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Procyanidin dimer B2-mediated IRAK-M induction negatively regulates TLR4 signaling in macrophages

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ABSTRACT

Polyphenolic compounds have been found to possess a wide range of physiological activities that may contribute to their beneficial effects against inflammation-related diseases; however, the molecular mechanisms underlying this anti-inflammatory activity are not completely characterized, and many features remain to be elucidated. In this study, we investigated the molecular basis for the down-regulation of toll-like receptor 4 (TLR4) signal transduction by procyanidin dimer B2 (Pro B2) in macrophages. Pro B2 markedly elevated the expression of the interleukin (IL)-1 receptor-associated kinase (IRAK)-M protein, a negative regulator of TLR signaling. Lipopolysaccharide (LPS)-induced expression of cell surface molecules (CD80, CD86, and MHC class I/II) and production of pro-inflammatory cytokines (tumor necrosis factor-α, IL-1β, IL-6, and IL-12p70) were inhibited by Pro B2, and this action was prevented by IRAK-M silencing. In addition, Pro B2-treated macrophages inhibited LPS-induced activation of mitogen-activated protein kinases such as extracellular signal-regulated kinase 1/2, p38, and c-Jun N-terminal kinase and the translocation of nuclear factor κB and p65 through IRAK-M. We also found that Pro B2-treated macrophages inactivated naïve T cells by inhibiting LPS-induced interferon-γ and IL-2 secretion through IRAK-M. These novel findings provide new insights into the understanding of negative regulatory mechanisms of the TLR4 signaling pathway and the immune-pharmacological role of Pro B2 in the immune response against the development and progression of many chronic diseases.

1. Introduction

Inflammation is a process by which the human body attempts to counteract potential injurious agents such as invading bacteria, viruses, and other pathogens [1,2]. Toll-like receptors (TLRs) are pathogen-recognition proteins that play important roles in detecting microbes and initiating inflammatory responses, and they are important in the activation of the innate immune response [3]. The intracellular signaling pathways activated by TLRs are mediated through the Toll/interleukin-1 receptor homology (TIR) domains. Activation of signaling through TIR domains results in the recruitment of the adapter molecule myeloid differentiation factor 88 (MyD88), and ultimately leads to degradation of inhibitory κB (Iκ-B) and translocation of nuclear factor κB (NF-κB) to the nucleus [3]. Recently, negative TLR regulators have become an important topic in research on the innate immune system. Several studies have reported that TLR-mediated overactivation of the host immune response is regulated by several intracellular negative regulators of TLRs, including suppressor of cytokine signaling 1 (SOCS1), Toll-interacting protein (TOLLIP), and interleukin (IL)-1 receptor-associated kinase (IRAK)-M, and transmembrane negative regulator of TLRs such as single immunoglobulin interleukin-1-related receptor (SIGIRR) [4]. IRAK-M inhibits the dissociation of IRAK1/IRAK4 complexes from the receptor and the formation of IRAK/tumor necrosis factor (TNF) receptor-associated factor 6 complexes [5]. SOCS1 inhibits lipopolysaccharide (LPS)-induced NF-κB activation through inhibition of IRAK1 [6], and SIGIRR binds to TLR4 and IRAK and terminates the downstream TLR signaling

Abbreviations: DTT, dithiothreitol; FITC, fluorescein isothiocyanate; IFN, interferon; Iκ-B, inhibitor of κB; IL, interleukin; IRAK, IL-1 receptor-associated kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; PE, phycoerythrin; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; SOCS1, suppressor of cytokine signaling 1; TIR, toll/interleukin-1 receptor homology; TLR, toll-like receptor; TNF-α, tumor necrosis factor-α; TOLLIP, toll-interacting protein; TRIF, TIR domain-containing adapter-inducing interferon-β.

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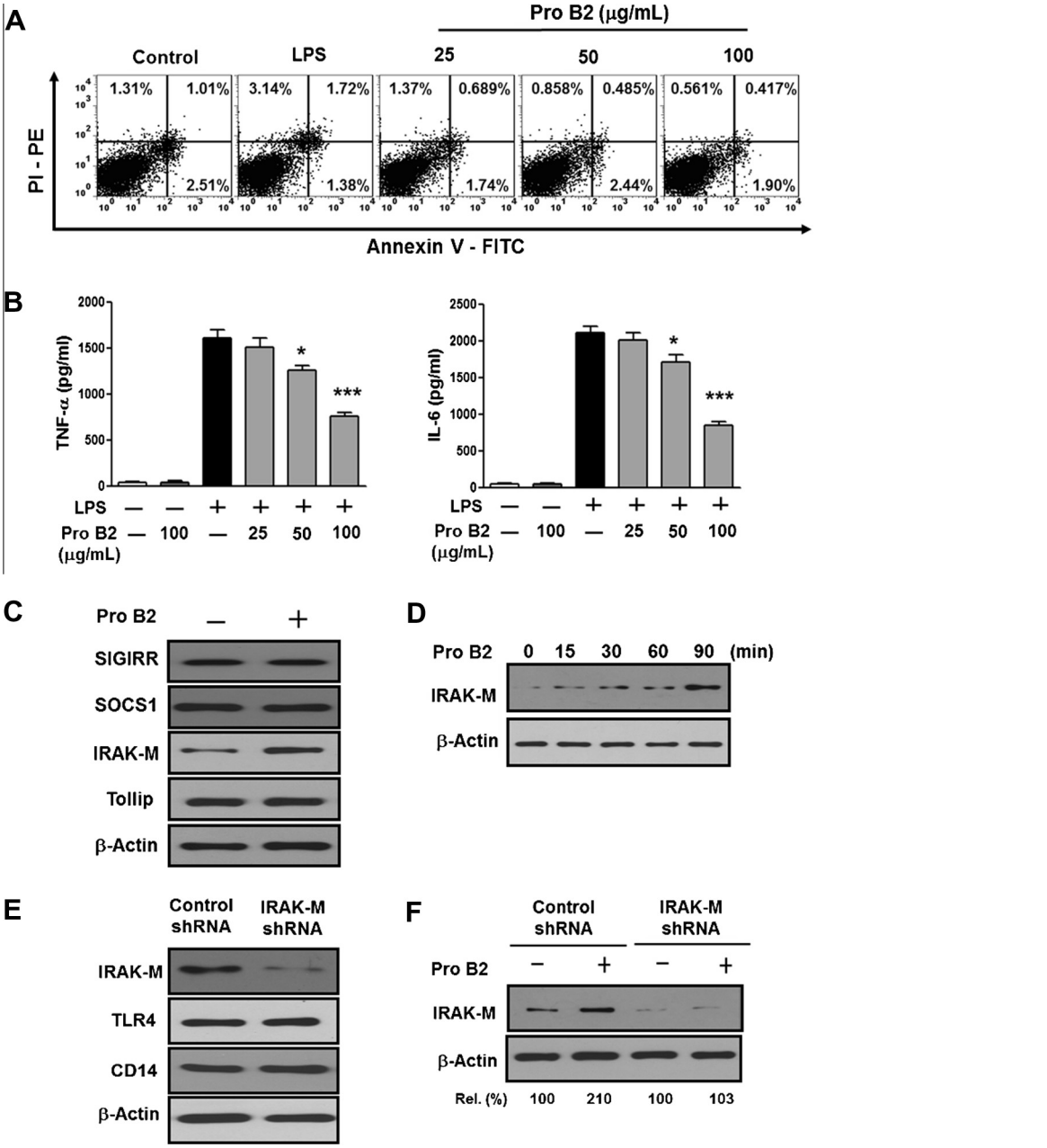

Fig. 1. Effect of Pro B2 on the metabolic activity of LPS-induced RAW264.7 macrophage cells. (A) RAW264.7 cells were treated with Pro B2 for 24 h, and cell viability was analyzed by flow cytometry. RAW264.7 cells were stained with anti-CD11b, annexin V, and PI. The percentage of positive cells (annexin V- and PI-stained cells) in each quadrant is indicated. The results are representative of 3 experiments. (B) The cells were then pretreated with Pro B2 (25–100 μg/mL) for 1 h before exposure to LPS (50 ng/mL) for 24 h. The concentrations of TNF-α and IL-6 in the culture medium were measured by ELISA. All data are expressed as the mean ± SD ($n = 3$) values, and statistical significance ($^{*}p < 0.05$; $^{***}p < 0.001$) is shown for treatments compared to the controls. The value of *n.s.* was defined as no significant effect. (C) RAW264.7 cells were treated with 100 μg/mL Pro B2 for 1 h. Total cellular proteins were resolved by SDS–PAGE, and the separated proteins were transferred to nitrocellulose membranes and detected using specific SIGIRR, SOCS1, IRAK-M, or TOLLIP antibodies. The data are shown as mean ± S.D. ($n = 3$) values. (D) RAW264.7 cells without LPS stimulation were treated with Pro B2 at 100 μg/mL for the indicated periods. (E) RAW264.7 cells were transfected with the IRAK-M shRNA vector. Protein levels of IRAK-M, TLR4, and CD14 were detected by immunoblotting. (F) Cells were treated with 100 μg/mL Pro B2 for 1 h. Total cellular proteins were resolved by SDS–PAGE, and the separated proteins were transferred to nitrocellulose membranes and detected with anti-IRAK-M antibody.

pathways [7]. TOLLIP interacts with IRAK1, and the level of IRAK phosphorylation and NF-κB activation decreases in the presence of TOLLIP [8].

Various polyphenolic compounds have attracted increasing attention in the fields of nutrition and medicine because of their potential health benefits [9–11]. In particular, procyanidins, which are phenolic compounds from the flavonoid group, have a wide range of antioxidant and anti-inflammatory activities [12]. The flavan-3-ol dimer procyanidin B2 (Pro B2) from apples (*Malus* spp., Rosaceae) is the primary source of this anti-inflammatory activity [13]. Pro B2 has been shown to inhibit constitutively active NF-κB by preventing its binding to DNA [14].

While it is known that multiple signal transduction pathways are correlated with inflammation, the underlying mechanisms are not well understood. Therefore, in this study, we tried to elucidate the molecular basis for the downregulation of TLR4 signal

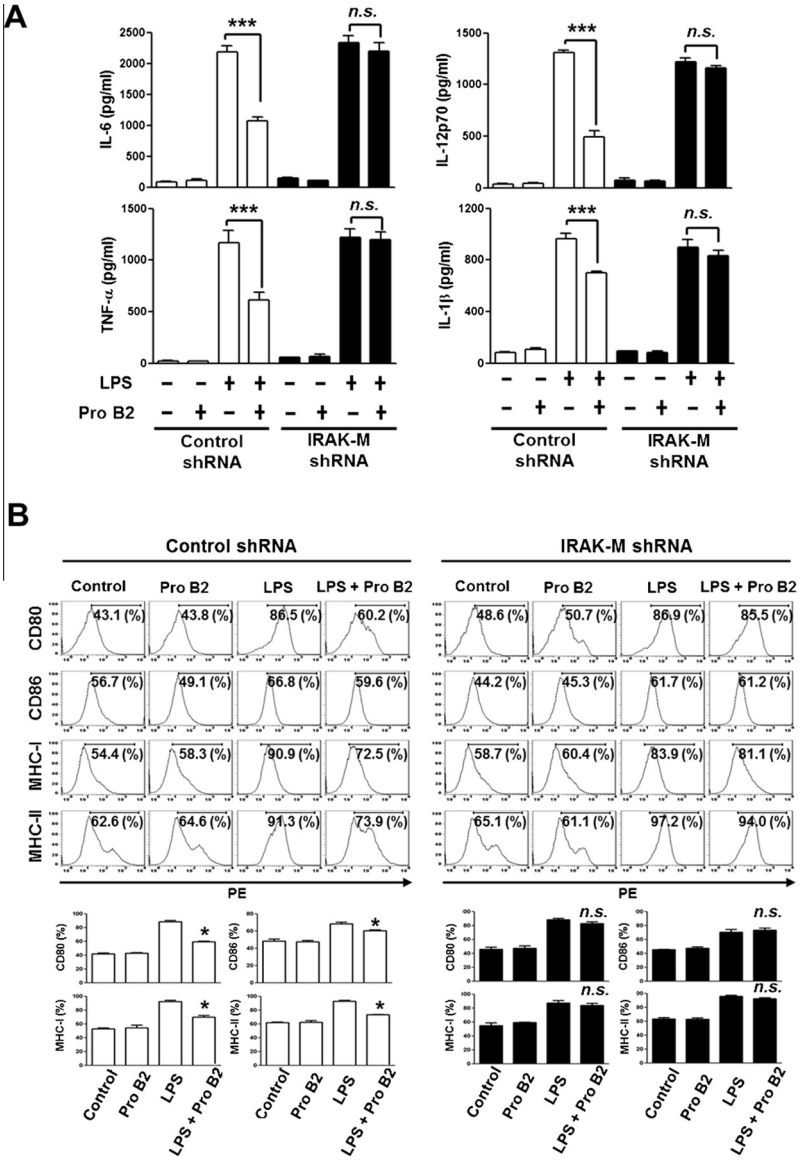

Fig. 2. Pro B2 suppresses the activation of RAW264.7 cells through IRAK-M. (A and B) RAW264.7 cells were pretreated with Pro B2 (100 μg/mL) for 1 h before exposure to LPS (50 ng/mL) for 24 h. (A) The concentrations of TNF-α, IL-6, IL12p70, and IL-1β in the culture medium were measured by ELISA. All the data are expressed as the mean ± SD ($n = 3$) values, and statistical significance ($^{***}p < 0.001$) is shown for treatments compared to the controls. The value of *n.s.* has been defined as no significant effect. (B) The cells were gated for CD11b. Macrophages were stained with anti-CD80, anti-CD86, or anti-MHC class I/II. The percentage of positive cells is shown for each panel.

transduction by Pro B2 in macrophages. Here, we have shown that IRAK-M is essential for mediating the anti-inflammatory activity of Pro B2 in LPS-stimulated macrophages.

2. Materials and methods

2.1. Materials

The fluorescein isothiocyanate (FITC)-annexin V/propidium iodide (PI) kit was purchased from R&D Systems (Minneapolis, MN, USA). Pro B2 was purchased from Sigma–Aldrich (St. Louis, MO, USA). LPS from *Escherichia coli* O111:B4 was purchased from Sigma and Invivogen (San Diego, CA). Anti-TLR4 polyclonal Ab, anti-CD14 polyclonal Ab, anti-phosphorylated ERK1/2 monoclonal Ab, anti-phosphorylated JNK monoclonal Ab, anti-phosphorylated p38 monoclonal Ab, anti-NF-κB (p65) polyclonal Ab, anti-phosphorylated inhibitor of κB (IκB)-α monoclonal Ab, anti-SOCS1 polyclonal Ab, anti-SIGIRR polyclonal Ab, anti-IRAK-M monoclonal Ab, anti-TOLLIP monoclonal Ab, horseradish peroxidase (HRP)-conjugated anti-goat donkey IgG Ab, and anti-lamin B polyclonal Ab were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). HRP-conjugated anti-mouse IgG Ab and HRP-conjugated anti-rabbit Ab were obtained from Calbiochem (San Diego, CA), and anti-β-actin mAb (AC-15) was purchased from Sigma–Aldrich. FITC-conjugated mAb to CD11b, phycoerythrin (PE)-conjugated mAb to CD80, CD86, and MHC class I/II were purchased from eBioscience (San Diego, CA). IL-6, IL-1β, IL-12p70, IFN-γ, IL-2, and TNF-α enzyme-linked immunosorbent assay (ELISA) kits were obtained from BD Biosciences (San Diego, CA). Female BALB/c (body weight, 19–21 g), 7-week-old, mice were obtained from Orient Inc. (Charles River Technology, Seoul, Republic of Korea). The experimental protocols were approved by the Animal Ethics Committee of the Korea Atomic Energy Research Institute (KAERI-IACUC-2010-014).

2.2. Cell culture

RAW264.7 cells were cultured in DMEM supplemented with antibiotics (100 U/mL penicillin and 100 U/mL streptomycin), and

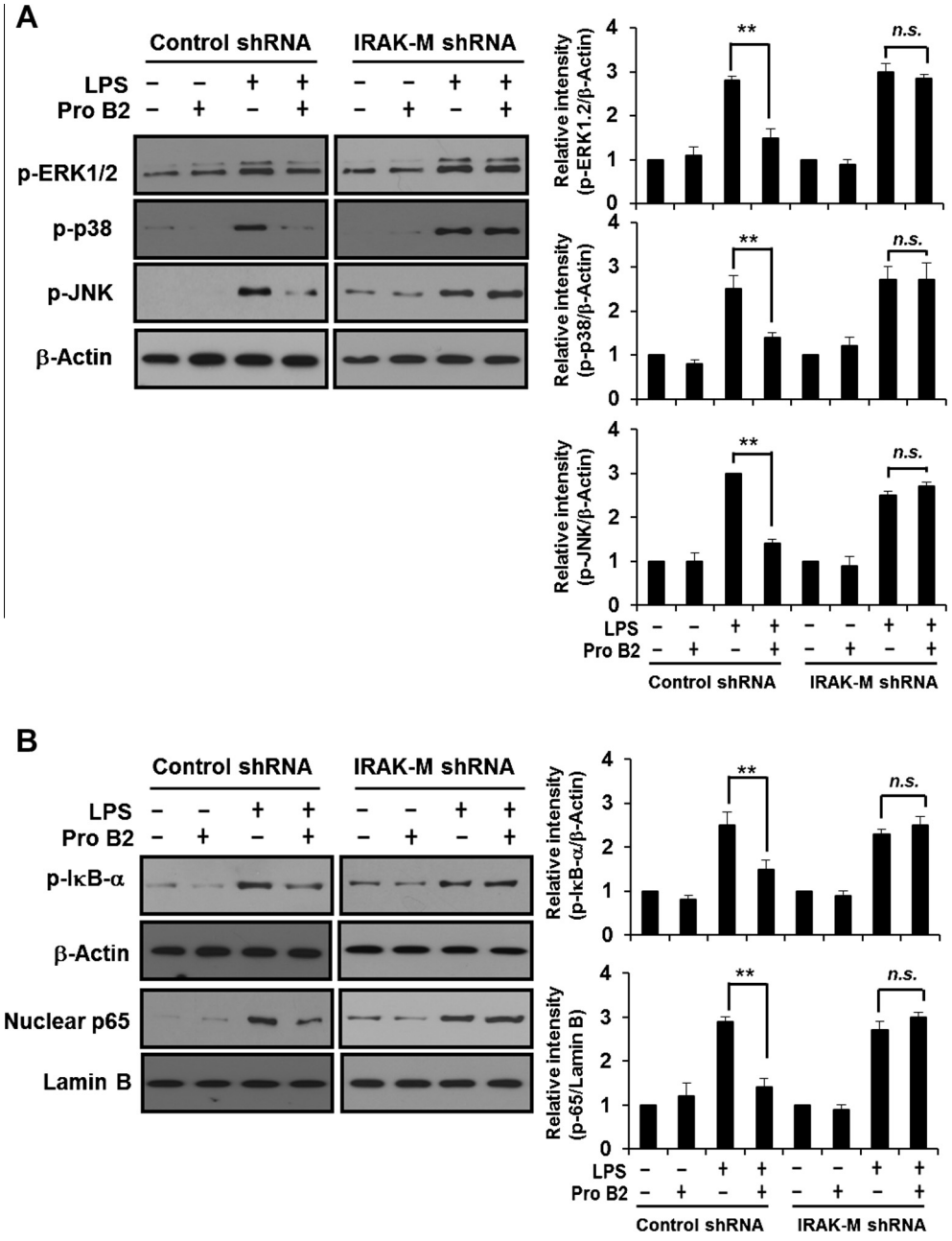

Fig. 3. Pro B2 inhibits LPS-induced activation of MAPK and NF-κB signaling pathways through IRAK-M. The cells were pretreated with the indicated concentrations of Pro B2 (100 μg/mL) for 1 h before exposure to LPS (50 ng/mL) for 45 min. Cells lysates were subjected to SDS–PAGE, and immunoblot analysis was performed using specific Abs to phospho-p38 (p-p38), phospho-ERK1/2 (p-ERK1/2), phospho-JNK (p-JNK), phospho-IκB-α (p-IκB-α), and p65 NF-κB. β-Actin and lamin B were used as loading controls for cytosolic and nuclear fractions, respectively. The results shown are representative of 3 experiments conducted under each condition. The data are shown as mean ± SD ($n = 3$) values. Relative band intensity of each protein was expressed. Statistical significance ($^{**}p < 0.01$) was indicated for LPS only versus LPS plus Pro B2. The value of *n.s.* was defined as no significant effect.

10% (v/v) fetal bovine serum (Intergen, Purchase, NY). The cells were maintained at 37 °C in a humidified incubator containing 5% CO_2. In all experiments, cells were allowed to acclimate for 24 h before any treatments.

2.3. Construction of IRAK-M-suppressed cells

IRAK-M short hairpin RNA expression vector was purchased from Santa Cruz Biotechnology. shRNA plasmids consist of a pool of 3–5 lentiviral vector plasmids each encoding target-specific 19–25 nt (plus hairpin) shRNAs designed to knockdown gene expression. For each transfection, we added 0.8 mL shRNA plasmid transfection medium to the well, incubated the cells for 7 h, and added neomycin to select stably transfected cells.

2.4. Cytotoxicity analysis

To investigate the cytotoxic effect of Pro B2 on RAW264.7 cells, we analyzed the cell death pattern of these cells after treatment with Pro B2. After 24 h of treatment, harvested RAW264.7 cells were washed with phosphate-buffered saline (PBS) and stained by FITC-annexin V/PI (BD Biosciences). Subsequently, cytotoxicity against RAW264.7 cells was analyzed by using FACSCanto flow cytometer (BD Biosciences).

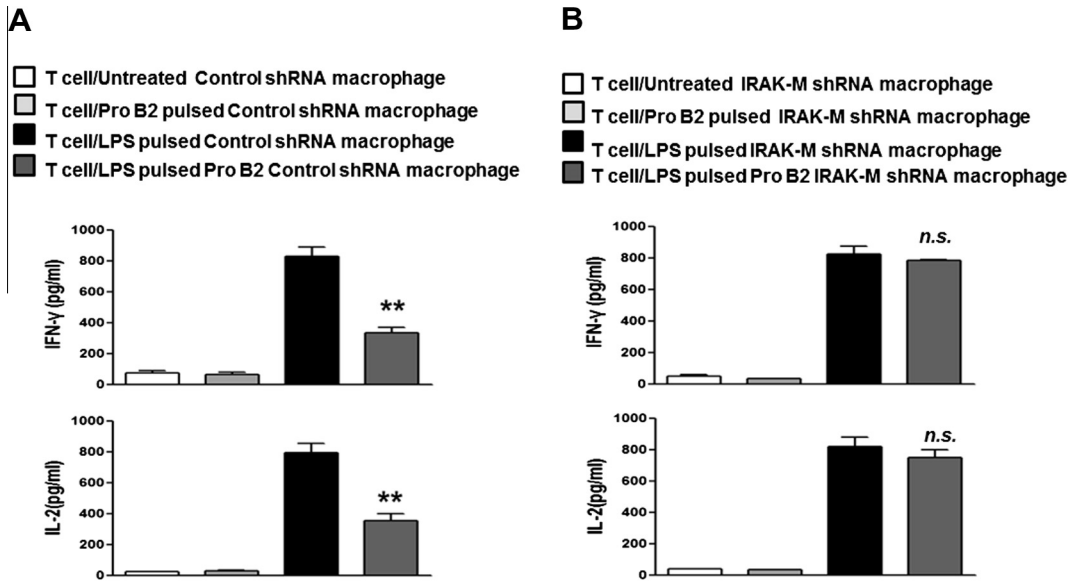

Fig. 4. Pro B2 decreased IFN-γ and IL-2 production from T cells through IRAK-M. (A and B) RAW264.7 cells were cultured in medium with or without 100 μg/mL Pro B2 for 24 h. The treated RAW264.7 cells were harvested and washed extensively to remove Pro B2. A mixed lymphocyte reaction (MLR) was performed for 3 d, as described in Materials and Methods. The culture supernatants obtained for the conditions described for A and B were harvested, and IFN-γ and IL-2 were measured by ELISA. The data are shown as mean ± SD ($n = 3$) values, and statistical significance ($^{**}p < 0.01$) is shown for treatments when compared to the appropriate controls (T cell/LPS pulsed RAW264.7 cells). The value of *n.s.* has been defined as no significant effect.

2.5. Measurement of cytokines

ELISA was used for detecting IL-6, IL-1β, IL-12p70, TNF-α, IFN-γ, and IL-2 in culture supernatants as described previously [15].

2.6. Analysis of the expression of surface molecules by flow cytometry

Cell were harvested, washed with PBS, and re-suspended in fluorescence-activated cell sorter washing buffer (2% FBS and 0.1% sodium azide in PBS). The cells were stained with PE-conjugated anti-I-Ab (MHC class I/II), anti-CD80, and anti-CD86 along with FITC-conjugated anti-CD11b for 45 min at 4 °C. The cells were washed 3 times with PBS and resuspended in 500 μL PBS. Fluorescence was measured by flow cytometry, and the data were analyzed using the CellQuest data analysis software.

2.7. Immunoblotting analysis

Cells were lysed in 100 μL lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Triton-X100, 1 mM EDTA, 50 mM NaF, 30 mM Na_4PO_7, 1 mM phenylmethanesulfonyl fluoride (PMSF), 2 μg/mL aprotinin, and 1 mM pervanadate. Whole-cell lysates were resolved by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and then transferred onto a nitrocellulose membrane. The membranes were blocked in 5% skim milk and incubated with the respective Ab for 2 h, followed by incubation with HRP-conjugated secondary Ab for 1 h at room temperature. Epitopes on target proteins, including mitogen-activated protein kinases (MAPKs) and NF-κB, recognized specifically by the used Abs were visualized using the ECL advance kit (GE Healthcare, Little Chalfont, UK).

2.8. Nuclear extract preparation

Nuclear extracts from cells were prepared as follows. Cells were treated with 100 μL lysis buffer (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol (DTT), 0.5 mM PMSF) on ice for 10 min. Following centrifugation at 4000 rpm for 5 min, the pellet was re-suspended in 100 μL extraction buffer (20 mM HEPES [pH 7.9], 400 mM NaCl, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF) and incubated on ice for 30 min. After centrifugation at 12,000 rpm for 10 min, the supernatant containing nuclear extracts was collected and stored at −80 °C until required.

2.9. Mixed lymphocyte reaction

Responder T cells, which participate in naïve T cell reactions, were isolated using a MACS column (Miltenyi Biotec) from total mononuclear cells prepared from BALB/c mice. RAW264.7 cells (2×10^5 cells per well) treated with Pro B2 or LPS for 24 h were co-cultured with T cells (2×10^6) at a ratio of 1:10 (macrophage:T cell). On day 3, the supernatants were harvested and the production of IFN-γ and IL-2 was measured by ELISA.

2.10. Statistical analysis

All the experiments were repeated at least 3 times with consistent results. The levels of significance for comparison between samples were determined by Tukey's multiple comparison test distribution using statistical software (GraphPad Prism Software, version 4.03; GraphPad Software, San Diego, CA). The data in the graphs are expressed as mean ± SEM values. Each value for which $^{*}p < 0.05$, $^{**}p < 0.01$, or $^{***}p < 0.001$ was considered to be statistically significant.

3. Results

3.1. Cytotoxicity and anti-inflammatory action of Pro B2

As shown in Fig. 1A, treatment with Pro B2 did not result in any cellular toxicity against macrophages. This finding suggests that, at concentrations below 100 μg/mL, Pro B2 is not cytotoxic to RAW264.7 cells and does not contain significant amounts of endotoxin that would potentially interfere with our studies using concentrations below 100 μg/mL. To examine the anti-inflammatory

effect of Pro B2 on the production of inflammatory cytokines from RAW264.7 cells, the cells were pretreated for 1 h with Pro B2 (25–100 μg/mL) prior to exposure to LPS. As shown in Fig. 1B, production of TNF-α and IL-6 in culture supernatants from RAW264.7 cells significantly increased upon treatment with LPS, and the LPS-induced production of these inflammatory cytokines was significantly inhibited by treatment with 100 μg/mL Pro B2; therefore, 100 μg/mL was used as the maximum dose of Pro B2 throughout subsequent experiments.

3.2. Pro B2 up-regulates IRAK-M protein

To elucidate additional mechanisms underlying the Pro B2-mediated anti-inflammatory activity, we examined whether Pro B2 induces negative TLR regulators. Control cells were treated with 100 μg/mL Pro B2 for 1 h, and the expression of negative TLR regulators was then measured by immunoblot analysis. Intriguingly, we found that the protein level of IRAK-M in control cells was up-regulated by Pro B2 treatment, whereas the levels of other proteins were not affected (Fig. 1C). Furthermore, we found that Pro B2 elevated the expression of IRAK-M in a time dependent manner in LPS-untreated control cells (Fig. 1D). Next, to establish whether IRAK-M is indeed involved in the suppressive effect of Pro B2 on LPS-induced inflammatory responses, we used stable RNAi to silence IRAK-M expression in RAW264.7 cells. Immunoblot analysis indicated that stable RNAi against IRAK-M specifically silenced IRAK-M protein expression without affecting the expression of TLR4 and CD14 (Fig. 1E). This result suggested that the silencing of IRAK-M did not affect the expression of TLR4 or CD14 and that the binding of LPS to TLR4 may be equal in both the IRAK-M-downregulated cells and the control cells. In addition, in control cells treated with Pro B2 for 1 h, the level of IRAK-M protein markedly increased, whereas silencing of IRAK-M prevented Pro B2-induced up-regulation of IRAK-M (Fig. 1F).

3.3. IRAK-M mediates the TLR4 inhibitory action of Pro B2

To examine the effect of Pro B2 mediated through IRAK-M on the production of inflammatory cytokines from macrophages, we stimulated IRAK-M-downregulated RAW264.7 cells with LPS. As shown in Fig. 2A, LPS-induced production of TNF-α, IL-6, IL-12p70, and IL-1β was significantly inhibited upon treatment with 100 μg/mL Pro B2. However, this inhibitory effect was not observed in IRAK-M-downregulated cells.

3.4. Pro B2 suppresses LPS-induced maturation of macrophages through IRAK-M

To investigate whether Pro B2 suppresses macrophage maturation through IRAK-M, we measured the expression of macrophage maturation markers such as CD80, CD86, and MHC classes I/II. IRAK-M-downregulated cells and control cells were pretreated for 1 h with 100 μg/mL Pro B2 prior to exposure to LPS and analyzed for the expression of surface markers. LPS was used as the positive control. Pro B2 was found to downregulate the LPS-induced expression of the macrophage maturation markers; however, Pro B2 did not affect IRAK-M-downregulated cells (Fig. 2B).

3.5. Effect of IRAK-M down-regulation on Pro B2-induced inactivation of the MAPK signaling pathway

We examined whether Pro B2 suppresses LPS-induced MAPK activation through IRAK-M. The phosphorylation of MAPKs, including ERK1/2, p38, and JNK, was measured by immunoblot analysis using specific Abs. LPS-induced phosphorylation of ERK1/2, p38, and JNK was inhibited by treatment with Pro B2, whereas in IRAK-M-downregulated cells, the inhibitory effect of Pro B2 on LPS-induced up-regulation of phosphorylation was attenuated (Fig. 3A).

3.6. Pro B2 inhibits LPS-induced NF-κB activation through IRAK-M

As shown in Fig. 3B, Pro B2 inhibited LPS-induced phosphorylation of IκB-α in control cells, whereas the inhibitory effect of Pro B2 was attenuated in IRAK-M-downregulated cells, suggesting that Pro B2 inhibits LPS-induced phosphorylation and degradation of IκB-α through IRAK-M. Next, we examined the effect of Pro B2 on LPS-induced nuclear translocation of p65. In parallel with the phosphorylation of IκB-α, treatment of the cells with LPS increased nuclear translocation of p65. We found that Pro B2 significantly suppressed LPS-induced nuclear translocation of p65 in control cells, whereas this effect was not observed in IRAK-M-downregulated cells (Fig. 3B).

3.7. Pro B2 diminished the stimulatory capacity of macrophages through IRAK-M

To determine whether Pro B2 had any effect on naïve T-cell stimulation, RAW264.7 cells were treated for 24 h with Pro B2, and co-cultured with T cells for 3 d. T-cell activation was assessed by IFN-γ and IL-2 release. Because macrophages do not produce IFN-γ and IL-2, activated T cells in the cocultures constitute the only source of IFN-γ and IL-2. As shown in Fig. 4A, IFN-γ and IL-2 secretion from T cells significantly increased upon treatment with LPS, and the LPS-induced production of these cytokines was significantly inhibited by treatment with 100 μg/mL Pro B2 in control cells. However, in IRAK-M-downregulated cells, the inhibitory effects of Pro B2 were not observed (Fig. 4B).

4. Discussion

TLRs are key components of the innate immune system [3]. Their activation in response to microbial infection and inflammation triggers NF-κB and MAPK signaling and culminates in the induction of host defense genes, including those involved in the production of numerous cytokines, chemokines, adhesion molecules, and enzymes [3,16,17]. Recently, negative TLR regulators have become a focus area in research on the innate immune system. Several studies have reported that TLR-mediated overactivation of the host immune response is regulated by multiple intracellular negative regulators of TLRs, including SOCS1, TOLLIP, and IRAK-M [18,19]. Among the negative TLR regulators, IRAK-M controls the magnitude of inflammatory cytokine production in response to IL-1β and LPS. Furthermore, IRAK-M overexpression impairs TLR4-triggered NF-κB and MAPK signaling pathways [20]. IRAK-M also interacts with IRAK-1 prior to stimulation and suppresses its kinase activity [20,21], indicating that IRAK-M negatively regulates TLR4 signaling.

In addition, it was recently reported that mice lacking IRAK-M are hyperresponsive to LPS and negatively associated with the magnitude of inflammatory response [22], suggesting that IRAK-M also possesses a potential role in pro-inflammatory signaling [5]. However, it is not clear whether this negative TLR regulator is involved in the inhibitory effect of Pro B2 on inflammatory activities. Therefore, in this study, we investigated whether the negative TLR regulators mediate the inhibitory action of Pro B2 on TLR4 signaling.

Our results supported that LPS-induced production of TNF-α and IL-6 was significantly inhibited by Pro B2 treatment, and Pro B2 enhances IRAK-M expression. In addition, the present study has indicated that the ability of Pro B2 to reduce production of inflammatory cytokines is mediated through IRAK-M, as is the LPS-induced expression of the macrophage-maturation markers

[23]. These results confirm that IRAK-M mediates the suppressive effect of Pro B2 on LPS-induced maturation of macrophages. Next, we examined whether Pro B2 suppresses LPS-induced MAPK and NF-κB activation through IRAK-M. Pro B2-treated macrophages inhibited LPS-induced activation of MAPKs and NF-κB through IRAK-M, as reported by other groups [24]. IRAK-M plays a role in modulating cell surface molecule expression and release of pro-inflammatory cytokines in response to various TLR ligands [5,25]. One previous study showed that IRAK-M, as a potent negative regulator of TLR signaling, decreasing TLR4 signaling through inhibition the NF-κB inhibition [5]. It has been demonstrated that activation of LPS-stimulated TLR4 ultimately leads to MAPKs and NF-κB-mediated production of pro-inflammatory cytokines (e.g., IL-6 and TNF-α) as well as surface molecule expression (e.g., CD80, CD86, and MHC class II) in macrophage [3]. Some previous report demonstrated that IRAK-M is expressed in myeloid cells and was initially shown to induce NF-κB signaling when overexpressed [26]. We also found that Pro B2-treated macrophages inactivated naïve T cells through IRAK-M. Finally, we found that Pro B2-treated macrophage inactivated naïve T cells by inhibition of LPS-induced IFN-γ and IL-2 secretion through IRAK-M. Here we showed that Pro B2 up-regulated the expression of IRAK-M and the elevation was regulated at the transcriptional level. From these results, we confirmed that Pro B2-mediated IRAK-M induction negatively regulates TLR4 signaling in macrophages, which provide new insights into the understanding of negative regulatory mechanisms of the TLR4 signaling pathway and the immune-pharmacological role of Pro B2 in the immune response against the development of inflammation.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

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