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\textit{J Immunol} published online 12 October 2012
http://www.jimmunol.org/content/early/2012/10/12/jimmunol.1103156

Supplementary Material

http://www.jimmunol.org/content/suppl/2012/10/12/jimmunol.1103156.6.DC1

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p16INK4a Exerts an Anti-Inflammatory Effect through Accelerated IRAK1 Degradation in Macrophages

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Induction of cyclin-dependent kinase (CDK) inhibitor gene p16INK4a into the synovial tissues suppresses rheumatoid arthritis in animal models. In vitro studies have shown that the cell-cycle inhibitor p16INK4a also exerts anti-inflammatory effects on rheumatoid synovial fibroblasts (RSF) in CDK activity-dependent and -independent manners. The present study was conducted to discern how p16INK4a modulates macrophages, which are the major source of inflammatory cytokines in inflamed synovial tissues. We found that p16INK4a suppresses LPS-induced production of IL-6 but not of TNF-α from macrophages. This inhibition did not depend on CDK4/6 activity and was not observed in RSF. p16INK4a gene transfer accelerated LPS-triggered IL-1R–associated kinase 1 (IRAK1) degradation in macrophages but not in RSF. The degradation inhibited the AP-1 pathway without affecting the NF-κB pathway. Treatment with a proteosome inhibitor prevented the acceleration of IRAK1 degradation and downregulation of the AP-1 pathway. THP-1 macrophages with forced IRAK1 expression were resistant to the p16INK4a-induced IL-6 suppression. Senescent macrophages with physiological expression of p16INK4a upregulated IL-6 production when p16INK4a was targeted by specific small interfering RNA. These findings indicate that p16INK4a promotes ubiquitin-dependent IRAK1 degradation, impairs AP-1 activation, and suppresses IL-6 production. Thus, p16INK4a senescence gene upregulation inhibits inflammatory cytokine production in macrophages in a different way than in RSF. The Journal of Immunology, 2012, 189: 000-000.

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovial inflammation, hyperplasia, and destruction of the cartilage and bone. In rheumatoid synovial tissues, lymphocytes and macrophages are recruited and activated. These cells, especially activated macrophages, release a large amount of inflammatory cytokines. In response to these cytokines, synovial fibroblasts proliferate vigorously and form villous hyperplastic synovial tissues called pannus. These fibroblasts also secrete inflammatory mediators that further attract inflammatory cells and stimulate growth of the synovial fibroblasts as well as vascular endothelial cells (1). The pannus becomes the source of tissue-degrading proteinases and activators of osteoclasts, leading to destruction of the affected joints (2, 3).

The pannus formation results from proliferation of the synovial fibroblasts driven by the inflammatory processes in RA. This fact led us to explore a new therapeutic approach that directly controls the cell cycle of synovial fibroblasts. If the synovial fibroblasts become refractory to the proliferative stimuli, no pannus should develop (4). It has been known that the master molecules that control cell cycling are cyclin-dependent kinases (CDK) (5). Especially CDK4/6 phosphorylates retinoblastoma gene product (pRb), which inhibits active E2F transcription factors for cell-cycle progression. CDK inhibitors (CDKI) are intracellular proteins that inhibit kinase activity of cyclin/CDK complexes. CDKI p16INK4a inhibits CDK4/6 specifically, whereas CDK p21CIP1 inhibits a broad spectrum of CDK (5). The intra-articular gene transfer of p16INK4a as well as p21CIP1 suppressed RA in animal models (4, 6). It inhibited histological findings characteristic to RA: synovial hyperplasia, mononuclear cell infiltration, and destruction of the bone and cartilage of the joints. Comparable therapeutic effects were observed when the small-molecule (sm)CDKI was administered orally or i.p. (7). A separate series of our experiments disclosed that cell-cycle progression is not the only function of CDK (8). CDK kinase activity also regulates production of inflammatory molecules in a pRb-independent manner. Also, it has been suggested that CDKI can affect expression of inflammatory molecules in a CDK kinase-independent manner.

The above studies were all carried out with synovial fibroblasts. In rheumatoid synovial tissues, macrophages are the major source of inflammatory cytokines that are critical for the resultant pathology (9–11). The present study was conducted to explore how p16INK4a affects expression of inflammatory cytokines from activated macrophages. We found that p16INK4a suppresses IL-6 production in macrophages. The effect was mediated by accelerated degradation of IL-1R–associated kinase 1 (IRAK1).
Materials and Methods

Reagents

Anti-p-p38 MAPK, anti-p-JNK, anti-p-IκB kinase (IKK)α/β, anti-p-MAPK kinase 4 (MKK4), and anti-iBeα Abs were purchased from Cell Signaling Technology (Danvers, MA). Biotin-labeled anti-TLR4 Ab and PE-labeled streptavidin were purchased from eBioscience (San Diego CA). Biotin-labeled IgG1 was purchased from Beckman Coulter (Tokyo, Japan). Anti-actin Ab and MGI32 were purchased from Sigma-Aldrich (St. Louis, MO). Anti-p16INK4a Ab was purchased from Millipore (Billerica, MA). Anti-IRAK1 Ab was kindly provided by Dr. Shizuo Akira [Osaka University, Osaka, Japan (12)]. An smCDK4/6 selective inhibitor, PD0332991, was provided by Pfizer (Boston, MA) (13). IRAK1 wild-type and knockout bone marrow-derived macrophage (BMM) lysates were kindly provided by Dr. James A. Thomas (University of Texas Southwestern Medical Center, Dallas, TX).

Cells

BMM were isolated from 6–8-wk-old DBA1/J mice (Charles River Laboratories, Yokohama, Japan) and cultured as described previously (14). They were cultured in RPMI 1640 medium containing rM-CSF (50 ng/ml) or 10% CMG14-12-conditioned media as a source of M-CSF (15). Human synovial tissues were derived from RA patients undergoing total joint replacement surgery at Shimoshizu National Hospital. Consent forms were completed by the patients prior to the surgery. RA was diagnosed according to the 1988 criteria of the American College of Rheumatology (16). Human synovial tissues were prepared as described previously (8). Human acute monocytic leukemia cell line THP-1 cells were cultured and differentiated to macrophages as described elsewhere (17). RAW264.7 cells were cultured as described elsewhere (18). For activation, cells were stimulated with the optimal doses of LPS, which were minimum dose to induce maximum IL-6 production in each cell type.

![Image](http://www.jimmunol.org/article-pdf/2/3/2_2008/2_2008.pdf)

**FIGURE 1.** Effect of p16INK4a expression on IL-6 production in LPS-stimulated macrophages. BMM, THP-1, and RAW264.7 cells were infected with pMX-IP (control) and pMX-p16INK4a (p16INK4a) retroviruses. (A) Cellular proteins were harvested, p16INK4a and actin expression were detected with Western blot analyses. (B) [3H]thymidine was added to culture media of the BMM transductants. Incorporation of [3H]thymidine was quantified with an scintillation counter. (C) BMM transductants were stimulated with LPS (10 ng/ml). Total RNA were harvested for TNF-α and IL-6, and mRNA levels at 3 h and IL-6 levels at 6 h after stimulation were depicted because they were highest during the observation. (D) The BMM transductants were stimulated with LPS (10 ng/ml) for 24 h for quantification of IL-6 in the culture supernatants. (E) TLR4 surface expression on BMM transductants was detected by flow cytometry analyses. (F) Differentiated THP-1 macrophages were stimulated with LPS (1 μg/ml) for 24 h for quantification of IL-6 in the culture supernatants. (G) RAW264.7 cells were treated in the same way with LPS (100 ng/ml). Data are representative of three independent experiments and expressed as the mean ± SD of triplicate wells. *p < 0.01.
Also suppressed significantly in BMM expressing p16INK4a (Fig. 1D). Our previous study demonstrated that p16INK4a inhibited matrix LPS-stimulated macrophages. CDK4/6 inhibition did not affect IL-6 expression in murine and human macrophages.

Results

p16INK4a suppressed IL-6 expression in LPS-stimulated macrophages

To study the effect of p16INK4a on macrophages, BMM were infected with pMX-p16 or control pMX-IP retroviruses. Ectopic p16INK4a protein expression in the pMX-p16INK4a-infected cells had been confirmed with Western blots analyses (Fig. 1A). [3H]Thymidine uptake by the p16INK4a-expressing BMM was almost completely suppressed compared with control virus-treated BMM (Fig. 1B). Quantitative PCR showed that p16INK4a gene transfer significantly suppressed IL-6 mRNA expression by BMM stimulated with LPS, whereas TNF-α mRNA expression was not affected (Fig. 1C). IL-6, but not TNF-α, production at the protein level was also suppressed significantly in BMM expressing p16INK4a (Fig. 1D). LPS recognition receptor TLR4 expression was not modified by p16INK4a overexpression, showing that IL-6 reduction was not due to the TLR4 downmodulation (Fig. 1E). The murine macrophage cell line RAW264.7 cells as well as human THP-1 cells that had been induced to differentiate to macrophages produce IL-6 in response to LPS. This response was also reduced by the p16INK4a gene transfer (Fig. 1F, 1G). Thus, p16INK4a gene transfer suppressed IL-6 expression in murine and human macrophages.

CDK4/6 inhibition did not affect IL-6 expression in LPS-stimulated macrophages

Our previous study demonstrated that p16INK4a inhibited matrix metalloproteinase (MMP)-3 expression in RSF by suppressing CDK4/6 kinase activity (8). To determine if the inhibitory effect of p16INK4a on LPS-induced IL-6 production in BMM depends on CDK4/6 kinase activity, CDK4/6 selective inhibitor (PD0332991) was added to the BMM culture. The CDK4/6 inhibitor suppressed [3H]thymidine uptake of BMM in a dose-dependent manner. Maximal inhibition was observed at 2 μM PD0332991 (Fig. 2A). The specific band was observed in wild-type BMM but not in IRAK1 knockout cells, demonstrating that IL-6 reduction was not due to CDK4/6 kinase inhibition by PD0332991. CDK6 inhibitor did not modulate LPS-induced IL-6 expression and production by BMM, but it completely suppressed BMM proliferation (Fig. 2B, 2C). To suppress CDK4 specifically, a retroviral vector containing shCDK4 was prepared. In contrast with shNC transfer, shCDK4 transfer suppressed CDK4 expression significantly, but not CDK6 expression, by BMM (Fig. 2D). As the [3H]thymidine uptake by the p16INK4a-expressing BMM was comparable to that in control cells at the unstimulated status. Upon LPS stimulation, the IRAK1 expression was significantly downmodulated in p16INK4a-expressing cells as compared with control cells (Fig. 3A). The specific band was observed in wild-type BMM but not in IRAK1 knockout cells, dem-

Quantification of cytokine and IRAK1 expression

Specific ELISA kits to quantify human IL-6 and TNF-α in the culture supernatants were purchased from R&D Systems (Minneapolis, MN). Quantitative real-time PCRs for IL-6, TNF-α, IRAK1, and GAPDH were carried out as previously described (21, 22). A p16INK4a gene-specific primer set was purchased from Qiagen (Tokyo, Japan).

Small interfering RNA transfection

To introduce small interfering RNA (siRNA) into BMM, 2 × 10⁵ BMM was incubated with 200 μl 1.2 μM siRNA containing 24 μl FuGENE-HD transfection reagent (Roche) in Opti-MEM for 6 h. p16INK4a specific siRNA (si-p16A and B) and control siRNA were purchased from Qiagen.

Statistical analyses

[3H]Thymidine uptake, IRAK1 and cytokine mRNA measurements, and IL-6 concentrations in the supernatants were compared with the Mann–Whitney U test.

FIGURE 2. Effects of direct CDK4/6 inhibition on IL-6 production in LPS-stimulated BMM. (A) BMM were treated with or without indicated concentrations of smCDK4/6-selective inhibitor PD0332991 (smCDKI) for 24 h, and [3H]thymidine uptake was assessed for the last 8 h. (B) BMM were treated with or without 2 μM smCDKI for 1 h prior to LPS stimulation (10 ng/ml) for 6 h. IL-6 mRNA in the treated BMM were quantified with real-time PCR. The amounts of the mRNA were normalized to that of GAPDH mRNA and presented as fold change relative to nontreated cells. (C) BMM were pretreated in the same manner and then stimulated with LPS (10 ng/ml) for 24 h. IL-6 in the culture supernatants was measured with ELISA. (D) BMM were transduced with shCDK4 or shNC. CDK4 and CDK6 mRNA in the BMM were quantified with real-time PCR. (E) BMM infected with shCDK4 and shNC were examined for [3H]thymidine uptake during 8 h incubation. (F) BMM infected with shCDK4 and shNC were stimulated with LPS (10 ng/ml) for 24 h. IL-6 in the culture supernatants was measured with ELISA. Data are representative of three independent experiments and expressed as the mean ± SD of triplicate wells. *p < 0.01.
onstrating that binding of anti-IRAK1 Abs was specific (Fig. 3A). p16INK4a expression did not affect IRAK1 mRNA levels, arguing that the decrease of the IRAK1 protein was not due to the decrease in gene transcription (Fig. 3B).

To discern if the same suppression is observed in RSF, p16INK4a or control LacZ were transduced using an adenoviral vector to RSF and IL-6 production was measured after LPS stimulation. Although [3H]thymidine uptake was inhibited in the p16INK4a-infected RSF, IL-6 production was not altered (Supplemental Fig. 1A, 1B). In accordance with this, IRAK1 expression in RSF in p16INK4a-expressing and control RSF was comparable throughout the LPS stimulation (Supplemental Fig. 1C).

p16INK4a suppressed the AP-1 signaling pathway without suppressing the NF-κB signaling pathway

Upon LPS stimulation of macrophages, activated IRAK1 triggers phosphorylation of p38 MAPK/JNK and IKKα/β, which leads to activation of the AP-1 and NF-κB transcription factors (24). Western blot analyses illustrated that ectopic p16INK4a expression suppressed phosphorylation of p38 MAPK and JNK but not of IKKα/β in BMM stimulated with LPS (Fig. 4A). Phosphorylation of MKK4, which is upstream of JNK, was suppressed by p16INK4a (Fig. 4A). Downstream AP-1 binding activity was also reduced in p16INK4a-expressing BMM (Fig. 4B). In contrast, IκB degradation, which is downstream of IKKα/β, as well as NF-κB–binding activity were not affected by p16INK4a overexpression (Fig. 4A, 4B).

We assumed that the impairment of the AP-1 pathway in p16INK4a-expressing BMM should be directly due to reduced IRAK1 protein expression. To substantiate this, shIRAK1 was retrovirally transduced in BMM. IRAK1 protein was downregulated in the shIRAK1-transduced cells in comparison with control cells (Fig. 4C). When these cells were stimulated with LPS, AP-1 activation, but not IκB degradation, was significantly suppressed in the shIRAK1-

FIGURE 3. Promotion of IRAK1 degradation by exogenous p16INK4a. BMM transduced with pMX-IP (control) and pMX-p16INK4a (p16INK4a) were stimulated with LPS (10 ng/ml). (A) Cellular proteins were harvested at the indicated time points. IRAK1 and actin expression was detected with Western blot analyses (top panel). The density of the IRAK1 bands were normalized to that of actin and presented as fold change relative to nontreated control cells (bottom panel). Data are representative of three experiments and expressed as the mean ± SD of triplicate wells.

FIGURE 4. Suppression of phosphorylation of p38 MAPK and JNK without affecting the IKKα/β–IκB pathway by p16INK4a. (A) Transduction of BMM with pMX-IP (control) and pMX-p16INK4a (p16INK4a) retroviruses was followed by LPS (10 ng/ml) stimulation. Total cell lysates were collected at the indicated time points and examined for p-p38, p-JNK, p-MKK4, IκBα, and actin expression with Western blot analyses. (B) Nuclear extracts of the control and p16INK4a-expressing BMM stimulated with LPS (10 ng/ml) for 1 h were examined for AP-1 and NF-κB binding activity with EMSA. (C) shIRAK1 or shNC were transduced retrovirally into BMM. Protein levels of IRAK1 and actin in total cell lysates were determined with Western blot analyses. (D) Cells were stimulated with LPS (10 ng/ml). After 1 h, the cells were lysed, and AP-1 binding activity in the nuclear extracts was examined with EMSA. After 30 min stimulation, the protein levels of IκBα in total cell lysates were determined with Western blot analyses. Data are representative of three independent experiments.
transduced cells (Fig. 4D). Thus, reduction of the IRAK1 protein in BMM resulted in inhibition of the AP-1 signaling pathway without affecting the NF-κB signaling pathway.

**p16**<sup>INK4a</sup>-induced IRAK1 degradation was mediated by proteosome pathway

It was reported that IRAK1 degradation is modulated by the ubiquitin-dependent proteosome pathway in LPS-stimulated monocyte/macrophages (23). To inhibit this degradation pathway, a proteosome-specific inhibitor, MG132, was added to the p16<sup>INK4a</sup>-expressing BMM culture. This treatment prevented acceleration of IRAK1 degradation in p16<sup>INK4a</sup>-transduced cells (Fig. 5A). It also restored p38 MAPK and JNK phosphorylation in p16<sup>INK4a</sup>-expressing BMM, indicating that p16<sup>INK4a</sup>-induced IRAK1 degradation depends on the proteosome degradation pathway.

To determine if the observed IL-6 downregulation is a direct consequence of accelerated IRAK1 degradation, the p16<sup>INK4a</sup> gene was retrovirally transferred to THP-1 macrophages with or without the exogenous IRAK1 gene. THP-1 macrophages were used because primary BMM are too sensitive to neomycin and puromycin treatment to select the transduced cells. As in BMM, p16<sup>INK4a</sup> suppressed LPS-induced IL-6 production by THP-1 cells. This suppression was abrogated by cotransduction of the exogenous IRAK1 gene. Thus, IL-6 production was not downregulated without reduction of IRAK1 (Fig. 5B).

**Inhibitory effect of endogenous p16**<sup>INK4a</sup> **on IL-6 production**

To discern if expression of endogenous p16<sup>INK4a</sup> exerts the same inhibitory effects, BMM were cultured to senescence as described previously (25). No endogenous p16<sup>INK4a</sup> protein was detectable in log-phase growing BMM, whereas the senescent BMM substantially upregulated the protein and mRNA of p16<sup>INK4a</sup> (Fig. 6A). These cells were then treated with two different siRNAs against p16<sup>INK4a</sup> to reduce p16<sup>INK4a</sup> expression levels (Fig. 6B). IL-6 production from BMM was upregulated by LPS stimulation and inversely proportional to p16<sup>INK4a</sup> mRNA expression levels (Fig. 6C). Thus, endogenous physiological p16<sup>INK4a</sup> expression can contribute to the suppression of the LPS-induced IL-6 production.

**Discussion**

p16<sup>INK4a</sup> expression in macrophages suppressed LPS-induced production of IL-6 but not of TNF-α in a CDK4/6-independent manner. This was not observed in synovial fibroblasts. Molecular analyses disclosed that it was due to the acceleration of proteosome-mediated IRAK1 degradation and following suppression of the AP-1 signaling pathway. Thus, p16<sup>INK4a</sup> gene transfer or its induction in synovial cells inhibits production of a part of macrophage-derived cytokines in addition to proliferation of synovial cells.

Recently, it was shown that TLR triggering is relevant in rheumatoid inflammation. Endogenous ligands for TLR-2 and TLR-4 are found in RA joints. These include gp96, fibrinogen, Hsp60, Hsp70, hyaluronic acid, myeloid-related protein 8/14, and high mobility group box chromosomal protein 1 (26). Indeed, TLR inhibition by a dominant-negative form of the Toll/IL-1R domain containing adaptor protein molecules suppressed the spontaneous production of proinflammatory cytokines and MMPs from RSF (27). LPS was used as a TLR stimulator in this study.

p16<sup>INK4a</sup> gene transfer suppressed IL-6 production via the promoted IRAK1 reduction, because IRAK1 coexpression prevented the IL-6 reduction. Indeed, the upstream molecule of IRAK1, IKKα/β, was phosphorylated equally in p16<sup>INK4a</sup>-expressing cells and control cells. These findings also indicated that neither downstream nor upstream molecule of IRAK1 was affected by p16<sup>INK4a</sup>.

Studies of IRAK1-deficient cells showed that IRAK1 activation is an essential step in the TLR signaling pathways that leads to NF-κB and AP-1 activation and subsequent production of proinflammatory cytokines (23, 28, 29). IRAK1 activation causes degra-
and B) and siNC. p16INK4a mRNA expression in the treated BMM was measured with real-time PCR. The amounts of the p16INK4a mRNA were presented as fold change relative to siNC-treated cells. (C) These cells were stimulated with LPS (100 ng/ml) for 24 h. IL-6 in the culture supernatants was measured with ELISA. Data are representative of two independent experiments and expressed as the mean ± SD of triplicate wells. *p < 0.01.

**FIGURE 6.** Inhibitory effect of endogenous p16INK4a on IL-6 production. (A) BMM cultured for 6 d were still in a logarithmic growth phase (6 d), whereas those cultured for 17 d reached replicative senescence (17 d). Endogenous p16INK4a and actin were detected with Western blotting analyses. p16INK4a mRNA expression in these cells was quantified with real-time PCR. The amounts of the p16INK4a mRNA were normalized to that of GAPDH mRNA and presented as fold change relative to BMM cultured for 6 d. (B) BMM were transfected with siRNA against p16INK4a (si-p16A and B) and siNC. p16INK4a mRNA expression in the treated BMM was measured with real-time PCR. The amounts of the p16INK4a mRNA were presented as fold change relative to siNC-treated cells. (C) These cells were stimulated with LPS (100 ng/ml) for 24 h. IL-6 in the culture supernatants was measured with ELISA. Data are representative of two independent experiments and expressed as the mean ± SD of triplicate wells. *p < 0.01.
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