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p16\(^{INK4a}\) Exerts an Anti-Inflammatory Effect through Accelerated IRAK1 Degradation in Macrophages

Yousuke Murakami,*,† Fumitaka Mizoguchi,* Tetsuya Saito,* Nobuyuki Miyasaka,*,† and Hitoshi Kohsaka*§†

Induction of cyclin-dependent kinase (CDK) inhibitor gene p16\(^{INK4a}\) into the synovial tissues suppresses rheumatoid arthritis in animal models. In vitro studies have shown that the cell-cycle inhibitor p16\(^{INK4a}\) 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 p16\(^{INK4a}\) modulates macrophages, which are the major source of inflammatory cytokines in inflamed synovial tissues. We found that p16\(^{INK4a}\) suppresses LPS-induced production of IL-6 but not of TNF-\(\alpha\) from macrophages. This inhibition did not depend on CDK4/6 activity and was not observed in RSF. p16\(^{INK4a}\) 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-\(\kappa\)B 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 p16\(^{INK4a}\)-induced IL-6 suppression. Senescent macrophages with physiological expression of p16\(^{INK4a}\) upregulated IL-6 production when p16\(^{INK4a}\) was targeted by specific small interfering RNA. These findings indicate that p16\(^{INK4a}\) senescence gene upregulation inhibits inflammatory cytokine production in macrophages in a different way than in RSF. The Journal of Immunology, 2012, 189: 000–000.
**Materials and Methods**

**Reagents**

Anti-p-p38 MAPK, anti-p-JNK, anti-p-IκB kinase (IKKα/β), anti-p-MAPK kinase 4 (MKK4), and anti-IκBα 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 MG132 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 or synovectomy at Shimoshizu National Hospital. Consent forms were obtained from all patients according to the 1988 criteria of the American College of Rheumatology (16). Human synovial cells were prepared as described previously (8). Human acute monocytic leukemia cell line THP-1 cells were cultured 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.

**Western blot analyses and electrophoresis mobility shift assay**

Total cell lysate of BMM and rheumatoid synovial fibroblasts (RSF) were subject to Western blot analyses with specific Abs. A primary Ab against mouse actin was used for loading control. Peroxidase-conjugated anti-mouse or rat IgG Abs were used as secondary Abs. After preparation of nuclear lysates with the Nuclear Extraction kit (Active Motif, Carlsbad, CA), EMSA was performed with the second-generation gel shift assay kit (Roche, Tokyo, Japan). AP-1 and NF-κB consensus sequence was purchased from Promega (Madison, WI).

**Proliferation assay**

Measurement of [³H]thymidine uptake by RSF and BMM was performed as described elsewhere (8, 14).

**Flow cytometry analysis**

BMM were stained with biotin-labeled anti-TLR4 mAb or biotin-labeled isotype-matched IgG1 followed by PE-labeled streptavidin. Data were acquired with the FACSCalibur system (BD Biosciences, San Jose, CA) and analyzed by CellQuest (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR).

**Preparation of retroviral and adenoviral vectors**

The human p16INK4a and IRAK1 genes were cloned into the retroviral expression vectors, pMX-IP and pMX-IN, respectively (pMX-p16INK4a and pMX-IRAK1). They had an internal ribosomal entry site and a resistance gene for pharmacological selection (19). Using pSilencer5.1 (Applied Biosystems, Tokyo, Japan), recombinant retroviral vectors containing murine CDK4- and IRAK1-specific short hairpin (sh)RNA sequences (IRAK1 sense, 5′-GATCCGAGGCCCCTCCCTGTTCAAGAGACGGGGAGGGTTGGCTTCTTTTGGAAAA3′; IRAK1 antisense, 5′-AGCTTTCCAAAAAGAAGCCATCCCTCCCGTTCTTGAAAC-3′) were constructed and used for infection of BMM and synovial fibroblasts.

**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 assessed after 8 h. (C) The BMM transductants were stimulated with LPS (10 ng/ml). Total RNA were harvested for TNF-α and IL-6, and mRNA was quantified with real-time PCR. The amounts of the cytokine mRNA were normalized to that of GAPDH mRNA and presented as fold change relative to nontreated control cells. The TNF-α 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. Culture supernatants were collected, and IL-6 and TNF-α levels quantified with ELISA. (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.
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 \( \mu \)M PD0332991, whereas TNF-\( \alpha \), production at the protein level was significantly suppressed IL-6 mRNA expression by BMM stimulated with pMX-p16 or control pMX-IP retroviruses. Ectopic p16INK4a overexpression, showing that IL-6 reduction was not due to the TLR4 downmodulation (Fig. 1E). The murine macrophage p16INK4a suppressed IL-6 expression in LPS-stimulated macrophages, but it completely suppressed IL-6 mRNA expression 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.

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 blot 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-\( \alpha \) mRNA expression was not affected (Fig. 1C). IL-6, but not TNF-\( \alpha \), 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

To introduce small interfering RNA (siRNA) into BMM, 2 \( \times \) \( 10^5 \) BMM was incubated with 200 \( \mu \)g/mL siRNA containing 24 \( \mu \)M FuGENE-HD transfection reagent (Roche) in Opti-MEM for 16 h. p16 INK4a-LPS-stimulated macrophages

Quantitative of cytokine and IRAK1 expression

Specific ELISA kits to quantify human and murine IL-6 and TNF-\( \alpha \) in the culture supernatants were purchased from R&D Systems (Minneapolis, MN). Quantitative real-time PCRs for IL-6, TNF-\( \alpha \), IRAK1, and GAPDH were carried out as previously described (21, 22).

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 mM 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.

p16INK4a expression in BMM promoted IRAK1 degradation

TLR4 signaling is mediated by quite a few signaling molecules. To explore how p16INK4a inhibits IL-6 production in LPS-stimulated BMM, we studied the expression of signaling molecules with Western blot analyses and found that p16INK4a gene transfer affected IRAK1 protein expression (Fig. 3A). Upon ligand binding to TLR, MyD88 is recruited to the receptor and brings IRAK1 and MyD88 to the receptor. This leads to phosphorylation of IRAK1, which activates kinase activity of IRAK1 and then triggers degradation of IRAK1 itself (23). The IRAK1 protein level in 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-
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-
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-kB signaling pathway.

**p16INK4a-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 p16INK4a-expressing BMM culture. This treatment prevented acceleration of IRAK1 degradation in p16INK4a-transduced cells (Fig. 5A). It also restored p38 MAPK and JNK phosphorylation in p16INK4a-expressing BMM, indicating that p16INK4a-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 p16INK4a 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, p16INK4a 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 p16INK4a on IL-6 production**

To discern if expression of endogenous p16INK4a exerts the same inhibitory effects, BMM were cultured to senescence as described previously (25). No endogenous p16INK4a protein was detectable in log-phase growing BMM, whereas the senescent BMM substantially upregulated the protein and mRNA of p16INK4a (Fig. 6A). These cells were then treated with two different siRNAs against p16INK4a to reduce p16INK4a expression levels (Fig. 6B). IL-6 production from BMM was upregulated by LPS stimulation and inversely proportional to p16INK4a mRNA expression levels (Fig. 6C). Thus, endogenous physiological p16INK4a expression can contribute to the suppression of the LPS-induced IL-6 production.

**Discussion**

p16INK4a 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, p16INK4a 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.

p16INK4a 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 p16INK4a-expressing cells and control cells. These findings also indicated that neither downstream nor upstream molecule of IRAK1 was affected by p16INK4a.

Studies of IRAK1-deficient cells showed that IRAK1 activation is an essential step in the TLR signaling pathways that leads to NF-kB 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 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.

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.

The degradation of IRAK1 itself, which induces tolerance against restimulation of TLR. Tolerant cells do not express IRAK1 and thus fail to activate both the NF-κB and AP-1 signaling pathways in response to repeated TLR ligation (30, 31). In contrast with IRAK1-deficient cells and LPS-induced tolerant cells, p16INK4a-expressing macrophages express a reduced but significant level of the IRAK1 protein. These cells as well as macrophages with partial IRAK1 knockdown by siRNA had selective impairment of AP-1 signaling pathway. These results are in concordance with previous observations that siRNA against IRAK1 downregulated IL-1β-induced production of inflammatory cytokines without interfering with IκB degradation and that IRAK1 knockdown impaired the AP-1 signaling pathway (32, 33). It was of interest that different transcription factors depend differentially on the IRAK1 level.

IL-6 expression in monocytes/macrophages depends on NF-κB and AP-1 pathways because loss of either pathway severely impaired IL-6 expression (34, 35). In contrast, TNF-α expression in monocytes/macrophages depends primarily on NF-κB because mutation of the AP-1 binding site in the TNF-α promoter sequence did not alter its expression (36). This difference in AP-1 dependency should account for the differential effect on IL-6 and TNF-α expression in p16INK4a-expressing macrophages.

p16INK4a can make complexes not only with CDK4/6 but also with other signaling molecules. In mouse embryonic fibroblasts, p16INK4a bound to JNK prevents its interaction with c-Jun, which resulted in suppression of AP-1 activation induced by UV irradiation (37). In contrast to p16INK4a expression in macrophages, its expression in mouse embryonic fibroblasts did not disturb JNK phosphorylation. Another report demonstrated that p16INK4a in HeLa cells was associated with RelA, which is a component of NF-κB (38). This association inhibited NF-κB activation in HeLa cells, although we did not observe suppression of the NF-κB signaling pathway in macrophages. Thus, the inhibition of signal transduction by p16INK4a appeared to depend on the cell types.

The promoted IRAK1 degradation by p16INK4a could be accelerated by either IRAK1 ubiquitination or by recruitment to the proteasome. Polyubiquitination of signaling molecules triggers either proteosome-dependent protein degradation or activation of downstream signaling molecules (39). K48 ubiquitination causes the degradation of target molecules, whereas K63 ubiquitination activates downstream signaling molecules. K48 ubiquitination of IRAK1 appears to trigger degradation because treatment with a proteasome inhibitor, MG132, prevented the IRAK1 degradation in HEK293 cells stimulated with IL-1 (40). This agrees with the previous observation that MG132 treatment prevented LPS-induced IRAK1 degradation and enhanced activation of the AP-1 signaling pathway in THP-1 cells (17). These findings suggested that p16INK4a might enhance the K48 ubiquitination of IRAK1. Unlike the ubiquitination processes, processes of IRAK1 recruitment to the proteasome are unknown. Because some specific ubiquitin-receptor proteins may escort the ubiquitinated proteins to the proteasome, p16INK4a might enhance the function of the ubiquitin-receptor proteins. A recent report revealed that LPS-induced IRAK1 degradation was regulated partly by micro-RNA 146a (miR146a) (41). However, the miR146a level in p16INK4a-expressing cells was close to that in control cells (data not shown), suggesting that miR146a was not a primary contributor to the downregulation of IRAK1 in the p16INK4a-expressing cells.

Cellular senescence is a potent anticancer mechanism that arrests proliferation of cells at risk for neoplastic transformation. Fibroblasts in senescence develop a complex senescence-associated secretory phenotype in vitro and in vivo (42–45). An increase in IL-6 production is one of the principal indicators of senescence-associated secretory phenotype. However, p16INK4a senescence gene transfer did not affect IL-6 production from human fibroblasts (46). Another report demonstrated that IL-6 production was upregulated in oncogene-induced senescent fibroblasts even when p16INK4a induction was abolished with short hairpin RNA (47). These findings indicated that p16INK4a does not impact IL-6 production from senescent fibroblasts. In contrast, our present study has revealed that exogenous and endogenous p16INK4a expression suppresses IL-6 production in senescent macrophages, showing that p16INK4a has inhibitory effect in macrophages. Furthermore, induction of endogenous p16INK4a in RSF suppressed the arthritis model by systemic treatment of histone deacetylase inhibitors, suggesting that induction of endogenous p16INK4a in macrophages as well as RSF can be useful for therapy of RA (48).

Our previous studies demonstrated that p16INK4a could suppress proliferation and MCP-1 and MMP-3 production of RSF in a CDK4/6-dependent manner (8). Thus, p16INK4a should exert anti-inflammatory effects in multiple ways. Retroviral and adenoviral p16INK4a gene transfer have advantages and disadvantages in clinical settings. Pharmacological means to induce endogenous p16INK4a in synovial cells of the arthritis joints should be investigated in future studies.
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Disclosures

The authors have no financial conflicts of interest.

References


Figure S1. Effects of p16INK4a on the IRAK1 protein level in RSF.
RSF were infected with AxCA-LacZ (LacZ) and AxCA-p16INK4a (p16) adenoviruses. (A) ^3^H-thymidine uptake was assessed 3 days later. (B) They were stimulated with LPS (10 ng/ml) for 24 hours. IL-6 in the culture supernatant was measured with ELISA. (C) The expression of IRAK1 was determined with Western blot analyses. The density of the IRAK1 bands were normalized to that of actin and presented as fold change relative to non-treated control cells. Data are representative of two independent experiments and expressed as the mean ± SD of triplicate wells. * p<0.01