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PECAM-1 Ligation Negatively Regulates TLR4 Signaling in Macrophages[1]

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Uncontrolled TLR4 signaling may induce excessive production of proinflammatory cytokines and lead to harmful inflammation; therefore, negative regulation of TLR4 signaling attracts much attention now. PECAM-1, a member of Ig-ITIM family, can mediate inhibitory signals in T cells and B cells. However, the role and the mechanisms of PECAM-1 in the regulation of TLR4-mediated LPS response in macrophages remain unclear. In this study, we demonstrate that PECAM-1 ligation with CD38-Fc fusion protein negatively regulates LPS-induced proinflammatory cytokine TNF-α, IL-6, and IFN-β production by inhibiting JNK, NF-κB, and IFN regulatory factor 3 activation in macrophages. In addition, PECAM-1 ligation-recruited Src homology region 2 domain-containing phosphatase 1 (SHP-1) and Src homology region 2 domain-containing phosphatase 2 (SHP-2) may be involved in the inhibitory effect of PECAM-1 on TLR4 signaling. Consistently, silencing of PECAM-1 enhances the macrophage response to LPS stimulation. Taken together with the data that PECAM-1 is constitutively expressed in macrophages and its expression is up-regulated by LPS stimulation, PECAM-1 might function as a feedback negative regulator of LPS inflammatory response in macrophages. This study may provide a potential target for intervention of inflammatory diseases. ***The Journal of Immunology,*** **2007, 179: 7344–7351.**

As one of pathogen-associated molecular patterns, LPS can be recognized by APC including macrophages and then activate an innate immune response. Upon LPS recognition and activation of TLR4 signaling, macrophages produce proinflammatory cytokines including TNF-α, IL-6, IL-1β, and IFN-β through intracellular signaling cascades (1–4). These proinflammatory cytokines can mediate innate response, prime the adaptive immune response, and also induce inflammation (5, 6). Excessive or inappropriate inflammatory processes would be harmful or even fatal. Therefore, multiple negative regulators are required for the restricted control of the LPS response (7–9).

PECAM-1 is a 130-kDa transmembrane protein, first identified as an adhesion molecule (10). PECAM-1 has been proven to be a member of the Ig-ITIM family and can mediate signal transduction via its ITIMs to moderate or attenuate tyrosine kinase-mediated signaling pathways (11). More recently, PECAM-1-deficient mice were demonstrated to be more sensitive to LPS stimulation and the survivals of the mice was reduced during LPS-induced endotoxic shock. As compared with wild-type controls, PECAM-1-deficient mice have elevated levels of serum TNF-α, IL-6, and IFN-β but reduced levels of phosphorylated STAT3 (12), suggesting that PECAM-1 may play an important role in negative regulation of the LPS response. However, the mechanisms by which PECAM-1 inhibits LPS responses remain unclear. In the present study, we demonstrate that PECAM-1 significantly inhibits LPS-induced macrophage production of proinflammatory cytokine and IFN-β by suppressing activation of NF-κB, JNK, and IRF3[4] pathways. Therefore, PECAM-1 is a negative regulator of TLR4 signaling in macrophages.

Materials and Methods

Reagents and Abs

LPS and DEAE-dextran were obtained from Sigma-Aldrich. Disuccinimidyl suberate (DSS) was purchased from Pierce and DMSO was purchased from Amresco. Abs specific to IκBα, ERK, JNK, IRF3, and p-Tyr and Ab against TLR4 were obtained from Cell Signaling Technology; anti-mouse β-actin Ab was obtained from Sigma-Aldrich; anti-PECAM-1 Ab, agarose-conjugated Ab specific for SHP-1 (SHP-1 AC) or SHP-2 (SHP-2 AC) and HRP-conjugated anti-Fc Ab were obtained from Santa Cruz Biotechnology.

RT-PCR

RT-PCR was performed as described previously (13). Total RNA was isolated from macrophages using TRIzol reagent (Invitrogen Life Technologies) and subsequently used as a template for reverse transcription. To amplify the cytoplasmic domain of all possible mouse PECAM-1 isoforms, the following primers were used: mouse PECAM-1 5′-CCA AGG CCA AAC AGA-3′ (sense) and 5′-AAG GGA GCC TTC CGT TCT-3′ (antisense). Extracellular domain sequence of CD38 cDNA was cloned from mouse lymphoid nodes with primers 5′-GCG AAT TCC TGA GGC CGC GCT CAC-3′ (sense) and 5′-GCG GTA CCG TAT TAA GTC TAC ACG ATG-3′ (antisense). Primers for mouse β-actin were 5′-AGT GTG ACG

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[4] Abbreviations used in this paper: IRF3, IFN regulatory factor 3; SHP-1, Src homology 2 domain-containing tyrosine phosphatase 1; SHP-2, Src homology 2 domain-containing protein tyrosine phosphatase 2; DSS, disuccinimidyl suberate; siRNA, small interfering RNA.

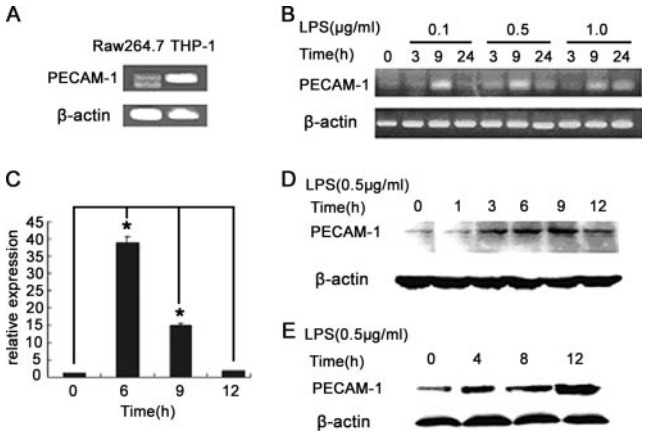

FIGURE 1. LPS up-regulates PECAM-1 expression in macrophages. *A*, PECAM-1 mRNA expression in RAW264.7 and THP-1 cells as detected by RT-PCR. *B–D*, RAW264.7 cells stimulated with the indicated doses of LPS for the indicated times. PECAM-1 expression was detected by RT-PCR (*B*), real-time quantitative PCR (*C*) or Western blot (*D*). The shown results are representative of three independent experiments. *E*, Thioglycolate-elicited mouse peritoneal macrophages were stimulated with 0.5 μg/ml LPS for the indicated times and PECAM-1 expression was detected by Western blot. Data are shown as mean ± SD of three independent experiments (*, $p < 0.05$).

TTG ACA TCC GT-3′ (sense) and 5′-GCA GCT CAG TAA CAG TCC GC-3′ (antisense). The PCR products were inserted into the PMD18-T vector (Pharmacia) and sequenced.

Expression and purification of soluble mouse extracellular CD38-Fc fusion protein

Coding regions of human IL-2 signal peptide, the extracellular domain (residues 158–938) of mouse CD38 and human IgG4 CH2 and CH3 fragments were cloned into pcDNA3.1/mic-His ¯ B for expression of secreted mouse extracellular CD38-Fc fusion protein (mexCD38-Fc) (14). COS-7 cells (*Cercopithecus aethiops*, African green monkey 7 cells) were transfected by the mexCD38-Fc expression vector using the DEAE-dextran method with minor modifications. After overnight recovery in DMEM supplemented with 10% FCS (PAA Laboratories), cells were cultured in DMEM without FCS for 48 h. The supernatant was harvested, and the secreted mexCD38-Fc protein was purified by using ion exchange columns (Bio-Rad). Then the mexCD38-Fc protein was identified by SDS-PAGE and Western blot. The contaminant endotoxin in mexCD38-Fc fusion protein was strictly removed by using the Detoxi-Gel endotoxin removing gel (Pierce). Endotoxin levels in the fusion protein were <0.015 endotoxin units/mg protein measured by the *Limulus* amebocyte lysate assay.

Silencing of mouse PECAM-1 in macrophages

Mouse macrophage cell line RAW264.7 was obtained from the American Type Culture Collection and cultured in DMEM supplemented with 10% FCS as described previously (15). To silence the *PECAM-1* gene, we designed a RNAi vector which was targeted to Ig-like domain 3 of extracellular PECAM-1. The small interfering RNA (siRNA)-expressing plasmid, PGCsi-H1/Neo/GFP/shRNA-PECAM-1 (PECAM1Si), containing a sequence matching PECAM-1 cDNA (TTGATTTCAAACTTGGGAG) was constructed and nucleofected into RAW264.7 cells by using Amaxa Nucleofector II Biosystems according to the manufacturer's instructions (16). The cells were selected by G418 (800 μg/ml) for 2–3 wk and pooled to generate PECAM-1-stably silenced RAW264.7 cells.

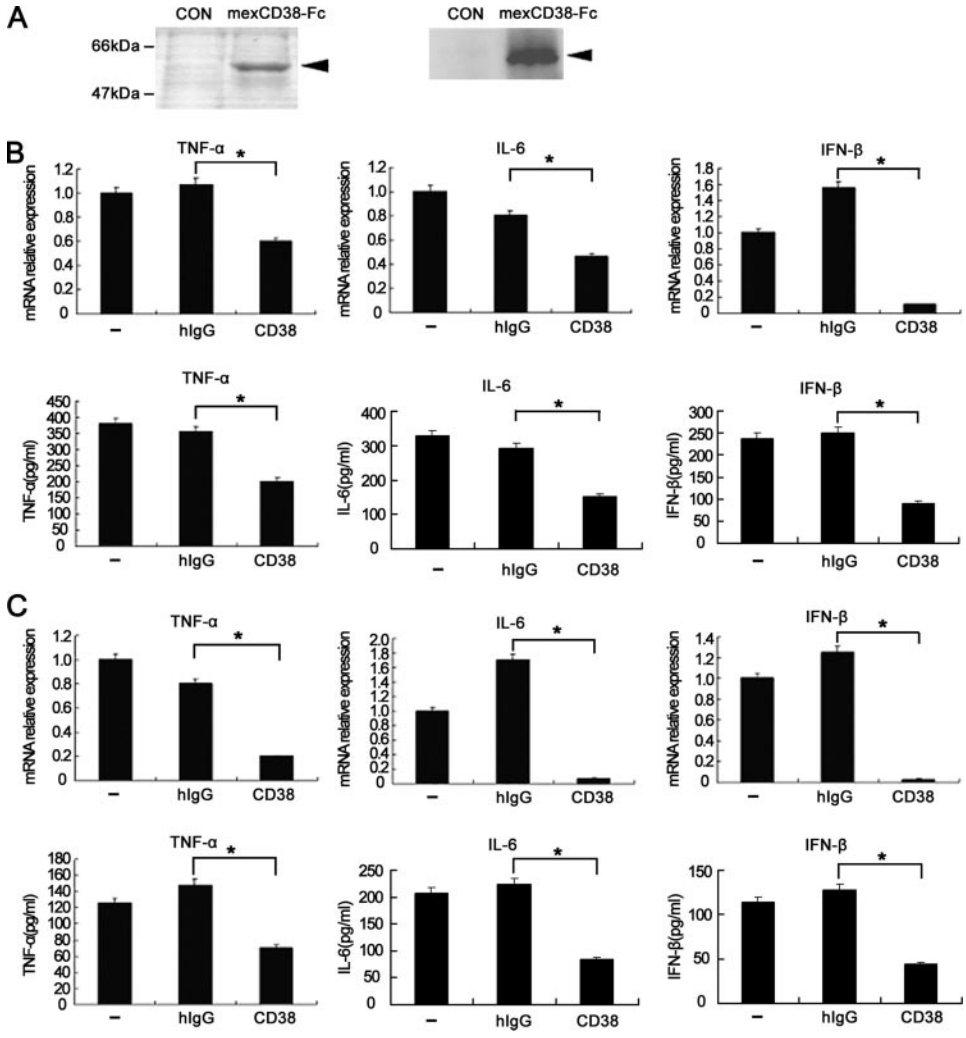

FIGURE 2. PECAM-1 ligation inhibits LPS-induced TNF-α, IL-6, and IFN-β production in macrophages. *A*, CD38-Fc fusion protein was harvested from the supernatants of COS-7 cells transiently transfected with the mexCD38-Fc expression vector and purified using ion exchange columns. The contaminant endotoxin in mexCD38-Fc fusion protein was strictly removed by using the Detoxi-Gel endotoxin removing gel. The purified mexCD38-Fc protein expression was analyzed by SDS-PAGE (*left panel*) and detected by Western blot using anti-Fc Ab (*right panel*). *B* and *C*, Two × 10^7/ml RAW264.7 cells (*B*) or peritoneal macrophages (*C*) were pretreated with 20 μg/ml CD38-Fc protein and 0.56 mM DSS to cross-linking PECAM-1 and CD38 and were then stimulated with 100 ng/ml LPS for 4–8 h. The production of TNF-α, IL-6, and IFN-β was detected by real-time quantitative PCR and ELISA. Data are shown as mean ± SD of four independent experiments (*, $p < 0.05$). CON, Control.

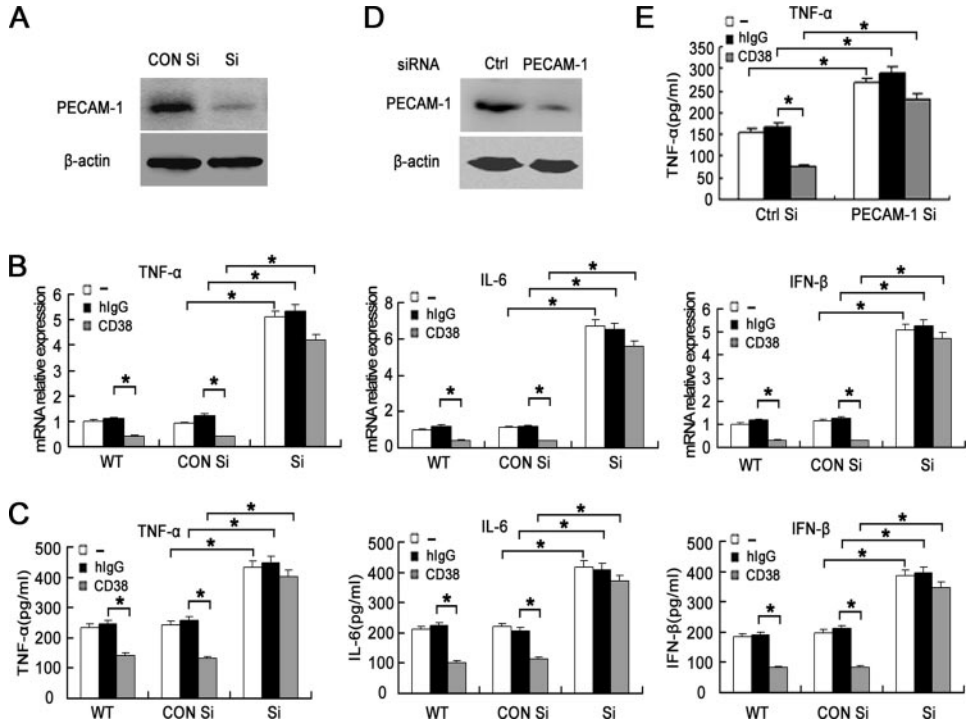

FIGURE 3. PECAM-1 knockdown enhances the production of LPS-induced TNF-α, IL-6, and IFN-β in macrophages. *A*, RAW264.7 cells were stably nucleofected with PECAM-1Si, plasmid-expressing interfering RNA targeting PECAM-1 (Si), or control plasmid (CON Si). PECAM-1 expression in the cells was detected by Western blot. *B* and *C*, PECAM-1-stably silenced RAW264.7 cells and control cells (2×10^7/ml) were pretreated with 20 μg/ml CD38-Fc protein and 0.56 mM DSS to cross-linking PECAM-1 and CD38, and then stimulated with 100 ng/ml LPS for 4–8 h. LPS-induced TNF-α, IL-6, and IFN-β expression was detected by real-time quantitative PCR (*B*) and ELISA (*C*). *D*, Mouse peritoneal macrophages were nucleofected with control siRNA (Ctrl) or PECAM-1 siRNA (PECAM-1). After 48 h, PECAM-1 expression in the cells was detected by Western blot. Similar results were obtained in three independent experiments. *E*, Mouse peritoneal macrophages were nucleofected with PECAM-1 siRNA (PECAM-1 Si) or control siRNA (Ctrl Si). After 48 h, the cells were pretreated with 20 μg/ml CD38-Fc protein and 0.56 mM DSS to cross-linking PECAM-1 and CD38, and then stimulated with 100 ng/ml LPS for 8 h. TNF-α in the supernatants was detected using ELISA. Data are shown as mean ± SD of four independent experiments (*, $p < 0.05$). WT, Wild type.

C57BL/6 mice were obtained from Joint Ventures Sipper BK Experimental Animal (Shanghai, China) and used at the age of 4–6 wk. All animal experiments were conducted according to guidelines of the Animal Use Committee of Second Military Medical University. Peritoneal macrophages were isolated from C57BL/6 mice 3 days after i.p. injection of 2 ml of thioglycolate medium as described previously (17). The isolated cells were cultured in RPMI 1640 medium containing 10% FCS and then nucleofected with siRNA targeted to PECAM-1 by using Amaxa Nucleofector II Biosystems according to the manufacturer's instructions. siRNA sequences 5′-UUGAUUUCAAACUUGGGAG-3′ were used to suppress PECAM-1 expression in macrophages. Nonsense sequences 5′-ACGUGA CACGUUCGGAGAA-3′ were used as control siRNA. The PECAM-1 gene expression in silenced macrophages was tested by Western blot.

Real-time quantitative PCR

SYBR Green-based quantitative real-time PCR analysis was performed using a PTC-200 DNA Engine thermal cycler (MJ Research) and chromo4 four-color real-time detector (Bio-Rad) as described previously (18). To standardize the PCR, we used serial dilutions of the pMD18-T vector contained in the cytoplasmic sequence of mouse PECAM-1 or mouse β-actin cDNA from 1.0×10^4–1.0×10^{10} copies as standard samples. The standard curves were determined by amplifying the standard samples. The primers used were identical to those used in RT-PCR. Amplification conditions were: 95°C (2 min), 32 cycles for 95°C (20 s), 57.2°C (30 s), and 72°C (30 s). The analysis of PCR results and the calculation of the absolute copy numbers of the starting templates were performed by Opticon monitor software (version 3.1.32; Bio-Rad). By normalization with the copy numbers of β-actin, the relative expression of mouse PECAM-1 in each sample was acquired. The mRNA levels of TNF-α, IL-6, and IFN-β were quantitated by real-time quantitative PCR. Primers used were as follows: TNF-α, sense: 5′-AAGCCTGTAGCCCACGTCGTA-3′, antisense: 5′-GGC ACCACTAGTTGGTTGTCTTTG-3′; IL-6, sense: 5′-ACAACCACGGCCT TCCCTACTT-3′, antisense: 5′-CACGATTTCCCAGAGAACATGTG-3′; and IFN-β, sense: 5′-AGCTCCAAGAAAGGACGAACAT-3′, antisense: 5′-GCCCTGTAGGTGAGGTTGATCT-3′.

Cross-linking of PECAM-1 by soluble mouse extracellular CD38-Fc fusion protein

RAW264.7 cells or primary peritoneal macrophages (2×10^7/ml) were incubated with 20 μg/ml mexCD38-Fc fusion protein or control human IgG protein for 1 h at 4°C. Then a 0.56 mM final concentration of the cross-linker reagent DSS prepared in DMSO was added into cell suspensions (19). After incubation for 30 min at 22°C, the cells were centrifuged (1.5 min at $2500 \times g$) and washed with PBS twice. Then the cells were cultured and stimulated with 100 ng/ml LPS for 4–8 h and the cytokine production was detected by real-time quantitative PCR or ELISA.

Immunoblot and immunoprecipitation

The harvested cells were lysed in cell lysis buffer (Cell Signaling Technology) supplemented with 1 mM PMSF proteinase inhibitor on ice. The nuclear and cytoplasmic extracts were prepared with NE-PER nuclear and cytoplasmic extraction reagents (Pierce) according to the manufacturer's instructions. Protein concentrations of the extracts were measured with a bicinchoninic acid assay (Pierce). Equal amounts of extracts were loaded on each lane of 10% SDS-PAGE, electrophoresed, and transferred to nitrocellulose membrane (Schleicher & Schuell BioScience). Membrane was blocked in 1× TBST with 5% nonfat milk for 2 h and hybridized with anti-PECAM-1 Ab (Santa Cruz) Biotechnology at 1/1000 overnight at 4°C. After five washes with 1× TBST for a total of 60 min, the membrane was incubated with HRP-conjugated anti-rabbit secondary Ab (Santa Cruz Biotechnology) for 1 h at room temperature. SuperSignal West Femto Maximum Sensitivity substrate (Pierce) was used for the chemiluminescent detection.

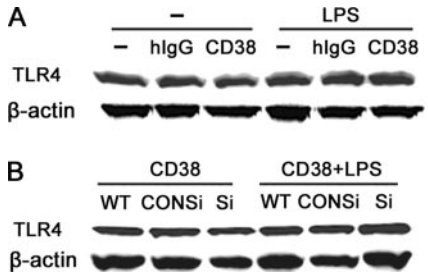

FIGURE 4. PECAM-1 ligation does not affect TLR4 expression in macrophages. After PECAM-1 ligation, 2×10^7/ml wild-type (WT) RAW264.7 cells (*A*) or PECAM-1-stably silenced RAW264.7 cells (*B*) were stimulated with 100 ng/ml LPS for 1 h. Then TLR4 expression was detected by Western blot. Similar results were obtained in three independent experiments. CONSi, Control siRNA.

Cell lysates were incubated with agarose-conjugated for SHP-1 (SHP-1 AC) or SHP-2 (SHP-2 AC) with gentle rocking overnight at 4°C. Immunoprecipitates were washed four times with immunoprecipitation buffer (50 mM Tris (pH 7.4) containing 150 mM NaCl, 2 mM Na_3VO_4, 1% Triton X-100, and 1 mM PMSF). Pelleted beads were boiled in SDS-sample loading buffer and then subjected to Western blot analysis.

Densitometric analysis

Densitometric analysis was done with Labworks Image Acquisition and Analysis Software (Ultraviolet Products). The background was subtracted, and the signals of the detected bands were normalized to the amount of loading control band. The relative values were presented as fold increase over control samples as indicated.

Statistical analysis

Statistical significance was determined by the Wilcoxon-signed rank test (see Figs. 2, *B* and *C*, and 3, *B*, *C*, and *E*) or ANOVA test (see Figs. 1*C* and 6), with a value of $p < 0.05$ considered to be statistically significant.

Results

Kinetics of PECAM-1 expression in LPS-activated macrophages

To explore the role of PECAM-1 in macrophage response to LPS stimulation, we first examined whether PECAM-1 was expressed in macrophages. As shown in Fig. 1*A*, PECAM-1 was expressed in the mouse macrophage-like cell line RAW264.7 and human monocytic cell line THP-1. We also examined whether the PECAM-1

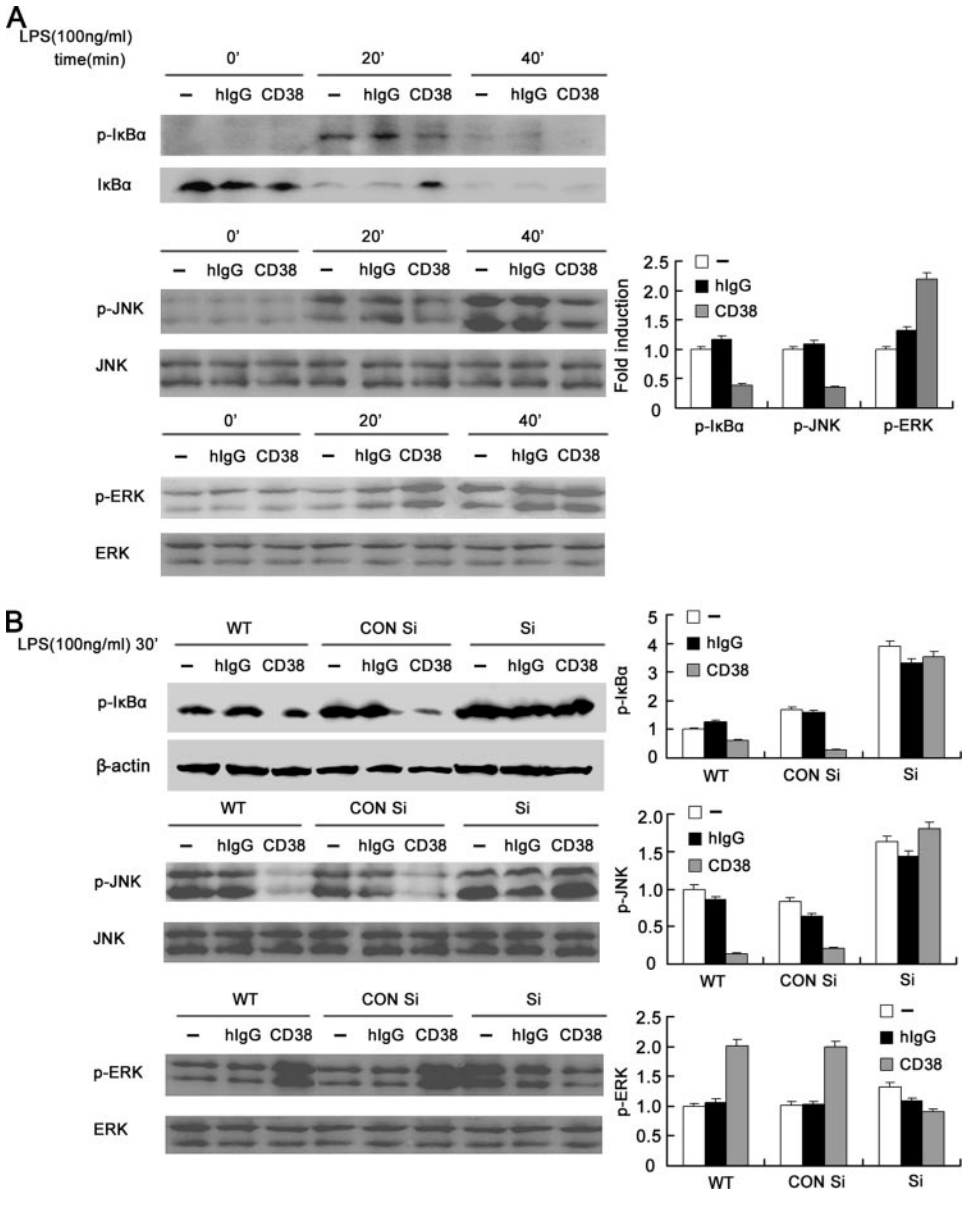

FIGURE 5. PECAM-1 ligation inhibits IκBα and JNK but enhances ERK activation in LPS-activated macrophages. *A*, After PECAM-1 ligation, wild-type (WT) RAW264.7 cells were stimulated with 100 ng/ml LPS for 10, 20, and 40 min, total expression and phosphorylation of IκBα, JNK, and ERK were detected by Western blot. The results shown are representative of three independent experiments. Phosphorylation levels of the proteins at the time point of 20 min after LPS stimulation were quantitated by measuring band intensities. The values were normalized to the total ERK or JNK signal, respectively. Relative phosphorylation levels of the proteins in the control cells are expressed as 1. *B*, After PECAM-1 ligation, the PECAM-1-stably silenced RAW264.7 cells were stimulated with 100 ng/ml LPS for 30 min. The total expression and phosphorylation of JNK and ERK as well as the phosphorylation of IκBα were detected by Western blot. The results shown are representative of three independent experiments. Phosphorylation levels of the proteins were quantitated by measuring band intensities. The values were normalized to the β-actin, total ERK, or JNK signal, respectively. Relative phosphorylation levels of the proteins in wild-type cells alone are expressed as 1. See other legends for the abbreviation definitions.

expression could be induced by LPS in RAW264.7 cells, as reported to be induced by LPS in endothelial cells (20, 21) or human monocytes (22). In brief, LPS at 0.1 μg/ml was enough to induce a significant increase in the PECAM-1 expression on RAW264.7 cells (Fig. 1*B*). A higher dose of LPS (0.5 or 1 μg/ml) did not further increase the expression level of PECAM-1 but prolonged the duration of the increased PECAM-1 expression (Fig. 1*B*). To investigate the kinetics of the up-regulation of PECAM-1 expression on macrophages by LPS, we detected PECAM-1 expression on macrophages stimulated with 0.5 μg/ml LPS for various times by real-time quantitative PCR and Western blot. Expression of PECAM-1 on RAW264.7 cells reached the peak level after stimulated with 0.5 μg/ml LPS for 6 h (by real-time quantitative PCR) or 9 h (by Western blot). Then the expression was decreased to the normal level (Fig. 1, *C* and *D*). PECAM-1 expression was up-regulated by LPS treatment in thioglycolate-elicited mouse primary peritoneal macrophages; however, PECAM-1 expression reached the peak level in these cells 12 h after LPS treatment (Fig. 1*E*). These results demonstrate that PECAM-1 is constitutively expressed in macrophages and LPS can up-regulate PECAM-1 expression, suggesting that PECAM-1 may be involved in feedback regulation of TLR4 signaling in macrophages.

Inhibitory effect of PECAM-1 ligation on LPS-induced inflammatory cytokine production by macrophages

To investigate the role of PECAM-1 in TLR4 signaling, we examined the effects of PECAM-1 ligation on the production of LPS-induced cytokines, including TNF-α, IL-6, and IFN-β in macrophages. Since CD38 is the ligand of PECAM-1 (23), we expressed the fusion protein (mexCD38-Fc) of the extracellular domain of CD38 fused with IgG4 CH2 and CH3 fragments in COS-7 cells, then purified the fusion protein and used it to cross-link PECAM-1 to activate PECAM-1 signaling. The expression of mexCD38-Fc protein was confirmed by SDS-PAGE and Western blot (Fig. 2*A*). As compared with the human IgG protein-pretreated control group, after mexCD38-Fc fusion protein (PECAM-1 ligation) pretreatment, both RAW264.7 cells (Fig. 2*B*) and peritoneal macrophages (Fig. 2*C*) produced less TNF-α, IL-6, and IFN-β in response to LPS stimulation. These results implied that PECAM-1 signaling can inhibit the LPS-induced inflammatory response in macrophages.

To confirm the effects of ligation of endogenous PECAM-1 on production of proinflammatory cytokines, we constructed interfering RNA-expressing vector PECAM1Si that specifically inhibited PECAM-1 expression. PECAM-1 expression in PECAM1Si-stably transfected RAW264.7 cells was decreased ~80%, as compared with PECAM-1 expression in control cells (Fig. 3*A*). As expected, the production of LPS-induced TNF-α, IL-6, and IFN-β in PECAM1Si-stably transfected RAW264.7 cells was significantly increased, as compared with that in wild-type cells or control RNA-expressing cells (Fig. 3*B*). The increase of LPS-induced TNF-α, IL-6, and IFN-β production in PECAM-1-stably silenced RAW264.7 cells was also confirmed by ELISA (Fig. 3*C*).

We further investigated the effect of PECAM-1 ligation on LPS-induced TNF-α production in PECAM-1-silenced peritoneal macrophages. As shown in Fig. 3*D*, PECAM-1-specific siRNA could inhibit ~70% endogenous PECAM-1 expression. Consistently, after PECAM-1 ligation, LPS-induced TNF-α production was increased significantly in PECAM-1-silenced peritoneal macrophages (Fig. 3*E*). Furthermore, we also observed the effect of PECAM-1 ligation on TLR4 expression and found that TLR4 expression remained unchanged in both wild-type (Fig. 4*A*) and PECAM-1-silenced macrophages after PECAM-1 ligation (Fig. 4*B*), indicating that the inhibitory effect of PECAM-1 ligation on TLR4 signaling was not due to the down-regulation of TLR4 expression on macrophages. Taken together, these results suggest that PECAM-1 functions as a negative regulator of LPS response in macrophages.

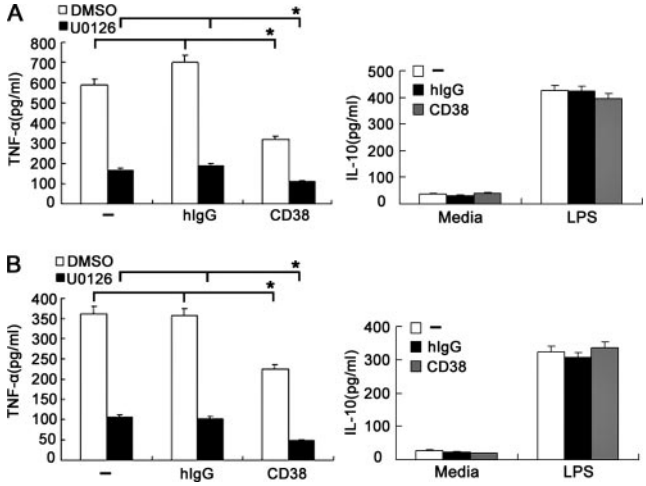

FIGURE 6. Inhibition of LPS-induced TNF-α production by PECAM-1 ligation is not dependent on ERK activation and IL-10 production. RAW264.7 cells (*A*) and peritoneal macrophages (*B*) were pretreated with U0126 (10 μM) for 30 min. After PECAM-1 ligation, cells were stimulated for 8 h in the presence or absence of U0126. TNF-α and IL-10 production was detected by ELISA. Data are shown as mean $\pm$ SD of three independent experiments (*, $p < 0.05$). See other legends for the abbreviation definitions.

PECAM-1 ligation inhibits IκBα, JNK, and IRF3 but enhances ERK activation in LPS-activated macrophages

To address the molecular mechanisms of PECAM-1 ligation-mediated negative regulation of LPS response in macrophages, we examined the effects of PECAM-1 ligation on the phosphorylation of the intermediators of TLR4 signal pathway in LPS-stimulated RAW264.7 cells. It has been shown that phosphorylation of IκBα, JNK, ERK, and IRF3 are important for LPS-induced cytokine production in macrophages. As shown in Fig. 5*A*, PECAM-1 ligation significantly decreased LPS-induced phosphorylation of IκBα and JNK1/2 as well as degradation of IκBα in RAW264.7 cells. Conversely, PECAM-1 knockdown increased LPS-induced phosphorylation of IκBα and JNK (Fig. 5*B*). However, p38 MAPK phosphorylation remained almost unchanged after PECAM-1 ligation and LPS stimulation (data not shown). These results suggested that PECAM-1 might inhibit LPS-induced cytokine production through inhibiting activation of NF-κB and JNK1/2 pathways.

Unlike the activation of IκBα and JNK1/2, LPS-induced ERK1/2 activation was significantly increased by the ligation of PECAM-1. To investigate the relationship between ERK activation and the negative regulation of TLR4-activated cytokine production by PECAM-1, we examined the effect of PECAM-1 ligation on LPS-induced TNF-α production in the presence of ERK inhibitor U0126. As shown in Fig. 6, U0126 (10 μM) pretreatment significantly inhibited LPS-induced TNF-α production, ignoring PECAM-1 ligation. On the other hand, PECAM-1 ligation could still inhibit LPS-induced TNF-α production in the presence of U0126. Because ERK activation might inhibit production of proinflammatory cytokines by increasing IL-10 production (24, 25), we also detected the effect of PECAM-1 ligation on the production of IL-10 from macrophages. As shown in Fig. 6, *A* and *B*, PECAM-1 ligation did not affect the production of IL-10 in LPS-activated

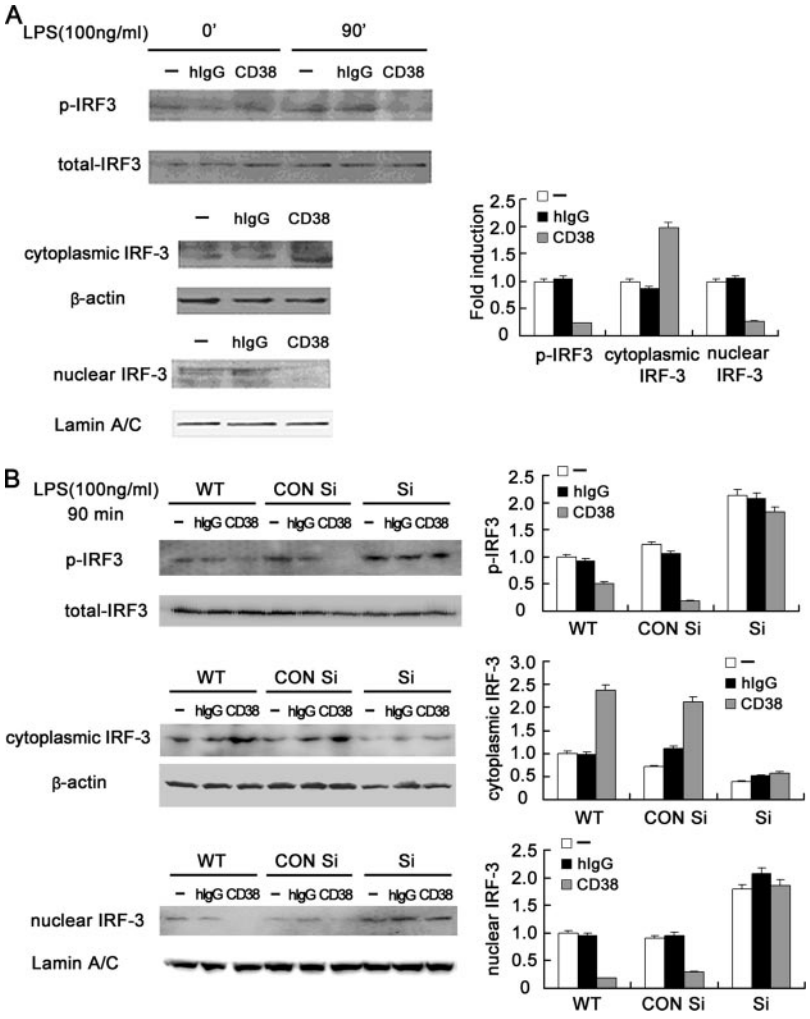

FIGURE 7. PECAM-1 ligation inhibits LPS-induced activation of IRF3 in macrophages. After PECAM-1 ligation, RAW264.7 cells (*A*) or the PECAM-1-stably silenced RAW264.7 cells (*B*) were stimulated with 100 ng/ml LPS for 90 min. Then the phosphorylated IRF3 and the total levels of cytoplasmic and nuclear IRF3 were detected by Western blot. Similar results were obtained in three independent experiments. Phosphorylation levels of IRF3 at the time point of 90 min after LPS stimulation and total levels of cytoplasmic and nuclear IRF3 were quantitated by measuring band intensities. The values were normalized to the total IRF3, β-actin, or Lamin A/C signal, respectively. Relative levels of the proteins in the control cells are expressed as 1. See other legends for abbreviation definitions.

RAW264.7 cells and primary macrophages. These results indicated that ERK activation was not involved in PECAM-1-mediated inhibition of TLR-activated TNF-α production.

In addition, we also detected the phosphorylation and expression of IRF3, a key signal intermediator in the MyD88-independent pathway. PECAM-1 ligation remarkably inhibited LPS-induced phosphorylation of IRF3 in RAW264.7 cells. Consistently, the total amount of cytoplasmic IRF3 was significantly increased and the amount of nuclear IRF3 was decreased in PECAM-1-ligated cells (Fig. 7*A*), suggesting that PECAM-1 ligation inhibited the translocation of IRF3 into the nucleus. These results were also confirmed in the PECAM-1-stably silenced RAW264.7 cells (Fig. 7*B*). Taken together, PECAM-1 may suppress LPS-induced production of IFN-β in macrophages by inhibiting the activation and nuclear translocation of IRF-3.

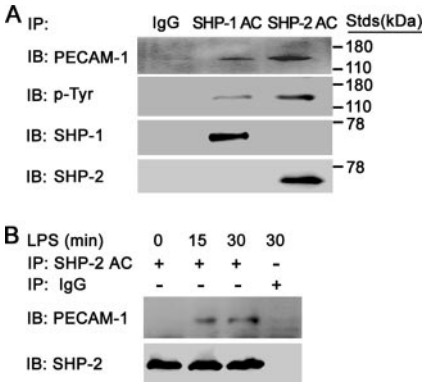

FIGURE 8. Association of PECAM-1 with SHP-1 and SHP-2 in LPS-activated macrophages after PECAM-1 ligation. After PECAM-1 ligation, macrophages were stimulated with 100 ng/ml LPS for 1 h (*A*) or 0, 15, and 30 min (*B*). Lysates of the cells were immunoprecipitated (IP) with SHP-1- or SHP-2-conjugated agarose (SHP-1 AC or SHP-2 AC) and then detected with anti-PECAM-1, anti-p-Tyr, and anti-SHP-1 as well as anti-SHP-2 Abs. The results shown are representative of three independent experiments. See other legends for abbreviation definitions.

Recruitment of SHP-1 and SHP-2 to PECAM-1 in LPS-activated macrophages after PECAM-1 ligation

PECAM-1 contains two ITIMs in its cytoplasmic region which may recruit tyrosine phosphatase SHP-1 and SHP-2 to transmit inhibitory signals. We investigated whether PECAM-1 could associate with tyrosine phosphatase SHP-1 and SHP-2 after mexCD38 cross-link. After being engaged by CD38 ligation, PECAM-1 was phosphorylated on tyrosine residues and associated with SHP-1 and SHP-2 (Fig. 8*A*). The coprecipitation between PECAM-1 and SHP-2 could be detected at 15 min after LPS stimulation (Fig. 8*B*). These results indicated that PECAM-1 might transduce its inhibitory signals by recruiting SHP-1 and SHP-2.

Discussion

In this study, we demonstrate that PECAM-1 is constitutively expressed in macrophages and its expression can be up-regulated by

LPS stimulation. LPS-induced inflammatory cytokine and IFN-β production in macrophages can be attenuated by cross-linking PECAM-1 with CD38. Consistently, silencing of PECAM-1 can enhance LPS-induced inflammatory cytokine production in macrophages. Suppression of NF-κB, JNK, and IRF3 activation may be responsible for PECAM-1 ligation-mediated inhibition of LPS-induced inflammatory cytokine and IFN-β production in macrophages. Furthermore, PECAM-1-mediated inhibition of TLR4 signaling might require the recruitment of SHP-1 and SHP-2 to its ITIM. Our present study provides evidence that PECAM-1 is a negative regulator of TLR4 response in macrophages.

Although LPS-induced production of proinflammatory cytokines is critical for host defense, uncontrolled or excessive production of proinflammatory cytokines may lead to harmful inflammation (26). PECAM-1 was originally assigned to be one member of the family of Ig-like cellular adhesion molecules based on the similarity of its extracellular domain with other members of cell adhesion molecules. Now, PECAM-1 is considered as an ITIM-containing inhibitory receptor. PECAM-1-negative $CD4^+$ T cells responded better to recall Ags and secreted more IL-4 than PECAM-1- positive $CD4^+$ T cells (27), suggesting that PECAM-1 may be an inhibitory receptor on T cells. Furthermore, in response to PECAM-1 and TCR coligation, PECAM-1 became tyrosine phosphorylated, recruited SHP-2, and attenuated cellular activation stimulated by TCR cross-linking. Using chicken DT-40 B cells stably transfected with FcγRIIBI-PECAM-1, coligation of the BCR complex with FcγRIIBI-PECAM-1 also attenuated cellular activation stimulated by BCR cross-linking (28). Similar results were obtained in the collagen-activated platelets (29–31). Moreover, the inhibitory effect of PECAM-1 required the tyrosine phosphorylation of PECAM-1 ITIM and recruitment and activation of SHP-2 and SHP-1. Compared with SHP-1 and SHIP, SHP-2 is the more preferential substrate to bind with PECAM-1, because SHP-2-deficient cells had a more significant reversal of PECAM-1-inhibitory signaling than SHP-1-deficient cells (32). Interestingly, the inhibitory function of PECAM-1 on TCR signal transduction even requires a member of the Src family of the protein tyrosine kinase $p56^{lck}$, because PECAM-1 failed to be tyrosine phosphorylated in $p56^{lck}$-deficient Jurkat T cells (33). The results obtained by using PECAM-1 knockout mice are also consistent with these findings in vitro. Taken together, PECAM-1 is a member of the Ig-ITIM family and may mediate negative regulation of the T and B cell responses. The results in this article also support this issue by showing the recruitment of SHP to PECAM-1.

TLR activates JNK and NF-κB pathways, both of which play important roles in the production of proinflammatory cytokine production. The inhibition of LPS-induced cytokine production by PECAM-1 ligation was coincident with the inhibition of NF-κB and JNK activation, suggesting that PECAM-1 might inhibit LPS-induced cytokine production through negatively regulating NF-κB and JNK activation. SHP-1 and SHP-2 function as negative regulators in various signal transduction pathways through their phosphatase activity. Since PECAM-1 ligation triggered the recruitment of SHP-1 and SHP-2, PECAM-1 might inhibit TLR signaling through SHP-1 and SHP-2.

Phosphorylation of ERK was significantly increased in PECAM-1-ligated macrophages. ERK activation is responsible for the production of IL-10 and IL-10 can inhibit the production of other cytokines in macrophage response to LPS (24, 25). However, PECAM-1 ligation did not affect the production of IL-10 and the inhibitory activities of PECAM-1 was not affected by ERK inhibitor, suggesting ERK activation might not be involved in the inhibition of cytokine production by PECAM-1 ligation.

PECAM-1-deficient mice have reduced survival during endotoxic LPS-induced shock and exhibited elevated levels of TNF-α, IL-6, and IFN-β in response to LPS stimulation (12). However, the inhibitory role of PECAM-1 might not be limited to the protection from endotoxin shock. CD38, the ligand of PECAM-1, is mainly expressed on the activated T and B cells. It is reasonable to suspect that the activated T and B cells might inhibit pathogen-associated molecule pattern-induced activation of immune cells through CD38-mediated PECAM-1 ligation, functioning as a feedback negative regulatory mechanism in both innate and adaptive immune responses.

In conclusion, our results demonstrate that PECAM-1 negatively regulates LPS-induced proinflammatory cytokine and type I IFN production by inhibiting JNK, NF-κB, and IRF3 activation in macrophages. The ERK activation and IL-10 production are not involved in the PECAM-1-mediated inhibition of LPS-induced proinflammatory cytokine production. Considering that PECAM-1 expression is up-regulated by LPS stimulation, PECAM-1 might function as a feedback negative regulator of LPS response in macrophages.

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Disclosures

The authors have no financial conflict of interest.

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