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Suppression of Inflammatory Cytokine Genes Expression in Vascular Endothelial Cells by Super-low Dose Lipopolysaccharide-activated Macrophages

TERUKO HONDA[1] and HIROYUKI INAGAWA[2,3]

[1]Department of Medical Technology, School of Life and Environmental Science, Azabu University, Kanagawa, Japan;
[2]Research Institute for Healthy Living, Niigata University of Pharmacy and Applied Life Sciences, Niigata, Japan;
[3]Control of Innate Immunity Collaborative Innovation Partnership, Kagawa, Japan

Abstract. *Background/Aim: Vascular endothelial cells play an important role in regulating immune responses and in keeping the balance between blood coagulation and fibrinolysis. Inflammatory cytokines produced by activated macrophages and vascular endothelial cells excessively activate vascular endothelial cells, leading to an imbalance in the expression of blood coagulation- and fibrinolysis-related factors. The dysfunction of vascular endothelial cells can lead to development of various diseases. In a previous study the increased expression of inflammatory cytokines in adipocytes was shown to be suppressed by the culture medium of macrophages activated by low-dose lipopolysaccharide (LPS). Suppressing inflammatory cytokine gene expression of low-dose LPS-activated macrophages may allow for the regulation of the dysfunction in vascular endothelial cells. Materials and Methods: Human monocytes THP-1 cells were differentiated into macrophages with phorbol 12-myristate 13-acetate (PMA) and were activated with LPS. The culture medium of the LPS-activated THP-1 was added to human aortic endothelial cells (HAoEC). After five days, the expression of inflammatory cytokine genes interleukin (IL)1B, IL6, IL8, and tumor necrosis factor (TNF)A, blood coagulation-related genes SERPINE1, tissue factor (TF), and thrombopoietin (TM), and fibrinolysis-related gene tissue-type plasminogen activator (t-PA) was analyzed using quantitative real-time PCR. Results: IL1B, IL8, SERPINE1, TF, and TM expression in HAoEC was significantly reduced in the culture medium of super-low dose (0.1 ng/ml) LPS-activated macrophages. Conclusion: Super-low dose LPS-activated macrophages can suppress vascular endothelial cell inflammation and may be useful in preventing various diseases caused by the dysfunction of activated vascular endothelial cells.*

Correspondence to: Teruko Honda, Department of Medical Technology, School of Life and Environmental Science, Azabu University, 1-17-71 Fuchinobe, Chuo-ku, Sagamihara, Kanagawa, 252-5201, Japan. Tel: +81 427547111, Fax: +81 427547611, e-mail: hondat@azabu-u.ac.jp

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Vascular endothelial cells form a monolayer cell covering the lumen of the blood vessels and possesses a variety of functions. They not only act as a barrier between the circulating blood and the subcutaneous tissue of blood vessels, but also regulate the immune response because they are in direct contact with blood components and blood cells (1, 2). In addition, they produce the factors involved in blood coagulation and fibrinolysis and play important roles in keeping their balance (1, 2).

Lipopolysaccharide (LPS) is an extracellular membrane component of gram-negative bacteria and induces an inflammatory response by binding to Toll-like receptor 4 (TLR4). Macrophages activated with high-dose LPS induce a variety of proinflammatory mediators (3-5). Vascular endothelial cells express TLR4. In the presence of high-dose LPS, such as during sepsis, vascular endothelial cells have been reported to induce an inflammatory response by activating the TLR4 signaling pathway (6). Inflammatory cytokines produced by activated macrophages and vascular endothelial cells excessively activate vascular endothelial cells (7, 8). Therefore, in diseases caused by excessive inflammatory reaction and in cancer and diabetes caused by chronic inflammation, the expression of blood coagulation- and fibrinolysis-related factors in activated vascular endothelial cells becomes imbalanced and promotes blood coagulation. Hence, vascular endothelial cell dysfunction can lead to development of thrombosis (9). It is necessary to suppress the increased expression of inflammatory cytokines

by activated macrophages and vascular endothelial cells to prevent the development of various diseases.

The function of macrophages changes depending on the surrounding environment (10, 11). Macrophages activated by intravenous administration of LPS increase the expression of inflammatory cytokines (3-5). Whereas it has been reported that oral administration of LPS induced anti-inflammatory effects in dementia and arteriosclerosis. The effect of oral administration of LPS has been shown to be different from that of intravenous administration (12, 13). However, the mechanism of the anti-inflammatory effects by oral administration of LPS remains unknown. Although an *in vitro* model has not been established, several reports have shown that low-dose LPS stimulation serves as a model for elucidating the mechanism (14, 15). We previously reported that pretreatment with super-low dose LPS could suppress the activation of inflammatory cytokine genes in monocytes/macrophages by coculturing with cancer cells or adipocytes using a transwell system (16-19). The effects of super-low dose LPS activation *in vitro* may be similar to the effects of oral administration of LPS.

It is known that the expression of plasminogen activator inhibitor (PAI)-1, a fibrinolytic inhibitor, is markedly promoted by inflammatory cytokines (20). The increased expression of *PAI-1* causes thrombosis and leads to development of arteriosclerosis (21-23). It has been shown that the increased expression of *PAI-1* in adipocytes can be suppressed through the culture medium of macrophages activated by super-low dose LPS (24). It has been suggested that super-low dose LPS-activated macrophages can suppress inflammation and may help prevent the development of various diseases by controlling the expression of blood coagulation- and fibrinolysis-related genes. We analyzed the changes in the expression of inflammation-related genes and blood coagulation- and fibrinolysis-related genes in vascular endothelial cells after treatment with LPS-activated macrophage culture medium to clarify the anti-inflammatory effects by super-low dose LPS activation.

Materials and Methods

Cells. Human monocytes THP-1 cells were obtained from the American Type Culture Collection. THP-1 in RPMI 1640 medium (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) containing 10% fetal calf serum supplemented with 100 units/ml each of penicillin and streptomycin (FUJIFILM Wako Pure Chemical Corporation) were incubated in 5% CO_2 at 37˚C. Human aortic endothelial cells (HAoEC) were obtained from PromoCell (PromoCell GmbH, Heidelberg, Germany). HAoEC in Endothelial Cell Growth Medium MV2 (PromoCell GmbH) were incubated in 5% CO_2 at 37˚C.

Addition of LPS-activated THP-1 culture medium. The THP-1 cells were seeded at a density of 5×10^5 cells/ml in a dish. The cells were treated with 5 ng/ml phorbol 12-myristate 13-acetate (PMA) (FUJIFILM Wako Pure Chemical Corporation) for 48 h and were activated with ultrapure *Escherichia coli* LPS (0.1 ng/ml, 10 ng/ml or 1,000 ng/ml) (InvivoGen Corporation, San Diego, CA, USA). The THP-1 culture medium was collected 24 h after activation with LPS and was centrifuged. The HAoEC culture medium was removed and was replaced with equal amounts of LPS-activated THP-1 culture medium and HAoEC culture medium. Then, the cells were incubated in 5% CO_2 at 37˚C for 5 days.

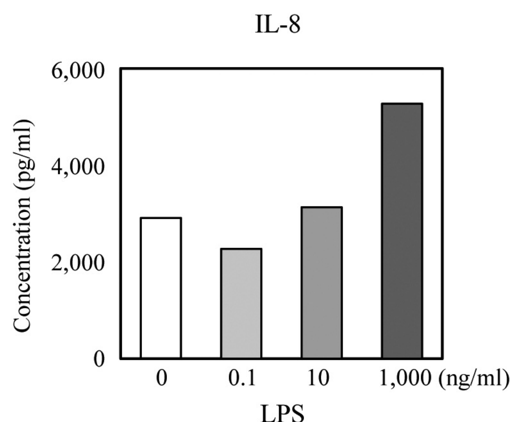

Figure 1. *IL-8 concentration in low-dose lipopolysaccharide (LPS)-activated THP-1 culture medium. THP-1 culture medium was collected 24 h after LPS-activated medium. The IL-8 concentration in THP-1 culture medium with and without LPS activation was measured.*

RNA extraction. Total RNA from the THP-1 cells and HAoEC was isolated using TRIzol® Reagent (Invitrogen Corporation), according to the manufacturer's protocol. RNA was quantified by measuring absorbance at 260 nm. cDNA was synthesized using reverse transcriptase with Oligo(dT)20 (TOYOBO Co., Ltd., Osaka, Japan).

Quantitative real-time PCR. The amplification reactions were performed with Thunderbird® SYBR® qPCR Mix (TOYOBO Co, Ltd.). The PCR conditions were set at 95˚C for 10 min, followed by 40 cycles of 95˚C for 10 s and 60˚C for 30 s. Relative quantification was performed by normalizing the target expression to that of the housekeeping gene, *ACTB* encoding β-actin.

Enzyme-linked immunosorbent assay (ELISA). THP-1 culture media were collected 24 h after activation with LPS and were stored at −20˚C until the assay. Interleukin (IL)-8 concentration in the THP-1 culture medium was measured using an ELISA kit (Funakoshi Co, Ltd., Tokyo, Japan), according to the manufacturer's protocol.

Statistical analysis. Statistical analyses were performed using Microsoft® office 365 Excel. Results were expressed as mean±standard deviation. The differences between stimulation with and without LPS were analyzed by Student's *t*-test. A *p*-value less than 0.05 was considered statistically significant.

Results

Measurement of IL-8 production in LPS-activated macrophage culture medium. We investigated the changes in the concentration of the inflammatory cytokine IL-8 in PMA-

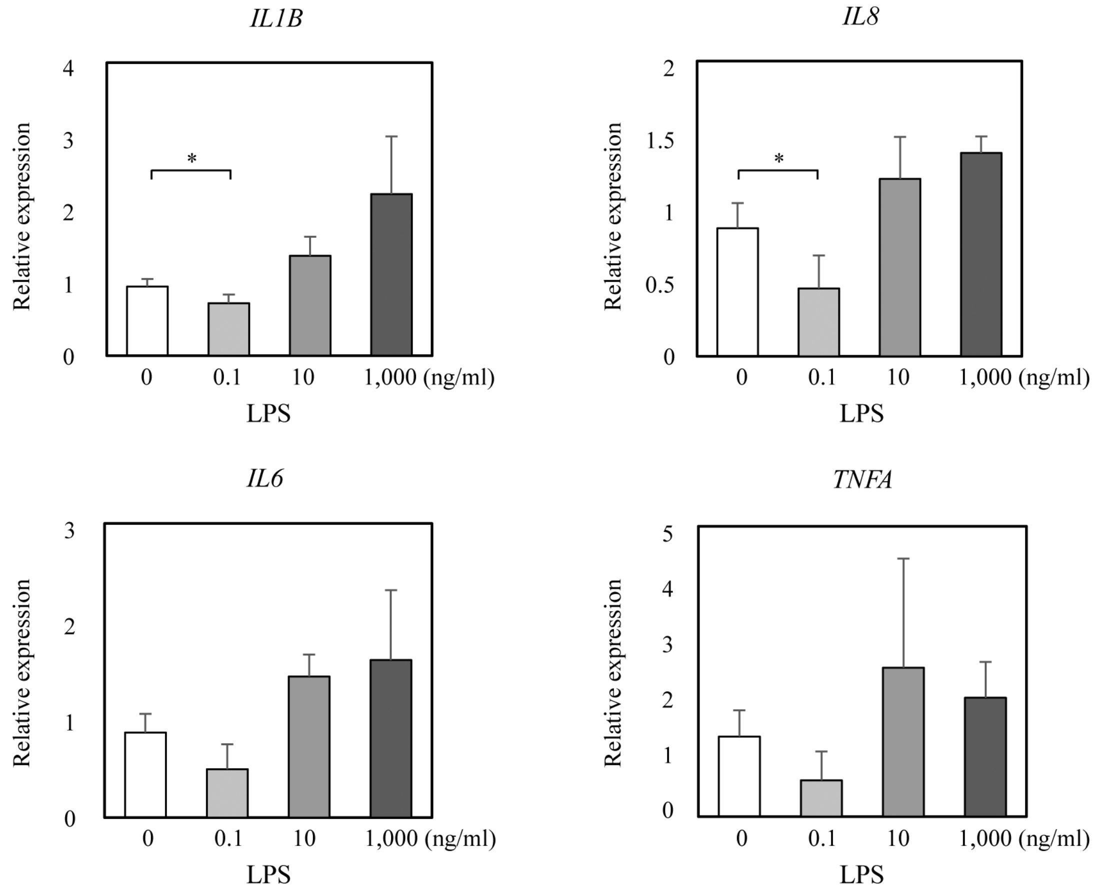

Figure 2. *Gene expression of IL1B, IL6, IL8, and TNFA in human aortic endothelial cells (HAoEC) through low-dose lipopolysaccharide (LPS)-activated macrophages. The mRNA expression of IL1B, IL6, IL8, and TNFA in HAoEC was analyzed using quantitative real-time PCR. Relative quantification was performed by normalizing the target expression to that of ACTB. *p<0.05.*

treated THP-1 culture medium according to the LPS concentration. IL-8 concentration was 2,910 pg/ml without LPS, 2,270 pg/ml with 0.1 ng/ml LPS, 3,130 pg/ml with 10 ng/ml LPS, and 5,270 pg/ml with 1 μg/ml LPS 24 h after stimulation (Figure 1). IL-8 production in macrophages activated with 0.1 ng/ml LPS was lower than in nonactivated macrophages. Macrophages activated by super-low dose LPS were shown to not increase the production of the inflammatory cytokine IL-8. It was demonstrated that the effect of super-low LPS activation was different from the effect of high-dose LPS activation in macrophages. The results indicated that super-low dose LPS activation may regulate the production of inflammatory cytokines in macrophages.

Expression of inflammation-related genes in HAoEC through super-low dose LPS-activated macrophages. We investigated the changes in the expression of inflammatory cytokine genes *IL1B*, *IL6*, *IL8*, and tumor necrosis factor *(TNF)A* and anti-inflammatory cytokine gene *TGFB1* in HAoEC after

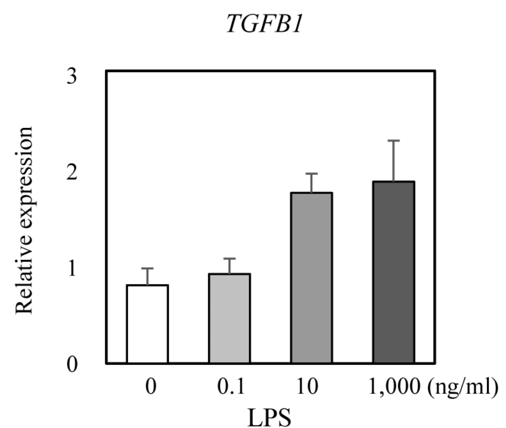

Figure 3. *Gene expression of TGFB1 in human aortic endothelial cells (HAoEC) through low-dose lipopolysaccharide (LPS)-activated macrophages. The mRNA expression of TGFB1 in human aortic endothelial cells (HAoEC) was analyzed using quantitative real-time PCR. Relative quantification was performed by normalizing the target expression to that of ACTB.*

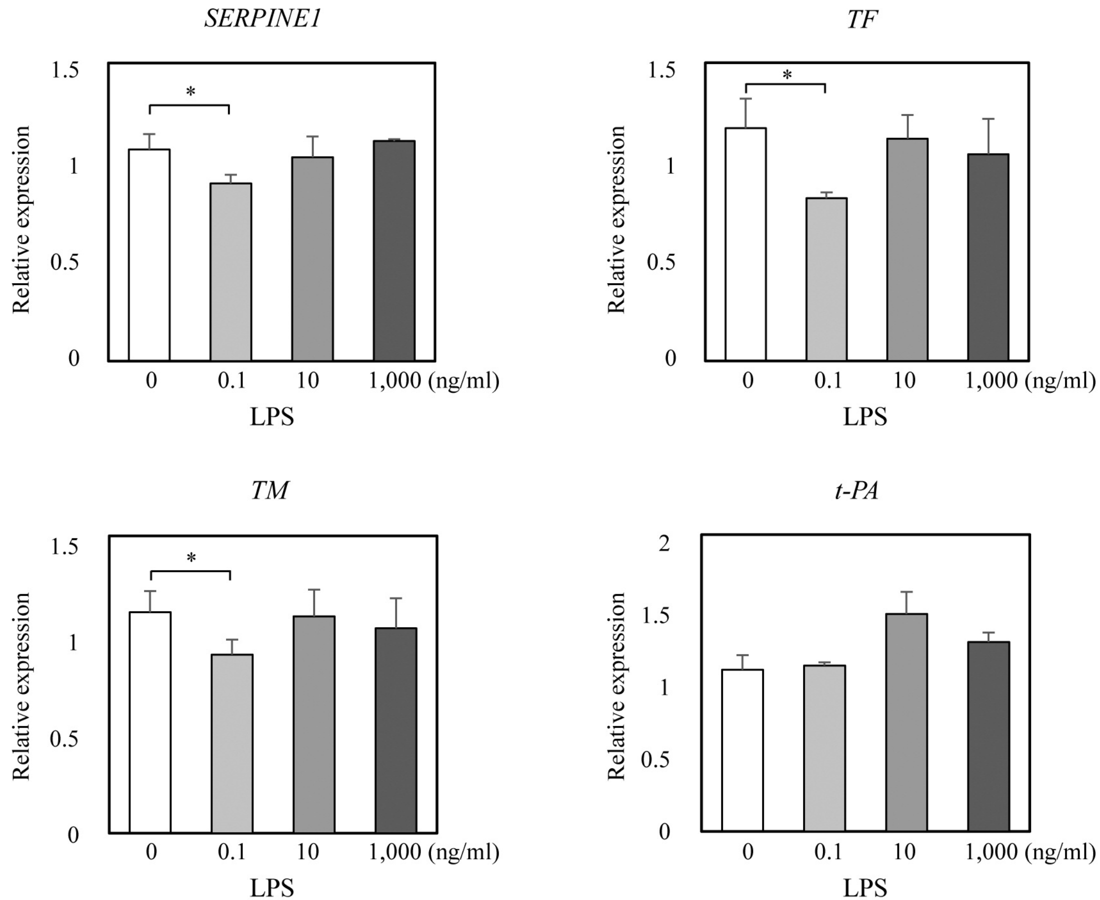

Figure 4. *Gene expression of SERPINE, TF, TM, and t-PA in human aortic endothelial cells (HAoEC) through low-dose lipopolysaccharide (LPS)-activated macrophages. The mRNA expression of SERPINE, TF, TM, and t-PA in HAoEC was analyzed using quantitative real-time PCR. Relative quantification was performed by normalizing the target expression to that of ACTB. *p<0.05.*

treatment with PMA-treated and LPS-activated THP-1 culture medium. *IL1B* and *IL8* expression was significantly suppressed by the culture medium activated with 0.1 ng/ml LPS (*p*=0.03). *IL6* and *TNFA* expression was suppressed by the culture medium activated with 0.1 ng/ml LPS (*p*=0.06). In contrast, *IL1B, IL6, IL8,* and *TNFA* expression increased by the culture medium activated with 10 ng/ml and 1,000 ng/ml LPS (Figure 2). *TGFB1* expression in the culture medium activated with 0.1 ng/ml LPS was the same as that in the control culture medium. *TGFB1* expression increased by the culture medium activated with 10 ng/ml and 1,000 ng/ml LPS (Figure 3). The increased expression of inflammation-related genes *IL1B*, *IL6*, *IL8*, and *TNFA* was suppressed, whereas the expression of antiinflammation-related gene *TGFB1* was not increased in vascular endothelial cells after treatment with super-low dose LPS-activated macrophages culture medium. The results indicate that macrophages transformed by super-low dose LPS suppress inflammation in vascular endothelial cells.

Expression of blood coagulation-related genes in HAoEC through super-low dose LPS-activated macrophages. We investigated the changes in the expression of blood coagulation-related genes *SERPINE1*, tissue factor *(TF)*, and thrombopoietin *(TM)* and fibrinolysis-related gene tissue-type plasminogen activator *(t-PA)* in HAoEC after treatment with PMA-treated and LPS-activated THP-1 culture medium. *SERPINE1*, *TF*, and *TM* expression was significantly suppressed by the culture medium activated with 0.1 ng/ml LPS (*p*=0.02, *p*=0.03, and *p*=0.02, respectively). *t-PA* expression in the culture medium activated with 0.1 ng/ml LPS was the same as that in the control culture medium (Figure 4). The increased expression of blood coagulation-related genes *SERPINE1*, *TF*, and *TM* in vascular endothelial cells was significantly suppressed after treatment with super-low dose LPS-activated macrophages culture medium. The increased expression of blood coagulation-related factors in vascular endothelial cells is known to be promoted by inflammatory cytokines. The results indicate that macrophages transformed

by super-low dose LPS may regulate blood coagulation in vascular endothelial cells.

Discussion

Intravenous administration of LPS is known to induce an inflammatory response and cause severe fever and shock (3-5). On the other hand, we found that oral administration of LPS was effective in the anti-inflammation effects, the anticancer effects, and improvement of cognitive function. However, the mechanism of the anti-inflammatory effects by oral administration of LPS is not clear. Previous studies were demonstrated that the effects of super-low dose LPS activation *in vitro* may be similar to the effects of oral administration of LPS (16-19). It is known that the function of vascular endothelial cells is reversible. In this study, we investigated the changes in the expression of inflammation-related genes in vascular endothelial cells after treatment with LPS-activated macrophage culture medium to clarify the anti-inflammatory effects by super-low dose LPS activation. It was demonstrated that the increased expression of inflammation-related genes *IL1B*, *IL6*, *IL8,* and *TNFA* in vascular endothelial cells was suppressed after treatment with macrophages culture medium activated with super-low dose LPS (Figure 2). Whereas the expression of antiinflammation-related gene *TGFB1* was not increased after treatment with super-low dose LPS-activated macrophages culture medium (Figure 3). It was confirmed that super-low dose LPS-activated macrophage can suppress inflammatory response in vascular endothelial cells. It was clarified that super-low dose LPS activation have the anti-inflammatory effects. Therefore, super-low dose LPS activation *in vitro* may be useful as a model for elucidating the mechanism of the anti-inflammatory effects by oral administration of LPS.

Vascular endothelial cells contribute to maintaining homeostasis by balancing blood coagulation and fibrinolysis (1, 2). However, inflammatory cytokines excessively activate vascular endothelial cells (7, 8), and the activated vascular endothelial cells lead to an imbalance in the expression of blood coagulation- and fibrinolysis-related factors. The dysfunction of activated vascular endothelial cells promotes thrombus formation by promoting blood coagulation, which contributes to the development of lifestyle-related diseases, such as arteriosclerosis. Super-low dose LPS-activated macrophages may be able to suppress functional changes in vascular endothelial cells due to the overexpression of inflammatory cytokines and regulate the dysfunction of vascular endothelial cells. Therefore, super-low dose LPS-activated macrophages may help prevent the development of lifestyle-related diseases, such as arteriosclerosis caused by chronic inflammation.

The expression of blood coagulation-related gene *SERPINE1* in vascular endothelial cells was significantly suppressed after treatment with the culture medium activated with super-low dose LPS (Figure 4). The expression of *SERPINE1*, which encodes PAI-1, was demonstrated to be promoted significantly by inflammatory cytokines (20). The significant suppression of *SERPINE1* expression in vascular endothelial cells may reflect a lack of the increased expression of the inflammatory cytokine gene. Increased *SERPINE1* expression is associated with the insulin-resistant syndrome (25). Therefore, suppressing *SERPINE1* expression may suppress the development of lifestyle-related diseases, such as diabetes.

The expression of blood coagulation-related genes *TF* and *TM* in vascular endothelial cells was significantly suppressed after treatment with the culture medium activated with super-low dose LPS (Figure 4). TF is a blood coagulation factor. TM is a factor involved in blood coagulation control. The significant suppression of *TF* and *TM* expression in vascular endothelial cells may reflect that blood coagulation is not promoted. Moreover, the expression of the fibrinolysis-related gene *t-PA* in vascular endothelial cells was unchanged after treatment with the culture medium activated with super-low dose LPS (Figure 4). PAI-1 inactivates the fibrinolysis-related factor t-PA. In this study, the expression of *SERPINE1* in vascular endothelial cells was significantly suppressed. The expression of *t-PA* in vascular endothelial cells did not change because blood coagulation was not promoted. Suppressing the inflammation of super-low dose LPS-activated macrophages may be useful in preventing thrombosis caused by the dysfunction of vascular endothelial cells.

Conclusion

It was demonstrated that super-low dose LPS activation has anti-inflammatory effects. The results indicated that the effects of super-low dose LPS activation *in vitro* were similar to the effects of oral administration of LPS. Therefore, super-low dose LPS activation *in vitro* may be useful as a model for elucidating the mechanism of the anti-inflammatory effects by oral administration of LPS. Moreover, the effects of macrophages transformed by super-low dose LPS may be effective in preventing the development of various diseases caused by the dysfunction of activated vascular endothelial cells. It is suggested that macrophages transformed by super-low dose LPS may contribute to the maintenance of homeostasis.

Conflicts of Interest

The Authors have no conflicts of interest for this article.

Authors' Contributions

All Authors have contributed to data collection and interpretation. TH performed experiments and drafted the manuscript, HI contributed to provide critical revision of the manuscript.

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