Pterostilbene reduces colonic inflammation by suppressing dendritic cell activation and promoting regulatory T cell development

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Abstract
Dendritic cells (DCs) and T cells play important roles in immune regulation, and modulating their function is an approach for developing preventive or therapeutic strategies against immune disorders. Herein, the effect of pterostilbene (PSB) (3’,5’-dimethoxy-resveratrol)—a resveratrol-related polyphenol found in blueberries—on immune regulation was evaluated. Using an in vitro co-culture system, PSB was found to exert the strongest inhibitory effect among all tested resveratrol derivatives on DC-mediated T cell proliferation; moreover, PSB treatment decreased the Th1 and Th17 populations and increased the regulatory T cell (Treg) population. Upon co-stimulation with anti-CD3 and anti-CD28 antibodies, PSB inhibited CD4+ T cell proliferation and differentiation into Th1 cells. Additionally, PSB acted on DCs to suppress the lipopolysaccharide-induced transactivation of genes encoding antigen presentation-related molecules and inflammatory cytokines by attenuating the DNA-binding ability of the transcription factor PU.1. Furthermore, PSB promoted DC-mediated Foxp3+ Treg differentiation, and PU.1 knockdown increased DC-induced Treg activity. Oral administration of PSB alleviated the symptoms of dextran sulfate sodium-induced colitis and decreased tumor necrosis factor-α expression in mice. Thus, PSB treatment ameliorates colonic inflammation.

Keywords
colitis, immunosuppression, pterostilbene, resveratrol

Abbreviations: BMDC, bone marrow-derived dendritic cell; CFSE, carboxyfluorescein succinimidyl ester; ChIP, chromatin immunoprecipitation; DAI, disease activity index; DC, dendritic cell; DSS, dextran sulfate sodium; ELISA, enzyme-linked immunosorbent assay; GM-CSF, granulocyte macrophage-colony stimulating factor; IBD, inflammatory bowel disease; LPS, lipopolysaccharide; MHCII, major histocompatibility complex class II; OVA, ovalbumin; PSB, pterostilbene; RSV, resveratrol; TCR, T cell receptor; Treg, regulatory T cell; siRNA, small interfering RNA.

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1 | INTRODUCTION

Although adequate immune responses are intrinsic to conferring protection against pathogens and environmental factors, excessive immune responses can lead to allergic and autoimmune diseases. Dendritic cells (DCs) and T cells play essential roles in the regulation of immune responses. To initiate an adaptive immune response, DCs present captured antigens on major histocompatibility complex (MHC) molecules and express the costimulatory molecules CD80 and CD86, which bind to CD28 expressed on T cells. Recognition of the cognate antigen by T cells via their T cell receptor (TCR) and CD28 ligation results in T cell activation. Activated T cells produce interleukin (IL)-2 to facilitate their proliferation. After clonal expansion, naïve CD4+ T cells differentiate into various effector helper T cells such as Th1, Th2, Th17, or regulatory T cells (Treg), depending on the surrounding cytokine milieu. Aberrant activation of Th1, Th2, and Th17 cells can lead to immune disorders, such as inflammatory bowel diseases (IBD), atopic dermatitis, and psoriasis. In contrast, Treg cells produce IL-10 to inhibit disease progression.

IBD is a chronic inflammatory disorder of the gastrointestinal tract, and its types include ulcerative colitis and Crohn’s disease. Although the pathogenesis of IBD remains unclear, excessive production of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, IL-1β, and IL-6 is often observed in IBD patients. The use of immunosuppressants and monoclonal antibodies against TNF-α are effective strategies for the management of IBD; however, a complete cure for IBD is currently unavailable. Therefore, there is an urgent need to develop preventive methods as well as novel therapeutic strategies for the treatment of IBD patients.

Polyphenols are natural products that are consumed by humans as vegetables, fruits, and herbs. Plant polyphenols possess antioxidant, anti-inflammatory, and anti-cardiogenic properties. Various mechanisms have been proposed to explain the beneficial effects of polyphenols. Resveratrol (RSV), a polyphenol found in grapes, is known to exert pleiotropic effects. Several studies have shown that RSV may exert therapeutic effects in neurological, cardiovascular, and metabolic disorders in humans. Moreover, RSV has been shown to possess immunomodulatory activity and reduce inflammation in a murine colitis model. Although natural analogs of RSV have been identified, their physiological functions remain unknown. Herein, the immunosuppressive effect of RSV derivatives on immune regulation was studied, and pterostilbene (PSB) (3′,5′-dimethoxy-resveratrol), an RSV-related polyphenol found in blueberries, was identified. PSB has a higher bioavailability than RSV due to the presence of two methoxy groups that increase its lipophilic and oral absorption. For disease prevention, it is important to identify the components of functional foods and understand their underlying mechanism of action.

2 | MATERIALS AND METHODS

2.1 | Reagents

Pterostilbene, trans-RSV, and cis-RSV were purchased from Cayman Chemical (Ann Arbor, MI, USA). Lipopolysaccharide (LPS, from Escherichia coli O111:B4) and trans-stilbene were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2 | Mice

OT-II mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). C57BL/6J mice were purchased from Japan SLC, Inc (Hamamatsu, Japan). All animal experiments were performed in accordance with approved guidelines of the Institutional Review Board of Tokyo University of Science, Tokyo, Japan.

2.3 | Cell preparation

Naïve CD4+ T cells were isolated from the splenocytes of C57BL/6J or OT-II mice using a mouse naïve CD4 T cell isolation kit and autoMACS Pro Separator (both from Miltenyi Biotec, Tubingen, Germany). For carboxyfluorescein succinimidyl ester (CFSE) staining, naïve CD4+ T cells were incubated with 2 μM CFSE (eBioscience, Inc, San Diego, CA, USA) for 10 minutes. Bone marrow-derived DCs (BMDCs) were generated as previously described.

2.4 | Cell culture

Naïve OT-II CD4+ T cells (5 × 10^5 cells) and OVA-pulsed BMDCs (1 × 10^5 cells) were seeded into round-bottomed 96-well plates and incubated for 3 days. Naïve CD4+ T cells (5 × 10^5 cells) were cultured for 3 days in flat-bottomed 96-well plates coated with anti-CD3ε and anti-CD28 antibodies (both from Tonbo Biosciences, San Diego, CA, USA). The following cytokines and antibodies were added to the medium under polarizing conditions: 10 ng/mL IL-12 (PeproTech, Inc, Rocky Hill, NJ, USA) and 10 μg/mL anti-IL-4 antibody (BioLegend, San Diego, CA, USA) for Th1 cells; 20 ng/mL IL-4 (PeproTech, Inc) and 10 μg/mL anti-IL12 antibody (BioLegend) for Th2 cells; and 1 ng/mL TGFB-β (PeproTech, Inc), 10 ng/mL IL-6
(BioLegend), 10 µg/mL anti-IFN-γ antibody (BioLegend), 10 µg/mL anti-IL-4 antibody for Th17, 1 ng/mL TGF-β, and 10 ng/mL IL-2 (PeproTech, Inc) for Treg cells.

2.5 Foxp3 staining

CD4+ T cells were stained with fluorescein isothiocyanate-labeled anti-CD4 antibody and PerCP-labeled anti-CD3ε antibody and then, fixed and permeabilized using a Foxp3/Transcription Factor Staining Buffer Kit (Tonbo Biosciences). The permeabilized cells were stained with allophycocyanin-labeled anti-FOXP3 antibody.

2.6 Intracellular cytokine staining

Cells were stimulated with 50 ng/mL phorbol myristate acetate (PMA) and 1 µg/mL ionomycin (both from Wako, Osaka, Japan) in the presence of 5 µg/mL brefeldin A and 2 µM monensin (both from BioLegend). After 12 hours, the cells were stained with FITC-labeled anti-CD4 antibody and PerCP-labeled anti-CD3ε antibody, and fixed and permeabilized with Fixation Buffer and Intracellular Staining Perm Wash Buffer (both from BioLegend). The permeabilized cells were stained with PECy7-labeled IFN-γ antibody, PE-labeled IL-4 antibody, or PE-labeled IL-17A antibody.

2.7 Flow cytometry

Flow cytometry was performed to detect cell-surface or intracellular molecules, as described previously.13 BMDCs were stained with PerCP-labeled MHC class II (MHCII) antibody and PE-labeled CD86 antibody (BioLegend).

2.8 Quantitative RT-PCR

The mRNA levels were determined as previously described.14 The TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) and sequences of synthesized oligonucleotide primers are listed in Table S1.

2.9 Western blotting

Western blotting was performed as previously described.15 Anti-PU.1 antibody (D19, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-β-actin antibody (AC-15, Sigma-Aldrich) were used as primary antibodies. The band intensity was measured using Image J software version 1.51 (NIH, Bethesda, MD, USA).

2.10 Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) kits for IL-2, TNF-α, IL-6, and IL-12 p40 were purchased from BioLegend. The cytokine concentrations in the culture medium were determined according to the manufacturer’s instructions.

2.11 Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed as previously described.16,17 Anti-PU.1 Ab (no. D-19) and Goat IgG (no. 02-6202; Invitrogen, Carlsbad, CA, USA) were used. The amount of precipitated DNA was determined by quantitative PCR using an Applied Biosystems Step-One Real-time PCR system. The nucleotide sequences of the PCR primer sets are listed in Table S1.

2.12 siRNA knockdown

PU.1 siRNA (Stealth Select RNAi, Spil-MSS247676) and control siRNA (Stealth Negative Control) were purchased from Thermo Fisher Scientific (Waltham, MA, USA) and transfected into BMDCs by Nucleofector 2b (Lonza, Basel, Switzerland) using the Amaxa Mouse Dendritic Cell Nucleofector Kit (Lonza), as previously described.13

2.13 Dextran sulfate sodium-induced colitis

PSB (Tokyo Chemical Industry, Tokyo, Japan) was suspended in methylcellulose (Wako) containing 0.2% Tween 20 (Tokyo Chemical Industry). This solution was orally administered to 8-week-old C57BL/6J female mice (100 mg/kg body weight) once daily from days −3 to 10. The mice were given ad libitum access to 2.5% of dextran sulfate sodium (DSS) dissolved in drinking water from days 0 to 10.18 Their body weight was monitored daily, and fecal samples were collected every 2 days. The mice were sacrificed on day 10 and their colon lengths were measured following dissection. Colonic RNA was extracted using Isogen (Nippon Gene, Tokyo, Japan), and subjected to reverse transcription and quantitative PCR. The disease activity index (DAI) was calculated by averaging the scores for body weight loss, stool consistency, and bleeding. The scoring pattern was as follows: change in body weight loss (0: <1%, 1:1%-5%, 2:5%-10%, 3:10%-15%, 4:> 15%), stool consistency (0: normal, 2: loose stool, 4: diarrhea), and bleeding (0: negative, 2: moderate, 4: severe).19
2.14 | Statistical analysis

Comparisons between multiple groups were analyzed using the one-way ANOVA and the Tukey-Kramer test. The difference between two groups was analyzed using the F test and the unpaired student’s t test. \( P < .05 \) was considered as statistically significant.

3 | RESULTS

3.1 | Effect of RSV derivatives on antigen-dependent T cell proliferation and differentiation

To evaluate the immunosuppressive activity of RSV derivatives, we co-cultured OVA-pulsed BMDCs and naïve CD4+ T cells derived from OT-II mice, which are OVA-specific TCR transgenic mice, in the presence or absence of RSV-related polyphenols. While trans-RSV (tRSV) slightly suppressed T cell proliferation at 10 μM, PSB showed stronger inhibition at the same concentration (Figure 1A). cis-RSV (cRSV) and trans-stilbene (tSB) did not exert any effect on T cell proliferation (Figure 1A). Therefore, we focused on the effect of PSB in subsequent experiments. As shown in Figure 1B, OVA-dependent T cell-proliferation was suppressed by PSB in a dose-dependent manner, indicating that PSB exhibits immunosuppressive activity during antigen-presenting cell-mediated T cell activation. We next examined whether PSB affects the CD4+ effector T cell population. Flow cytometric analysis showed that the population of Th1 and Th17 cells co-cultured with OVA-pulsed DCs under each polarizing condition was significantly decreased in the presence of PSB (Figure 1C,E). However, treatment with PSB did not affect the population of Th2 cells (Figure 1D). Interestingly, Foxp3+ Treg differentiation was markedly increased in the presence of PSB (Figure 1F). These results indicate that PSB exhibits immunosuppressive activity by promoting T cell differentiation toward Treg cells rather than toward Th1 and Th17 cells.

FIGURE 1 Effect of resveratrol derivatives on antigen-dependent T cell proliferation and differentiation. A, B, OVA-pulsed BMDCs and CFSE-labeled OT-II naïve CD4+ T cells were co-cultured for 72 hours in the presence of 10 μM RSV derivatives (A) or 0-25 μM PSB (B). CFSE dilution was analyzed using flow cytometry. Representative histograms and mean fluorescence intensity (MFI) are shown. C-F, OVA-pulsed BMDCs and OT-II naïve CD4+ T cells were co-cultured for 72 hours with 25 μM PSB under (C) Th1-, (D) Th2-, (E) Th17-, and (F) Treg-skewing conditions. Cells were treated with PMA, ionomycin, brefeldin A, and monensin for 12 hours (C-E). After fixation and permeabilization, intracellular cytokines or nuclear FOXP3 was stained and detected by flow cytometry. Representative dot plots are shown. Data are presented as the mean + SD (n = 3). A, B, *P < .05, Tukey-Kramer test. C-F, *P < .05, two-tailed student’s t test analysis.
3.2 | Effect of PSB on antigen-independent T cell proliferation and differentiation

Although the results showed the effects of PSB on T cell differentiation in a co-culture system, it was unclear whether PSB directly acts on T cells, and/or suppresses T cell proliferation by modulating DC function. To determine the effect of PSB on T cell proliferation in the absence of DCs, naïve CD4+ T cells were cultured with plate-bound anti-CD3ε and anti-CD28 antibodies to induce T cell proliferation in a DC-independent manner. PSB completely inhibited TCR stimulation-dependent T cell proliferation (Figure 2A). Consistent with this finding, PSB significantly decreased IL-2 mRNA and protein expression (Figure 2B,C). These results indicate that PSB directly suppresses T cell proliferation. We next assessed whether PSB directly affects helper T cell differentiation. PSB significantly inhibited Th1 differentiation (Figure 2D) but did not affect Th2, Th17, or Treg differentiation (Figure 2E-G). These results indicate that PSB, at least in part, directly inhibits the proliferation and differentiation of T cells into Th1.

3.3 | Effect of PSB on DCs

To determine the effect of PSB on DCs, we evaluated the gene expression levels and activity of PSB-treated DCs. The expression levels of antigen presentation-related genes such as H2-ea, Citta, Cd86, Ccr7, and Pdcd1lg2 in DCs was significantly decreased upon treatment with PSB (Figure 3A). We next investigated the effect of PSB on TLR-mediated activation of DCs. As shown in Figure 3B, LPS-induced upregulation of cell surface expression of MHC class II and CD86 was significantly suppressed upon pretreatment with PSB. LPS-induced secretion of TNF-α, IL-6, and IL-12 p40 was also significantly reduced upon PSB pretreatment (Figure 3C,D). We previously reported that the transcription of DC-specific genes, including Citta, Cd86, Ccr7, Tnf, and Pdcd1lg2, is positively regulated by PU.1. Therefore, we hypothesized that PSB modulates PU.1 activity in DCs. Western blotting analysis revealed that the total protein level of PU.1 in PSB-treated DCs was comparable to that in control DCs (Figure 3E). Consistent with this finding, PU.1 mRNA expression was not affected by PSB.
pretreatment ("Spi1" in Figure 3A). Next, we analyzed the effect of PSB treatment on the recruitment of PU.1 to chromosomal DNA. As shown in Figure 3F, the amount of PU.1 that remained bound to the promoters of these genes was significantly reduced upon PSB treatment. These results suggest that PSB downregulates the expression of antigen presentation-related and pro-inflammatory genes by decreasing PU.1-binding to the promoters of PU.1 target genes.

We then examined whether reduced PU.1 activity and subsequent downregulation of PU.1-target genes in DCs affect T cell proliferation and differentiation. PU.1 siRNA-transfected BMDCs were pulsed with OVA 323-339 peptide and co-cultured with naive CD4+ T cells from OT-II mice. T cell proliferation was observed in the co-culture with control siRNA-transfected BMDCs, but was significantly decreased in PU.1 siRNA-transfected BMDCs (Figure 4A). Furthermore, we co-cultured CD4+ OT-II T cells with PU.1-knockdown DCs under Treg-polarizing conditions to confirm whether PU.1 knockdown accelerates Treg induction in DCs, similar to in PSB-treated DCs. Treg differentiation in PU.1 siRNA-transfected BMDCs was higher than that in control BMDCs (Figure 4B). This result suggests that downregulation of PU.1-target genes in PSB-treated BMDCs enhances Treg differentiation.

**FIGURE 3** Effect of PSB on BMDCs. A, BMDCs were treated with 0, 10, 25, and 50 µM PSB for 48 hours. Relative mRNA expression was determined by qPCR and normalized to Gapdh mRNA expression. B-D, BMDCs were pretreated with 25 µM PSB for 30 minutes and stimulated with LPS for 6 hours. B, Cell surface protein expression was determined by staining with specific antibodies and analyzed using flow cytometry. Representative dot plots are shown. C, Relative mRNA expression was determined as described in A. D, Protein expression in the culture medium was determined using ELISA. E, F, BMDCs were treated with 25 µM PSB for 48 hours. E, Cellular protein expression was determined by western blotting. Band intensity was quantified with Image J software. F, ChIP assays were performed using anti-PU.1 (PU.1 Ab) or goat IgG (isotype) antibody. Co-immunoprecipitated DNA was quantified using qPCR with specific primer sets for the promoter region of the indicated genes. Data are presented as the mean ± SD (n = 3). A, *p < .05, Tukey-Kramer test. B-F, *p < .05, two-tailed student's t test analysis.
3.4 | Immunosuppressive activity of PSB in vivo

To understand the physiological relevance of the immunosuppressive activity of PSB, we determined the effect of PSB administration on colonic inflammation using a DSS-induced colitis mouse model. To demonstrate the protective effect of PSB, mice were orally administered with 100 mg/kg/day PSB daily from 3 days prior to ad libitum feeding with drinking water containing 2.5% (w/v) DSS (Figure 5A). DSS-induced weight loss, DAI score, and colon length were significantly improved upon PSB treatment compared with the corresponding parameters in the vehicle group (CTL) (Figure 5B-D). Moreover, Tnf mRNA expression in the colon was decreased upon PSB treatment (Figure 5E). These results indicate that oral administration of PSB can significantly ameliorate DSS-induced colitis in mice.

4 | DISCUSSION

Our in vitro and in vivo data showed that PSB exhibits robust immunosuppressive activity by modulating DC function and CD4+ T cell activation. PSB exerts its immunosuppressive effect by suppressing the proliferation and differentiation of CD4+ T cells into Th1 cells and promoting differentiation into Treg cells.

The immunosuppressive activity of PSB against T cell proliferation was higher than that of tRSV. This may be due to differences in their affinity for the receptor involved in modulating T cell proliferation. A previous study showed that PSB exhibits higher hypolipidemic activity than tRSV because of its highly efficient binding to PPARα in hepatocytes.23 Another study showed that PPARα agonists can improve the pathology of autoimmune diseases; however, the effect of PPARα agonists on T cells was not investigated.24 Furthermore, PSB and tRSV exert their biological effects by targeting sirtuin and adenosine monophosphate kinase (AMPK).25 AMPK activation inhibits T cell proliferation by altering glycolysis and lipid metabolism.26 These data suggest that PSB inhibits T cell proliferation by binding to PPARα and/or regulating AMPK activity.

Chang et al demonstrated that PSB inhibits extracellular signal-regulated kinase (ERK)1/2 and induces cell cycle arrest in T cell leukemia/lymphoma; however, they did not investigate whether PSB-induced cell cycle arrest is mediated
by ERK inhibition.\textsuperscript{27} In naïve CD4\textsuperscript{+} T cells, binding of TCR and CD28 to MHCII and CD80/86, respectively, activates various signaling pathways such as the calcineurin-nuclear factor of activated T-cells (NFAT), mitogen-activated protein kinase (MAPK), and phosphatidylinositol-3 kinase (PI3K)-protein kinase B (PKB/Akt)-mammalian target of rapamycin (mTOR) pathways.\textsuperscript{28-31} These pathways activate the transcription factors NFAT, AP-1, and NF-κB, respectively, for the transcription of \textit{Il2}.\textsuperscript{32,33} Immunosuppressants such as tacrolimus and cyclosporine, which are used to prevent graft rejection in allogeneic transplantation, can suppress T cell proliferation by inhibiting calcineurin-NFAT signaling.\textsuperscript{34} In addition, the immunosuppressant rapamycin can suppress T cell proliferation by inhibiting mTOR signaling.\textsuperscript{35} PSB may inhibit these signaling pathways, as \textit{Il2} mRNA expression was decreased in PSB-treated CD4\textsuperscript{+} T cells.

PU.1 regulates the expression of antigen-presentation-related molecules and inflammatory cytokines such as MHCII, CD86, CCR7, and TNF-α in DCs.\textsuperscript{12,14,20,21} We demonstrated that PSB decreases the expression of these genes by attenuating the DNA binding of PU.1 to regulatory elements. PU.1 is phosphorylated by PKCδ during the differentiation of hematopoietic stem cells into DCs.\textsuperscript{36} However, PSB treatment did not affect the phosphorylation level of PU.1 (data not shown). Although further studies are required to fully understand the relationship between PSB treatment and the DNA-binding ability of PU.1, we demonstrated that DC-mediated inflammation was suppressed by PSB treatment through PU.1 inhibition.

PSB suppressed Th1 differentiation in a DC-independent T cell culture system, suggesting that PSB directly inhibits the differentiation of T cells into Th1. As we added IL-12 to the culture medium under polarizing conditions, downstream signaling effectors of the IL-12 receptor, such as STAT4 or T-bet, can be inactivated by PSB. Additionally, strong TCR signaling is required for Th1 differentiation.\textsuperscript{37,38} Therefore, PSB-induced decreased expression of MHCII and CD86 on DCs can inhibit Th1 differentiation in the co-culture system.

Our results showed that PSB promotes Treg differentiation in the co-culture system but not in the DC-independent T cell culture system. This implied that PSB promotes Treg differentiation by modulating DC activity. Additionally, it was demonstrated that the downregulation of PU.1 target genes in DCs effectively promotes Treg differentiation. Furthermore, considering that weak TCR signaling is required for Treg differentiation, PSB-induced decreased expression of MHCII and CD86 on DCs promotes Treg differentiation.\textsuperscript{38}

Oral administration of PSB significantly ameliorated DSS-induced colitis by decreasing the expression of TNF-α, which is one of the primary cytokines involved in IBD progression. Our in vitro experiments using BMDCs showed that PSB treatment significantly inhibited TNF-α expression. PSB was previously shown to inhibit TNF-α and IL-6 production in RAW264.7 macrophages.\textsuperscript{39} Orally administered PBS may suppress DSS-induced TNF-α production by acting mainly on submucosal DCs and macrophages.

Additionally, Th17 contributes to IBD progression by releasing IL-17, whereas Treg inhibits by counteracting Th1\textsuperscript{40} Orally administered PBS may have promoted Treg differentiation by inhibiting PU.1 function in DCs and suppressed Th17 differentiation in the gut. PSB-induced inhibition of DSS-induced colitis may be mediated by repressing Th1/Th17 and promoting Treg.

Although several effective therapeutic agents have been developed to retard IBD progression, a complete cure for the disease is still not available.\textsuperscript{7} Several studies have shown that RSV mitigated intestinal inflammation in rodent models and, more importantly, improved the quality of life of IBD patients in human clinical trials.\textsuperscript{10} Considering that PSB exerted stronger immunosuppressive effects than RSV in the present study, PSB supplementation may either inhibit the pathology of IBD or delay its onset. Whether PSB executes similar immunosuppressive effects in human immune cells requires further analysis.

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CONFLICT OF INTEREST
The authors have stated explicitly that there are no conflicts of interest in connection with this article.

AUTHOR CONTRIBUTIONS
T. Yashiro designed research, performed experiments, analyzed data, and wrote the paper; S. Yura, A. Tobita, and Y. Toyoda performed experiments and analyzed data; K. Kasakura provided experimental tools; C. Nishiyama designed research and wrote the paper.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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