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Mechanisms of LPS-induced CD40 Expression in Human Peripheral Blood Monocytic Cells

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Abstract

CD40 plays important roles in cell-mediated and humoral immune responses. In this study, we explored mechanisms underlying lipopolysaccharide (LPS)-induced CD40 expression in purified human peripheral blood monocytic cells (PBMCs) from healthy volunteers. Exposure to LPS induced increases in CD40 mRNA and protein expression on PBMCs. LPS stimulation caused I κ B α degradation. Inhibition of NF κ B activation abrogated LPS-induced CD40 expression. LPS stimulation also resulted in phosphorylation of mitogen-activated protein kinases, however, only Jun N-terminal kinase (JNK) was partially involved in LPS-induced CD40 expression. In addition, LPS exposure resulted in elevated interferon γ (IFN γ) levels in the medium of PBMCs. Neutralization of IFN γ and IFN γ receptor using specific antibodies blocked LPS-induced CD40 expression by 44% and 37%, respectively. In summary, LPS induced CD40 expression on human PBMCs through activation of NF κ B and JNK, and partially through the induction of IFN γ production.

Keywords

CD40; interferon γ ; LPS; mitogen-activated protein kinase; NF κ B

CD40 is a member of the tumor necrosis factor (TNF) receptor family and is expressed on a variety of cell types, such as monocytes, B cells, macrophages, basophils, eosinophils, dendritic cells and endothelial cells [1]. The CD40 molecule is a phosphorylated glycoprotein with the structure of a typical type I transmembrane protein that is composed of 277 amino acids with a 193 amino acid extracellular domain, a 22 amino acid transmembrane domain, and a 62 amino acid intracellular tail [2]. Ligation of CD40 by its ligand, CD154, activates numerous signaling pathways leading to changes in gene expression and function. These signaling pathways include nuclear factor κ B (NF κ B), mitogen-activated protein kinases (MAPKs), TNF receptor-associated factor proteins, phosphoinositide 3-kinase (PI3K), and the janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway [3]. Signaling through CD40 up-regulates the expression of cell surface molecules such as class II major histocompatibility complex (MHC II), CD80, and CD86, adhesion molecules, secretion of cytokines and

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chemokines such as interleukin-1 (IL-1), IL-6, IL-10, TNF α , and promotes prothrombotic activities and immunoglobulin class-switching to IgE [4,5]. Thus, CD40 is not only critical in mediating normal immune responses, it also participates in a wide range of immune responses that have been associated with a variety of pathogenic processes in chronic inflammatory diseases that include asthma, autoimmune diseases, and atherosclerosis [6,7].

In comparison to studies of CD40 signaling and function, less is known about the mechanisms that regulate human CD40 gene expression [4]. The CD40 gene is mapped to chromosome 20q11-q13 and expressed as a single 1.5 kb mRNA species. The CD40 promoter is a TATA-less promoter that contains three potential interferon (IFN) gamma activated sequence (GAS) elements. Other potentially relevant *cis*-regulatory elements in the human CD40 5'-flanking region are consensus sequences for NF κ B, Ets family, activator protein-1 (AP-1), *c-Myc*, and Sp1 [8]. In mouse macrophages, transcription factors, such as NF κ B, STAT-1 α , PU.1, and Spi-B, have been shown to mediate CD40 induction after endotoxin or cytokine stimulation [4,9,10]. The human CD40 gene shares 62% amino acid identity in the complete coding sequence with the mouse CD40 gene [1,11]. A previous study has shown that coarse, fine, and ultrafine air pollution particles increased immune costimulatory receptors including CD40, CD80, and CD86, and MHC class II molecules (HLA-DR) on human blood-derived monocytic cells [12]. Ensuing studies in our laboratory have indicated that LPS was an important component on air particles and responsible for particle-induced immune responses. The present study aimed to uncover the events regulating LPS-induced CD40 expression in normal human peripheral blood monocytic cells (PBMCs). Human PBMCs (composed of 93% monocytes and 7% lymphocytes) were isolated from healthy volunteers. We observed that LPS induced CD40 expression on human blood PBMCs through activation of NF κ B and JNK, and partially through secreted IFN γ .

Materials and Methods

Materials and reagents

LPS (*Escherichia coli* 0127:B8) was purchased from Sigma-Aldrich Co. (St. Louis, MO). SDS-PAGE supplies such as molecular mass standards and buffers were obtained from Bio-Rad (Richmond, CA). The mouse anti-human CD40-phycoerythrin (PE) and IgG1-PE antibodies were purchased from Beckman Coulter-Immunotech (Marseille, France). IFN γ neutralizing antibodies and ELISA assay kit were purchased from eBioscience (San Diego, CA). IFN γ receptor neutralizing antibody was obtained from R&D Systems, Inc. (Minneapolis, MN). Phospho-specific and pan antibodies against ERK, JNK, and p38, and I κ B α antibody were purchased from Cell Signaling Technology (Beverly, MA). β -actin antibody was purchased from USBiological (Swampscott, MA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit and goat anti-mouse IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polymyxin B, the ERK kinase inhibitor PD98059, the p38 kinase inhibitor SB203580, the JNK inhibitor SP600125, and the NF κ B activation inhibitor Bay11-7082 were purchased from EMD Biosciences (San Diego, CA).

PBMC isolation and cell culture

PBMCs were isolated from heparinized peripheral blood from healthy volunteers using Lymphoprep (Nycomed Pharma AS, Oslo, Norway) and further purified using a discontinuous 35/51% percoll gradient (Sigma, St Louis, MO) yielding monocytes of 93% purity, as described previously [13]. The remaining 7% cells of these PBMCs were microscopically identified as lymphocytes with Hema 3 stain. PBMCs were incubated in RPMI 1640 medium containing 10% fetal bovine serum and 100 μ g/ml penicillin/streptomycin at 37 $^{\circ}$ C in 5% CO $_2$. Cells were incubated in ultra low attachment plates (Corning Inc., Corning, NY) or polypropylene tubes for LPS stimulation studies.

Immunoblotting

PBMCs treated with LPS were washed twice with cold phosphate-buffered saline (PBS), and then lysed in RIPA buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors: 20 µg/ml leupeptin, 20 µg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, 200 µM sodium orthovanadate, and 20 mM sodium fluoride). Supernatants of cell lysates were subjected to SDS-PAGE, as described before [14]. Proteins were transferred onto nitrocellulose membrane. Membrane was blocked with 5% nonfat milk, washed briefly, incubated with primary antibody at 4°C overnight, followed by incubating with corresponding HRP-conjugated secondary antibody for 1 h at room temperature. Immunoblot images were detected using chemiluminescence reagents and the Gene Gynome Imaging System (Syngene, Frederick, MD).

Flow cytometry

3×10^5 PBMCs were used for measurement of CD40 protein expression. Flow cytometry was performed with a FACSORT (Becton Dickinson, Miami, FL) using an Argon-ion laser (wavelength 488nm) [15]. The FACSORT was calibrated with Calibrite beads before each use, and 10,000 events were counted for all sample runs. PE-conjugated non-specific antibody of the same isotype as the receptor antibody was used as control to establish background fluorescence and non-specific antibody binding. The mean fluorescence intensity (MFI) of the cells stained with control antibody was subtracted from the MFI of cells stained with receptor antibody to provide a measure of receptor-specific MFI. Relative cell size and density/granularity were quantified by analyzing light-scatter properties using Cell Quest software (Becton Dickinson), namely forward scatter for cell size and side scatter for density/granularity.

Real-time reverse transcriptase/polymerase chain reaction (RT-PCR)

1×10^6 blood PBMCs were exposed to LPS. Cells were washed with ice-cold PBS and then lysed with TRIZOL reagent (Invitrogen Corporation, Carlsbad, CA). Total RNA was isolated according to manufacturer-provided instructions. RNA (200ng) was reverse transcribed into cDNA. Quantitative PCR was performed using Platinum Quantitative PCR SuperMix-UDG (Invitrogen Corporation, Carlsbad, CA) and an ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA). mRNA levels of *CD40* and *IFN γ* were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA. Relative amounts of *CD40* or *IFN γ* and *GAPDH* mRNA were based on standard curves prepared by serial dilution of cDNA from human airway epithelial cells. The oligonucleotide primers and probes were purchased from Applied Biosystems (Foster City, CA).

Enzyme linked immunosorbent assay (ELISA)

5×10^5 PBMCs were treated with LPS. Supernatants were collected by centrifugation. Measurement of released IFN γ from PBMC culture with ELISA was conducted following the manufacturer's instructions.

Statistics

Differences in CD40 expression or cytokine production were evaluated using nonparametric paired *t* tests with the overall α level set at 0.05. Data were presented as means $\pm$ SEMs.

Results and Discussion

LPS exposure increases CD40 expression on the monocytes of human PBMCs

CD40 expression is observed in a wide range of cells, including macrophages and dendritic cells. In this present study, CD40 expression was determined on freshly isolated human PBMCs at both mRNA and cell surface protein levels. As shown in Fig.1A, PBMCs exposed to 1 ng/

ml LPS developed increased *CD40* mRNA expression at 4 h and a further increase at 8 h of exposure. Exposure of PBMCs to 0.001–1000 ng/ml LPS for 24 h induced a dose-dependent increase in CD40 cell surface protein expression (Fig.1B). Stimulation of PBMCs with 1 ng/ml LPS for 6, 12, and 24 h caused a time-dependent increase in CD40 protein expression that became significant at 12 h (Fig.1C). From flow analysis (gated on lymphocytes) we observed little increase of CD40 protein expression on the 7% residual lymphocytes. To confirm this with a larger number of cells, lymphocytes (99% purity) were collected through the same procedure for PBMC isolation and exposed to 1 ng/ml LPS for 24 h. It was found that LPS did not induce CD40 cell surface expression on these lymphocytes (data not shown). These data suggested that LPS-induced CD40 expression only occurred on the monocytes and not on the residual lymphocytes of PBMCs.

Activation of NFκB is required for LPS-induced CD40 expression on PBMCs

Because of the importance of NFκB activation in LPS-induced signaling [16,17], the involvement of NFκB in LPS-induced *CD40* gene transcription was investigated in this study. NFκB transcription factors are present in the cytoplasm in an inactive state, complexed with inhibitory IκB proteins, which cover the nuclear localization structures of NFκB. Phosphorylation of cytoplasmic IκBα at two conserved NH2-terminal serine residues (Ser32 and Ser36) and its subsequent proteosomal degradation by the 26S proteasome allows NFκB nuclear translocation and activation of NFκB-dependent transcriptional activity [18]. NFκB has been proposed as an important transcription factor in LPS-induced CD40 expression in murine macrophages and microglia [9]. Analysis of the CD40 promoter demonstrates that NFκB regulatory elements is conserved between the human and mouse CD40 promoters. Within the CD40 promoter, there are four NFκB sites that are designated as κB, mκB, m2κB, and pκB [19]. To determine whether NFκB was activated by LPS stimulation in our system and to examine its involvement in LPS-induced CD40 expression, IκBα levels were determined in PBMCs exposed to LPS. As shown in Fig. 2A, LPS (1 ng/ml) induced a rapid time-dependent reduction of IκBα protein, indicative of NFκB activation. Recent studies have investigated the upstream signaling pathways responsible for LPS-induced NFκB activation. It was shown that LPS could induce NFκB activation through transforming growth factor β (TGFβ)-activated kinase (TAK1), which was a member of the mitogen-activated protein kinase kinase kinase (MAPKKK) family [16]. PI3K was also demonstrated to mediate LPS-induced NFκB activation through induction of p65 phosphorylation[20].

To investigate whether NFκB activation was involved in LPS-induced CD40 expression on human monocytes, we pretreated PBMCs with the potent inhibitor of NFκB activation Bay11-7082 [21] prior to LPS addition. PBMCs were pretreated with 2 μM Bay11-7082 for 30 min before addition of 1 ng/ml LPS. At this dose Bay11-7082 was not cytotoxic to PBMCs. As shown in Fig. 2B, Bay11-7082 strongly suppressed LPS-induced CD40 expression on monocytes at 24 h. Thus, NFκB activation was required for LPS-induced CD40 expression on the monocytes of human PBMCs as in murine macrophages and microglia [3,9].

Signaling through CD40 is known to activate the NFκB pathway, and in this study we have demonstrated that CD40 expression is also regulated by NFκB. Taken together, the upregulation of CD40 expression might be regulated through a positive feedback mechanism using this CD40/NFκB activation pathway.

Involvement of JNK in LPS-induced CD40 expression

The MAPKs are generally expressed in all cell types. Three groups of MAPKs have been identified in mammals: the extracellular signal-regulated protein kinases (ERKs), the c-Jun N-terminal kinases (JNKs), and the p38 stress-activated protein kinases. The ERK pathway appears mainly to respond to mitogens and growth factors that regulate cell proliferation and

differentiation. The JNK and p38 pathways are predominantly activated by stress, such as osmotic changes and heat shock, but also by inflammatory cytokines. LPS is a potent activator of all three MAPKs [22]. Previous studies have demonstrated that MAPK-mediated LPS effects were cell type specific and depended on the surface molecules studied [23–25]. In this study, we examined the effect of LPS on activities of MAPKs and their involvement in CD40 expression on PBMCs. Activation of MAPKs occurs through phosphorylation of specific residues of these kinases. Thus, phosphorylation of MAPKs was measured using phospho-specific antibodies against JNK (Thr183/Tyr185), p38 (Thr180/Tyr182), and ERK (Thr202/Tyr204), respectively. PBMCs were exposed to 1 ng/ml LPS for 15, 30, 60, and 120 min. As shown in Fig. 3, LPS stimulation resulted in a rapid increase in phosphorylation of JNK, p38, and ERK in PBMCs, reaching a maximum for JNK and ERK at 30 min whereas phosphorylation of p38 continued to increase throughout the period of observation.

To determine whether activated MAPKs participated in LPS-induced CD40 expression on blood monocytes, we used selective kinase inhibitors, 20 μ M PD98059, SP600125, and SB203580, which specifically inhibited ERK kinase, JNK, and p38 activities, respectively, to pretreat PBMCs for 30 min prior to LPS stimulation (1 ng/ml) for 24 h. It was found that only the JNK inhibitor SP600125 partially inhibited LPS-induced CD40 expression on the monocytes (Fig. 3D). Whether JNK modulates CD40 gene transcription through activation of transcription factors such as AP-1 in the CD40 promoter remains to be investigated.

IFN γ released from the residual lymphocytes of PBMCs is partially involved in LPS-induced CD40 expression

Since 7% lymphocytes were presented in the PBMCs and lymphocytes were well-known for their production of IFN γ [26], a potent CD40 inducer [19], we next used ELISA to determine the levels of IFN γ , in the medium of PBMCs exposed to 0.01, 1, and 100 ng/ml LPS for 24 h. As shown in Fig. 4A, LPS stimulation induced dose-dependent increase in IFN γ protein levels in the medium of PBMCs. Exposure of PBMCs to 1 ng/ml LPS increased IFN γ protein production in a time-dependent fashion (Fig. 4B). In addition, IFN γ mRNA expression was also determined in PBMCs treated with 1 ng/ml LPS for 8 h (Fig. 4C), which showed that IFN γ mRNA levels were increased by LPS stimulation. Since freshly purified human monocytes do not produce detectable IFN activity when stimulated with LPS [27,28], the IFN γ proteins detected in the culture medium must be secreted from the residual 7% lymphocytes. We confirmed this using a human monocytic cell line (THP-1) where no IFN γ production after LPS treatment was observed (data not shown).

IFN γ is a dimerized soluble cytokine that is the only member of the type II class of IFN [29]. The major biological responses to IFN γ are mediated through the JAK/STAT pathway [30]. Seven mammalian STATs have been identified thus far, and STAT-1 is the major transcription factor in the IFN γ signal transduction pathway [30]. Binding of IFN γ to its receptor induces a series of events, which ultimately results in tyrosine phosphorylation of STAT-1, followed by homodimerization, nuclear translocation, and binding to GAS elements in the promoter regions of IFN γ -inducible genes. To ascertain the magnitude of the role played by IFN γ in LPS-induced CD40 expression, neutralizing antibodies against human IFN γ (anti-IFN γ) or IFN γ receptors (anti-IFN γ R) were employed to pretreat PBMCs. To first characterize the specificities of these two neutralizing antibodies, PBMCs were pretreated with 20 μ g/ml anti-IFN γ and anti-IFN γ R, respectively, prior to addition of 500 pg/ml recombinant human IFN γ . It was shown that anti-IFN γ and anti-IFN γ R significantly inhibited IFN γ -induced CD40 expression on PBMCs by 85% and 82%, respectively, at 24 h. Then, PBMCs were pre-incubated with 20 μ g/ml IgG (vehicle), anti-IFN γ , or anti-IFN γ receptor (IFN γ R) at 37°C for 2 h prior to stimulation with 1 ng/ml LPS for 24 h. We observed that anti-IFN γ and anti-IFN γ R inhibited LPS-induced CD40 expression by 44% and 37%, respectively (Fig. 4D). Therefore, these data

indicated that IFN γ released from the lymphocytes of PBMCs was only partially responsible for LPS-induced CD40 expression. Additional studies have demonstrated that both NF κ B and JNK were required for LPS-induced IFN γ production (data not shown). And the interaction between lymphocytes and monocytes in PBMC culture facilitated LPS-induced IFN γ production from the residual lymphocytes of PBMCs since the same number of isolated pure lymphocytes as in PBMCs produced less IFN γ after LPS stimulation.

In summary, LPS induces CD40 expression on human peripheral blood monocytes through activation of NF κ B and JNK, and through IFN γ production by lymphocytes.

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Fig 1. LPS exposure results in increase in CD40 expression on human monocytes

A, Human PBMCs were treated with 1 ng/ml LPS for 4 and 8 h. Cells were then lysed with TRIZOL reagent. RNA was extracted and reverse transcribed before further analysis of CD40 mRNA expression using quantitative PCR. CD40 mRNA levels were normalized using the expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA. B, Human PBMCs were treated with 0.001–1000 ng/ml LPS for 24 h. C, PBMCs were incubated with 1 ng/ml LPS for 6, 12, and 24 h. CD40 protein expression was measured with flow cytometry using isotype and anti-CD40 antibodies, respectively, as described in Materials and Methods. * $P < 0.05$, compared to control (Ct).

Fig 2. NFκB activation is required for LPS-induced CD40 expression

A, PBMCs were treated with 1 ng/ml LPS for 0, 15, 30, 60, and 120 min. IκBα protein levels were measured with Western blotting using anti-IκBα antibody. B, PBMCs were pretreated with DMSO or 2 μM Bay11-7082 for 30 min prior to 1 ng/ml LPS stimulation for 24 h, respectively. CD40 expression (MFI) was measured with flow cytometry. * $P<0.05$, compared to vehicle (DMSO) LPS.

Fig 3. JNK is partially involved in LPS-induced CD40 expression

PBMCs were stimulated with 1 ng/ml LPS for 15–120 min. Cells were lysed with RIPA buffer. Supernatants of cell lysates were subjected to SDS-PAGE and immunoblotting. Phosphorylated JNK (A), p38 (B), and ERK (C) kinases were detected using phospho-specific antibodies. D, PBMCs were pretreated with DMSO (vehicle), 20 μ M SB203580, 20 μ M SP600125, or 20 μ M PD98059 for 30 min, respectively, prior to 1 ng/ml LPS treatment for 24 h. CD40 expression was determined using flow cytometry. * $P < 0.05$, compared to vehicle (DMSO) LPS. Data shown are representative of three separate experiments.

Fig 4. LPS-induced IFN γ production is involved in CD40 expression

A, PBMCs were treated with 0.01, 1, and 100 ng/ml LPS for 24 h, respectively. Supernatants of cell media were collected for measurement of IFN γ protein with ELISA. * P <0.05, compared to control (Ct). B, PBMCs were treated with 1 ng/ml LPS for 8 and 24 h, respectively. Supernatants of cell media were collected for measurement of IFN γ protein with ELISA. Increase in IFN γ protein release was expressed as fold over control. * P <0.05, compared to control (Ct) at the same time point. C, PBMCs were treated with 1 ng/ml LPS for 8 h prior to lysis with TRIZOL reagent. RNA was extracted and reverse transcribed before further analysis of IFN γ mRNA expression using quantitative PCR. D, PBMCs were pretreated with 20 μ g/ml IgG (vehicle), anti-IFN γ , or anti-IFN γ R for 2 h before treatment with 1 ng/ml LPS for 24 h,

respectively. CD40 expression was determined and expressed as fold over control. $*P<0.05$, compared to vehicle LPS.