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# V. 論　文　別　刷

（研究分担者　名簿順）

# $p16^{INK4a}$ Exerts an Anti-Inflammatory Effect through Accelerated IRAK1 Degradation in Macrophages

**Yousuke Murakami,*,[1] 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-α 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-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 $p16^{INK4a}$-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 $p16^{INK4a}$ promotes ubiquitin-dependent IRAK1 degradation, impairs AP-1 activation, and suppresses IL-6 production. Thus, $p16^{INK4a}$ senescence gene upregulation inhibits inflammatory cytokine production in macrophages in a different way than in RSF.** ***The Journal of Immunology*, 2012, 189: 5066–5072.**

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 liberates active E2F transcription factors for cell-cycle progression. CDK inhibitors (CDKI) are intracellular proteins that inhibit kinase activity of cyclin/CDK complexes. CDKI $p16^{INK4a}$ inhibits CDK4/6 specifically, whereas CDK $p21^{CIP1}$ inhibits a broad spectrum of CDK (5). The intra-articular gene transfer of $p16^{INK4a}$ as well as $p21^{CIP1}$ 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 $p16^{INK4a}$ affects expression of inflammatory cytokines from activated macrophages. We found that $p16^{INK4a}$ suppresses IL-6 production in macrophages. The effect was mediated by accelerated degradation of IL-1R–associated kinase 1 (IRAK1).

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*Abbreviations used in this article:* BMM, bone marrow-derived macrophage; CDK, cyclin-dependent kinase; CDKI, cyclin-dependent kinase inhibitor; IKK, IκB kinase; IRAK1, IL-1R–associated kinase 1; miR146a, micro-RNA 146a; MKK4, MAPK kinase 4; MMP, matrix metalloproteinase; pRb, retinoblastoma gene product; RA, rheumatoid arthritis; RSF, rheumatoid synovial fibroblast; shCDK4, murine cyclin-dependent kinase 4-specific short hairpin RNA; shIRAK1, murine IL-1R–associated kinase 1-specific short hairpin RNA; shNC, short hairpin RNA vector for negative control; siRNA, small interfering RNA; sm, small molecule.

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## 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-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-$p16^{INK4a}$ 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 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 cells 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.

### *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 [$^3$H]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 $p16^{INK4a}$ and IRAK1 genes were cloned into the retroviral expression vectors, pMX-IP and pMX-IN, respectively (pMX-$p16^{INK4a}$ 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′-GATCCAGAGCCCATCCCTCCCCGTTTCAAGAGAACGG-GGAGGGATGGGCTCTTTTTTGGAAA-3′, and IRAK1 antisense, 5′-AGCTTTTCCAAAAAAGAGCCCATCCCTCCCCGTTCTCTTGAAAC-

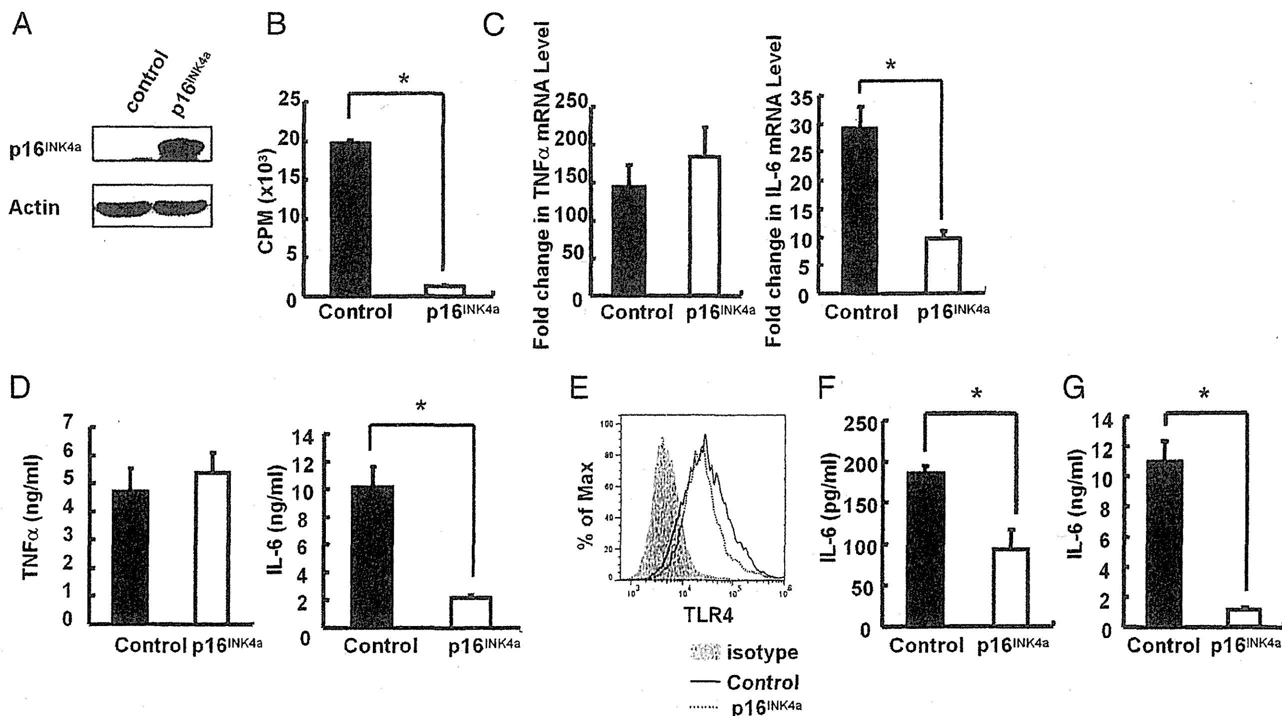


**FIGURE 1.** Effect of $p16^{INK4a}$ expression on IL-6 production in LPS-stimulated macrophages. BMM, THP-1, and RAW264.7 cells were infected with pMX-IP (control) and pMX-$p16^{INK4a}$ ($p16^{INK4a}$) retroviruses. (**A**) Cellular proteins were harvested. $p16^{INK4a}$ and actin expression were detected with Western blot analyses. (**B**) [$^3$H]thymidine was added to culture media of the BMM transductants. Incorporation of [$^3$H]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. $*p < 0.01$.

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GGGGAGGGATGGGCTCTG-3′; CDK4 sense, 5′-GATCCGGTTGAGGTCATTGAGGATCTTCCTGTCAATCCTTAATGGTCTCAACCGGTTTTTTGGAAA-3′, and CDK4 antisense 5′-AGCTTTTCCAAAAAACCGGTTGAGACCATTAAGGATTGACAGGAAGATCCTCAATGACCTCAACCG-3′) were constructed (murine CDK4 short hairpin [shCDK4] and murine IRAK1 short hairpin [shIRAK1]). Short hairpin RNA vector for negative control (shNC) was purchased from Applied Biosystems. Recombinant retroviruses were prepared as described previously (20). Cells were infected with the retroviruses in the presence of 6 μg/ml polybrene. One day after the infection, they were exposed to puromycine (6 μg/ml) or neomycine (500 μg/ml).

Replication-defective adenoviruses containing a human p16 gene (AxCA-p16) and LacZ gene (AxCA-LacZ) were prepared as described previously (8).

*Quantification of cytokine and IRAK1 expression*

Specific ELISA kits to quantify human and murine 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 p16$^{INK4a}$ gene-specific primer set was purchased from Qiagen (Tokyo, Japan).

*Small interfering RNA transfection*

To introduce small interfering RNA (siRNA) into BMM, $2 \times 10^5$ BMM was incubated with 200 μl 1.2 μM siRNA containing 24 μl FuGENE-HD transfection reagent (Roche) in Opti-MEM for 16 h. p16$^{INK4a}$-specific siRNA (si-p16A and B) and control siRNA were purchased from Qiagen.

*Statistic analyses*

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

## Results

### *p16$^{INK4a}$ suppressed IL-6 expression in LPS-stimulated macrophages*

To study the effect of p16$^{INK4a}$ on macrophages, BMM were infected with pMX-p16 or control pMX-IP retroviruses. Ectopic p16$^{INK4a}$ protein expression in the pMX-p16$^{INK4a}$-infected cells had been confirmed with Western blot analyses (Fig. 1A). [$^3$H]Thymidine uptake by the p16$^{INK4a}$-expressing BMM was almost completely suppressed compared with control virus-treated BMM (Fig. 1B). Quantitative PCR showed that p16$^{INK4a}$ 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 p16$^{INK4a}$ (Fig. 1D). LPS recognition receptor TLR4 expression was not modified by p16$^{INK4a}$ 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 p16$^{INK4a}$ gene transfer (Fig. 1F, 1G). Thus, p16$^{INK4a}$ 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 p16$^{INK4a}$ inhibited matrix metalloproteinase (MMP)-3 expression in RSF by suppressing CDK4/6 kinase activity (8). To determine if the inhibitory effect of p16$^{INK4a}$ 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 [$^3$H]thymidine uptake of BMM in a dose-dependent manner. Maximal inhibition was observed at 2 μM PD0332991 (Fig. 2A), which did not affect viability of BMM (data not shown). The CDK4/6 inhibitor did not modulate LPS-induced IL-6 expression and production by BMM, but it completely suppressed BMM proliferation

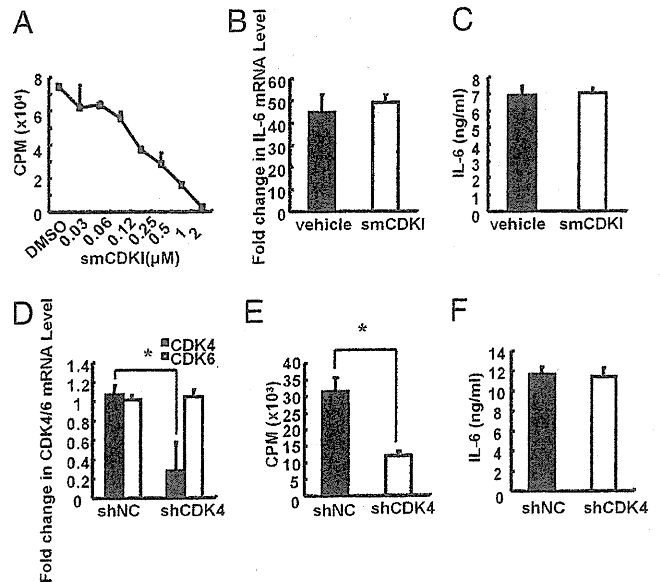


**FIGURE 2.** Effects of direct CDK4/6 inhibition on IL-6 production in LPS-stimulated BMM. (**A**) BMM were treated with or without indicated concentrations of smCDK4/6-selective inhibitor PD0332991 (smCDKI) for 24 h, and [$^3$H]thymidine uptake was assessed for the last 8 h. (**B**) BMM were treated 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 [$^3$H]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. $^*p < 0.01$.

(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 [$^3$H]thymidine incorporation by BMM was inhibited by shCDK4, BMM depended primarily on CDK4 for phosphorylation of pRb (Fig. 2E). Consistent with the effect of PD0332991, CDK4 knockdown by shCDK4 did not affect LPS-induced IL-6 production (Fig. 2F). Thus, inhibition of LPS-induced IL-6 production by p16$^{INK4a}$ did not depend on the CDK kinase activity.

### *p16$^{INK4a}$ expression in BMM promoted IRAK1 degradation*

TLR4 signaling is mediated by quite a few signaling molecules. To explore how p16$^{INK4a}$ inhibits IL-6 production in LPS-stimulated BMM, we studied the expression of signaling molecules with Western blot analyses and found that p16$^{INK4a}$ gene transfer affected IRAK1 protein expression (Fig. 3A). Upon ligand binding to TLR, MyD88 is recruited to the receptor and brings IRAK1 and IRAK4 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 p16$^{INK4a}$-expressing BMM was comparable to that in control cells at the unstimulated status. Upon LPS stimulation, the IRAK1 expression was significantly downmodulated in p16$^{INK4a}$-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-

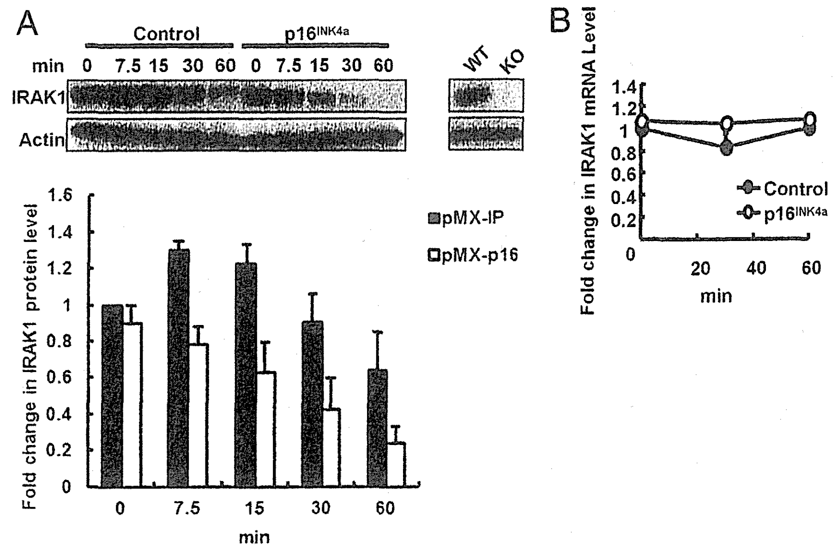


**FIGURE 3.** Promotion of IRAK1 degradation by exogenous p16$^{INK4a}$. BMM transduced with pMX-IP (control) and pMX-p16$^{INK4a}$ (p16$^{INK4a}$) were stimulated with LPS (10 ng/ml). (**A**) Cellular proteins were harvested at the indicated time points. IRAK1 and actin expression was detected with 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 three independent experiments. (**B**) IRAK1 mRNA in the BMM was quantified with real-time PCR. The amount of the IRAK1 mRNA were normalized to that of GAPDH mRNA and presented as fold change relative to nontreated control cells. Data are representative of three experiments and expressed as the mean ± SD of triplicate wells.

onstrating that binding of anti-IRAK1 Abs was specific (Fig. 3A). p16$^{INK4a}$ 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, p16$^{INK4a}$ or control LacZ were transduced using an adenoviral vector to RSF and IL-6 production was measured after LPS stimulation. Although [$^3$H]thymidine uptake was inhibited in the p16$^{INK4a}$-infected RSF, IL-6 production was not altered (Supplemental Fig. 1A, 1B). In accordance with this, IRAK1 expression in RSF in p16$^{INK4a}$-expressing and control RSF was comparable throughout the LPS stimulation (Supplemental Fig. 1C).

### *p16$^{INK4a}$ 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 p16$^{INK4a}$ 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 p16$^{INK4a}$ (Fig. 4A). Downstream AP-1 binding activity was also reduced in p16$^{INK4a}$-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 p16$^{INK4a}$ overexpression (Fig. 4A, 4B).

We assumed that the impairment of the AP-1 pathway in p16$^{INK4a}$-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-

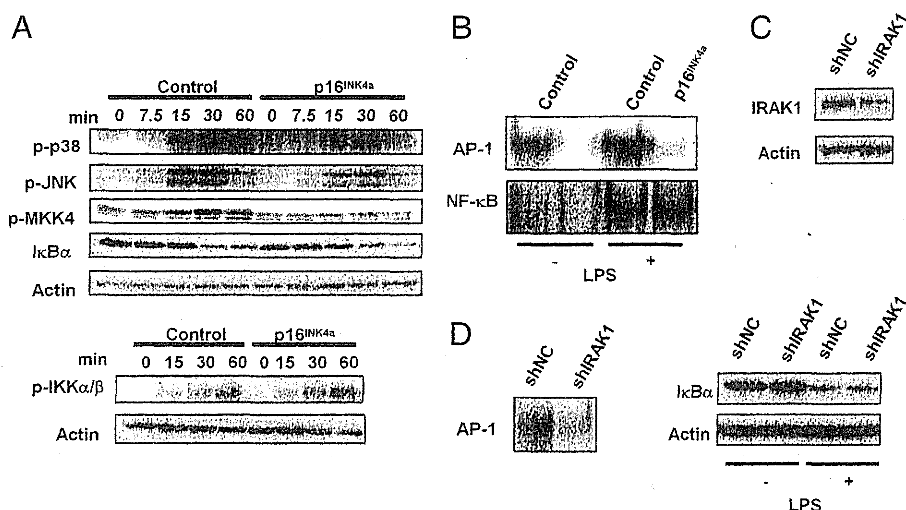


**FIGURE 4.** Suppression of phosphorylation of p38 MAPK and JNK without affecting the IKKα/β–IκB pathway by p16$^{INK4a}$. (**A**) Transduction of BMM with pMX-IP (control) and pMX-p16$^{INK4a}$ (p16$^{INK4a}$) retroviruses was followed by LPS (10 ng/ml) stimulation. Total cell lysates were collected at the indicated time points and examined for p-p38 MAPK, p-JNK, p-MKK4, p-IKKα/β, IκBα, and actin expression with Western blot analyses. (**B**) Nuclear extracts of the control and p16$^{INK4a}$-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.



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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$^{INK4a}$-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$^{INK4a}$-expressing BMM culture. This treatment prevented acceleration of IRAK1 degradation in p16$^{INK4a}$-transduced cells (Fig. 5A). It also restored p38 MAPK and JNK phosphorylation in p16$^{INK4a}$-expressing BMM, indicating that p16$^{INK4a}$-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$^{INK4a}$ 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$^{INK4a}$ 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$^{INK4a}$ on IL-6 production*

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

## Discussion

p16$^{INK4a}$ 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$^{INK4a}$ 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$^{INK4a}$ 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$^{INK4a}$-expressing cells and control cells. These findings also indicated that neither

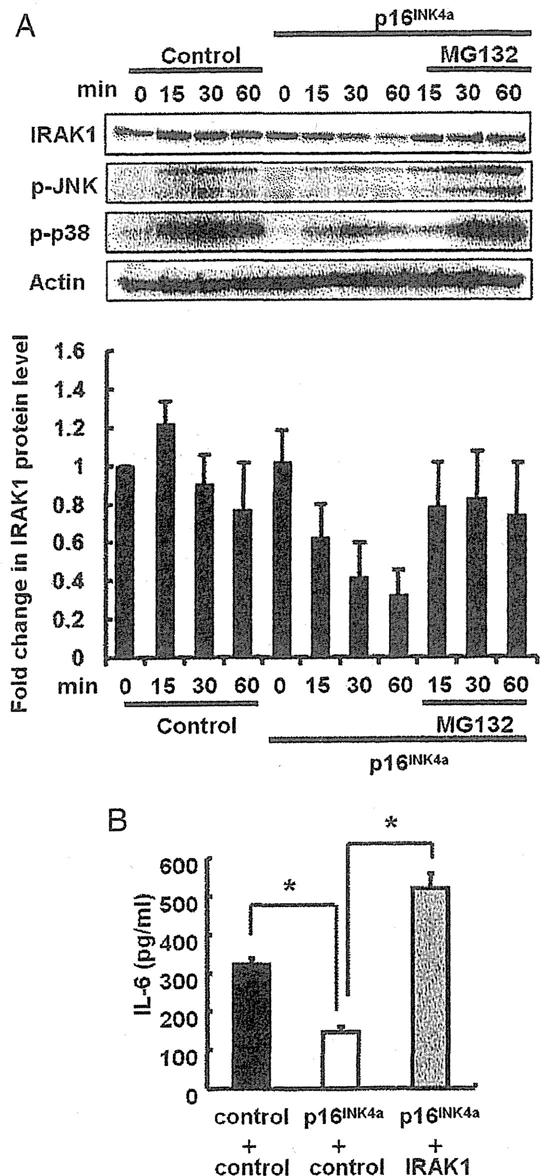


**FIGURE 5.** Proteome dependency of p16$^{INK4a}$-induced suppression of IL-6 production. (**A**) BMM transduced with pMX-IP (control) and pMX-p16$^{INK4a}$ (p16$^{INK4a}$) retroviruses were pretreated with the proteasome inhibitor MG132 (20 μM) for 1 h then stimulated with LPS (10 ng/ml) for the indicated times. p-p38 MAPK, p-JNK, IRAK1, and actin in the total cell lysates were 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 three independent experiments. (**B**) THP-1 cells were double-transduced with empty vectors (pMX-IP and pMX-IN controls; black bar), pMX-p16$^{INK4a}$ with pMX-IN empty vector control (white bar), or pMX-p16$^{INK4a}$ and pMX-IRAK1 (gray bar). THP-1 cells were treated with PMA for 2 d to induce macrophage differentiation and then stimulated with LPS (1 μg/ml) for 24 h. IL-6 in the culture supernatants was measured with ELISA. Data are representative of three experiments and expressed as the mean ± SD of triplicate wells. *$p < 0.01$.

downstream nor upstream molecule of IRAK1 was affected by p16$^{INK4a}$.

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-

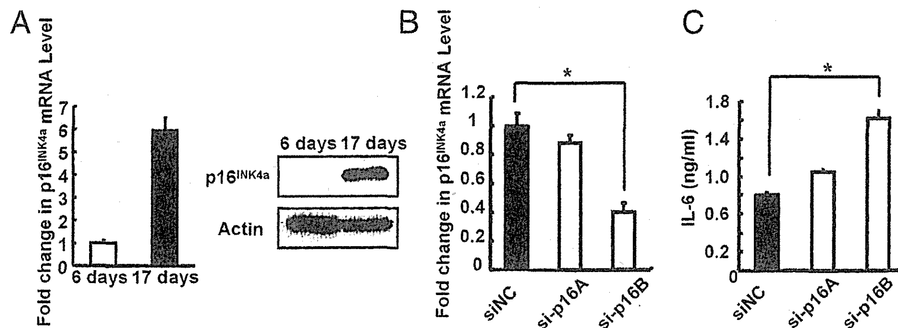


**FIGURE 6.** Inhibitory effect of endogenous p16$^{INK4a}$ on IL-6 production. (**A**) BMM cultured for 6 d were still in a logarithmic growth phase (6 d), whereas those cultured for 17 d reached replicative senescence (17 d). Endogenous p16$^{INK4a}$ and actin were detected with Western blotting analyses. p16$^{INK4a}$ mRNA expression in these cells was quantified with real-time PCR. The amounts of the p16$^{INK4a}$ 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 p16$^{INK4a}$ (si-p16A and B) and siNC. p16$^{INK4a}$ mRNA expression in the treated BMM was measured with real-time PCR. The amounts of the p16$^{INK4a}$ 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. *$p < 0.01$.

dation of IRAK1 itself, which induces tolerance against restimulation of TLR. Tolerant cells do not express IRAK1 and thus fail to activate both the NF-B and AP-1 signaling pathways in response to repeated TLR ligation (30, 31). In contrast with IRAK1-deficient cells and LPS-induced tolerant cells, p16$^{INK4a}$-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 p16$^{INK4a}$-expressing macrophages.

p16$^{INK4a}$ can make complexes not only with CDK4/6 but also with other signaling molecules. In mouse embryonic fibroblasts, p16$^{INK4a}$ 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 p16$^{INK4a}$ expression in macrophages, its expression in mouse embryonic fibroblasts did not disturb JNK phosphorylation. Another report demonstrated that p16$^{INK4a}$ in HeLa cells was associated with RelA, which is a component of NK-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 p16$^{INK4a}$ appeared to depend on the cell types.

The promoted IRAK1 degradation by p16$^{INK4a}$ 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 proteosome 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 p16$^{INK4a}$ 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, p16$^{INK4a}$ 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 p16$^{INK4a}$-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 p16$^{INK4a}$-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, p16$^{INK4a}$ 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 p16$^{INK4a}$ induction was abolished with short hairpin RNA (47). These findings indicated that p16$^{INK4a}$ does not impact IL-6 production from senescent fibroblasts. In contrast, our present study has revealed that exogenous and endogenous p16$^{INK4a}$ expression suppresses IL-6 production in senescent macrophages, showing that p16$^{INK4a}$ has inhibitory effect in macrophages. Furthermore, induction of endogenous p16$^{INK4a}$ in RSF suppressed the arthritis model by systemic treatment of histone deacetylase inhibitor, suggesting that induction of endogenous p16$^{INK4a}$ in macrophages as well as RSF can be useful for therapy of RA (48).

Our previous studies demonstrated that p16$^{INK4a}$ could suppress proliferation and MCP-1 and MMP-3 production of RSF in a CDK4/6-dependent manner (8). Thus, p16$^{INK4a}$ should exert anti-arthritic effects in multiple ways. Retroviral and adenoviral p16$^{INK4a}$ gene transfer have advantages and disadvantages in clinical settings. Pharmacological means to induce endogenous p16$^{INK4a}$ 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.

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ORIGINAL ARTICLE

# Time-Dependent Increased Risk for Serious Infection From Continuous Use of Tumor Necrosis Factor Antagonists Over Three Years in Patients With Rheumatoid Arthritis

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***Objective.*** **To investigate associations between continuous treatments with tumor necrosis factor (TNF) antagonists and risk for developing serious infections (SIs) over 3 years in Japanese patients with rheumatoid arthritis (RA) enrolled in the Registry of Japanese RA Patients for Long-Term Safety (REAL) database.**
***Methods.*** **We analyzed 727 RA patients who had started either infliximab or etanercept (the anti-TNF group; 1,480.1 patient-years [PY]) and 571 RA patients who had started conventional nonbiologic disease-modifying antirheumatic drugs (the unexposed group; 1,104.1 PY) at the time of enrollment in the REAL. We assessed the occurrence of SIs within a 3-year observation period, including the period after switching to other TNF antagonists, and all SIs, unlimited to the first one in each patient as reported in other studies, to evaluate the real safety of TNF antagonists in daily practice.**
***Results.*** **The incidence rate of SIs per 100 PY was 5.54 (95% confidence interval [95% CI] 4.44–6.84) in the anti-TNF group and 2.72 (95% CI 1.87–3.83) in the unexposed group. Poisson regression analysis revealed that the relative risk (RR) of continuous use of TNF antagonists for SIs after adjusting for baseline and time-dependent covariates was significantly elevated both overall (1.97, 95% CI 1.25–3.19) and for the first year (2.40, 95% CI 1.20–5.03), but not for the second and third years combined (1.38, 95% CI 0.80–2.43). The adjusted RR for SIs of etanercept compared to infliximab was not significantly elevated.**
***Conclusion.*** **Continuous anti-TNF therapy was significantly associated with increased risks for developing SIs during, but not after, the first year.**

## INTRODUCTION

Biologic disease-modifying antirheumatic drugs (DMARDs) have been widely used to treat patients with rheumatoid arthritis (RA) whose response to conventional DMARD therapy was inadequate (1–4). In Japan, 6 biologic DMARDs (infliximab, etanercept, adalimumab, tocilizumab, abatacept, and golimumab) have been approved and widely used in clinical practice. The criterion for indication for

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## Significance & Innovations

- Using a Japanese rheumatoid arthritis (RA) patient registry, we show for the first time in Asia that the continuous use of tumor necrosis factor (TNF) antagonists over a 3-year observation period was associated with a 2-fold increased risk for serious infections (SIs) compared to nonbiologic disease-modifying antirheumatic drugs (DMARDs). This elevation, however, was time dependent and significant only for the first year, not for the second and third years combined.
- To redeem methodologic shortcomings in previous reports, we examined all SIs occurring during treatment with TNF antagonists, including those after switching to other TNF antagonists. We used not only baseline but also time-dependent variables as candidates for risk factors for SIs in multivariate analysis because disease activity of RA and the dose of drugs such as corticosteroids and methotrexate are subject to change during treatment.
- Over 3 years, the incidence rate of SIs in the etanercept group was numerically higher than that of the infliximab group, but the risk for SIs from treatment with etanercept was not significantly different from that of infliximab after adjusting for covariates.

infliximab or the other 5 biologic DMARDs, according to Japanese labeling, consists of inadequate response to methotrexate (MTX) or nonbiologic DMARDs, respectively. In addition, Japanese rheumatologists follow the guidelines proposed by the Japan College of Rheumatology (5,6).

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Although biologic DMARDs have superior clinical efficacy for patients with RA, there are concerns about increased risk for infection (7). Prevention of infections in RA patients who are treated with immunosuppressive drugs is relevant because the incidence rate (IR) of infections is already higher in patients with RA than in the general population, and infection is a major factor hampering proper management of the disease and influencing prognosis (8–10). Infection was the most frequent serious adverse event (SAE) reported in postmarketing surveillance programs for infliximab and etanercept in Japan: the most prevalent infectious disease was pneumonia, and higher IRs of tuberculosis and *Pneumocystis jiroveci* pneumonia (PCP) were reported compared to Western countries (11–13). We established the Registry of Japanese RA Patients for Long-Term Safety (REAL) in 2005 and, utilizing this database, recently reported that treatment with either tumor necrosis factor (TNF) antagonist infliximab or etanercept for up to 1 year was associated with increased risk for serious infections (SIs) compared to treatment with nonbiologic DMARDs (14). Recent data from prospective observational studies in Europe and the US also suggest that the risk for infection was higher in RA patients treated with biologic DMARDs, at least in the short term (15–18), and disappeared with increasing treatment duration (15,16,18–20).

In clinical practice, rheumatologists often switch from the initial TNF antagonist to an alternative TNF antagonist when the patient shows insufficient efficacy or develops an adverse event. Some patients also experience more than one adverse event during treatment with TNF antagonists. In previous reports from prospective cohort studies, observation was stopped after switching to another TNF antagonist or after the first adverse event (18,21–23); therefore, second or third adverse events and those occurring after switching TNF antagonists were not analyzed (18,21–23). In addition, the time dependency of covariates such as corticosteroid dose and disease activity was not included in some studies (14,15,19–24). To understand the real safety of TNF antagonists for patients with RA, it is essential to design an epidemiologic study that evaluates all adverse events during continuous treatment with these agents. However, in Japan, as well as in Asia overall, there are no safety data from prospective cohort studies with an observation period longer than 1 year in RA patients receiving TNF antagonists. Because differences in genetic, environmental, and medical factors in each geographic region may influence the safety of biologic DMARDs (25), it is prudent to compare the safety of biologic DMARDs from various countries or regions. The primary purpose of this study was to assess the risk for SIs associated with continuous use of infliximab or etanercept for 3 years, including the period after switching to other TNF antagonists, and its trend over time, and to identify independent risk factors after adjusting for time-dependent covariates. In a secondary analysis, we focused on the first TNF antagonist used in each patient to investigate differences in the risk for SIs among the agents.

## PATIENTS AND METHODS

**Database.** The REAL is an ongoing prospective cohort established to investigate the long-term safety of biologic DMARDs in patients with RA. Details of the REAL have been previously described (14,26). In brief, 27 institutions participated in the REAL, including 16 university hospitals and 11 referring hospitals. The criteria for enrollment in the REAL include those patients meeting the 1987 American College of Rheumatology criteria for RA (27) with written informed consent and starting or switching treatment with biologic DMARDs (the biologics exposed group) or starting, adding, or switching nonbiologic DMARDs (the biologics unexposed group) at the time of study entry. Until the end of 2007, patients already receiving treatment with nonbiologic DMARDs at the time of study entry were also enrolled in the unexposed group. To facilitate enrollment in the REAL, participating physicians were asked to enroll their patients already registered to postmarketing surveillance programs previously implemented by pharmaceutical companies for biologic DMARDs (11,12). In addition, our investigators were also encouraged to enroll as many patients as possible who fulfilled the inclusion criteria (14). For this study, data were retrieved from the REAL database on November 30, 2009. This study was in compliance with the Declaration of Helsinki (revised in 2008). The REAL study was approved by the ethics committees of the Tokyo Medical and Dental University Hospital and the other participating institutions (see Appendix A for members of the REAL Study Group and their affiliates).

**Data collection.** Each patient's recorded baseline data included demography, disease activity, comorbidities, treatments, and laboratory data at the start of the observation period. A followup form was submitted every 6 months by the participating physicians to the REAL Data Center at the Department of Pharmacovigilance of Tokyo Medical and Dental University to report the occurrence of SAEs, current RA disease activity, treatments, and clinical laboratory data. We collected the Steinbrocker class (28) as the measurement for patient physical disability instead of the Health Assessment Questionnaire disability index at baseline (29). Using this protocol, SAEs were reported at regular followup times every 6 months. The REAL Data Center checked all of the data sent by attending physicians to improve the quality of data, and the participating physicians in each hospital confirmed them on the web site of the REAL.

**Anti-TNF group.** In the biologics exposed group, there were 727 patients with RA who started infliximab or etanercept at enrollment in the REAL (anti-TNF group; 1,480.1 patient-years [PY]); 335 started infliximab (infliximab group) and 392 started etanercept (etanercept group). In the infliximab group, 67 patients were switched to either etanercept (58 patients), tocilizumab (8 patients), or adalimumab (1 patient), and 74 patients stopped treatment with infliximab during the study observation period. The remaining patients continued treatment with infliximab

throughout. In the etanercept group, 60 patients were switched to either infliximab (27 patients), tocilizumab (25 patients), or adalimumab (8 patients), and 62 patients stopped administration of etanercept during the study observation period. The remaining patients continued etanercept treatment throughout. The overall survival rates of the first biologic agent at year 3 were 0.48 (95% confidence interval [95% CI] 0.41–0.55) for infliximab and 0.61 (95% CI 0.55–0.66) for etanercept. Our analysis was restricted to infliximab or etanercept because few patients receiving adalimumab or tocilizumab were registered in the REAL database and golimumab and certolizumab pegol were not approved in Japan at the time this study was conducted.

**Unexposed group.** Among 574 RA patients in the biologics unexposed group, 3 patients had received biologic DMARDs within 90 days before their enrollment in the REAL. These 3 patients were excluded from our analysis in consideration of the pharmacokinetic and pharmacodynamic property of biologic DMARDs and their possible effects on development of infection. Fifteen patients who had received biologic DMARDs and stopped them over 90 days before their enrollment in the REAL were included in this analysis. Therefore, 571 RA patients who initiated or were receiving nonbiologic DMARDs and not receiving biologic DMARDs at enrollment in the REAL were included in the unexposed group (1,104.1 PY). At enrollment, 347 patients (60.8%) of the patients in the unexposed group were being treated with MTX, 127 patients (22.4%) with sulfasalazine, 103 patients (18.0%) with tacrolimus, 95 patients (16.6%) with bucillamine, and 29 patients (5%) with other nonbiologic DMARDs.

**Followup.** For those patients who initiated nonbiologic DMARDs or biologic DMARDs at entry, the start of the observation period was the date these agents were first administered. For those patients enrolled in the unexposed group already receiving treatment with nonbiologic DMARDS at the time of study entry, the observation period started from the date of their enrollment in the REAL database.

Observation was stopped either 3 years after the start of the observation period, the day a patient died or met the exclusion criteria (14), or on November 30, 2009, whichever came first. For the unexposed group, stopping all nonbiologic DMARDs or starting any biologic DMARDs stopped followup. For the anti-TNF group, stopping therapy with either infliximab or etanercept ended observation. Patients were followed even after development of SAEs, as long as they did not meet the above criteria for stopping observation. The date of the last administration of infliximab or etanercept was retrieved from medical records and reported by the participating physicians. The mean ± SE followup was 2.04 ± 0.92 years for the anti-TNF group and 1.93 ± 0.99 years for the unexposed group. Figure 1 shows the number of patients for each year and the number who dropped out from each group during observation. Four hundred forty-two patients (34%) of all patients (n = 1,298) were followed up for 3 years.

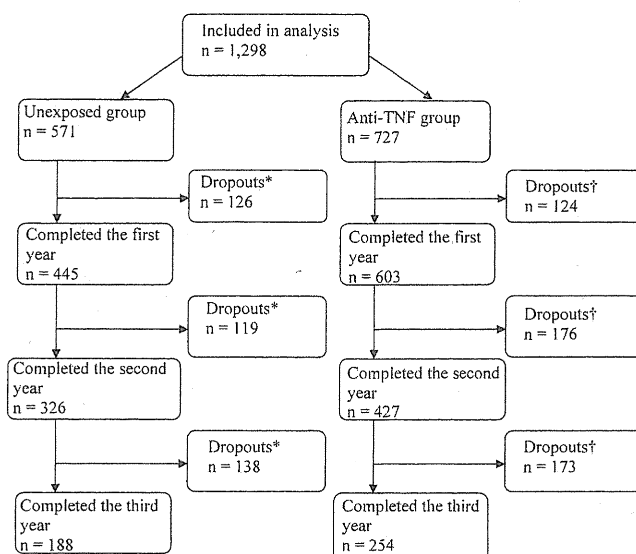


**Figure 1.** Distribution of numbers of patients with rheumatoid arthritis during the 3-year observation period. * = dropouts from the unexposed group include patients who started biologic disease-modifying antirheumatic drugs (DMARDs) or patients whose observation did not complete the next 1 year; † = dropouts from the anti–tumor necrosis factor (anti-TNF) group include patients who stopped infliximab or etanercept or switched to biologic DMARDs, except infliximab and etanercept, or patients whose observation did not complete the next 1 year.

**Definition of SAEs.** Our definition of an SAE, including an SI, was based on the report by the International Conference on Harmonisation (30). In addition, bacterial infections that required intravenous administration of antibiotics, as well as opportunistic infections, were also regarded as SAEs (14) (see Supplementary Table 1, available in the online version of this article at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)2151-4658).

**Statistical analysis.** Crude IRs per 100 PY and crude IR ratios (IRRs) with their 95% CIs were calculated. We conducted 2 analyses in this study. In the primary analysis (analysis 1), risk factors for SIs during continuous treatment with infliximab or etanercept for up to 3 years were identified. We also calculated the risk of TNF antagonists for SIs in the first year and in the second and third years combined to investigate time dependence of the risk. In the secondary analysis (analysis 2), the risks for SIs were compared between treatment with infliximab and etanercept.

*Analysis 1.* We included both patient groups and the entire observation period for each patient as described above for analysis 1 and added risk windows as follows. When a patient no longer received either infliximab or etanercept, the patient was excluded from the study on the day of the last administration of the agents and a 90-day postdiscontinuation risk window was applied (14). Any SAEs occurring within the risk window were attributed to the effects of the TNF antagonists. No risk window was needed for the unexposed group. For multivariate analysis, Poisson regression models were employed to estimate

**Table 1. Comparison of RA patients treated with or without TNF antagonists infliximab or etanercept at the start of the observation period***

| | Unexposed group (n = 571) | All anti-TNF groups (n = 727) | Infliximab group (n = 335) | Etanercept group (n = 392) | *P*† |
|---|---|---|---|---|---|
| Age, years | 59.3 ± 13.1 | 56.3 ± 13.4‡ | 53.7 ± 13.9 | 58.5 ± 12.7 | < 0.001 |
| Women, % | 83.2 | 82.0 | 79.3 | 85.1 | 0.045 |
| Disease duration, years | 8.9 ± 9.3 | 9.5 ± 8.6‡ | 8.1 ± 8.0 | 10.6 ± 9.0 | < 0.001 |
| Steinbrocker class 3 or 4, %§ | 10.7 | 30.7‡ | 28.4 | 32.7 | 0.211 |
| Steinbrocker stage III or IV, %§ | 39.6 | 53.0‡ | 45.1 | 59.7 | < 0.001 |
| DAS28-CRP | 3.4 ± 1.2 | 4.5 ± 1.2‡ | 4.6 ± 1.1 | 4.5 ± 1.3 | 0.197 |
| N | 567 | 723 | 335 | 388 | |
| MTX use, % | 60.8 | 68.8‡ | 99.1 | 42.9 | < 0.001 |
| MTX dosage, mg/week | 6.4 ± 2.0 | 7.6 ± 2.2‡ | 7.9 ± 2.2 | 7.0 ± 2.1 | < 0.001 |
| MTX dosage >8 mg/week, % | 4.4 | 10.6‡ | 18.2 | 4.1 | < 0.001 |
| Use of immunosuppressive drugs except for MTX, %¶ | 20.1 | 4.3‡ | 1.2 | 6.9 | < 0.001 |
| Oral corticosteroid use, % | 58.3 | 71.5‡ | 69.0 | 73.7 | 0.16 |
| Prednisolone or equivalent dosage of corticosteroids, mg/day | 4.6 ± 2.1 | 5.7 ± 3.0‡ | 5.3 ± 2.7 | 6.0 ± 3.2 | 0.006 |
| Prednisolone or equivalent dosage of corticosteroids ≥10 mg/day, % | 1.9 | 9.1‡ | 5.7 | 12.0 | 0.003 |
| No. of previous DMARDs | 2.2 ± 1.2 | 2.5 ± 1.2‡ | 2.3 ± 1.1 | 2.7 ± 1.2 | < 0.001 |
| Chronic pulmonary disease, %# | 18.7 | 21.6 | 11.9 | 29.8 | < 0.001 |
| Diabetes mellitus, % | 5.8 | 12.0‡ | 8.7 | 14.8 | 0.011 |

* Values are the mean ± SD unless otherwise indicated. For univariate analysis, the chi-square test for categorical variables and the Student's *t*-test or Mann-Whitney test were used to compare continuous variables among groups. RA = rheumatoid arthritis; TNF = tumor necrosis factor; DAS28-CRP = 3-variable Disease Activity Score including 28-joint counts using the C-reactive protein level; MTX = methotrexate; DMARDs = disease-modifying antirheumatic drugs.
† Between the 2 anti-TNF antagonists.
‡ $P < 0.05$ versus the unexposed group.
§ Steinbrocker classification (28) was used to define RA disease stages and classes.
¶ Immunosuppressive drugs include tacrolimus, leflunomide, mizoribine, and cyclosporine.
# Chronic pulmonary diseases include interstitial pneumonia, chronic obstructive pulmonary disease, bronchial asthma, prior pulmonary tuberculosis, and bronchiectasis.

the risk for SIs with TNF antagonist treatment. To analyze the time-dependent risk for SIs, observation periods were divided into the first year and the second and third years combined.

*Analysis 2.* To compare the risk for SIs between the use of infliximab and etanercept in the anti-TNF group, the treatment period with the first TNF inhibitor for each patient was evaluated without setting a risk window because most of the patients who had stopped the first biologic agent started treatment with the second one immediately. We applied propensity score (PS) methodology to calculate the likelihood of being treated with TNF antagonists. First, we made a multivariate logistic regression model with the use of TNF antagonists as the dependent variable and the following as independent variables: age, sex, the 3-variable Disease Activity Score including 28-joint counts using the C-reactive protein level (DAS28-CRP), the presence of chronic pulmonary comorbidity, diabetes mellitus, calendar year of entry in the REAL, Steinbrocker stage (III or IV), MTX (≤8 or >8 mg/week), and oral corticosteroid (prednisolone or equivalent dosage <10 or ≥10 mg/day) at enrollment. We applied the Hosmer-Lemeshow goodness-of-fit test to assess how effectively the model described the outcome variable (i.e., the use of TNF antagonist: yes/no). We used the PS to select representative patients receiving TNF antagonist treatment: the patients with a PS >0.4 were included in analysis 2 and different cutoff values for PS were used for sensitivity analyses (31). To compare the risk for SIs between etanercept and infliximab, we employed Poisson regression models in the anti-TNF group patients with various combinations of adjusting factors, including the PS, to calculate the relative risks (RRs) of etanercept with 95% CIs, using infliximab as the reference.

These statistical analyses were conducted using SPSS, version 16.0, and R statistical language software, version 2.8.1. All *P* values were 2-tailed and *P* values less than 0.05 were considered statistically significant.

## RESULTS

**Baseline characteristics of patients.** This study included a total of 1,298 patients: 727 in the anti-TNF group and 571 in the unexposed group. Baseline data for the patients are shown in Table 1. Compared to the unexposed group, the anti-TNF group was younger ($P < 0.001$), had more severe disease activity ($P < 0.001$), and was treated with higher doses of MTX ($P < 0.001$) and oral corticosteroids ($P < 0.001$). Significantly more patients with diabe-

Table 2. Number and IRs of SAEs in RA patients treated with and without the TNF antagonists infliximab or etanercept*

| | Unexposed group (n = 571) | Anti-TNF group | | | Etanercept vs. infliximab, crude IRR (95% CI) | Anti-TNF vs. unexposed group, crude IRR (95% CI) |
|---|---|---|---|---|---|---|
| | | All (n = 727)† | Infliximab (n = 335)‡ | Etanercept (n = 392)§ | | |
| Patient-years (PY) | 1,104.1 | 1,480.1 | 583.31 | 787.94 | | |
| All SAEs | | | | | 1.49 (1.10–2.03) | 1.67 (1.31–2.13) |
| No. of events | 95 | 213 | 61 | 123 | | |
| IR/100 PY (95% CI) | 8.60 (7.00–10.47) | 14.39 (12.55–16.42) | 10.46 (8.07–13.34) | 15.61 (13.03–18.56) | | |
| Serious infection | | | | | 1.16 (0.72–1.87) | 2.04 (1.34–3.10) |
| No. of events | 30 | 82 | 28 | 44 | | |
| IR/100 PY (95% CI) | 2.72 (1.87–3.83) | 5.54 (4.44–6.84) | 4.80 (3.26–6.84) | 5.58 (4.11–7.42) | | |
| Serious respiratory tract infection | | | | | 1.20 (0.65–2.24) | 1.96 (1.10–3.48) |
| No. of events | 17 | 42 | 16 | 26 | | |
| IR/100 PY (95% CI) | 1.45 (0.86–2.30) | 2.84 (2.07–3.80) | 2.74 (1.63–4.35) | 3.30 (2.21–4.76) | | |
| Serious infection leading to death | | | | | NA | 0.75 (0.15–3.69) |
| No. of events | 3 | 3 | 0 | 3 | | |
| IR/100 PY (95% CI) | 0.27 (0.08–0.72) | 0.20 (0.06–0.54) | 0 | 0.38 (0.11–1.02) | | |

* Note that the number of severe adverse events (SAEs) in the All column is not the sum of the Infliximab and Etanercept columns. IRs = incidence rates; TNF = tumor necrosis factor; IRR = IR ratio; 95% CI = 95% confidence interval; NA = not applicable.
† The continuous treatment period with infliximab or etanercept for each patient was evaluated.
‡ Patients with rheumatoid arthritis (RA) given infliximab as the first TNF inhibitor in the Registry of Japanese RA Patients for Long-Term Safety (REAL) were included. The treatment period with infliximab for each patient was evaluated.
§ Patients with RA given etanercept as the first TNF inhibitor in the REAL were included. The treatment period with etanercept for each patient was evaluated.

tes mellitus ($P < 0.001$) were seen in the anti-TNF group compared to the unexposed group. In the anti-TNF group, the etanercept group compared to the infliximab group was older ($P < 0.001$), had a longer disease duration ($P < 0.001$), used MTX less frequently ($P < 0.001$), was treated with higher doses of oral corticosteroids ($P = 0.006$), and had higher percentages of chronic pulmonary comorbidity ($P < 0.001$) (see Table 1 for definition) and diabetes mellitus ($P = 0.011$) (Table 1).

**Types and occurrence of SAEs.** Among the 1,298 patients, 308 SAEs were reported during the observation period, 95 in the unexposed group and 213 in the anti-TNF group. The crude IRR comparing the anti-TNF group with the unexposed group for SAEs was 1.67 (95% CI 1.31–2.13) and for SIs was 2.04 (95% CI 1.34–3.10); both of these IRRs were significantly elevated. The IRs of SAEs, SIs, and serious respiratory tract infections in the infliximab group and the etanercept group are shown in Table 2. The crude IRR comparing the infliximab group with the etanercept group for SAEs was 1.49 (95% CI 1.10–2.03) and for SIs was 1.16 (95% CI 0.72–1.87). The IRs of SAEs, SIs, serious respiratory tract infections, and SIs leading to death are summarized in Table 2.

In the anti-TNF group, there were 82 SIs, including 21 opportunistic (14 cases of herpes zoster, 4 PCP, 3 pulmonary cryptococcosis, and 1 pulmonary nontuberculous mycobacterial infection) and 61 other infections. In the unexposed group, 30 SIs occurred, including 12 opportunistic (4 cases of herpes zoster, 3 PCP, 2 pulmonary tuberculosis, and 3 pulmonary nontuberculous mycobacterial infections) and 18 other infections. The names of the SIs in each site of infection are listed in Table 3. The respiratory system was the most frequent site of infection (n = 59), followed by skin and subcutaneous tissue (n = 24), gastrointestinal (n = 6), urinary tract (n = 5), and bone and joints (n = 5). Four of the latter 5 patients had histories of joint surgery. Three patients in each group died from SIs.

**Continuous treatment with TNF antagonists and other risk factors contributing to the development of SIs (analysis 1).** We initially performed univariate analyses to compare patients who did and did not develop SIs (data not shown) and selected the following variables for multivariate analysis: age, sex, chronic pulmonary comorbidity, diabetes mellitus, disease duration, calendar year, the number of previous DMARDs, Steinbrocker class, the use of immunosuppressive drugs, mean DAS28-CRP, and the mean dose of MTX and oral corticosteroids during the observation period. We used Poisson regression models and identified continuous use of TNF inhibitors as an independent risk factor for the development of SIs (RR 1.97, 95% CI 1.25–3.19; $P = 0.0045$) (Table 4). Among the confounding factors, we found that increasing age (RR 1.45 per 10-year increment, 95% CI 1.20–1.77; $P < 0.001$), chronic pulmonary comorbidity (RR 1.77, 95% CI 1.15–2.70; $P = 0.009$), mean DAS28-CRP score (RR 1.33, 95% CI 1.05–1.66; $P = 0.015$), mean dosage of MTX >8 mg/week (RR 2.14, 95% CI 1.15–3.87; $P = 0.013$), and mean dosage of oral prednisolone $\geq$10 mg/day (RR 2.49, 95% CI 1.08–5.50; $P = 0.027$) were significantly associated with SIs. The

**Table 3. Classification of serious infections***

| Site and name of infection | No. of infections: Anti-TNF group | No. of infections: Unexposed group | No. of deaths: Anti-TNF group | No. of deaths: Unexposed group |
|---|---|---|---|---|
| Pulmonary | | | | |
| Bacterial pneumonia | 27 | 9 | 1 | 2 |
| Fungal pneumonia† | 7 | 3 | 0 | 1 |
| Bronchitis | 4 | 0 | 0 | 0 |
| Nontuberculous mycobacterial infection | 1 | 3 | 0 | 0 |
| Empyema | 1 | 0 | 0 | 0 |
| Tuberculosis | 0 | 2 | 0 | 0 |
| Aspiration pneumonia | 1 | 0 | 1 | 0 |
| Infectious pneumatocele | 1 | 0 | 0 | 0 |
| Total | 42 | 17 | 2 | 3 |
| Skin | | | | |
| Herpes zoster | 14 | 4 | 0 | 0 |
| Cellulitis | 4 | 2 | 0 | 0 |
| Total | 18 | 6 | 0 | 0 |
| Gastrointestinal | | | | |
| Infectious gastroenteritis | 3 | 0 | 0 | 0 |
| Acute suppurative cholangitis | 1 | 0 | 0 | 0 |
| Appendicitis | 1 | 0 | 0 | 0 |
| Infection due to drain replacement‡ | 0 | 1 | 0 | 0 |
| Total | 5 | 1 | 0 | 0 |
| Urinary | | | | |
| Pyelonephritis | 3 | 1 | 0 | 0 |
| Urinary tract infection | 1 | 0 | 0 | 0 |
| Total | 4 | 1 | 0 | 0 |
| Bone and joints | | | | |
| Infectious arthritis | 3 | 1 | 0 | 0 |
| Osteomyelitis | 0 | 1 | 0 | 0 |
| Total | 3 | 2 | 0 | 0 |
| Others | | | | |
| Sepsis | 4 | 1 | 0 | 0 |
| Surgical wound infection | 0 | 2 | 0 | 0 |
| Bacteremia | 1 | 0 | 0 | 0 |
| Bacterial meningitis | 1 | 0 | 1 | 0 |
| Sinusitis | 1 | 0 | 0 | 0 |
| Viral meningitis | 1 | 0 | 0 | 0 |
| Unidentified | 2 | 0 | 0 | 0 |
| Total | 10 | 3 | 1 | 0 |

* Anti-TNF = anti–tumor necrosis factor.
† Fungal pneumonia included *Pneumocystis jiroveci* pneumonia and cryptococcal pneumonia.
‡ For the treatment of cholangiocellular carcinoma.

Poisson regression analysis also revealed that the RR of TNF inhibitors in the first year was significantly elevated (RR 2.40, 95% CI 1.20–5.03), but not in the second and third years combined (RR 1.38, 95% CI 0.80–2.43).

**Comparison of risk for SIs between infliximab and etanercept (analysis 2).** We next investigated possible differences between the TNF inhibitors in their contribution to risk for development of SIs. The PS of each patient was calculated by logistic regression model as described in the Methods. The model fit well; the Hosmer-Lemeshow goodness-of-fit statistics did not show a significant difference between observed and predicted frequencies ($P = 0.164$). The patients with a PS of <0.4 (17.6% of the infliximab group and 20.9% of the etanercept group) were considered not representing those receiving TNF antagonists and we excluded them from the following analysis. We constructed 3 Poison regression models to calculate the RR from the use of etanercept for the development of SIs compared to infliximab. In the first model, we adjusted for age, sex, Steinbrocker class, chronic pulmonary comorbidity, diabetes mellitus, observation period, and the PS. The second model added the mean dosage of MTX (≤8 or >8 mg/week) and the mean dosage of oral corticosteroids (<10 or ≥10 mg prednisolone or equivalent/day) to the adjusting factors in the first model. The third model added the calendar year and the number of previous nonbiologic DMARDs to the adjusting factors in the second

**Table 4. Multivariate analysis of independent risk factors for serious infections during continuous use of TNF antagonists in the Registry of Japanese Rheumatoid Arthritis Patients for Long-Term Safety database***

| | RR (95% CI)† | *P* |
|---|---|---|
| TNF antagonist (infliximab or etanercept) | 1.97 (1.25–3.19) | 0.0045 |
| Age by decade | 1.45 (1.20–1.77) | $< 0.001$ |
| Chronic pulmonary disease | 1.77 (1.15–2.70) | 0.009 |
| Diabetes mellitus | 1.20 (0.69–1.97) | 0.49 |
| Mean DAS28-CRP (per 1.0 increment) | 1.33 (1.05–1.66) | 0.015 |
| Mean MTX dosage $>8.0$ mg/week‡ | 2.14 (1.15–3.87) | 0.013 |
| Mean prednisolone dosage $\geq 10$ mg/day‡ | 2.49 (1.08–5.50) | 0.027 |

* TNF = tumor necrosis factor; RR = relative risk; 95% CI = 95% confidence interval; DAS28-CRP = 3-variable Disease Activity Score including 28-joint counts using the C-reactive protein level; MTX = methotrexate.
† The RRs of biologic agents for development of serious infection for up to 3 years of the observation period were calculated using the Poisson regression model after adjusting for confounding factors of age, sex, disease duration, chronic pulmonary disease, diabetes mellitus, Steinbrocker class (28), calendar year, number of previous disease-modifying antirheumatic drugs, observation period, disease activity, immunosuppressive drugs, corticosteroid dose, and MTX dose.
‡ Mean dosage during the observation period.

model. The RR for using etanercept compared to infliximab in the first model was 1.28 (95% CI 0.73–2.30, *P* = 0.41), for the second model was 1.39 (95% CI 0.69–2.76, *P* = 0.35), and for the third model was 1.32 (95% CI 0.65–2.66, *P* = 0.44). We performed sensitivity analyses using different cutoffs for PS and observed essentially the same results.

## DISCUSSION

This is the first epidemiologic study of patients with RA that uses a prospective cohort from an Asian country to investigate the association of SIs and use of TNF antagonists during 3 years and includes patients that changed to a second agent. In addition, we performed a head-to-head comparison of the risk for SIs between infliximab and etanercept. We demonstrated that the continuous use of TNF antagonists for up to 3 years was an independent risk factor for SIs (RR 1.97, 95% CI 1.25–3.19), but the risk was time dependent. We also revealed that the RR for SIs comparing the etanercept group with the infliximab group after adjusting for covariates was not significantly different.

Studies from European biologics registries analyzed the association of TNF antagonists with infections in patients with RA (32,33). There are some reports indicating that the risk for SIs was not increased by TNF antagonists (21–24), but other studies show significant associations between the use of these agents and development of SIs (14–20,34–36). Several of the latter studies revealed time dependence of the risk for SIs (15,16,18–20,34), which is compatible with our results where the risk for SIs was significantly elevated only in the first year and declined in the second and third years combined. The decrease in risk might be explained in part by the effect of dropout patients who developed SIs and stopped the TNF antagonist (34). Of 68 patients who developed SIs in the anti-TNF group, 22 discontinued the biologic agents. Patients who were not susceptible to SIs were more likely to remain in the cohort, which could contribute to reduced risk with increasing observation period.

Increasing age, presence of chronic pulmonary comorbidity, higher mean DAS28-CRP, mean dosage of MTX $>8$ mg/week, and mean dosage of oral prednisolone $\geq 10$ mg/day were identified as independent risk factors for SIs in this study. Most previous studies have reported that increasing age, pulmonary comorbidity, and use of oral prednisolone were risk factors for infections (14,21–23,35,36) and for PCP (37) in RA patients treated with TNF antagonists. Conflicting results, however, have been reported regarding the association of disease activity and risk for SIs (23,36). Because disease activity is often improved rapidly and significantly by treatment with biologic agents, including TNF antagonists, it seems reasonable that baseline disease activity may not accurately predict infectious events. Mean disease activity during the observation period may serve as a better predictor, as our study indicates.

In Japan, the data from postmarketing surveillance programs conducted by pharmaceutical companies showed that the IRs of pneumonia, PCP, and tuberculosis occurring during the first 6 months of treatment with infliximab were numerically higher than those of etanercept (11–13). In the present study, however, we show that the risk for SIs of treatment with etanercept during the longer observation period was not significantly different from that of infliximab after adjusting for covariates. Some observational studies directly (23) or indirectly (17,20) compared the risk for SIs between treatment with infliximab and etanercept, and found no statistically significant difference. A recent meta-analysis including randomized controlled trials and their extension studies also supports the results of our study; the odds ratio of etanercept treatment for SIs indirectly compared with infliximab was 0.73 (95% CI 0.46–1.15), which was not statistically significant (38).

There are a number of limitations to our study. First, we have to consider possible selection bias in our study. All of

the patients were enrolled from university hospitals or referral hospitals that are dedicated to the treatment of RA. The number of the unexposed group was smaller than that of the anti-TNF group in this study, which did not reflect the real world and may indicate unidentified selection bias. Although we estimated the risk of SIs after adjusting for variables that were clinically important, we had to interpret our data under these conditions. A second limitation is the effect of prevalent users on the analyses. In the exposed group, there were 273 prevalent nonbiologic DMARD users who had already been receiving the nonbiologic DMARDs at enrollment in the REAL database, and the rest were incident nonbiologic DMARD users. Inclusion of these prevalent nonbiologic DMARD users in our cohort might lead to the underestimation of the incidence of SIs. However, the majority of these patients started new nonbiologic DMARDs or underwent dose escalations of nonbiologic DMARDs during the observation period (data not shown), reducing the degree of underestimation. Third, the mean observation periods for both groups were approximately 2 years; it is possible that we underestimated the rate of SIs in the third year. Fourth, the mean dose of MTX of our database is lower than those of Western cohorts. In Japan, the maximum approved dosage of MTX for RA has been increased since February 2011 and Japanese rheumatologists can now officially prescribe MTX up to 16 mg/week for patients with RA. Therefore, in the future, we will be able to conduct further studies to examine the risk of TNF antagonists in patients receiving a higher dose of MTX.

In conclusion, we have shown that the continuous use of TNF therapy for up to 3 years in Japanese patients with RA, including cases where a clinical switch to a second TNF antagonist was employed, time dependently increased the risk for SIs compared to treatment with nonbiologic conventional DMARDs. A comparison of actual long-term safety among different classes of biologic DMARDs using registry data will be necessary for choosing the appropriate treatment of RA and needs to be performed.

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## AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Harigai had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Study conception and design.** Sakai, Komano, Michi Tanaka, Nanki, Ryuji Koike, Miyasaka, Harigai.

**Acquisition of data.** Komano, Michi Tanaka, Nanki, Ryuji Koike, Nagasawa, Amano, Nakajima, Atsumi, Takao Koike, Ihata, Ishigatsubo, Saito, Yoshiya Tanaka, Ito, Sumida, Tohma, Tamura, Fujii, Sugihara, Kawakami, Hagino, Ueki, Hashiramoto, Nagasaka, Miyasaka, Harigai.

**Analysis and interpretation of data.** Sakai, Komano, Michi Tanaka, Nanki, Ryuji Koike, Miyasaka, Harigai.

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## APPENDIX A: MEMBERS OF THE REAL STUDY GROUP AND THEIR AFFILIATES

Members of the Registry of Japanese Rheumatoid Arthritis Patients for Long-Term Safety (REAL) Study Group and their affiliates who contributed to this work were as follows: Hideto Kameda (Saitama Medical University), Shinsuke Yasuda (Hokkaido University), Mitsuhiro Takeno (Yokohama City University), Shintaro Hirata (University of Occupational and Environmental Health), Taichi Hayashi (University of Tsukuba), Yoshinari Takasaki (Juntendo University), Tsuneyo Mimori (Kyoto University), Hiroaki Ida, Katsumi Eguchi (Nagasaki University), Kazuhiko Yamamoto (University of Tokyo), Shunichi Shiozawa, Yasushi Miura (Kobe University), Tetsuji Sawada (Tokyo Medical University Hospital), Hiroaki Dobashi (Kagawa University Hospital), Sae Ochi (Tokyo Metropolitan Bokutoh Hospital), Ayako Nakajima, Hisashi Yamanaka (Tokyo Women's Medical University), Kiyoshi Migita (National Hospital Organization Nagasaki Medical Center), and Hayato Yamazaki, Kaori Watanabe (Tokyo Medical and Dental University).

The following university and hospitals are also members of the REAL Study Group, but were not involved in the present study: Keio University, Kurashiki Kohsai Hospital, Tokyo Kyosai Hospital, and Yokohama City Minato Red Cross Hospital.

EXTENDED REPORT

# Drug retention rates and relevant risk factors for drug discontinuation due to adverse events in rheumatoid arthritis patients receiving anticytokine therapy with different target molecules

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▸ Additional supplementary data are published online only. To view these files please visit the journal online (http://ard.bmj.com/content/early/recent)

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## ABSTRACT

**Objective** To compare reasons for discontinuation and drug retention rates per reason among anticytokine therapies, infliximab, etanercept and tocilizumab, and the risk of discontinuation of biological agents due to adverse events (AE) in patients with rheumatoid arthritis (RA).

**Method** This prospective cohort study included Japanese RA patients who started infliximab (n=412, 636.0 patient-years (PY)), etanercept (n=442, 765.3 PY), or tocilizumab (n=168, 206.5 PY) as the first biological therapy after their enrolment in the Registry of Japanese Rheumatoid Arthritis Patients for Long-term Safety (REAL) database. Drug retention rates were calculated using the Kaplan–Meier method. To compare risks of drug discontinuation due to AE for patients treated with these biological agents, the Cox proportional hazard model was applied.

**Results** The authors found significant differences among the three therapeutic groups in demography, clinical status, comorbidities and usage of concomitant drugs. Development of AE was the most frequent reason for discontinuation of biological agents in the etanercept and tocilizumab groups, and the second most frequent reason in the infliximab group. Discontinuation due to good control was observed most frequently in the infliximab group. Compared with etanercept, the use of infliximab (HR 1.69; 95% CI 1.14 to 2.51) and tocilizumab (HR 1.98; 95% CI 1.04 to 3.76) was significantly associated with a higher risk of discontinuation of biological agents due to AE.

**Conclusions** Reasons for discontinuation are significantly different among biological agents. The use of infliximab and tocilizumab was significantly associated with treatment discontinuation due to AE compared with etanercept.

Biological disease-modifying antirheumatic drugs (biological agents) are a standard treatment for rheumatoid arthritis (RA).[1 2] A number of clinical trials have demonstrated that biological agents significantly improve signs and symptoms of RA patients with both early and established disease, and that remission of RA can be achieved with biological agents not only in early RA patients, but also in established RA patients who have shown inadequate responses to conventional non-biological disease-modifying antirheumatic drugs (DMARD).

In Japan, six biological agents have been approved for the treatment of RA, infliximab in 2002, etanercept in 2005, tocilizumab and adalimumab in 2008, abatacept in 2010 and golimumab in 2011. These drugs are widely used in clinical practice according to treatment guidelines for biological agents by the Japan College of Rheumatology[3 4] and Japanese drug package inserts. Postmarketing surveillance and some clinical studies have shown short-term effectiveness and safety of these biological agents for Japanese RA patients.[5–8] The European League Against Rheumatism recommendations for the management of RA state that a tumour necrosis factor (TNF) antagonist should be administered as the first biological DMARD for patients who fail to respond to non-biological DMARD, including methotrexate,[9] whereas Japanese guidelines do not clearly specify the precedence of biological agents.

Some RA patients treated with biological agents are compelled to stop the administration of these drugs because of lack of efficacy (LOE), adverse events (AE), or financial reasons. In addition, some RA patients discontinue biological agents in the hope of a biological-free remission or biological-free low disease activity status.[10–12] In general, drugs with high retention rates have a good balance between long-term effectiveness and tolerability, reflecting the satisfaction of patients and doctors with the treatment. Because treatment for RA continues for many years or is life-long in the majority of patients, the examination of long-term drug retention rates using a prospective cohort study is important for the evaluation of biological agents.

To establish better treatment strategies for RA, it is important to identify reasons and risk factors causing the discontinuation of a drug, especially for biological agents. Several studies have shown that