

decreased in the RAWvec cells after LPS stimulation for 6 h; however, this level was not decreased in the RAWar cells (Fig. 3B). To further confirm the role of  $\alpha_2$ AR in LPS-stimulated NF- $\alpha$ B activation, the effects of forced  $\alpha_2$ AR expression on NF- $\alpha$ B-dependent gene transcription were analyzed. NF- $\alpha$ B-mediated-luciferase reporter activity (Fig. 3C) and NOS II promoter activity (Fig. 3D) after stimulation with LPS were inhibited in cells co-transfected with the pcDNA4- $\alpha_2$ ar construct (AR) as well as in cells co-transfected with pCMV-I $\alpha$ B $\alpha$ M (DN- $\alpha$ B). These findings suggested that  $\alpha_2$ AR functions as a negative regulator of NF- $\alpha$ B activation by inhibiting I $\alpha$ B $\alpha$  degradation in LPS-stimulated macrophages. Previously, it has been shown that PDTC blocks NF- $\alpha$ B activation by inhibiting I $\alpha$ B $\alpha$  degradation and subsequently the translocation of NF- $\alpha$ B subunits to the nucleus [22]. Thus, to elucidate the effects of NF- $\alpha$ B activation on the expression of the responsive gene, *Nos2*, PDTC was added to the RAW264 cell cultures at several time points after the addition of LPS, and NO accumulation in the supernatants was analyzed after LPS stimulation for 24 h. As illustrated in Fig. 3E, when PDTC was added to cultures at 0-9 h after the addition of LPS, the NO concentrations in these cultures were markedly lower than those in cultures stimulated with LPS for 24 h without PDTC (right column), indicating that continuous NF- $\alpha$ B activation is essential for adequate NOS II induction.

#### *$\kappa_2$ AR Regulates NF- $\kappa$ B Activation through $\kappa$ -arrestins.*

As  $\alpha$ -arrestin 2 has been reported to interact with I $\alpha$ B $\alpha$  [15, 16], we examined whether  $\alpha$ -arrestin 2 participates in the  $\alpha_2$ AR-mediated regulation of I $\alpha$ B $\alpha$  degradation and NF- $\alpha$ B activation in response to LPS.  $\alpha$ -Arrestin 2 expression was also down-regulated in the LPS-stimulated RAW264 cells (Fig. 4, left panels). Forced  $\alpha_2$ AR expression abolished the down-regulation of  $\alpha$ -arrestin 2 expression (middle panels), suggesting that  $\alpha$ -arrestin 2 expression was regulated by  $\alpha_2$ AR. Deletion of  $\alpha_2$ AR by siRNA decreased  $\alpha$ -arrestin 2 expression (data not shown), supporting that  $\alpha$ -arrestin 2 expression is regulated by  $\alpha_2$ AR. To investigate the role of  $\alpha$ -arrestin 2 down-regulation in response to LPS, a stable  $\alpha$ -arrestin 2 transfectant (RAWarr2) was established (right panels). Since transfection with the vector did not influence NO production (Fig. 1C), cells transfected with  $\alpha$ -arrestin 2 were compared with RAW264 cells. As shown in the RAWar cells (Fig. 2), NO production (Fig. 5A) and NOS II protein and mRNA expressions (Fig. 5B) were definitely decreased in the RAWarr2 cells.

Anti- $\alpha$ -arrestin 2 Abs co-immunoprecipitated I $\alpha$ B $\alpha$  in RAW264 cells before but not after LPS stimulation for 6 h (Fig. 6). On the other hand, the amount of I $\alpha$ B $\alpha$  co-precipitated by anti- $\alpha$ -arrestin 2 Abs was not reduced but rather increased in the RAWar and RAWarr2 cells after LPS stimulation, indicating that the LPS-stimulated down-regulation of  $\alpha_2$ AR and  $\alpha$ -arrestin 2 is essential for I $\alpha$ B $\alpha$  degradation.

## Discussion

In this study, we investigated the role played by  $\alpha_2$ AR in the anti-microbial responses of macrophages was investigated. First, we demonstrated that  $\alpha_2$ AR expression is decreased by LPS stimulation. To directly investigate the role of  $\alpha_2$ AR down-regulation in response to LPS, we established a macrophage cell line, RAWar. Prevention of the down-regulation of  $\alpha_2$ AR expression in RAWar cells resulted in reduced NO production, suggesting that the LPS-associated down-regulation of  $\alpha_2$ AR expression plays an important role in NO production in macrophages.

Decreases in NOS II mRNA expression were observed in the RAWar cells, indicating that NOS II expression was transcriptionally down-regulated by forced  $\alpha_2$ AR expression. Prevention of the down-regulation of  $\alpha_2$ AR expression in the RAWar cells resulted in a marked decrease in NF- $\alpha$ B activation and inhibited cytosolic I $\alpha$ B $\alpha$  degradation, indicating that the forced  $\alpha_2$ AR expression inhibited LPS-induced NF- $\alpha$ B activation by I $\alpha$ B $\alpha$  stabilization.

On the other hand,  $\alpha$ -arrestins, which are universally expressed members of the arrestin family, are the major regulators of GPCR signaling that bind to activated GPCRs causing receptor desensitization and internalization [14]. Recently,  $\alpha$ -arrestins have been shown to play functional roles in the regulation of a variety of signaling pathways and in the mediation of cross-talk between signaling pathways. Moreover, there is accumulating evidence that  $\alpha$ -arrestin 2, which is expressed abundantly in the spleen, is functionally involved in some important immune responses [23-26]. We have demonstrated that  $\alpha$ -arrestin 2 is down-regulated in LPS-stimulated RAW264 cells.  $\alpha$ -Arrestin 2 down-regulation was abolished in RAWar cells, suggesting that  $\alpha$ -arrestin 2 expression is regulated by  $\alpha_2$ AR. These findings suggest that  $\alpha_2$ AR participates in signal transduction pathways from TLR4 by regulating the level of  $\alpha$ -arrestin 2 expression. Meanwhile, the amount of I $\alpha$ B $\alpha$  co-immunoprecipitated by anti- $\alpha$ -arrestin 2 Abs was decreased in the RAW 264 cells after their stimulation with LPS but not in the RAWar or RAWarr2 cells, suggesting that  $\alpha_2$ AR inhibited LPS-induced NF- $\alpha$ B activation by stabilizing I $\alpha$ B $\alpha$  through  $\alpha$ -arrestin 2. The release of NF- $\alpha$ B following the degradation of I $\alpha$ B $\alpha$  proteins is an essential step in the generation of transcriptionally competent NF- $\alpha$ B. In addition, NF- $\alpha$ B activity following stimulation is dependent on the level of cytoplasmic NF- $\alpha$ B/I $\alpha$ B $\alpha$  complexes free from stabilizing factors. Therefore, the following appear likely: (1) LPS-stimulated signals suppress  $\alpha_2$ AR expression, (2) the reduction of  $\alpha_2$ AR results in the down-regulation of  $\alpha$ -arrestin 2 expression, (3)  $\alpha$ -arrestin 2 stabilizes cytoplasmic I $\alpha$ B $\alpha$  and inhibits NF- $\alpha$ B activation; thus, reduction in the level of  $\alpha$ -arrestin 2 accelerates I $\alpha$ B $\alpha$  degradation and NF- $\alpha$ B activation in LPS-stimulated cells, and (4) nuclear translocation of NF- $\alpha$ B enhances NOS II expression.

The cross-talk between  $\alpha_2$ AR and TLR signaling pathways is schematically summarized in Fig. 7. Catecholamines increase cAMP *via*  $\alpha_2$ AR activation, and PKA activation inhibits NF- $\alpha$ B-induced

transcription by phosphorylating cAMP responsive element binding protein (CREB), which competes with p65 for the limited amounts of CREB-binding protein (CBP) (Fig. 7A(a)) [27]. However,  $\alpha_2$ AR agonists did not suppress NO production (unpublished observation). In the present study, we demonstrated that LPS stimulation suppressed the cAMP accumulation in RAWvec cells stimulated with  $\alpha_2$ AR agonist. In addition, we showed that prevention of the down-regulation of  $\alpha_2$ AR inhibits the degradation of I $\alpha$ B $\alpha$  through  $\alpha$ -arrestin 2, which stabilizes I $\alpha$ B $\alpha$  in the steady state (Fig. 7A (b)). Therefore, the down-regulation of  $\alpha_2$ AR and  $\alpha$ -arrestin 2 expressions by the TLR4-dependent pathway might provide a mechanism for "escaping" anti-proinflammatory signals, such as the  $\alpha_2$ AR-cAMP-PKA pathway [27] or the  $\alpha_2$ AR- $\alpha$ -arrestin 2-I $\alpha$ B $\alpha$  pathway. As the levels of  $\alpha_2$ AR ligands vary under different conditions, understanding the cross-talk between TLRs and  $\alpha_2$ AR pathways may have both physiological and pathophysiological importance. Taken together, the observations of the present study regarding the regulation of TLR4 signaling through  $\alpha_2$ AR appear to provide another therapeutic target for the regulation of inflammatory disease conditions.

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### Legends for figures

Fig. 1. LPS stimulation down-regulates  $\alpha_2$ AR expression. (A) RAW264 cells were stimulated with LPS. The protein levels of  $\alpha_2$ AR and GAPDH (loading control) in the plasma membrane were analyzed by Western blotting (left panel). The  $\alpha_2$ AR mRNA and 18S rRNA (loading control) were analyzed by RT-PCR (right upper panel). Bar graphs show the relative intensity of the PCR bands from three separate experiments (mean  $\pm$  SEM) (right lower panel). \*P < 0.01 vs. 0 h. (B) RAW264 cells were transfected with the  $\alpha_2ar$  construct or vector alone. The protein levels of  $\alpha_2$ AR and GAPDH (left panel) and mRNA expressions of  $\alpha_2$ AR and 18S rRNA (right upper panel) were analyzed as in A. Bar graphs show the relative intensity of the PCR bands from three separate experiments (mean  $\pm$  SEM) (right lower panel). \*P < 0.01 vs. 0 h. (C) mRNA expressions of  $\alpha_2$ AR and 18S rRNA (upper panel) were analyzed as in A. Bar graphs show the relative intensity of the PCR bands from three separate experiments (mean  $\pm$  SEM) (lower panel). \*P < 0.01 vs. 0 h. (D) Cells were cultured with or without LPS for 6 h and were stimulated with Salbutamol ( $1 \times 10^{-6}$  M) for final 30 min. Then, intracellular cAMP concentrations were analyzed. \*P < 0.05 vs. without LPS. (E) Cell size was measured by flow cytometric analysis of forward light scatter characteristics (FSC).

Fig. 2. Forced  $\alpha_2$ AR expression suppresses NO production and NOS II expression. (A) Cells were stimulated with LPS for 24 h, and nitrite accumulation in the supernatants was measured using the Griess reagent. The results are expressed as means  $\pm$  SEM from three-well cultures. \*P < 0.001 vs. LPS-stimulated RAW264 or RAWvec cells. (B) The protein levels of NOS II and GAPDH (left panel) and mRNA expressions of NOS II and 18S rRNA were analyzed as in A (right upper panel). Bar graphs show the relative intensity of the PCR bands from four separate experiments (mean  $\pm$  SEM) (right lower panel). \*P < 0.01 vs. corresponding RAWvec cells. Data shown are representative of three–four separate experiments.

Fig. 3. Forced  $\alpha_2$ AR expression suppresses NF- $\kappa$ B activation.

(A) The vector control cells and  $\alpha_2$ AR transfectant were stimulated with LPS, and NF- $\kappa$ B activation was analyzed by EMSA. (B) The vector control cells and  $\alpha_2$ AR transfectant were stimulated with LPS, and cytoplasmic I $\kappa$ B $\alpha$  and GAPDH (loading control) was analyzed by Western blotting. (C, D) RAW264 cells were co-transfected with the pNF- $\kappa$ B-Luc vector (C) or NOS II promoter-luciferase construct (D) and vector (Vec), pcDNA4- $\alpha_2$ AR (AR) or pCMV-I $\kappa$ B $\alpha$ M (DN-I $\kappa$ B). The cells were cultured with LPS for 24 h, and luciferase activities were determined. The results are expressed as means  $\pm$  SEM from six-well cultures. \*P < 0.001 vs. cells co-transfected with Vec. (E) PDTC was

added to the cultures at the indicated time points after addition of LPS. Nitrite accumulation in the supernatants at 24 h of culture was measured using the Griess reagent. The results are expressed as means  $\pm$  SEM from three-well cultures. The error bars are too small to be distinguishable in the figure (numeric data from the left bar:  $3.75 \pm 0.18$ ,  $5.07 \pm 0.22$ ,  $4.22 \pm 0.07$ ,  $5.69 \pm 0.12$ ,  $10.38 \pm 0.06$ ,  $15.00 \pm 0.05$ , and  $25.20 \pm 0.28$ ). \* $P < 0.001$  vs. LPS-stimulated cells without PDTC. Data shown are representative of two–three separate experiments.

Fig. 4. LPS stimulation down-regulates  $\alpha$ -arrestin 2 expression. RAW264, RAWar, and RAWarr2 cells were stimulated with LPS, and the protein levels of  $\alpha$ -arrestin 2 and GAPDH (upper panel) and mRNA expressions of  $\alpha$ -arrestin 2 and 18S rRNA (middle panel) were analyzed as in Fig. 1A. Bar graphs show the relative intensity of the band from three separate experiments (mean  $\pm$  SEM) (lower panel). \* $P < 0.01$  vs. 0 h.

Fig. 5. Forced  $\alpha$ -arrestin 2 expression suppresses NO production and NOS II expression.

(A) Cells were stimulated with LPS for 24 h, and nitrite accumulation in the supernatants was measured using the Griess reagent. The results are expressed as means  $\pm$  SEM from three-well cultures. \* $P < 0.001$  vs. LPS-stimulated RAW264 cells. (B) The protein levels of NOS II and GAPDH (left panel) and mRNA expressions of NOS II and 18S rRNA (light upper panel) were analyzed as in Fig. 1A. Bar graphs show the relative intensity of the PCR bands from three separate experiments (mean  $\pm$  SEM) (right lower panel). \* $P < 0.01$  vs. corresponding RAW264 cells. Data shown are representative of three–four separate experiments.

Fig. 6.  $\alpha$ -arrestin 2 interacts with cytosolic I $\alpha$ B $\alpha$ . Before and after stimulation with LPS for 6 h, cells were lysed and immunoprecipitated with anti- $\alpha$ -arrestin 2 Abs. Western blotting analysis was performed using anti-I $\alpha$ B $\alpha$  Abs (upper panel). The protein levels of GAPDH in equal amounts of lysates were used for control (lower panel).

Fig. 7. Cross-talk between  $\alpha_2$ AR and TLRs signaling pathways.

(A)  $\alpha_2$ AR agonists suppress NF- $\kappa$ B activation by increasing cytoplasmic  $\alpha$ -arrestin 2, which stabilizes the NF- $\kappa$ B/I $\alpha$ B $\alpha$  complexes in cytoplasm (a) or by activating CREB which competes CBP with NF- $\kappa$ B in the nucleus (b). (B) TLR4-dependent signals lead to the following steps both in the presence or absence of  $\alpha_2$ AR agonists: ① TLR4-dependent down-regulation of  $\alpha_2$ AR expression, ② down-regulation of  $\alpha$ -arrestin 2, ③ release of NF- $\kappa$ B/I $\alpha$ B $\alpha$  complexes in the cytoplasm, ④ degradation of I $\alpha$ B $\alpha$ , and ⑤ translocation of NF- $\kappa$ B to the nucleus and transcription of its target genes.



Figure 1

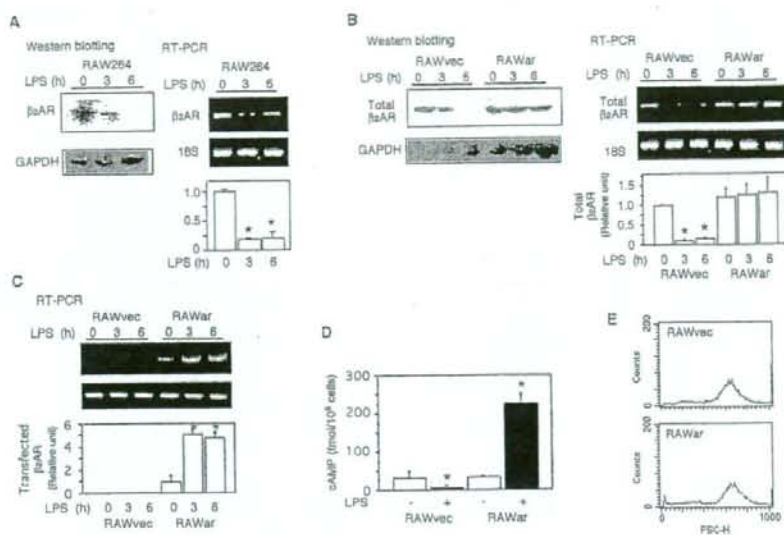


Figure 2

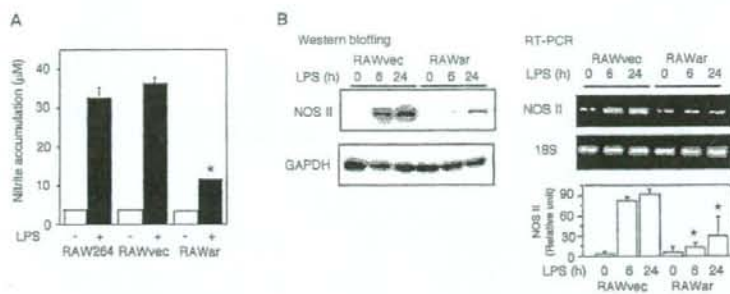


Figure 3

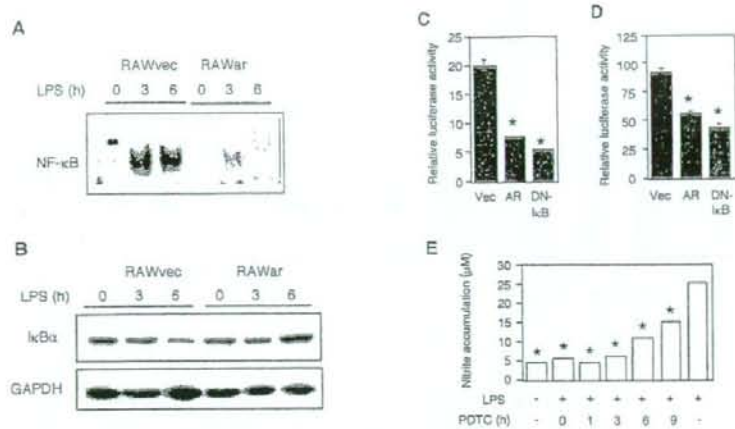


Figure 4

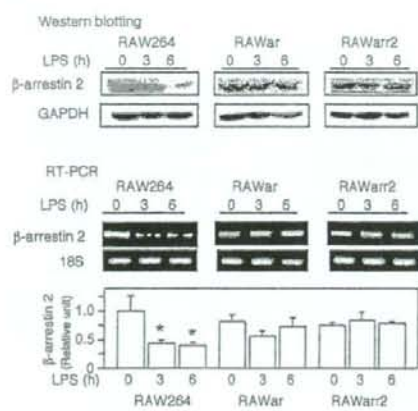




Figure 5

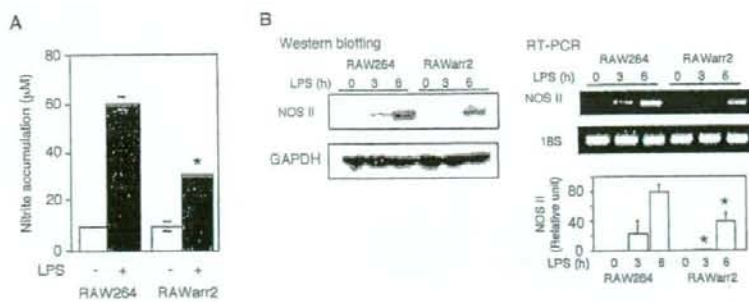


Figure 6

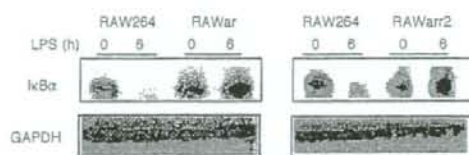
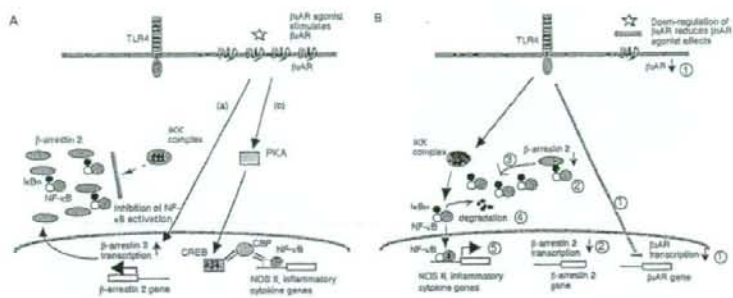


Figure 7



## Original article

Title: White matter lesions as a feature of cognitive impairment, low vitality, and other symptoms of the geriatric syndrome in the elderly

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Running title: White matter lesions in the elderly

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**Abstract**

White matter lesions (WMLs) are common findings on MRI in elderly persons. In this study, we analyzed the relation of WMLs with global cognitive function, depression, vitality/volition, and nineteen symptoms of the geriatric syndrome in Japanese elderly patients who attended three university geriatric outpatient clinics. Two hundred and eighty six subjects (103 men and 183 women, mean±SD age 74.5±7.8 years old) were included in this study. MRI scans were performed for the diagnosis of WMLs, and the severity of periventricular and subcortical white matter hyperintensities (PVHs and DWMHs) was rated semi-quantitatively. Concurrently, all subjects underwent tests of cognitive function, depressive state, and vitality, and were examined for nineteen symptoms of the geriatric syndrome. The study subjects showed cognitive decline, depression, and low vitality, all to a mild extent. Univariate linear regression analysis showed a negative correlation between the severity of WMLs and cognitive function or vitality. Multiple logistic analysis revealed that the severity of WMLs was a significant determinant of cognitive impairment and low vitality, after adjustment for confounding factors such as age, sex, and concomitant diseases. PVH and/or DWMH score was significantly greater in

subjects who exhibited 13 out of 19 symptoms of the geriatric syndrome. Logistic regression analysis indicated that WMLs were associated with psychological disorders, gait disturbance, urinary problems, and parkinsonism. In conclusion, WMLs were associated with various symptoms of functional decline in older persons. Evaluating WMLs in relation to functional decline would be important for preventing disability in elderly people.

Key words: white matter lesion, periventricular hyperintensity, deep white matter hyperintensity, geriatric syndrome

## Introduction

Brain magnetic resonance imaging (MRI) has markedly enhanced the chance of detecting characteristic hyperintense signals in the periventricular and subcortical areas on T2-weighted images, even in asymptomatic older persons.<sup>1</sup> This lesion is known as white matter lesions (WMLs), leukoaraiosis, or white matter (periventricular and subcortical) hyperintensities.<sup>2-4</sup> WMLs, which accompany symptoms of gait abnormalities,<sup>5-7</sup> urinary symptoms,<sup>5,8,9</sup> and cognitive impairment,<sup>4,10,11</sup> are reported to be associated with aging,<sup>12-14</sup> hypertension,<sup>14</sup> diabetes,<sup>15</sup> and atherosclerosis.<sup>5</sup> There is poor understanding of the pathogenesis of the lesions, and it remains unknown whether WMLs are mere innocuous radiological changes that appear as a result of the aging process,<sup>2,3,10</sup> or whether they are one of the causal factors of the functional decline in elderly people.

The geriatric syndrome is a group of symptoms that are related to daily life, and the comorbidity triggers the loss of independence of elderly persons. Hence, evaluation of the geriatric syndrome is important for the physical and mental care of the elderly. To address the pathological significance of WMLs in the global cognitive and psychological functions, and in the geriatric syndrome in the