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The Inhibitory Effect of IFN- γ on Protease HTRA1 Expression in Rheumatoid Arthritis

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The high temperature requirement A1 (HTRA1) is a potent protease involved in many diseases, including rheumatoid arthritis (RA). However, the regulatory mechanisms that control HTRA1 expression need to be determined. In this study, we demonstrated that IFN- γ significantly inhibited the basal and LPS-induced HTRA1 expression in fibroblasts and macrophages, which are two major cells for HTRA1 production in RA. Importantly, the inhibitory effect of IFN- γ on HTRA1 expression was evidenced in collagen-induced arthritis (CIA) mouse models and in human RA synovial cells. In parallel with the enhanced CIA incidence and pathological changes in IFN- γ -deficient mice, HTRA1 expression in the joint tissues was also increased as determined by real-time PCR and Western blots. IFN- γ deficiency increased the incidence of CIA and the pathological severity in mice. Neutralization of HTRA1 by Ab significantly reversed the enhanced CIA frequency and severity in IFN- γ -deficient mice. Mechanistically, IFN- γ negatively controls HTRA1 expression through activation of p38 MAPK/STAT1 pathway. Dual luciferase reporter assay and chromatin immunoprecipitation analysis showed that STAT1 could directly bind to HTRA1 promoter after IFN- γ stimulation. This study offers new insights into the molecular regulation of HTRA1 expression and its role in RA pathogenesis, which may have significant impact on clinical therapy for RA and possibly other HTRA1-related diseases, including osteoarthritis, age-related macular degeneration, and cancer. *The Journal of Immunology*, 2014, 193: 130–138.

Rheumatoid arthritis (RA), a common autoimmune disease with a prevalence of ~1% worldwide, is characterized by chronic inflammation and high levels of destructive mediators in the synovium, which leads to cartilage and bone destruction (1). Cytokines such as TNF- α , IL-1, IL-6, IL-17, IL-15, IL-18, GM-CSF, and various chemokines, produced mainly by T cells, macrophages, and fibroblasts localized in the rheumatoid synovium, are known to be associated with the development and progression of RA in humans and mice (2, 3). In contrast, cytokines such as IFN- γ have been suggested to be protective (4–6). Many patients respond well to biologic agents that inhibit proinflammatory cytokines in the short term. However, a considerable

number of patients (30–50%) respond poorly to such interventions in the long term (7). Therefore, exploring long-lasting efficacious treatment is desired. The key to achieving this goal is identifying the factors that drive prolonged expression of inflammatory and destructive mediators as well as advancing our understanding of the molecular mechanisms involved in the pathogenesis of RA.

High temperature requirement A1 (HTRA1, PRSS11, or L56), a secreted serine protease, has a highly conserved trypsin-like protease domain and a C-terminal PDZ domain (8). The role of protease HTRA1 in degrading proteins such as fibronectin, aggrecan, decorin, biglycan, fibromodulin, nidogen 1 and 2 (structural constituents of basement membranes), E-cadherin, talin, fascin,

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Y.H. designed and performed the major experiments with cells and mice, analyzed data, and contributed to drafting of the manuscript; H.L. designed the experiments, analyzed data, and contributed to drafting of the manuscript; L. Zhu prepared the CIA

animal models and related assays; Z. Liu performed the ChIP assays; F.H. collected clinical samples and performed related assays; J.S. analyzed histological data; T.Y. established cell lines; X.S. performed ELISA assays; H.G. analyzed clinical data and revised the manuscript; X.T. performed PCR assays; L. Zhang provided animal models and revised the manuscript; Q.W. performed the ChIP assays, analyzed data, and contributed intellectually to the manuscript; Z. Li performed clinical assays and contributed intellectually to the manuscript; and Y.Z. designed experiments, analyzed data, and drafted and finalized the manuscript.

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Abbreviations used in this article: B6, C57BL/6; CIA, collagen-induced arthritis; DP, downstream primer; HPRT, hypoxanthine phosphoribosyltransferase; HTRA1, high temperature requirement A1; KO, knockout; MEF, mouse embryonic fibroblast; MTA, 5'-deoxy-5'-(methylthio)-adenosine; RA, rheumatoid arthritis; RNAi, RNA interference; shRNA, short hairpin RNA; UP, upstream primer; WT, wild-type.

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chloride intracellular channel protein 1, and TGF- β family members (9–15) makes it central to extracellular matrix homeostasis and turnover, intercellular adhesion, and cell migration. It is reported that the elevated HTRA1 levels were associated with RA, osteoarthritis, age-related macular degeneration, Duchenne muscular dystrophy, and aging in humans (16–22). Locally enhanced HTRA1 was recently identified as one of the key molecular mediators in arthritic diseases, as it directly degrades cartilage through proteolytic cleavage of extracellular matrix components and stimulates overproduction of matrix metalloproteinase by synovial fibroblasts (9, 10, 17, 23). In contrast, decreased HTRA1 expression contributes to the aggressiveness, metastatic ability, and chemoresistance of tumors (20, 24). Recently, we have demonstrated that TLR4 activation by LPS or its endogenous ligand tenascin-C significantly induced HTRA1 expression through the classic NF- κ B pathway in fibroblasts and macrophages (25). Furthermore, the enhanced HTRA1 expression is closely involved in the enhancing effects of LPS on the incidence and severity of arthritis in a standard collagen-induced arthritis (CIA) mouse model (25). However, the microenvironmental extracellular factors and intracellular upstream events that negatively regulate HTRA1 expression in mammalian cells have not been determined.

In the present study, we investigated 13 cytokines and discovered that only IFN- γ significantly inhibited HTRA1 expression in control or LPS-treated fibroblasts and macrophages. The antagonistic relationship between LPS and IFN- γ on HTRA1 expression was also detected in RA mouse models and human RA patient samples. These observations offer new insights into the molecular pathophysiology of RA and the interaction of infection and inflammation with tissue-destructive mediators in RA pathogenesis. These findings may have potential impacts on exploring new clinical therapies for RA.

Materials and Methods

Animals and reagents

C57BL/6 (B6) mice were purchased from the Beijing University Experimental Animal Center (Beijing, China). IFN- γ knockout (KO) and IFN- γ R1 KO mice were provided by Dr. Lianfeng Zhang. All mice were maintained in a specific pathogen-free facility and were housed in microisolator cages containing sterilized feed, autoclaved bedding, and water. All experimental manipulations were undertaken in accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals, Institute of Zoology (Beijing, China).

Recombinant mouse cytokines and human IFN- γ were purchased from PeproTech (Rocky Hill, NJ). Recombinant mouse IL-21, IL-23, and HTRA1 were obtained from R&D Systems (Minneapolis, MN). Bacterial LPS (*Escherichia coli* 055:B5) was purchased from Sigma-Aldrich (St. Louis, MO). SB203580 (559389), LY294002 (440202), and 6-amino-4-(4-phenoxyphenylethylamino)quinazoline (481406) were from the Merck Group (Darmstadt, Germany); SP600125 (S5567), U0126 (U120), and 5'-deoxy-5'-(methylthio)-adenosine (MTA, D5011) were from Sigma-Aldrich.

The reagents were used at the indicated or following concentrations: IL-1 β (100 ng/ml), IL-2 (100 U/ml), IL-4 (1000 U/ml), IL-6 (20 ng/ml), IL-10 (20 ng/ml), IL-12 (10 ng/ml), IL-17 (100 ng/ml), IL-21 (100 ng/ml), IL-23 (100 ng/ml), IL-33 (100 ng/ml), TNF- α (100 ng/ml), IFN- γ (50 ng/ml), TGF- β 1 (5 ng/ml), recombinant human IFN- γ (50 ng/ml), LPS (500 ng/ml), SB203580 (10 μ M), LY294002 (1 μ M), SP600125 (10 μ M), and MTA (10 μ M).

Cells

Freshly isolated mouse macrophages were obtained from the peritoneal exudates as described (26). Mouse embryonic fibroblasts (MEFs) were isolated in our laboratory. RAW264.7 (TIB-71), a murine macrophage-like cell line, was purchased from the American Type Culture Collection (Manassas, VA). Human synovial cells of RA patients were provided by Prof. Z.G. Li (People's Hospital, Peking University, Beijing, China). Cells were cultured in DMEM (HyClone Laboratories) containing 10% FBS and 1% penicillin/streptomycin at 37°C, 5% CO₂.

Immunofluorescent staining

Cells were cultured on coverslips for the indicated time and then fixed in 4% paraformaldehyde for 10 min and stored in PBS at 4°C. Cells were permeabilized in 0.2% Triton X-100/PBS for 10 min at room temperature and blocked for 1 h in 5% BSA/PBS. Cells were incubated in BSA/PBS solution containing indicated mAbs (1:100 dilution in blocking buffer) overnight at 4°C. Following PBS washes, secondary Ab (goat anti-rabbit, Alexa Fluor 546, Invitrogen; 1:500 dilution) was applied for 1 h and Hoechst 33342 (2 μ g/ml) for 10 min before the coverslips were washed in PBS and mounted. Photomicrographs were taken using a LSM 510 Meta laser scanning microscope (Carl Zeiss, Jena, Germany).

Western blotting

Total cell lysates and immunoblot analysis were performed as described (27). Protein bands were visualized by adding HRP membrane substrate (Millipore) and then scanned using the Tanon 1600R gel image system (Tanon, Shanghai, China). The Abs used were as follows: anti-HTRA1 (sc-50335; Santa Cruz Biotechnology, Santa Cruz, CA), anti-p-p38 MAPK (9211), anti-p38 MAPK (9218), anti-p-Erk1/2 (9101), anti-Erk1/2 (4695), anti-p-JNK (Thr¹⁸³/Tyr¹⁸⁵; 4668), anti-JNK (9252), anti-p-AKT (Ser⁴⁷³; 9271), anti-AKT (9272), anti-p-STAT1 (Tyr⁷⁰¹/Ser⁷²⁷; 9167/9177), and anti-STAT1 (9172) (Cell Signaling Technology, Beverly, MA). GAPDH mAb (Proteintech Group) was used to normalize for the amount of loaded protein.

ELISA to detect HTRA1

HTRA1 protein levels within synovial fluid and culture media were determined using ELISA (25). Briefly, ELISA plates were coated overnight with anti-HTRA1 Ab (1:200; SAB1300009; Sigma-Aldrich) and blocked with 5% BSA/PBS. Plates were washed with 0.05% Tween 20/PBS and incubated with samples for 2 h at 30°C. After washing, anti-HTRA1 Ab (1:100; sc-15465; Santa Cruz Biotechnology) was added for 1 h at 30°C followed by an HRP-conjugated donkey anti-goat IgG (1:5000; sc-2020; Santa Cruz Biotechnology) for 1 h at 30°C. Plates were developed using 3,3',5,5'-tetramethylbenzidine in 100 mM citric acid, 0.1% H₂O₂ (pH 3.95). The reaction was stopped with 2 M H₂SO₄, and ODs were determined at 450 nm using a plate reader. Purified recombinant HTRA1 (2916SE; R&D Systems) was tested at concentrations ranging from 1 to 128 ng/ml to generate a standard curve.

Real-time PCR for HTRA1 mRNA

mRNAs were isolated using TRIzol (Invitrogen) according to the manufacturer's instructions. A real-time PCR kit (SYBR Premix Ex Taq, DRR041A) was purchased from TaKaRa. Bio-PCR was performed using a CFX96 (Bio-Rad). Housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT) was used as an internal control. The primers for human HPRT were 5'-CAGTATAATCCAAAGATGGTCAA-3' for the upstream primer (UP) and 5'-TTAGGCTTTGTATTTTGCTTTTCC-3' for the downstream primer (DP); human HTRA1, 5'-CAAGGATGTGGATGA-GAAGCAGACA-3' for UP and 5'-ATGATGGCGTCGGTCTGTGATG-TAGTC-3' for DP; mouse HPRT, 5'-AGTACAGCCCCAAATGGTT-AAG-3' for UP and 5'-CTTAGGCTTTGTATTTGGCTTTTTC-3' for DP; and mouse HTRA1, 5'-CAAGGATGTGGATGAAAAGGC-3' for UP and 5'-ATGATAGCGTCTGTCTGAATGTAGTC-3' for DP.

Induction and assessment of CIA

Twelve- to 14-wk-old male B6 and IFN- γ KO mice were used to induce CIA by chicken collagen II (Sigma-Aldrich, C9301) (28). For the treatment, i.p. injections of IFN- γ (5 μ g/mouse) were given every other day from day 1 and/or with LPS (30 μ g/mouse) at day 23. Additionally, some IFN- γ KO mice also received an i.v. injection of anti-HTRA1 Ab (100 μ g; Santa Cruz Biotechnology) on days 20 and 23.

Macroscopic assessment of arthritis was assessed by the thickness of hindpaws two to three times per week with microcalipers. The reported diameter was an average of the inflamed hindpaws per mouse. Animals were also scored for clinical signs of arthritis as follows (29): 0, normal; 1, slight swelling and/or erythema; 2, pronounced edematous swelling; and 3, joint rigidity. Each limb was graded, allowing a maximum score of 12 per mouse. At the end of the experiment, the hindpaws of the mice were removed, fixed, decalcified, and paraffin embedded. Sections (5 μ m) were stained with H&E and examined for the histological changes of inflammation, pannus formation, and cartilage and bone damage.

To detect the plasma anti-chicken collagen II Ab levels, ELISAs were performed as described (28). HRP-conjugated goat anti-mouse IgG (Sigma-Aldrich) or isotype-specific (IgG1, IgG2a, or IgG2b) Abs (BD Biosciences) were used.

RNA interference

A gene-knockdown lentiviral construct was generated by subcloning a gene-specific short hairpin RNA (shRNA) sequence into vector plasmid (pLL3.7) (30). The following gene-specific targeting sequences were used: p38 MAPK, 5'-GAACCTTCGCAAATGTATT-3'; and STAT1, 5'-GCCGA-GAACATACCAGAGAAT-3'. Lentiviruses were harvested from culture supernatant of 293T cells transfected with 4 μ g shRNA vector, 3 μ g psPAX2, and 3 μ g pMD2.G. Then, RAW264.7 cells were infected with recombinant lentivirus, and GFP-expressing cells were isolated using fluorescence sorting 48 h later. The expressions of the above genes were confirmed using Western blots.

DNA transfection

RAW264.7 cells were grown overnight to obtain 70–80% confluent monolayer cells in 24-well plates. DNA plasmids (0.2–0.5 μ g) were transfected using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol. Opti-MEM I (50 μ l) and Lipofectamine 2000 (1.5 μ l) were incubated for 5 min at room temperature. DNA (0.2–0.5 μ g) was added to the mixture and incubated for an additional 20 min. After the medium was removed, the DNA mixture and Opti-MEM I (250 μ l) were then added to each well and incubated at 37°C for 4 h. Subsequently, complete DMEM (1 ml) was added to the mixture. Transfected cells were then incubated for 24–72 h in the medium (1.3 ml). The cells were then processed to be used in assays.

Dual-Luciferase reporter assays

Dual-Luciferase reporter assays were performed as previously described (31). RAW264.7 cells (4×10^5 /well in 24-well plates) were cotransfected with expression plasmid DNA (0.1–0.3 μ g), Fluc reporter plasmid (0.1 μ g), and the internal control vector pRL-TK (0.1 μ g) using Lipofectamine 2000 transfection reagent according to the manufacturer's protocol. Twenty-four hours after transfection, the cells were stimulated with described reagents for another 24 h and then collected, lysed with 50 μ l 1 \times passive lysis buffer, and subsequently assayed for luciferase activity using the Dual-Luciferase reporter assay system (E1910; Promega, Madison, WI).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed from $\sim 2 \times 10^7$ RAW264.7 cells according to a previously described protocol with slight modifications (32). Briefly, cells were crosslinked with 1% formaldehyde for 10 min at room temperature and the reaction was quenched by

addition of glycine to a final concentration of 0.125 M. Chromatin was sonicated to an average size of 0.5–1 kb using a Bioruptor (Diagenode). A total of 3–5 μ g Ab (anti-NF- κ B or anti-STAT1, Cell Signaling Technology) was added to the sonicated chromatin and incubated overnight at 4°C. Ten percent of chromatin used for each ChIP reaction was kept as input DNA. Subsequently, 75 μ l protein A or protein G Dynal magnetic beads were added to the ChIP reactions and incubated for an additional 4 h at 4°C. Magnetic beads were washed and chromatin eluted, followed by reversal of the crosslinkings and DNA purification. Resultant ChIP DNA was dissolved in water. ChIP DNA was next used as a template for PCR using the appropriate primers: STAT1/NF- κ B BS-1, 5'-CCACGGCGGCGT-CAAGTTCA-3' for UP and 5'-TGGCTCAGTTCTCATTCTA-3' for DP; STAT1/NF- κ B BS-2, 5'-CCCCTTGATGTGTGGGACTT-3' for UP and 5'-GCACCTGCCATAA CACACC-3' for DP; and nonspecific BS, 5'-GAACCAGAAGGAACACAAGC-3' for UP and 5'-AATCCTCAGAC-TACAGAAAC-3' for DP.

Statistical analysis

Data are presented as mean \pm SD. A Student unpaired *t* test for comparison between two groups was used. Two-way ANOVA analysis was used for comparison among multiple groups with SPSS 16.0 software. A *p* value < 0.05 was considered to be statistically significant.

Results

Different effects of cytokines on HTRA1 expression in MEFs and macrophages

To identify the potential cytokines regulating HTRA1 expression in fibroblasts and macrophages, which are the major HTRA1 producers in RA (17), we screened 13 cytokines, which were previously demonstrated to be involved in RA progression (1, 33), for HTRA1 mRNA and protein expression. As shown in Fig. 1, IL-21 slightly but significantly increased HTRA1 expression in MEFs ($p < 0.001$). We were unable to detect any changes in expression of HTRA1 with other incubated cytokines, including TNF- α , IL-1 β , IL-6, IL-2, IL-12, IL-4, IL-10, TGF- β 1, IL-17, IL-23, and IL-33 (Fig. 1A, Supplemental Fig. 1A), even with increasing cytokine levels (Supplemental Fig. 1C). These cytokines were functional as determined by other analyses, including the

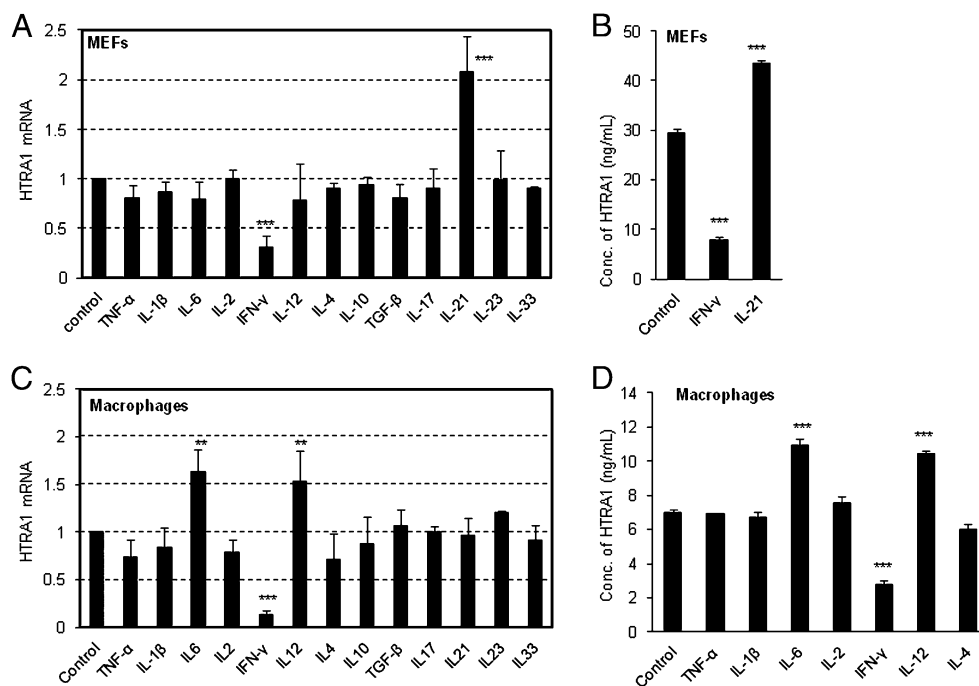


FIGURE 1. Different effects of cytokines on HTRA1 mRNA and protein expression in MEFs and macrophages. MEFs (**A** and **B**) and freshly isolated mouse macrophages (**C** and **D**) were cultured with different cytokines for 24 h as described in *Materials and Methods*. HTRA1 mRNA levels (**A** and **C**) were determined by real-time PCR, and secreted HTRA1 protein concentrations from the culture media (**B** and **D**) were detected by ELISA. Data were shown as means \pm SD ($n = 3$), which represent one of at least three independent experiments with similar results. ** $p < 0.01$, *** $p < 0.001$ compared with the control.

enhanced ERK phosphorylation by a TNF- α assay in macrophages (Supplemental Fig. 1D and data not shown). In contrast, IFN- γ significantly inhibited HTRA1 expression in MEFs in a dose- and time-dependent manner ($p < 0.001$; Fig. 1A, 1B and data not shown).

Consistent with our observations in MEFs, IFN- γ markedly inhibited HTRA1 mRNA and protein expression in macrophages ($p < 0.001$; Fig. 1C, 1D). The inhibiting effect of IFN- γ is specific and mediated by IFN- γ R, as IFN- γ R-deficient macrophages did not respond to IFN- γ in this assay (data not shown). IL-6 and IL-12 increased HTRA1 mRNA and protein expression in macrophages (Fig. 1C, 1D). Other screened cytokines, including TNF- α , IL-1 β , IL-2, IL-4, IL-10, TGF- β 1, IL-17, IL-21, IL-23, and IL-33, failed to show a significant effect on HTRA1 expression (Fig. 1C, 1D, Supplemental Fig. 1B, 1E). Thus, IFN- γ was identified as an important cytokine for controlling HTRA1 expression in fibroblasts and macrophages, the major cell types involved in HTRA1 expression in RA (17). Additionally, note that IL-21 increased HTRA1 expression in MEFs but not in macrophages, whereas IL-6 and IL-12 increased HTRA1 expression in macrophages but not in MEFs.

IFN- γ significantly inhibits LPS-induced HTRA1 expression in vitro and in vivo

To see whether IFN- γ has antagonistic regulatory effects on LPS-induced HTRA1 expression, which was recently determined in our laboratory (25), we measured HTRA1 mRNA and protein levels in MEFs and macrophages after LPS stimulation in the presence or absence of IFN- γ . IFN- γ strikingly inhibited LPS-induced HTRA1 expression in both MEFs and macrophages in vitro ($p < 0.001$; Fig. 2A–D). Consistent with the in vitro results, injection of IFN- γ significantly decreased LPS-induced HTRA1 mRNA and protein expression in joint tissues ($p < 0.001$; Fig. 2E, 2F), and LPS promoted significantly more HTRA1 production in

joints of IFN- γ KO mice compared with wild-type (WT) mice ($p < 0.001$; Fig. 2G, 2H). However, other tested cytokines did not show strong cooperative or antagonistic effects with LPS in inducing HTRA1 synthesis as determined by in vitro assays (Supplemental Fig. 2). Thus, IFN- γ is the only cytokine among the detected cytokines identified to display the inhibitory ability on basal or LPS-induced HTRA1 expression in vitro and in vivo.

IFN- γ inhibits HTRA1 expression via the p38 MAPK/STAT1 pathway

The following studies were performed in an attempt to understand the intracellular signal pathways mediating the effects of IFN- γ on HTRA1 synthesis. Monocyte/macrophage RAW264.7 cells were potential model cell lines for these experiments. RAW264.7 cells showed similar responses to IFN- γ compared with the freshly isolated primary macrophages as determined by HTRA1 mRNA expression (data not shown). Thus, the following molecular and biochemical assays were performed mainly using RAW264.7 cells.

In RAW264.7 cells, LPS promoted significant phosphorylation of p38 MAPK, ERK, STAT1 (Ser⁷²⁷), and AKT (protein kinase B), whereas IFN- γ increased phosphorylation of STAT1 (both Tyr⁷⁰¹ and Ser⁷²⁷; Fig. 3A). IFN- γ cooperatively enhanced the LPS-induced phosphorylation of p38 MAPK, Erk, and STAT1 (Ser⁷²⁷; Fig. 3A), as previously reported (34). LPS and IFN- γ stimulation increased p65 levels in the nuclear fraction (Fig. 3A). Nuclear localization of increased p-STAT1 (Tyr⁷⁰¹ and Ser⁷²⁷) after LPS and/or IFN- γ stimulation was further confirmed by two-photon confocal microscopy (Fig. 3B).

To further understand the mechanism by which IFN- γ inhibits HTRA1 expression, we chose to systematically explore possible downstream mediators of the IFN- γ receptor. Initially, inhibiting PI3K and Erk, which blocked LPS-induced HTRA1 production, failed to reverse the inhibiting effects of IFN- γ on LPS-induced HTRA1 mRNA and protein expression, even at increasing inhib-

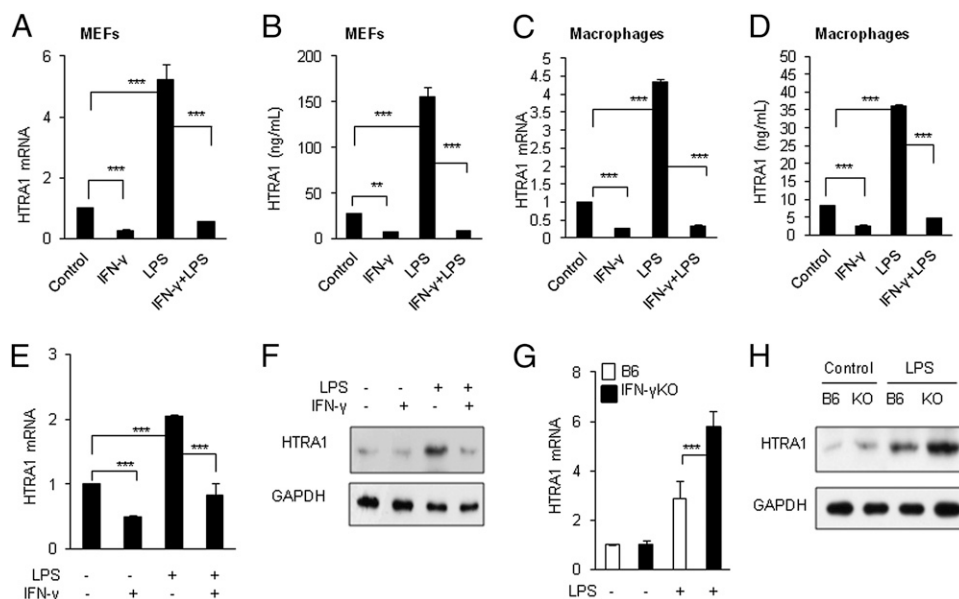


FIGURE 2. Antagonistic effects of LPS and IFN- γ on HTRA1 expression in vitro and in vivo. MEFs (A and B) and freshly isolated mouse macrophages (C and D) were cultured with LPS and/or IFN- γ for 24 h as described in *Materials and Methods*. HTRA1 mRNA levels (A and C) and HTRA1 protein concentrations in the culture media (B and D) were determined. Data were shown as means \pm SD ($n = 3$), which represent one of three independent experiments with similar results. B6 mice (E and F; $n = 5$) were injected i.p. with 50 μ g/mouse of LPS at day 1 and/or 5 μ g/mouse of IFN- γ every other day from day 1 as described in *Materials and Methods*. At day 5, HTRA1 mRNA (E) and HTRA1 protein expression in joint tissues (F) were detected by real-time PCR and Western blotting, respectively. B6 mice and IFN- γ KO mice (G and H; $n = 5$) were injected i.p. with LPS at day 1. At day 5, the expression of HTRA1 mRNA (G) and protein (H) in joint tissues was determined. Data are shown as means \pm SD, which represent one of at least three independent experiments with similar results. ** $p < 0.01$, *** $p < 0.001$ for comparisons between the indicated groups.

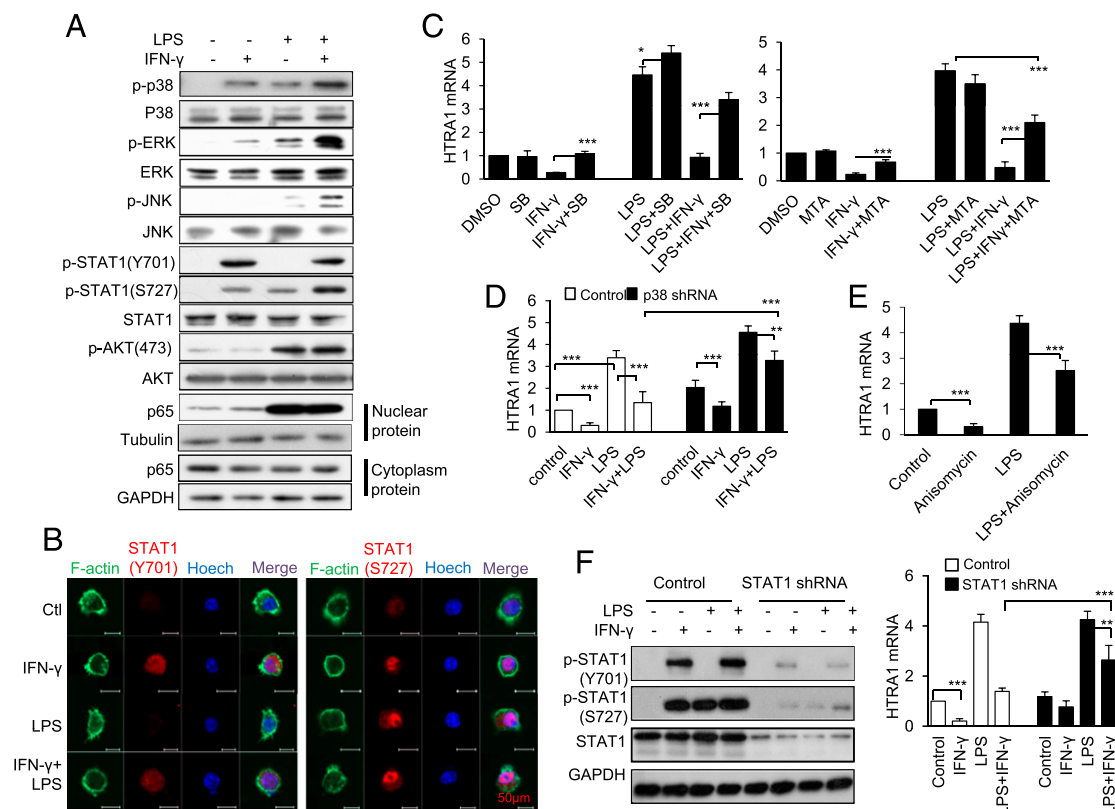


FIGURE 3. The inhibitory effect of IFN- γ on HTRA1 expression is mediated by the p38 MAPK/STAT1 pathway. **(A)** RAW264.7 cells were cultured with LPS and/or IFN- γ for 30 min at the concentrations described in *Materials and Methods*. The phosphorylation state of p38 MAPK, Erk, JNK, STAT1, AKT, and p65 as well as nuclear translocation of p65 were determined using Western blotting. **(B)** The intracellular location of p-STAT1 in RAW264.7 cells was detected by two-photon microscope 30 min after stimulation with LPS and/or IFN- γ . **(C)** HTRA1 mRNA expression was determined in RAW264.7 cells pretreated with inhibitors of p38 MAPK (SB203580) and STAT1 (MTA) for 30 min and then cocultured with LPS and/or IFN- γ for an additional 24 h. **(D)** RAW264.7 cells with ectopic overexpression of shRNA targeting p38 MAPK were cultured with LPS and/or IFN- γ for 24 h. HTRA1 mRNA expression was determined by real-time PCR. **(E)** RAW264.7 cells were pretreated with p38 activator for 30 min and then cultured with LPS for an additional 24 h. HTRA1 mRNA expression was determined by real-time PCR. **(F)** RAW264.7 cells with ectopic overexpression of shRNA targeting STAT1 were cultured with LPS and/or IFN- γ for 24 h. The levels of p-STAT1 and total STAT1 were determined by Western blots. The HTRA1 mRNA expression was determined by real-time PCR. Data are shown as means \pm SD ($n = 3$), which represent one of two independent experiments with similar results. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for comparisons between the indicated groups.

itor doses (data not shown). However, blocking p38 MAPK and STAT1 significantly reversed the inhibiting effects of IFN- γ on LPS-induced HTRA1 expression (Fig. 3C, Supplemental Fig. 3). Furthermore, utilizing specific activators or RNA interference (RNAi) to create gain or loss of function of the p38 MAPK/STAT1 pathway, respectively, we demonstrated the critical roles of p38 MAPK and STAT1 in mediating the inhibitory effects of IFN- γ on HTRA1 expression. Loss of p38 MAPK strikingly reversed the IFN- γ inhibiting effects on LPS-induced HTRA1 expression ($p < 0.001$; Fig. 3D, Supplemental Fig. 4A, 4B) whereas activation of p38 MAPK inhibited basal and LPS-induced HTRA1 expression (Fig. 3E, Supplemental Fig. 4C, 4D). Additionally, specific loss of STAT1 using RNAi significantly blocked the inhibitory effects of IFN- γ on LPS-induced HTRA1 expression ($p < 0.001$; Fig. 3F, Supplemental Fig. 4E). These data suggest that the p38 MAPK/STAT1 pathway is involved in the inhibitory effects of IFN- γ on HTRA1 expression and is consistent with a prior study establishing the importance of the p38 MAPK/STAT1 pathway in IFN- γ function (35).

We also wanted to determine whether STAT1, after activation by IFN- γ , can directly regulate HTRA1 gene transcription. We discovered two potential STAT1 binding sites upstream of the mouse HTRA1 transcription start site: AA-343 (STAT1 BS-1; Fig. 4E) and AA-1093 (STAT1 BS-2; Fig. 4A). Dual-Luciferase reporter gene assays revealed that STAT1-regulated HTRA1 expression was

critically dependent on binding at STAT1 BS-2 but not at STAT1 BS-1 (Fig. 4B). Furthermore, ChIP assays utilizing anti-STAT1 mAb demonstrated that STAT1 almost exclusively bound to STAT1 BS-2 with no detectable binding to STAT1 BS-1, as determined by PCR (Fig. 4C) and real-time PCR (Fig. 4D). In contrast, IFN- γ treatment did not alter the binding efficiency of NF- κ B to the HTRA1 promoter (Fig. 4C, 4D), indicating that STAT1 binding does not influence NF- κ B binding at the HTRA1 promoter. These data collectively indicate that a major pathway for the inhibition of HTRA1 expression occurs through IFN- γ -activated STAT1 directly binding to the HTRA1 promoter (at AA-1093) and subsequently downregulating HTRA1 expression in macrophages.

Because LPS and TNF- α share many common intracellular pathways, including NF- κ B and p38 MAPK/STAT1 pathways (36), we investigated the ability of TNF- α to induce HTRA1 expression in cells treated with p38 MAPK or STAT1 inhibitors or using RNAi to decrease p38 MAPK activity. TNF- α significantly induced HTRA1 expression in the absence of the p38 MAPK pathway (Supplemental Fig. 4F–H). The difference in the ability of LPS and TNF- α to activate the p38 MAPK pathway may partially explain how they act differently with respect to HTRA1 induction.

IFN- γ negatively regulates HTRA1 expression in RA

Because of the respective protective and destructive roles of IFN- γ and HTRA1 on joint tissues in RA (5, 6, 37–39), we investigated

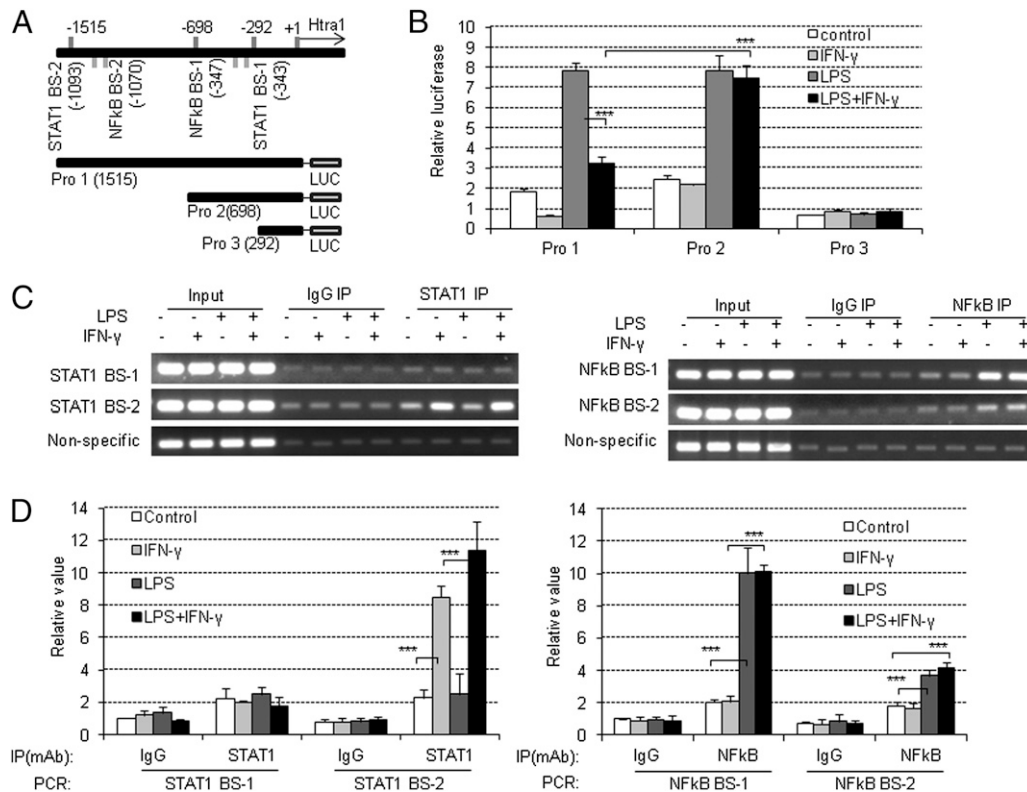


FIGURE 4. STAT1 directly binds to HTRA1 promoter range after IFN- γ treatment. **(A)** The STAT1 and NF- κ B binding sites in the HTRA1 promoter range are as indicated in the schematic diagram as well as the mouse HTRA1 promoter constructs for the reporter gene and ChIP assay. **(B)** Luciferase activity was determined using the Dual-Luciferase reporter assay system as described in *Materials and Methods*. **(C and D)** After RAW264.7 cells were treated with LPS and/or IFN- γ for 6 h, ChIP assays were performed using either anti-STAT1 or anti-NF- κ B mAbs as described in *Materials and Methods*. ChIP DNA was analyzed using either PCR (C) or real-time PCR (D). Input DNA was used as an internal control, and the data are shown as the relative fold increase over IgG control samples. Data are shown as means \pm SD ($n = 3$), which represent one of two independent experiments with similar results. *** $p < 0.001$ for comparisons between indicated groups.

the regulatory effects of IFN- γ on HTRA1 expression in a standard CIA mouse model. We found that IFN- γ KO mice had a higher CIA incidence ($p < 0.001$; Fig. 5A) and suffered from more severe CIA, as evaluated by clinical arthritic score (data not shown), hindpaw thickness (Fig. 5B and data not shown), and histological tissue examination (Fig. 5C) compared with WT control mice. In contrast, we were unable to detect a difference in anti-collagen Abs between IFN- γ KO and WT mice except for anti-collagen IgG2a, an observation made in a previous study (data not shown) (6). Consistent with observed arthritis severity patterns, joint tissue from CIA IFN- γ KO mice expressed significantly higher HTRA1 mRNA and protein levels as compared with CIA WT mice ($p < 0.001$; Fig. 5D, 5E). To determine whether the enhanced HTRA1 expression in IFN- γ KO mice contributes to the increased incidence and severity, we used anti-HTRA1 Ab to neutralize HTRA1 in IFN- γ KO mice during induction of CIA. Injection of anti-HTRA1 Ab significantly decreased the CIA incidence and hindpaw thickness in IFN- γ KO mice ($p < 0.05$; Fig. 5F–H). Meanwhile, the arthritic score of the anti-HTRA1 Ab-treated CIA mice was significantly lower than for control CIA mice ($p < 0.01$; Fig. 5I). These data indicate that HTRA1 likely contributes to the protective roles of IFN- γ in the process of CIA in mice.

To determine the clinical application of these observations, we treated CIA B6 mice with a low dose of LPS and/or IFN- γ during CIA induction as described in *Materials and Methods*. LPS significantly increased CIA incidence and arthritis clinical and histological severity, whereas supplying exogenous IFN- γ clearly prevented CIA occurrence regardless of whether the mice were treated with or without LPS (Fig. 5J–L and data not shown).

Importantly, LPS treatment significantly enhanced HTRA1 mRNA and protein expression in joints, whereas IFN- γ efficiently inhibited CIA- or CIA plus LPS-induced HTRA1 mRNA and protein expression in joints ($p < 0.001$; Fig. 5M, 5N). Thus, LPS and IFN- γ regulate HTRA1 production in both naive and CIA mice in an opposing manner.

IFN- γ inhibits HTRA1 expression in human RA synovial cells

To assess whether the results detected in mouse cells were reproducible in humans, we used human RA synovial cells to determine the role of IFN- γ on HTRA1 expression. As shown in Fig. 6, LPS induced significantly more HTRA1 mRNA and protein expression (Fig. 6A, 6B), whereas IFN- γ significantly inhibited HTRA1 expression in basal or LPS-induced conditions (Fig. 6A, 6B). We also found that IFN- γ inhibits HTRA1 mRNA and protein expression in human synovial cells also mainly through the p38 MAPK/STAT1 pathway, consistent with our results in mouse cells (Fig. 6C–F).

Discussion

Our present studies show that LPS and IFN- γ exert profound effects on RA. Whereas LPS exacerbates RA, IFN- γ protects animals from developing RA. Both pathways converge on the expression of HTRA1, a powerful protease that is known in joint damage. IFN- γ inhibits the expression of HTRA1 via the p38 MAPK/STAT1 pathway. These findings reveal novel insights or new molecular mechanisms in the pathogenesis of RA. Thus, the cytokine milieu or the inflammatory status that influences the presence of TLR4 ligands may have a significant impact on the

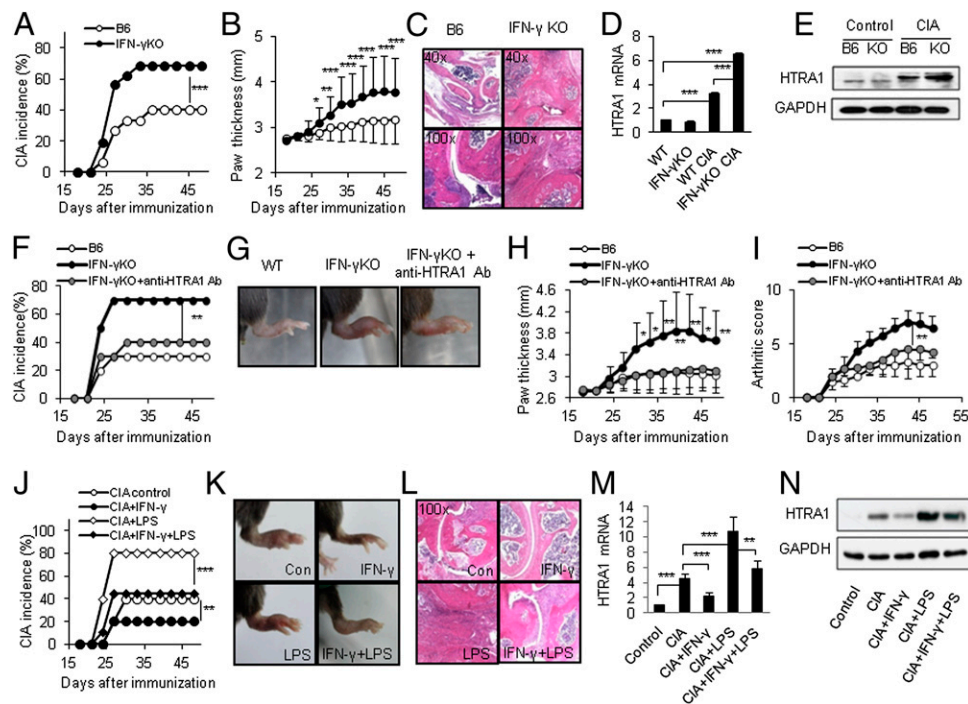


FIGURE 5. IFN- γ and LPS antagonistically regulate HTRA1 expression in the CIA mouse model. B6 and IFN- γ KO mice (A–E, $n = 15$ each) were induced for CIA as described in *Materials and Methods*. CIA incidence (A), hindpaw thickness (B), H&E staining of joint tissues (C), and HTRA1 mRNA (D) and protein (E) expression in joint tissues were determined. IFN- γ KO mice (J–L, $n = 10$ /group) were induced for CIA and treated with or without anti-HTRA1 Ab as described in *Materials and Methods*. CIA induced in B6 mice was used as an additional control. CIA incidence (F), imaging of the hindpaw (G), the summary of hindpaw thickness (H), and the arthritic score (I) of CIA mice are shown. B6 mice (J–L, $n = 10$ /group) were induced for CIA and treated with LPS and/or IFN- γ as described in *Materials and Methods*. CIA incidence (J), imaging of the hindpaw (K), and H&E staining of the joint tissues (L) were shown. HTRA1 mRNA levels in joint tissues (M) were determined by real-time PCR, and HTRA1 protein expression in joint tissues (N) was detected by Western blotting. Data are shown as mean \pm SD, which represent one of at least three independent experiments with similar results. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for comparisons between indicated groups or with the controls.

disease process. Additionally, absence of IFN- γ is as potent as LPS stimulation in promoting RA, indicating the biological protecting significance of endogenous IFN- γ in the LPS-promoted RA pathological process. Importantly, neutralization of HTRA1 by Ab significantly reversed the enhanced CIA frequency and severity in IFN- γ -deficient mice, indicating that HTRA1 likely contributes to the protective roles of IFN- γ in the process of CIA in mice.

With few exceptions, LPS and IFN- γ act cooperatively in macrophage function (40, 41). In our study, IFN- γ unexpectedly inhibited LPS-induced HTRA1 expression in both fibroblasts and macrophages. The inhibitory effect of IFN- γ on HTRA1 expression in joint tissues may be one of the reasons for the protective effect of IFN- γ against RA in mice and humans (4, 37). Furthermore, our studies showed that many downstream signaling molecules of major IFN- γ pathways, such as PI3K and ERK, are not involved in the inhibitory effect of IFN- γ on HTRA1 expression. We instead demonstrated that p38 MAPK/STAT1 is a key pathway for IFN- γ -mediated inhibition of HTRA1 expression. In this pathway, the transcription factor STAT1 binds directly to the HTRA1 promoter (AA-1093) and subsequently downregulates HTRA1 transcription. Additionally, the inhibitory effect of IFN- γ on HTRA1 expression is unlikely to be related to decreased NF- κ B binding to the HTRA1 promoter, as increased STAT1 binding to the HTRA1 promoter (AA-1093) did not cause significant alteration of NF- κ B binding to the promoter (AA-347). Identification of STAT1 as an important transcriptional inhibitor of HTRA1 expression offers a potential therapeutic target for RA and osteoarthritis.

Although the results of our study clearly delineate the function of TLR ligands and IFN- γ in RA, we recognize the complexity of RA as a disease entity and that TLR ligands and IFN- γ may be among

many contributing factors. A recent report implicated cytokines associated with Th17 cells, such as IL-17, IL-21, IL-22, and IL-23, in the pathogenesis of many human diseases, including RA (42). In our study, we failed to detect any effect of IL-17, IL-23, TGF- β 1, IL-1 β , IL-10, and IL-33 on HTRA1 expression, but we discovered that IL-21, IL-6, and IL-12 caused significantly more HTRA1 production in MEFs and macrophages. Based on these results, we propose that some proinflammatory cytokines such as IL-21, IL-6, and IL-12 might contribute to the RA pathogenesis, at least in part, via the HTRA1-dependent pathway.

TNF- α is a powerful NF- κ B activator (43). Despite activating the NF- κ B pathway, TNF- α did not induce HTRA1 expression in our experiments. Although this finding may appear to conflict with our observation of LPS induction of HTRA1 expression through the NF- κ B pathway, we think that these results can be explained by the role TNF- α plays in activating the p38 MAPK/STAT1 pathway (44). As demonstrated in our study, activation of the p38 MAPK/STAT1 pathway strongly inhibits HTRA1 expression. Therefore, simultaneous activation of the p38 MAPK/STAT1 pathway and classical NF- κ B pathway by TNF- α may act antagonistically with regard to HTRA1 expression and could yield no detectable changes in HTRA1 production. This explanation is further supported by our observation that TNF- α efficiently induces HTRA1 expression in RAW264.7 cells transfected with p38 MAPK shRNA and cells treated with p38 MAPK and/or STAT1 inhibitor.

Our findings may be of significance in the understanding and treatment of other HTRA1-related processes such as age-related macular degeneration, cancer, and aging. One study reported that loss of HTRA1 in ovarian and gastric cancers contributes to

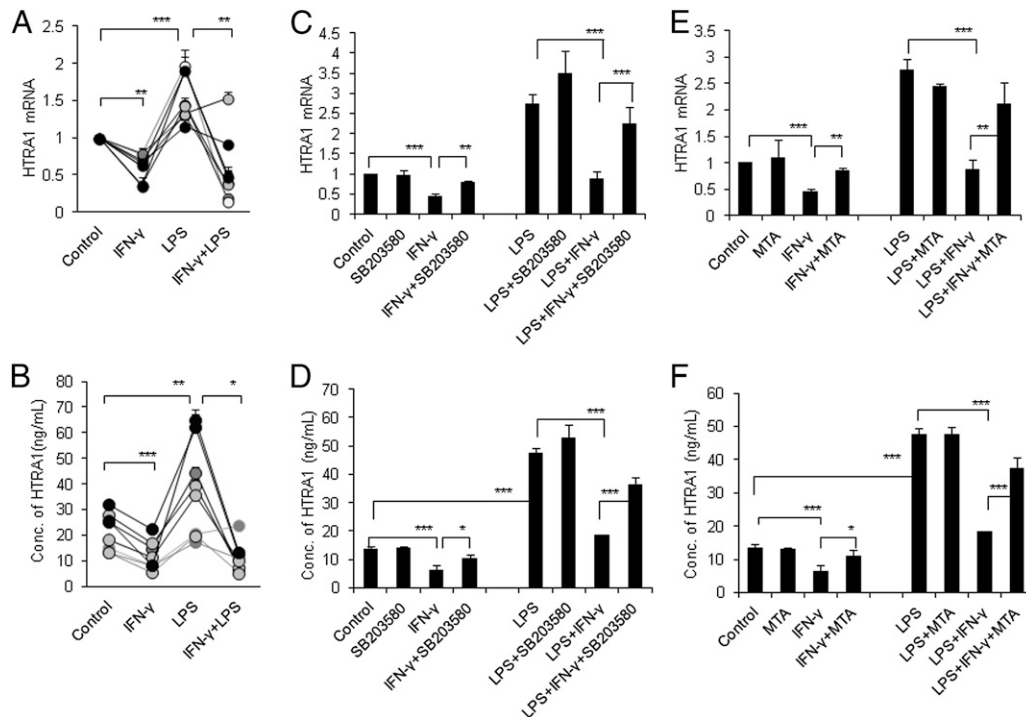


FIGURE 6. IFN- γ and LPS antagonistically regulate HTRA1 expression in human RA synovial cells via p38 MAPK/STAT1. Freshly isolated human RA synovial cells were cultured with LPS and/or IFN- γ for 24 h at concentrations described in *Materials and Methods*. HTRA1 mRNA levels (**A**) were determined by real-time PCR, and HTRA1 protein concentrations in the culture media (**B**) were measured by ELISA. Eight RA patients were studied individually. Freshly isolated human RA synovial cells were pretreated with p38 MAPK inhibitor (SB203580) for 30 min and then cocultured with LPS and/or IFN- γ for an additional 24 h. HTRA1 mRNA expression (**C**) and HTRA1 concentration in the medium (**D**) were determined by real-time PCR and ELISA assays. Freshly isolated human RA synovial cells were pretreated with STAT1 inhibitor (MTA) for 30 min and then cocultured with LPS and/or IFN- γ for an additional 24 h. HTRA1 mRNA expression (**E**) and HTRA1 concentration in the medium (**F**) were determined by real-time PCR and ELISA assays, respectively. Data are shown as means \pm SD. * p < 0.05, ** p < 0.01, *** p < 0.001 for comparisons between the indicated groups.

chemoresistance (24). Jones et al. (21) demonstrated that increased HTRA1 alone is sufficient to cause polypoidal choroidal vasculopathy and is a risk factor for choroidal neovascularization. Thus, the regulation of HTRA1 expression by TLR4 ligands and IFN- γ via their respective intracellular signaling pathways, the classical NF- κ B and p38 MAPK/STAT1 pathways, might also be important for other HTRA1-related pathological processes such as osteoarthritis, age-related macular degeneration, and cancer, which needs to be determined in the future.

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Disclosures

The authors have no financial conflicts of interest.

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