Our results demonstrated that the treatment with atRA is beneficial for host resistance against *L. monocytogenes* infection in the early phase and suggested a new therapeutic possibility of atRA in bacterial infections.

Vitamin A deficiency is one of the major nutritional deficiency syndromes in developing countries and is associated with an increased incidence of infectious diseases\(^1\). In populations where vitamin A availability from food is low, infectious diseases can precipitate vitamin A deficiency by decreasing intake\(^2\), decreasing absorption\(^3\), and increasing excretion\(^4\). The visual function of vitamin A depends on its metabolite 11-cis-retinal, but the other functions of vitamin A depend on retinoic acid (RA), principally all-trans-RA (atRA) and 9-cis-RA. atRA acts through RA receptors (RAR) to transcriptionally activate target genes\(^5\), such as cytochrome P450, CYP26, CRABP. Although atRA has some side effects such as teratogenicity, weight loss, bone fracture, anemia and liver damage, atRA is currently used to treat acute promyelocytic leukemia\(^7\) and inflammatory diseases such as psoriasis\(^8\), acne\(^9\) and rheumatoid arthritis\(^10\). Infectious diseases that induce the acute-phase response also impair the assessment of vitamin A status by transiently depressing serum retinol concentrations\(^11\). Clinical studies have shown that supplementation with vitamin A decreases the severity of diseases and the mortality rates\(^12\). Dietary vitamin A is an essential precursor of tissue retinol, which participates in a variety of biological processes including vision, reproduction, epithelial cell differentiation, bone development and immunity\(^13,14\). Vitamin A deficiency impairs innate immunity by impeding normal regeneration of mucosal barriers damaged by infection\(^15,16\), and by diminishing the function of neutrophils\(^17,18\), macrophages\(^19\), and natural killer (NK) cells\(^13,20\). Vitamin A is also required for adaptive immunity and plays a role in the development of T-helper (Th) cells and B cells. In particular, vitamin A

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deficiency diminishes antibody-mediated responses directed by Th2 cells\textsuperscript{15,21,23}, and several studies showed that Th1-mediated immunity is also diminished. For example, the DTH responses to dermal contact sensitization\textsuperscript{24} and subcutaneous ovalbumin administration\textsuperscript{25} were diminished in vitamin A-deficient rodents.

*Listeria monocytogenes* is a gram-positive bacterium that invades to host cells and proliferates intracellularly. *L. monocytogenes* causes sepsis and meningitis in immunocompromized hosts, and severe fetal infection in pregnancy. *L. monocytogenes* is widely used for analysis of infections with intracellular-growing pathogens, and previous studies using knock-out mice and monoclonal antibodies that deplete endogenous cytokines showed that multiple cytokines are involved in regulation of early infection of *L. monocytogenes*. These cytokines induce interferon (IFN)-γ production from NK cells and T cells and are involved in the activation of macrophages. The importance of innate immunity to *L. monocytogenes* was shown by studies using severe combined immunodeficient (SCID) mice and nude mice\textsuperscript{26,27}. Mice that lack both cellular and humoral immunity were found to be remarkably resistant to infection with *L. monocytogenes* at early time points. The early resistance to infection is attributed to the production of IFN-γ and the resultant activation of macrophages\textsuperscript{28}. IFN-γ is an effector cytokine that is produced mainly by NK and Th1 cells and signal through IFN signaling pathways and use signal transducer and activator of transcription (STAT) proteins as transcription factors\textsuperscript{29}. IFN-γ and tumor necrosis factor (TNF)-γ are essential for a primary defense against *L. monocytogenes* infection\textsuperscript{30,31}, and mice that lack these cytokines or their cognate receptors rapidly succumb to infection\textsuperscript{32–34}.

Recently, more attention has been drawn to potential interactions between the pathway involved in vitamin A signaling and that involved in IFN-γ signaling, since synergistic effects of atRA and IFN-γ have been observed in both animal models\textsuperscript{35} and cultured cell lines\textsuperscript{36,37}. However, the molecular events that are involved in atRA-dependent regulation of IFN-γ signaling are poorly understood. In this study, we assessed the role of vitamin A and atRA pre-treatment on host resistance against *L. monocytogenes* infection.

Female BALB/c mice and C57BL/6J mice were purchased from Clea Japan (Tokyo, Japan). Female C57BL/6J-SCID mice were obtained from The Jackson Laboratories (Bar Harbor, ME). Mice were maintained under specific pathogen-free condition at the Institute for Animal Experiments, Hiroasaki University School of Medicine. All mice were allowed free access to water and food and were studied at 8 to 10 weeks of age. This study was carried out in accordance with the Guidelines for Animal Experimentation of Hiroasaki University. BALB/c mice and C57BL/6J mice were bred, and gravid females received either a chemically defined diet that lacked vitamin A (the modified AIN-93M feed, Oriental Yeast, Tokyo, Japan) or vitamin A-sufficient (VAS) normal diet. These diets were started at 7 to 10 days of gestation. The pups were weaned at 4 weeks of age and maintained on the same diet at least until 10 weeks of age before analysis was performed. VAD mice showed no signs of inanition and had equivalent body weights. Serum retinol levels were analyzed by BML (Tokyo, Japan), and confirmed that serum retinol levels of VAD mice were below the detectable level. atRA was purchased from Sigma-Aldrich (St. Louis, MO). Mice were administered with 400 μg of atRA dissolved in 0.1 ml of olive oil intraperitoneally. As a control, olive oil with dimethyl sulfoxide (DMSO, Wako Pure Chemical Industries, Ltd., Osaka, Japan) was injected. Administration of atRA was carried out 3 times a week for 2 weeks.

*Listeria monocytogenes* 1b 1684 was prepared as previously described\textsuperscript{38}. The concentration of
washed cells was adjusted spectrophotometrically at 550 nm, and cells were stored at -80 °C until use. Mice were infected with $5 \times 10^3$ to $1 \times 10^6$ CFU of L. monocytogenes intravenously. The numbers of viable L. monocytogenes in the liver and spleen of infected mice were counted by plating serial 10-fold dilutions of organ homogenates in RPMI 1640 medium (Nissui, Tokyo, Japan) containing 1% 3-[[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS, Wako) on tryptic soy agar (BD Biosciences, Sparks, MD) plate. Colonies were routinely counted 24 h later.

Data were expressed as the mean±SD, and the Student's $t$ test was used to determine the significance of the differences of bacterial count, ELISA, and phagocytic activities in the specimens between the control and atRA pre-treated groups. The Logrank test was used to determine the significance of differences in the survival rates.

First, we investigated the effect of atRA pre-treatment on the susceptibility to a sublethal infection with L. monocytogenes in VAD mice. Oil or atRA pre-treated VAD mice were infected intravenously with $5 \times 10^3$ CFU of viable L. monocytogenes, and survival was observed until day 10 of infection. All of the control VAD mice died within 6 days, while all of atRA pre-treated VAD mice were survived for 10 days (Figure 1A). (P<0.01). Next, we investigated the kinetics of bacterial growth in the spleens and livers of control and atRA pre-treated VAD mice. Mice were infected with $5 \times 10^5$ CFU of L. monocytogenes, and the bacterial numbers in the organs of mice were determined at 2 and 4 days after infection (Figure 1B, 1C).

In both organs, the bacterial numbers of atRA pre-treated mice were significantly lower than those of oil pre-treated mice. Then, we assessed
Figure 2  The effect of atRA pre-treatment on VAS mice infected with *L. monocytogenes*. Mice were infected with 1 \times 10^6 CFU of *L. monocytogenes*. Survival rates of VAS mice that were pre-treated with atRA (square) and that were pre-treated with oil as control (diamond) were determined up to 10 days after infection (A). Each point represents a group of 11 mice. An asterisk indicates that the value is significantly different from the control group (P<0.01). Oil pre-treated or atRA pre-treated mice were infected with 5\times10^5 CFU of *L. monocytogenes*. Bacterial numbers in the spleens (B) and livers (C) were determined at 2 and 4 days after infection. Each result represents the mean\pmSD for a group of 7 mice. An asterisk indicates that the value is significantly different from the oil pre-treated control group (P<0.01).

Liver histology of control or atRA pre-treated VAD mice 2 days after infection with 5\times10^5 CFU of *L. monocytogenes*. Abscess formation and mononuclear cell infiltration were more prominent in the oil pre-treated mice than in the atRA pre-treated mice (data not shown). Moreover, the size of abscess in the atRA pre-treated mice was larger than that of oil pre-treated mice, and those were mostly consisted of necrotizing tissues. These results suggest that pre-treatment with atRA improved host resistance against *L. monocytogenes* infection compared with the control VAD mice. Pre-treatment with atRA to VAD mice recovered host resistance against listerial infection as almost the same level as oil pre-treated mice fed with vitamin A-sufficient normal diet (data not shown). Our results show that the resistance against *L. monocytogenes* infection is decreased in VAD mice, and atRA pre-treatment recovered the host resistance against listerial infection in VAD mice.

Next, we investigated the effect of atRA pre-treatment on the susceptibility to a lethal infection with *L. monocytogenes* in VAS mice. Oil or atRA pre-treated VAS mice were infected intravenously with 1\times10^6 CFU of viable *L. monocytogenes*, and survival was observed until day 10 of infection. All of the control VAS mice were died within 7 days, while all of atRA pre-treated VAS mice were survived for 10 days (Figure 2A). (P<0.01). We also investigated the kinetics of bacterial growth in the spleens and livers of control and atRA pre-treated VAS mice. Mice were infected with 5\times10^5 CFU of *L. monocytogenes*, and the bacterial numbers in the organs of mice were determined at 2 and 4 days after infection (Figure 2B, 2C).

In both organs, the bacterial numbers of
atRA pre-treated mice were significantly lower than those of oil pre-treated mice. Then, we assessed liver histology of control or atRA pre-treated VAD mice 2 days after infection with $5 \times 10^5$ CFU of *L. monocytogenes*. Abscess formation and mononuclear cell infiltration were more prominent in the oil pre-treated mice than in the atRA pre-treated mice (data not shown). These results suggested that atRA pre-treatment increased the host resistance against listerial infection in VAS mice.

IFN-γ and TNF-α are essential for primary defense against *L. monocytogenes* infection. Previous studies showed that the functions of macrophages, neutrophils, NK cells, and T cells and B cells are modulated by vitamin A and its metabolites in vitro. Therefore, we assessed the production of IFN-γ and TNF-α in atRA pre-treated VAS mice during *L. monocytogenes* infection. The spleens were obtained from mice infected with $5 \times 10^5$ CFU of *L. monocytogenes* at 24 h after infection, and the titers of IFN-γ and TNF-α in the organ homogenates were determined (Figure 3A, 3B). Titers of IFN-γ and TNF-α in the organ homogenates were determined by double-sandwich enzyme-linked immunosorbent assays (ELISAs) as previously described. The IFN-γ titers in the spleens from atRA pre-treated VAS mice were significantly lower compared with that in the control VAS mice (Figure 3A). The TNF-α titers in the spleens from atRA pre-treated VAS mice were also significantly lower compared with that in the control VAS mice (Figure 3B). Host resistance against *L. monocytogenes* is controlled by cell-mediated immunity and is regulated by endogenous cytokines. *L. monocytogenes* promotes the induction of the Th1 response, and IFN-γ plays a critical role in antilisterial resistance. TNF-α is also essential for primary host defense against infection with *L. monocytogenes*, because TNF-α can activate resident macrophages and production of reactive oxygen and reactive nitrogen intermediates by activated macrophages is important for bactericidal activity during *L. monocytogenes* infection. Although IFN-γ is essential for primary defense against infection with *L. monocytogenes*, the IFN-γ (Figure 3A) and TNF-α (Figure 3B) titers in the spleens from atRA pre-treated VAS mice were significantly lower compared with the control VAS mice. These results suggested that the atRA pre-treatment does not affect IFN-γ.

![Image](https://example.com/image.png)
and TNF-α production during infection. To investigate whether the effect of atRA pre-treatment on infection with \textit{L. monocytogenes} is dependent on T and B cells, we examined the bacterial numbers in the organ of wild and SCID C57BL/6J background mice those were infected with $5 \times 10^5$ CFU of \textit{L. monocytogenes} 2 days after infection. The numbers of viable \textit{L. monocytogenes} in the spleens and livers of atRA pre-treated VAS mice with C57BL/6J background (VAS-wild mice) were significantly lower than those of control VAS-wild mice (Figure 4; P<0.01). Host resistance against \textit{L. monocytogenes} infection in VAS mice with SCID background (VAS-SCID mice) was recovered by atRA pre-treatment compared with that in the control VAS-SCID mice (Figure 4; P<0.01). These results suggested that host resistance against listerial infection in VAS-SCID mice was increased by atRA pre-treatment, and also suggested that the effect of atRA pre-treatment on VAS mice depends on the population of leukocytes except T cells and B cells, for example, macrophages, neutrophils, and NK cells.

Activated macrophages are thought to be the principal mediators of the killing of \textit{L. monocytogenes}\textsuperscript{41}. Moreover, the functions of macrophages are impaired by vitamin A-deficiency\textsuperscript{10}. The previous study showed that vitamin A deficiency decreases the phagocytic activity and bactericidal activity of peritoneal macrophages for \textit{Staphylococcus aureus}\textsuperscript{19}. We supposed that atRA might be involved in the activation of macrophages. Therefore we performed the assessment of the function of macrophages from atRA pre-treated VAS mice. At first, we investigated whether atRA pre-treatment affects the phagocytic and listericidal activities of splenic macrophages in vitro. The phagocytic and bactericidal activities of splenic macrophages were determined by the method described previously\textsuperscript{42}. Splenic macrophages were prepared by adhering spleen cells twice in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) in a petri dish for 1 h at 37 °C in a 5% CO$_2$ condition. Splenic macrophages which were resuspended in antibiotic-free RPMI 1640 medium supplemented with 10% FCS at a concentration of $10^6$ cells/ml, were mixed with $10^7$ CFU of viable \textit{L. monocytogenes} and incubated at 37 °C for 30 min. Then they were washed three times with RPMI 1640 medium supplemented with 10% FCS and 5 μg/ml of gentamicin and were transferred into a 96-well flat-bottom microplate (Nunc, Roskilde, Denmark) at a density of $10^6$ cells in 100 μl per well. The infected cells were lysed by treatment with RPMI 1640 medium containing 1% (wt/vol) CHAPS at 0, 2, 4, and 6 h later. Lysates from three wells were pooled, and the number of viable intracellular bacteria in each sample was determined by culturing on tryptic soy agar plate. The phagocytic and listericidal activities of splenic macrophages from atRA pre-treated VAS mice were almost the same level.
compared with control VAS mice (Figure 5A). TNF-α is essential for defense against infection with L. monocytogenes, and macrophages produce TNF-α by stimulation with microbial products. We examined the effect of atRA pre-treatment on TNF-α production from splenic macrophages infected with L. monocytogenes to confirm upregulation of listericidal activity with atRA pre-treatment. TNF-α production by splenic macrophages were up-regulated by L. monocytogenes infection, but atRA pre-treatment significantly decreased TNF-α production by splenic macrophages compared with the controls (Figure 5B).

These results suggested that the atRA pre-treatment on VAS mice does not affect TNF-α production of macrophages during infection.

In conclusion, atRA pre-treatment with both of VAD and VAS mice increased host resistance against L. monocytogenes infection in the early phase through IFN-γ-independent manner. Our results showed that treatment with atRA is beneficial for host resistance against bacterial infections and may provide a new therapeutic possibility of atRA in bacterial infections.

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