

IL-4–Stat6 Signaling Induces Tristetraprolin Expression and Inhibits TNF- α Production in Mast Cells

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Abstract

Increasing evidence has revealed that mast cell–derived tumor necrosis factor α (TNF- α) plays a critical role in a number of inflammatory responses by recruiting inflammatory leukocytes. In this paper, we investigated the regulatory role of interleukin 4 (IL-4) in TNF- α production in mast cells. IL-4 inhibited immunoglobulin E–induced TNF- α production and neutrophil recruitment in the peritoneal cavity in wild-type mice but not in signal transducers and activators of transcription 6 (Stat6)–deficient mice. IL-4 also inhibited TNF- α production in cultured mast cells by a Stat6-dependent mechanism. IL-4–Stat6 signaling induced TNF- α mRNA destabilization in an AU-rich element (ARE)–dependent manner, but did not affect TNF- α promoter activity. Furthermore, IL-4 induced the expression of tristetraprolin (TTP), an RNA-binding protein that promotes decay of ARE-containing mRNA, in mast cells by a Stat6-dependent mechanism, and the depletion of TTP expression by RNA interference prevented IL-4–induced down-regulation of TNF- α production in mast cells. These results suggest that IL-4–Stat6 signaling induces TTP expression and, thus, destabilizes TNF- α mRNA in an ARE-dependent manner.

Key words: mast cell–derived TNF- α • mRNA destabilization • RNA interference • AU-rich element • IgE

Introduction

Mast cells are one of the major producers of TNF- α (1–3), and mast cell–derived TNF- α has been shown to play a critical role in several inflammatory responses (4–10). Mast cells produce TNF- α upon stimulation via the high-affinity receptors for IgE (2, 3), and the mast cell–derived TNF- α is believed to be involved in the induction of IgE-dependent allergic inflammation (4, 11).

Recent works have revealed that mast cell–derived TNF- α also plays important roles in other inflammatory processes of both innate and acquired immune responses. It has been shown that mast cell–derived TNF- α is involved in the protection against gram-negative bacteria in experimental peritonitis (5, 6), immune complex–mediated peritonitis (7), T cell–mediated delayed-type hypersensitivity reaction (8), and autoantibody–induced arthritis (9, 10). Although TNF- α is beneficial in some situations such as bacterial infection (5, 6), an excess of TNF- α seems harmful in other situations (4, 7–10). Therefore,

the production of TNF- α should be tightly controlled in mast cells.

IL-4 is a multifunctional cytokine that plays a central role in causing allergic Th2-type immune responses (12, 13). Binding of IL-4 to IL-4R results in the activation of signal transducers and activators of transcription 6 (Stat6) and induces the expression of IL-4–inducible genes, including class II major histocompatibility molecules, low-affinity IgE receptor (CD23), and IL-4R α chain (12, 13). IL-4–Stat6 signaling plays a central role in the commitment of CD4⁺ T cells to the Th2 phenotype and IgE isotype switching in B cells (12, 13). On the other hand, it has been shown that IL-4–Stat6 signaling enhances IL-10–induced apoptosis of IL-3–dependent mast cells (14) and decreases the expression of IgE receptors on mast cells (15). However, the regulatory role of IL-4 in TNF- α production in mast cells is still largely unknown.

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Abbreviations used in this paper: ARE, AU-rich element; DOX, doxycycline; GFP, green fluorescent protein; HSA, human serum albumin; MAP, mitogen-activated protein; RNAi, RNA interference; shRNA, short hairpin RNA; Stat6, signal transducers and activators of transcription 6; TTP, tristetraprolin.

In this paper, we investigated the molecular basis for IL-4-induced regulation of TNF- α production in mast cells. We found that IL-4-Stat6 signaling down-regulated TNF- α production in mast cells by destabilizing mRNA in an AU-rich element (ARE)-dependent manner. We also found that IL-4 induced the expression of tristetraprolin (TTP), an RNA-binding zinc-finger protein that promotes decay of ARE-containing mRNAs (16–18), by a Stat6-dependent mechanism and that the depletion of TTP by RNA interference (RNAi) prevented IL-4-induced down-regulation of TNF- α production. Our results indicate that IL-4-Stat6 signaling induces TTP expression and subsequent ARE-dependent mRNA destabilization, resulting in the down-regulation of TNF- α production in mast cells.

Materials and Methods

Mice. Stat6-deficient (Stat6^{-/-}) mice (19) were backcrossed for more than eight generations onto C57BL/6 mice (Japan SLC) or BALB/c mice (Charles River Laboratories), and the littermate WT mice were used as controls. C57BL/6 background mice were used except for the experiments of IgE-dependent late-phase reactions. Stat6^{-/-} mice were obtained from S. Akira and K. Takeda (both from Research Institute for Microbial Diseases, Osaka University, Osaka, Japan). Mice were housed in microisolator cages under pathogen-free conditions. All experiments were performed according to the guidelines of Chiba University.

Constructs. COOH-terminal-truncated Stat6 mutant at amino acid 673 (673 Stat6) was described previously (20). A constitutively active form of Stat6 (Stat6VT; reference 21), which has two alanine substitutions at amino acids 547 and 548, was generated using a PCR-based site-directed mutagenesis kit according to the manufacturer's instruction (Stratagene). All mutations were confirmed by DNA sequencing.

Cell Culture. BMMCs were prepared and maintained as described previously (20). More than 98% of cells obtained after 4 wk of culture were morphologically mast cells and positive for c-kit expression. CFTL-15 cells (obtained from M.A. Brown, Emory University School of Medicine, Atlanta, GA), a murine mast cell line, were cultured in RPMI 1640 medium containing 10% heat-inactivated FCS, 50 mM β -mercaptoethanol, 2 mM L-glutamine, antibiotics, and 10% (vol/vol) murine IL-3 transfectant X63 cell-conditioned medium as a source of IL-3 (complete RPMI 1640 medium). X63-IL-3 cells were obtained from H. Karasuyama (Tokyo Metropolitan Organization of Medical Science, Tokyo, Japan).

Stimulation of BMMCs and CFTL-15 Cells. For stimulation of BMMCs via Fc ϵ receptors, BMMCs were incubated with 1 μ g/ml mouse anti-DNP IgE (YAMASA) at 37°C for 2 h, washed twice with RPMI 1640 medium, and incubated with 50 ng/ml DNP-HSA (human serum albumin; Sigma-Aldrich) at 37°C for 6 or 24 h. In some experiments, BMMCs or CFTL-15 cells were stimulated with 500 ng/ml A23187 (Sigma-Aldrich) at 37°C for 24 h. Where indicated, 10 ng/ml murine recombinant IL-4 (R&D Systems) was added to the culture.

IgE-dependent Late-phase Reactions. IgE-dependent late-phase reactions in the mouse peritoneal cavity were induced as described previously (22). In brief, mouse anti-DNP IgE (100 μ g per mouse) or PBS (as a control) was injected intravenously to BALB/c mice or Stat6^{-/-} mice. Murine IL-4 (1 μ g per mouse) or PBS (as a control) was injected intraperitoneally to the mice

24 h after anti-DNP IgE or PBS injection. 1 h later, DNP-HSA (6 μ g in 0.2 ml of saline) or saline (as a control) was injected intraperitoneally to the mice. Peritoneal lavage was performed with 1 ml of ice-cold PBS 8 h after DNP-HSA injection. The number of total cells in the lavage fluid was counted with a hemocytometer, and differential cell counts were determined on the cytospin cell preparations stained with Wright-Giemsa solution. The amount of TNF- α in the peritoneal lavage fluid was determined by ELISA as described in the next paragraph.

Measurement of TNF- α by ELISA. The amount of TNF- α in the culture supernatant or in the peritoneal lavage fluid was measured using a murine TNF- α ELISA kit (BD Biosciences). The assay was performed in duplicate according to the manufacturer's instructions. The detection limit was 15 pg/ml.

Intracellular Staining for TNF- α . BMMCs were stimulated with IgE engagement or A23187 in the presence or absence of 10 ng/ml IL-4 at 37°C for 6 h, with 2 μ M monensin (Sigma-Aldrich) added for the final 4 h to prevent cytokine release. Cells were harvested, washed with PBS, and stained with anti-c-kit PE (2B8; BD Biosciences) for 30 min at 4°C. Cells were washed with PBS, fixed with IC Fix (Biosource International), permeabilized with IC Perm (Biosource International), and stained with anti-TNF- α allophycocyanin (MP6-XT22; BD Biosciences) for 30 min at 4°C. After washing, cells were analyzed on FACScalibur™ using CELLQuest™ software.

Retrovirus-mediated Gene Expression. To overcome the limited efficiency of transfection on BMMCs, we used a bicistronic retrovirus system, in which infected cells were identified by coexpressed green fluorescent protein (GFP; pMX-IRES-GFP vector; reference 23) or coexpressed Thy1.1 (MSCV-IRES-Thy1.1 vector, obtained from P. Marrack, National Jewish Medical and Research Center, Denver, CO; reference 24). pMX-IRES-GFP vector was obtained from T. Kitamura (Institute of Medical Science, University of Tokyo, Tokyo, Japan). Retroviral vectors pMX-WT Stat6-IRES-GFP, pMX-673 Stat6-IRES-GFP, and pMX-IRES-GFP (as a control) were described previously (20). Retrovirus-mediated gene expression for BMMCs was performed as described previously (20). Efficiency of infection to BMMCs was 8–15% in all viruses as assessed by FACS®.

CFTL-15 cells were infected with retroviruses of MSCV-Stat6VT-IRES-Thy1.1 or MSCV-IRES-Thy1.1 (as a control), and retrovirus-infected cells (Thy1.1⁺ cells) were purified by magnetic cell sorting as described previously (20). The purity of Thy1.1⁺ cells was routinely >95%.

TNF- α Promoter Assay. Reporter construct of TNF- α promoter (pGL3TNF; reference 25), in which full-length murine TNF- α promoter (26) drives the luciferase gene, was a gift from E.W. Gelfand (National Jewish Medical and Research Center, Denver, CO). CFTL-15 cells were transfected with pGL3TNF in the presence of pRL-TK (Promega) in 800 μ l of serum-free RPMI 1640 medium at 960 μ F/300 V. Where indicated, WT Stat6 expression vector (pcDNA3 Stat6), Stat6VT expression vector (pcDNA3 Stat6VT), or pcDNA3 (as a control) was cotransfected. After cells were cultured in complete RPMI 1640 medium at 37°C for 12 h, aliquoted cells were left treated or untreated with 10 ng/ml IL-4 for another 12 h. The luciferase activity was measured by the dual luciferase assay system (Promega) according to the manufacturer's instructions. Firefly luciferase activity of pGL3TNF was normalized by Renilla luciferase activity of pRL-TK. All values were obtained from experiments performed in triplicate and repeated at least three times.

Translational Activation Assay. 3'-UTR of TNF- α mRNA (709–1463 from the translation start site) was inserted into XbaI-

BamHI site of pGL3-promoter vector (Promega) to construct pGL3 TNF ARE(+). pGL3 TNF ARE(-), in which a 69-bp element (from 1128 to 1196) containing six reiterated repeats of the (T)TAAAT(AT) motif was deleted from pGL3 TNF ARE(+), was generated by site-directed deletion as described previously (27). CFTL-15 cells were transfected with either pGL3 TNF ARE(+) or pGL3 TNF ARE(-) in the presence of pRL-TK at 960 μ F/300 V. Where indicated, pcDNA3 Stat6, pcDNA3 Stat6VT, or pcDNA3 was cotransfected. After cells were cultured in complete RPMI 1640 medium at 37°C for 12 h, aliquoted cells were left treated or untreated with 10 ng/ml IL-4 for another 12 h. Firefly luciferase activity of pGL3 TNF ARE(+) or pGL3 TNF ARE(-) was normalized by Renilla luciferase activity of pRL-TK.

Analysis of mRNA Decay. pTet-BBB vector, in which tet-responsive element drives rabbit β -globin transcription, and pTet-BBB ARE^{TNF}, in which TNF- α ARE is inserted to pTet-BBB vector at downstream of rabbit β -globin gene, were gifts from A.B. Shyu (The University of Texas Houston Medical School, Houston, Texas; reference 28). To measure the rate of mRNA decay, we modified the experimental system that was developed by Loflin et al. (28). In brief, CFTL-15 cells were first infected with MSCV-Stat6VT-IRES-Thy1.1 retrovirus or MSCV-IRES-Thy1.1 retrovirus (as a control). Infected cells (Thy1.1⁺ cells) were purified by magnetic cell sorting and transfected with pTet-BBB ARE^{TNF} or pTet-BBB in the presence of pTet-Off vector,

which expresses the tet-responsive transcriptional activator (BD Biosciences and CLONTECH Laboratories, Inc.). G418-resistant clones were selected by limiting dilution and the presence of pTet-Off as well as pTet-BBB ARE^{TNF} or pTet-BBB vector in the clone was confirmed by PCR. These clones were cultured in the presence of 100 ng/ml doxycycline (DOX) for 16 h, and DOX was removed from the culture for 4 h to resume transcription from pTet-BBB ARE^{TNF} or pTet-BBB. These clones were added with DOX to block further transcription. At indicated times after the addition of DOX, total RNA was isolated and the amount of rabbit β -globin mRNA was determined by Taqman PCR analysis using ABI PRISM 7000 (Applied Biosystems). The following primers and a fluorogenic probe were used: sense primer, 5'-TCGCTGCAAATGCTGTTATGAAC-3'; antisense primer, 5'-GAATTCTTTGCCAAAATGATGAGA-3'; and probe, 5'-FAM-CTGGACAACCTCAAG-MGB-3'. The levels of rabbit β -globin mRNA were normalized to the levels of glyceraldehyde-3-phosphate dehydrogenase mRNA (Applied Biosystems).

RT-PCR Assay. Total RNA was prepared and RT-PCR was performed as described previously (29). PCR primers for TTP cDNA were used as follows: 5'-TCTCTGCCATCTAC-GAGAGCCTC-3' and 5'-GCTGATGCTTTGTCCGAGCATATG-3'. RT-PCR for β -actin was performed as a control. All PCR amplifications were performed at least three times with multiple sets of experimental RNAs.

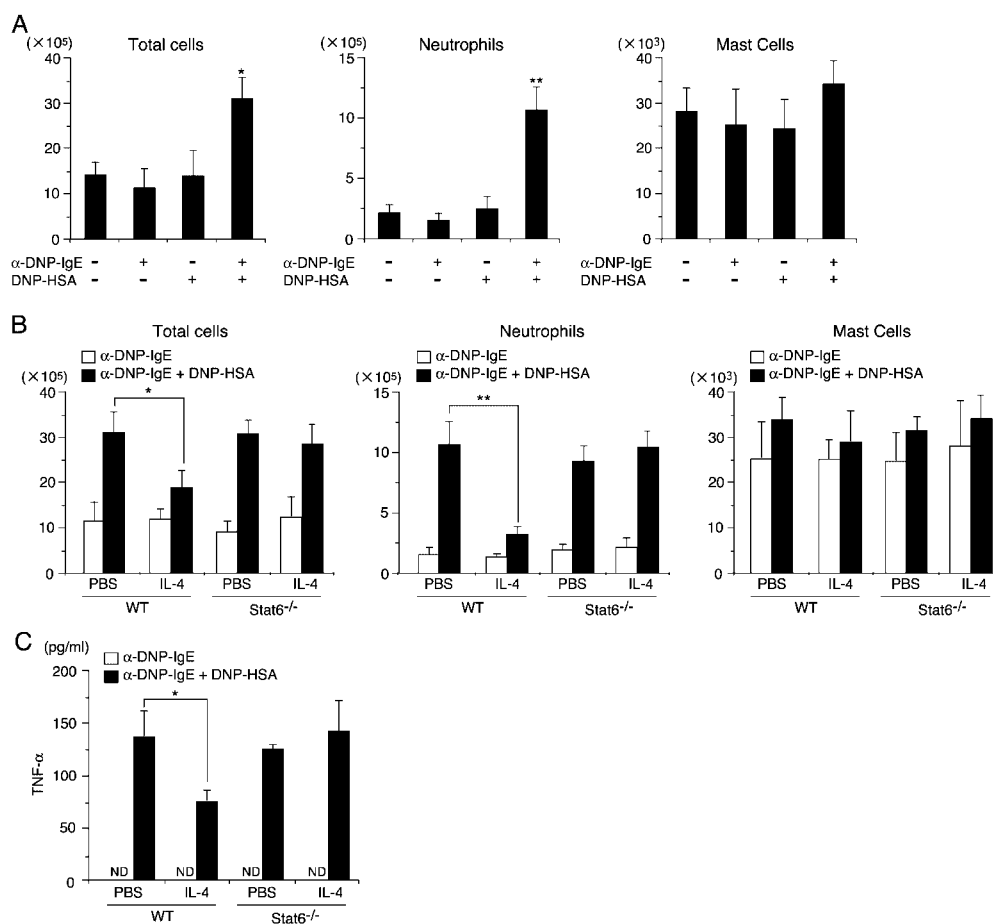


Figure 1. IL-4 inhibits IgE-induced neutrophil recruitment into the peritoneal cavity through a Stat6-dependent mechanism. (A) IgE engagement induces neutrophil recruitment into the peritoneal cavity. Anti-DNP IgE or PBS (as a control) was first injected intravenously to BALB/c mice. DNP-HSA or saline (as a control) was injected intraperitoneally to the mice 25 h after anti-DNP IgE sensitization. 8 h after DNP-HSA injection, the number of total cells, neutrophils, and mast cells in the peritoneal lavage fluid was determined. Data are mean \pm SD from five mice in each group. *, Significantly different from the mean value of control group (saline injection). *, $P < 0.05$. **, $P < 0.01$. (B and C) IL-4 inhibits IgE-induced neutrophil recruitment through a Stat6-dependent mechanism. Similar to A, anti-DNP IgE or PBS was injected intravenously to Stat6-deficient (Stat6^{-/-}) mice or the littermate wild-type (WT) mice. 1 μ g recombinant IL-4 or PBS (as a control) was injected intraperitoneally to the mice 24 h after anti-DNP IgE sensitization. 1 h later, DNP-HSA or saline was injected intraperitoneally to the mice. 8 h after DNP-HSA injection, the number of total cells, neutrophils, and mast cells (B) as well as the amount of TNF- α (C) in the peritoneal lavage fluid was determined. Data are mean \pm SD from four mice in each group. ND, not detectable. *, Significantly different from the mean value of control group (PBS). *, $P < 0.05$. **, $P < 0.01$.

Immunoblotting. Whole cell extracts were prepared and immunoblotting was performed as described previously (20). Antiserum to TTP (H-120) was purchased from Santa Cruz Biotechnology, Inc.

TTP Promoter Assay. Murine TTP promoter, either -691 to $+59$ or -524 to $+59$, was amplified by PCR using a 2.1-kb fragment of murine TTP promoter (a gift from P.J. Blackshear, National Institute of Environmental Health Sciences, Research Triangle Park, NC; reference 30) as a template and inserted into KpnI-XhoI site of pGL3-basic vector (Promega) to generate TTP-691Luc or TTP-524Luc. TTP-691mtLuc, in which Stat6 binding site (TTCctaaGAA from -576 to -567) was mutated to TTTctaaGAA, was generated using a PCR-based site-directed mutagenesis kit (Stratagene). CFTL-15 cells were infected with MSCV-Stat6VT-IRES-Thy1.1 retrovirus or MSCV-IRES-Thy1.1 retrovirus (as a control) and infected cells (Thy1.1⁺ cells) were purified by magnetic cell sorting. The purified cells were transfected with either TTP-691Luc, TTP-524Luc, or TTP-691mtLuc in the presence of pRL-TK at $960 \mu\text{F}/300 \text{V}$. After cells were cultured in complete RPMI 1640 medium at 37°C for 24 h, firefly luciferase activity of TTP-691Luc, TTP-524Luc, or TTP-691mtLuc was measured and normalized by Renilla luciferase activity of pRL-TK.

TTP Short Hairpin RNA (shRNA)-expressing CFTL-15 Cells. To deplete the expression of TTP, we used RNAi with shRNA expression vector (pSuppressor Neo; Imgenex). Targets of small interfering RNA are as follows: TTP shRNA A (5'-ACTCG-GACTCCATCCCGTCT-3', corresponding to 160–179 of the murine TTP mRNA); TTP shRNA B (5'-GCATCAGCT-TCTCCGGCTT-3', corresponding to 586–605); and TTP

shRNA C (5'-GAGACCCTAACCAGGCCTG-3', corresponding to 763–781). We confirmed that all sequences were unique for TTP (unpublished data). Construction of shRNA expression vectors was performed according to the manufacturer's instruction. CFTL-15 cells were transfected with these vectors and cultured with 0.8 mg/ml G418 at 37°C for 14 d. G418-resistant clones were selected by limiting dilution.

Data Analysis. Data are summarized as mean \pm SD. The statistical analysis of the results was performed by the unpaired Student's *t* test. *p*-values < 0.05 were considered significant.

Results

IL-4 Inhibits IgE-induced TNF- α Production and Neutrophil Recruitment through a Stat6-dependent Mechanism. First, we investigated the role of IL-4–Stat6 signaling in IgE-mediated inflammatory responses in vivo. We used a murine model of IgE-dependent late-phase reaction, in which neutrophil recruitment is induced into the peritoneal cavity upon IgE engagement through the activation of mast cells (22). Mice were passively sensitized with anti-DNP IgE, and subsequently IgE was engaged by an intraperitoneal injection of DNP-HSA. As shown in Fig. 1 A, at 8 h after DNP-HSA injection, the number of leukocytes in the peritoneal cavity was increased in the mice that were sensitized with anti-DNP IgE. The number of neutrophils recovered from the peritoneal cavity was significantly increased by DNP-HSA injection in sensitized mice (saline

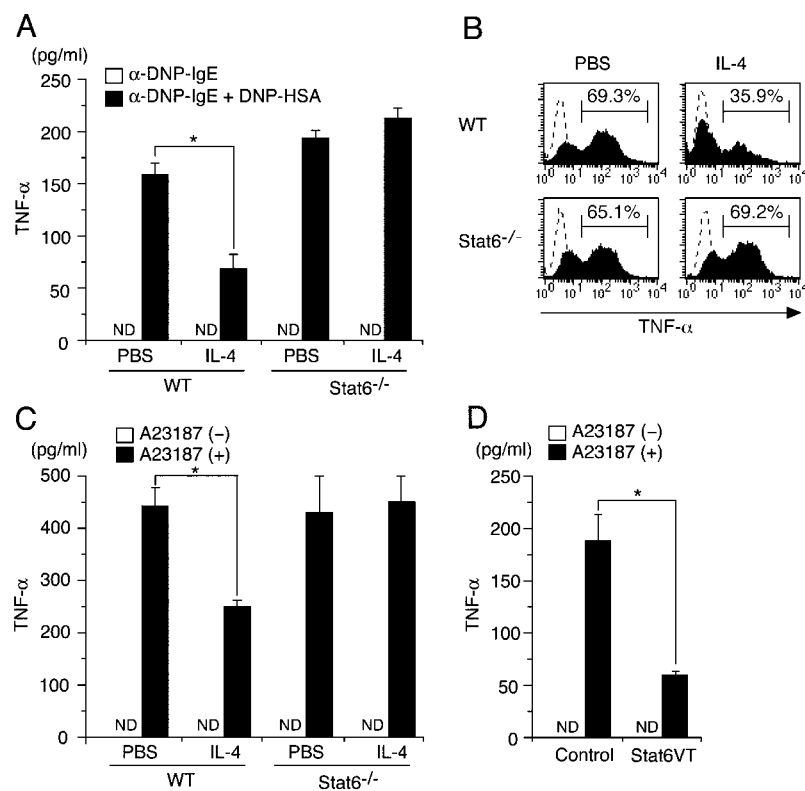


Figure 2. IL-4–Stat6 signaling inhibits TNF- α production in mast cells. (A) IL-4 inhibits IgE-induced TNF- α release from WT BMMCs but not from Stat6^{-/-} BMMCs. WT BMMCs or Stat6^{-/-} BMMCs were incubated with anti-DNP IgE for 2 h, and surface IgE was cross-linked with or without DNP-HSA in the presence or absence of 10 ng/ml IL-4. 24 h later, the amount of TNF- α in the supernatant was measured by ELISA. Data are mean \pm SD from five independent experiments. ND, not detectable. *, Significantly different from the mean value of control group (PBS). *, *P* < 0.01. (B) IL-4 inhibits IgE-induced TNF- α production from WT BMMCs but not from Stat6^{-/-} BMMCs. WT BMMCs or Stat6^{-/-} BMMCs were incubated with anti-DNP IgE and surface IgE was cross-linked with or without DNP-HSA for 6 h in the presence or absence of 10 ng/ml IL-4. Intracellular TNF- α was analyzed on FACS[®]. Representative FACS[®] profiles from five independent experiments are shown. (C) IL-4 inhibits A23187-induced TNF- α production from WT BMMCs but not from Stat6^{-/-} BMMCs. WT BMMCs or Stat6^{-/-} BMMCs were stimulated with or without 500 ng/ml A23187 in the presence or absence of 10 ng/ml IL-4. 24 h later, the amount of TNF- α in the supernatant was measured by ELISA. Data are mean \pm SD from five independent experiments. ND, not detectable. *, Significantly different from the mean value of control group (PBS). *, *P* < 0.01. (D) Expression of constitutively active Stat6 (Stat6VT) inhibits TNF- α production. CFTL-15 cells were infected with MSCV-Stat6VT-IRES-Thy1.1 or MSCV-IRES-Thy1.1 (as a control) retroviruses and infected cells (Thy1.1⁺ cells) were

sorted by MACS. Sorted cells were stimulated with or without 500 ng/ml A23187 for 24 h and the amount of TNF- α in the supernatant was measured by ELISA. Data are mean \pm SD from five independent experiments. ND, not detectable. *, *P* < 0.01.

[2.0 ± 0.4] vs. DNP-HSA [10.4 ± 2.4 × 10⁵]; mean ± SD; *n* = 5; *P* < 0.01) (Fig. 1 A), whereas the number of mast cells was not affected by DNP-HSA injection (Fig. 1 A). IL-4 significantly inhibited IgE-induced neutrophil recruitment in the peritoneal cavity by 67% in WT mice without affecting the number of mast cells (*n* = 4; *P* < 0.01) (Fig. 1 B). By contrast, IL-4 did not inhibit IgE-induced neutrophil recruitment in Stat6^{-/-} mice (Fig. 1 B). IL-4 also inhibited IgE-induced TNF-α production in the peritoneal cavity in WT mice but not in Stat6^{-/-} mice (Fig. 1 C). These results suggest that IL-4–Stat6 signaling inhibits TNF-α production and neutrophil recruitment during IgE- and mast cell-dependent late-phase reactions.

IL-4 Inhibits TNF-α Production in Mast Cells by a Stat6-dependent Mechanism. To determine whether IL-4 down-regulates TNF-α production in mast cells and whether Stat6 is involved in this regulation, next we examined the effect of IL-4 on IgE-induced TNF-α production in WT or Stat6^{-/-} BMMCs. When IgE receptors on WT BMMCs were engaged with anti-DNP IgE plus DNP-HSA, a considerable amount of TNF-α was released (Fig. 2 A). Stat6^{-/-} BMMCs also released a comparable amount of TNF-α to WT BMMCs upon IgE engagement (Fig. 2 A). IL-4 significantly inhibited IgE-induced TNF-α release from WT BMMCs by 57% (PBS [158.5 ± 11.0] vs. IL-4 [68.2 ± 13.7 pg/ml]; *n* = 5; *P* < 0.01) (Fig. 2 A), whereas IL-4 did not inhibit IgE-induced TNF-α release from Stat6^{-/-} BMMCs (PBS [192.9 ± 7.96] vs. IL-4 [211.3 ± 10.3 pg/ml]; *n* = 5) (Fig. 2 A). We evaluated TNF-α production of BMMCs using intracellular staining, which enabled us to measure de novo TNF-α production by adding monensin 2 h after IgE stimulation. As shown in Fig. 2 B, IL-4 decreased the number of TNF-α producing cells in IgE-stimulated WT BMMCs (PBS [69.3%] vs. IL-4 [35.9%]; percentage TNF-α-producing

cells; representative data from five independent experiments) but not in IgE-stimulated Stat6^{-/-} BMMCs (PBS [65.1%] vs. IL-4 [69.2%]; *n* = 5). These results suggest that IL-4 inhibits de novo TNF-α production in mast cells by a Stat6-dependent mechanism.

Because it has been demonstrated that IL-4–Stat6 signaling down-regulates IgE receptors on mast cells (15), it is possible that IL-4-induced down-regulation of TNF-α production in IgE-stimulated BMMCs results from the down-regulation of IgE receptors. To exclude this possibility, WT BMMCs or Stat6^{-/-} BMMCs were stimulated with calcium ionophore (A23187), which mimics IgE-mediated activation, in the presence or absence of IL-4, and the levels of TNF-α in the supernatant were determined. Again, IL-4 significantly inhibited TNF-α production from A23187-stimulated WT BMMCs (PBS [440.6 ± 34.5] vs. IL-4 [247.8 ± 14.6 pg/ml]; *n* = 5; *P* < 0.01) (Fig. 2 C) but not from Stat6^{-/-} BMMCs (PBS [427.5 ± 74.6] vs. IL-4 [449.2 ± 54.5 pg/ml]; *n* = 5) (Fig. 2 C). Intracellular TNF-α staining confirmed that IL-4 inhibited TNF-α production from A23187-stimulated WT BMMCs (unpublished data). IL-4 also inhibited TNF-α production from LPS-stimulated WT BMMCs but not from LPS-stimulated Stat6^{-/-} BMMCs (unpublished data).

Next, we examined whether Stat6 activation is sufficient to down-regulate TNF-α production in mast cells using a constitutively active form of Stat6 (Stat6VT) (21). As shown in Fig. 2 D, when Stat6VT was expressed in a mast cell line (CFTL-15 cells) using retroviruses, Stat6VT significantly inhibited A23187-induced TNF-α production (control virus [186.3 ± 26.0] vs. Stat6VT virus [58.6 ± 2.83 pg/ml]; *n* = 5; *P* < 0.01). These results indicate that Stat6 activation is sufficient for the down-regulation of TNF-α production in mast cells.

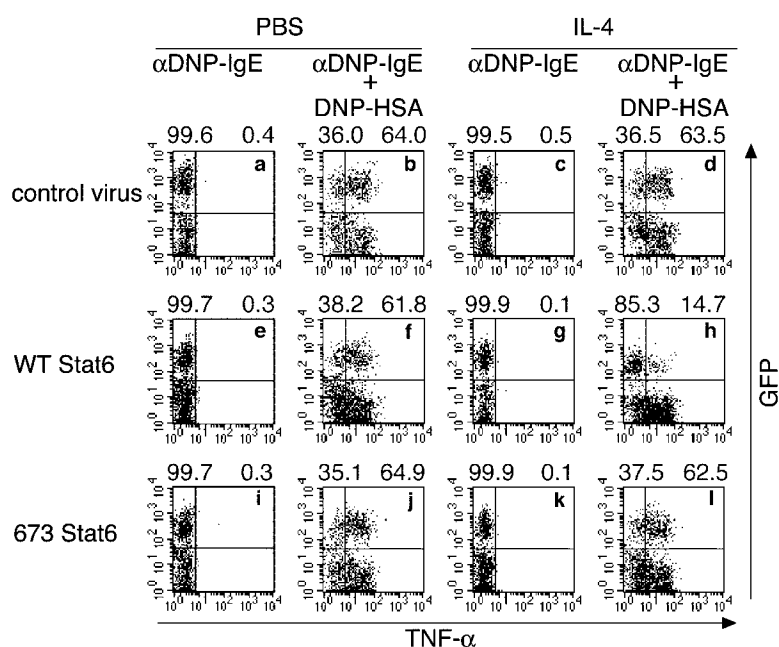


Figure 3. The transcriptional activation domain of Stat6 is essential for IL-4-induced down-regulation of TNF-α production. Stat6^{-/-} BMMCs were infected with retroviruses of pMX-WT Stat6-IRES-GFP, pMX-673 Stat6-IRES-GFP, or pMX-IRES-GFP (as a control). Cells were stimulated with anti-DNP IgE alone or anti-DNP IgE plus DNP-HSA for 6 h in the presence or absence of 10 ng/ml IL-4. Intracellular TNF-α was evaluated together with GFP by FACS[®]. Representative FACS[®] profiles from five independent experiments are shown.

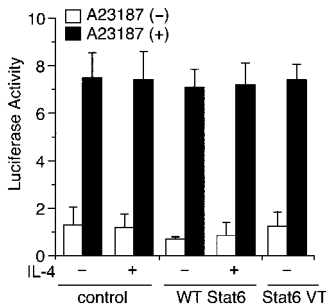


Figure 4. IL-4–Stat6 signaling does not inhibit TNF- α promoter activity. CFTL-15 cells were transfected with pGL3TNF and pRL-TK in the presence of pcDNA3 Stat6, pcDNA3 Stat6VT, or pcDNA3 (as a control). 12 h later, cells were stimulated with or without 500 ng/ml A23187 in the presence or absence of 10 ng/ml IL-4. The luciferase activity was measured by the dual luciferase reporter system another 12 h later. Data are mean \pm SD from five independent experiments.

Transcriptional Activation Domain of Stat6 Is Required for IL-4–induced Down-regulation of TNF- α Production. To determine whether transcriptional activity of Stat6 is required for IL-4–induced down-regulation of TNF- α production, we examined the effect of IL-4 on Stat6^{-/-} BMMCs that were reconstituted with WT Stat6 or 673 Stat6 (20), which lacks the transactivation domain. Stat6^{-/-} BMMCs were infected with retroviruses of pMX-WT Stat6-IRES-GFP, pMX-673 Stat6-IRES-GFP, or pMX-IRES-GFP (as a control) and stimulated with IgE engagement (anti-DNP IgE + DNP-HSA) in the presence or absence of IL-4. Consistent with the data depicted in Fig. 2 (A and B), IL-4 inhibited IgE-induced TNF- α production in WT Stat6-expressing Stat6^{-/-} BMMCs (61.8 vs. 14.7%; percentage TNF- α -producing cells in infected GFP⁺ populations) (Fig. 3, f vs. h). By contrast, IL-4 did not inhibit IgE-induced TNF- α production in 673 Stat6-expressing Stat6^{-/-} BMMCs (64.9 vs. 62.5%) (Fig. 3, j vs. l) or in control Stat6^{-/-} BMMCs (64.0 vs. 63.5%) (Fig. 3, b vs. d). In noninfected populations (GFP⁻ cells), IL-4 did not inhibit IgE-induced TNF- α productions even when WT Stat6-expressing cells coexisted in the culture (Fig. 3, f vs. h). These results indicate that the transcriptional activity of Stat6 is required for IL-4–induced down-regulation of TNF- α production.

IL-4 Does Not Inhibit Transcription from TNF- α Promoter. To further address molecular mechanisms of IL-4–induced down-regulation of TNF- α production in mast cells, we next examined the effect of IL-4 on TNF- α promoter activity. In this experiment, pGL3TNF (25) that contains full-length murine TNF- α promoter (26) was used as a reporter construct. CFTL-15 cells were transfected with pGL3TNF and the effect of IL-4 on A23187-induced transcription of pGL3TNF was examined. On the contrary to our expectation, IL-4 did not inhibit A23187-induced transcription of pGL3TNF (Fig. 4). IL-4 did not inhibit the transcription of pGL3TNF, even when WT Stat6 was coexpressed in CFTL-15 cells (Fig. 4). In addition, the expression of Stat6VT did not inhibit A23187-induced transcription of pGL3TNF (Fig. 4), although Stat6VT did inhibit A23187-induced TNF- α production in CFTL-15 cells (Fig. 2 D). These results suggest that IL-4–Stat6 signaling does not inhibit TNF- α promoter activity.

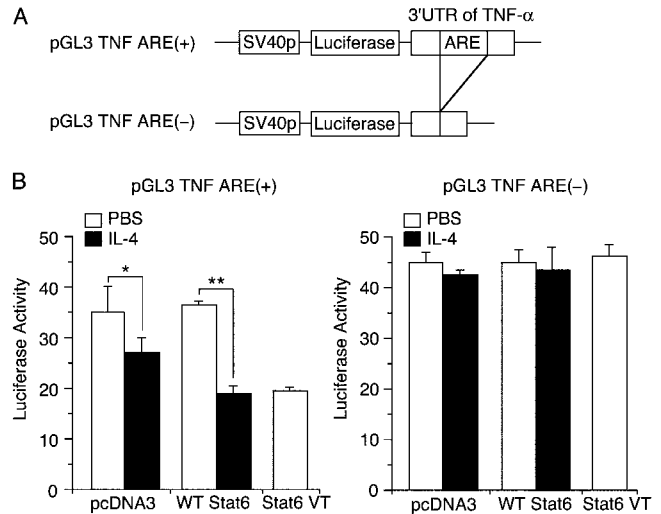


Figure 5. ARE in 3'UTR of TNF- α mRNA is essential for IL-4–induced down-regulation of TNF- α production. (A) Schema of reporter constructs of pGL3 TNF ARE(+) and pGL3 TNF ARE(-). (B) IL-4 inhibits the luciferase activity of pGL3 TNF ARE(+) but not of pGL3 TNF ARE(-) by a Stat6-dependent mechanism. CFTL-15 cells were transfected with pRL-TK and either pGL3 TNF ARE(+) or pGL3 TNF ARE(-) in the presence of pcDNA3 Stat6, pcDNA3 Stat6VT, or pcDNA3 (as a control). 12 h later, cells were stimulated with or without 10 ng/ml IL-4. The luciferase activity was measured by the dual luciferase reporter system another 12 h later. Data are mean \pm SD from five independent experiments. *, $P < 0.05$. **, $P < 0.01$.

IL-4–Stat6 Signaling Down-regulates TNF- α mRNA Stability by an ARE-dependent Mechanism. The amount of mRNA is controlled not only by the de novo transcription but also by the stability of mRNA (18, 31). Given that the AREs residing in the 3'UTR of TNF- α mRNA has been shown to be important for the regulation of gene expression (27, 32, 33), next we examined whether the ARE is involved in IL-4–induced down-regulation of TNF- α production in mast cells. We prepared two reporter constructs: pGL3 TNF ARE(+), in which 3'UTR of TNF- α mRNA was inserted just after the luciferase gene of pGL3-promoter vector and pGL3 TNF ARE(-), in which 69 bp of ARE was deleted from pGL3 TNF ARE(+) (Fig. 5 A). CFTL-15 cells were transfected with pGL3 TNF ARE(+) or pGL3 TNF ARE(-) in the presence of pcDNA3 Stat6, pcDNA3 Stat6VT, or pcDNA3 (as a control) and stimulated with or without IL-4. As shown in Fig. 5 B, IL-4 inhibited the luciferase activity of pGL3 TNF ARE(+). IL-4–induced down-regulation of pGL3 TNF ARE(+) was more profound when WT Stat6 was coexpressed in CFTL-15 cells (Fig. 5 B). The expression of Stat6VT also inhibited the activity of pGL3 TNF ARE(+) even in the absence of IL-4 stimulation (Fig. 5 B). In contrast, IL-4 stimulation or the expression of Stat6VT did not inhibit the activity of pGL3 TNF ARE(-) (Fig. 5 B). These results suggest that ARE residing in the 3'UTR of TNF- α mRNA is involved in IL-4–induced down-regulation of TNF- α production in mast cells.

Because it has been demonstrated that the ARE in the 3'UTR of TNF- α mRNA controls both mRNA stability

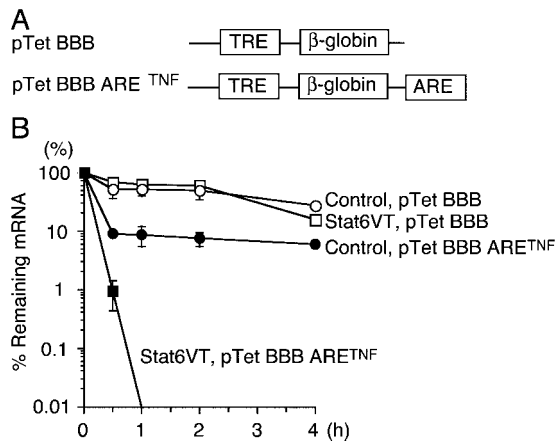


Figure 6. ARE in 3'UTR of TNF- α mRNA induces rapid decay of mRNA in Stat6VT expressing cells. (A) Schema of pTet-BBB ARE^{TNF} and pTet-BBB (28). (B) CFTL-15 cells were infected with MSCV-Stat6VT-IRES-Thy1.1 retrovirus or control MSCV-IRES-Thy1.1 retrovirus. Sorted Thy1.1⁺ cells were transfected with either pTet-BBB ARE^{TNF} or pTet-BBB in the presence of pTet-Off and G418-resistant clones were selected by limiting dilution. These clones were cultured in the absence of DOX for 4 h to resume transcription from pTet-BBB ARE^{TNF} or pTet-BBB, which was followed by the addition of 100 ng/ml DOX to block further transcription. At indicated times after the addition of DOX, total RNA was isolated and Taqman PCR analysis for rabbit β -globin and glyceraldehydes-3-phosphate dehydrogenase (as a control) was performed. Representative data from five independent experiments are shown.

and translation (27), next we examined the effect of Stat6 activation on the regulation of ARE-dependent mRNA stability using a more direct system established by Loflin et al. (28). CFTL-15 cells that were infected with Stat6VT retrovirus or control retrovirus were transfected with pTet-BBB ARE^{TNF} or pTet-BBB (Fig. 6 A) in the presence of pTet-Off vector. pTet-BBB ARE^{TNF}- or pTet-BBB-expressing cells were cultured in the absence of DOX for 4 h to resume transcription of rabbit β -globin from pTet-BBB ARE^{TNF} or pTet-BBB. After further transcription was blocked by adding DOX, the amount of rabbit β -globin mRNA was examined by Taqman PCR analysis (Fig. 6 B). Even in the absence of Stat6VT expression, the decay of rabbit β -globin mRNA was more rapid in pTet-BBB ARE^{TNF}-expressing cells than that in pTet-BBB-expressing cells (Fig. 6 B). Furthermore, the decay of rabbit β -globin mRNA was significantly enhanced by the expression of Stat6VT in pTet-BBB ARE^{TNF}-expressing cells (Fig. 6 B). On the other hand, the expression of Stat6VT did not affect the decay of rabbit β -globin mRNA in pTet-BBB-expressing cells (Fig. 6 B). These results indicate that Stat6 activation induces TNF- α mRNA destabilization in an ARE-dependent manner.

Stat6 Activation Induces the Expression of TTP in Mast Cells. Recently, it has been shown that TTP, the prototype member of a zinc-finger family of RNA binding proteins (16–18), regulates the expression of certain cytokines including TNF- α by destabilizing the mRNA in an ARE-dependent manner (16, 27, 33). To determine whether

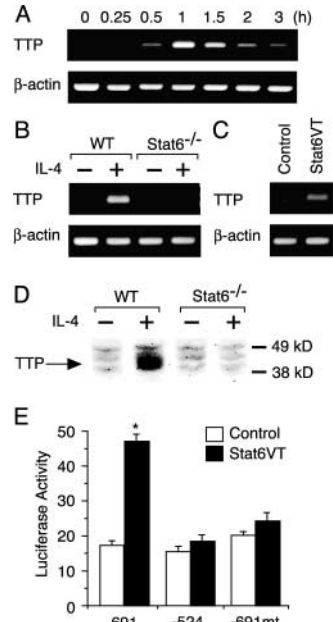


Figure 7. IL-4 induces the expression of tristetraprolin (TTP) by a Stat6-dependent mechanism. (A) IL-4 induces the expression of TTP mRNA in BMMCs. WT BMMCs were stimulated with 10 ng/ml IL-4 at 37°C. At indicated times after IL-4 stimulation, total RNA was prepared and RT-PCR for TTP and β -actin (as a control) was performed. Representative data from five independent experiments are shown. (B) IL-4-induced TTP mRNA expression requires Stat6. WT BMMCs or Stat6^{-/-} BMMCs were stimulated with 10 ng/ml IL-4 at 37°C. At indicated times after IL-4 stimulation, total RNA was prepared and RT-PCR for TTP and β -actin was performed. Representative data from five independent experiments are shown. (C) Stat6 activation is sufficient for the induction of TTP mRNA expression. CFTL-15 cells were infected with pMX-Stat6VT-IRES-Thy1.1 retrovirus or pMX-IRES-Thy1.1 retrovirus (as a control). Total RNA was prepared from sorted Thy1.1⁺ cells and subjected to RT-PCR analysis for TTP. Representative data from five independent experiments are shown. (D) IL-4 induces TTP expression at protein levels in WT BMMCs but not in Stat6^{-/-} BMMCs. WT BMMCs and Stat6^{-/-} BMMCs were stimulated with 10 ng/ml IL-4 for 4 h, and whole cell extracts were subjected to Western blotting with anti-TTP antibody. Representative data from four independent experiment are shown. (E) Stat6 directly activates TTP promoter in mast cells. CFTL-15 cells were infected with MSCV-Stat6VT-IRES-Thy1.1 retrovirus or MSCV-IRES-Thy1.1 retrovirus (as a control). Infected cells (Thy1.1⁺ cells) were purified and transfected with either TTP-691Luc, TTP-524Luc, or TTP-691mtLuc in the presence of pRL-TK. 24 h later, firefly luciferase activity of TTP-691Luc, TTP-524Luc, or TTP-691mtLuc was measured and normalized by Renilla luciferase activity of pRL-TK. Data are mean \pm SD of four independent experiments. *, P < 0.01.

TTP is involved in IL-4-induced down-regulation of TNF- α production, we first examined whether IL-4 induced the expression of TTP in mast cells. Interestingly, the expression of TTP mRNA was induced in WT BMMCs within 1 h after IL-4 stimulation (Fig. 7 A). However, the induction of TTP mRNA was absent in IL-4-stimulated Stat6^{-/-} BMMCs (Fig. 7 B), indicating that IL-4-induced TTP expression requires the presence of Stat6. Enforced expression of Stat6VT also induced TTP mRNA even in the absence of IL-4 stimulation (Fig. 7 C). IL-4-induced TTP expression was also detected at protein levels in WT BMMCs but not in Stat6^{-/-} BMMCs (Fig. 7 D).

We further examined whether Stat6-mediated TTP expression resulted from the direct activation of TTP promoter by Stat6. As shown in Fig. 7 E, TTP-691Luc, a reporter construct in which murine TTP promoter (-691 to +59) drives the luciferase gene, was significantly activated in CFTL-15 cells that expressed Stat6VT but not in control CFTL-15 cells (P < 0.01). In contrast, when the Stat6-binding site was mutated (TTP-691mtLuc), the expression of Stat6VT did not activate the reporter construct (Fig. 7 E). In addition, the expression of Stat6VT did not

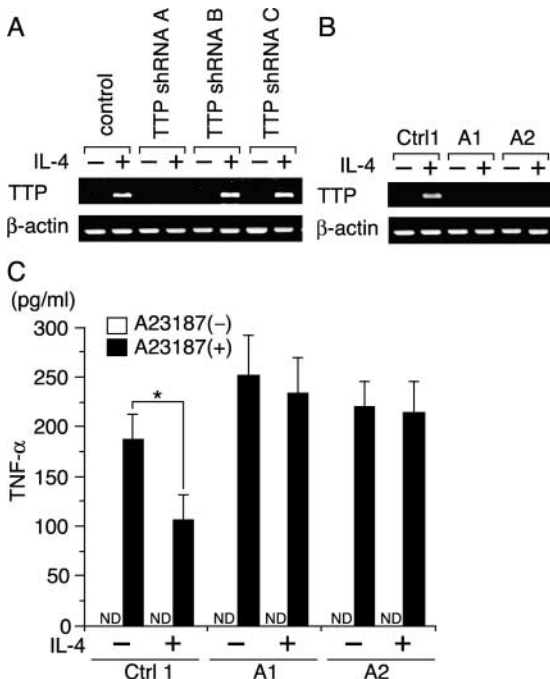


Figure 8. IL-4 does not inhibit TNF- α production in TTP-depleted cells. (A) TTP shRNA A inhibits IL-4-induced TTP expression. CFTL-15 cells were transfected with TTP short hairpin RNA (shRNA) vectors (TTP shRNA A, B, and C) or pSuppressor Neo (as a control) and cultured in the presence of IL-3 and G418 (0.8 mg/ml) for 14 d. G418-resistant cells were stimulated with or without IL-4 for 60 min, and the amount of TTP mRNA was evaluated by RT-PCR. Representative data from five independent experiments are shown. (B) IL-4-induced TTP expression is diminished in A1 and A2 cells. CFTL-15 cells were transfected with TTP shRNA A, and G418-resistant clones (A1 and A2) were selected by limiting dilution. As a control, CFTL-15 cells were transfected with pSuppressor Neo and G418-resistant clone (Ctrl 1) was selected by limiting dilution. These cells were stimulated with or without 10 ng/ml IL-4 for 60 min and the amount of TTP mRNA was evaluated by RT-PCR. Representative data from three independent experiments are shown. (C) IL-4 does not inhibit A23187-induced TNF- α production in A1 and A2 cells. A1 cells, A2 cells, and Ctrl1 cells were stimulated with or without 500 ng/ml A23187 for 24 h in the presence or absence of 10 ng/ml IL-4. The amounts of TNF- α in the supernatant were measured by ELISA. Data are mean \pm SD from five independent experiments. ND, not detectable. *, $P < 0.01$.

activate TTP-524Luc, a construct in which the Stat6-binding site was deleted (Fig. 7 E), although TTP-524Luc exhibited an equivalent baseline activity to TTP-691Luc (Fig. 7 E). These results suggest that the activated Stat6 directly induces the transcription from TTP promoter in mast cells.

TTP Is Required for IL-4-induced Down-regulation of TNF- α Production in Mast Cells. Finally, we examined the effect of TTP depletion on IL-4-induced down-regulation of TNF- α production in mast cells. We prepared several shRNA RNAi constructs and tested the efficiency of the depletion. As shown in Fig. 8 A, TTP shRNA A significantly inhibited the expression of TTP mRNA in IL-4-stimulated CFTL-15 cells. In contrast, TTP shRNA B or TTP shRNA C did not inhibit the expression of TTP mRNA at all (Fig. 8 A). We selected several clones that stably expressed TTP shRNA A and found that IL-4-induced

TTP expression was severely decreased in A1 and A2 cells (Fig. 8 B). In contrast, Ctrl 1 cells that were stably transfected with a control construct (pSuppressor Neo) expressed a significant amount of TTP mRNA upon IL-4 stimulation (Fig. 8 B). We compared the effect of IL-4 on A23187-induced TNF- α production in these clones. Interestingly, A1 and A2 cells, but not Ctrl1 cells, were resistant to IL-4-induced down-regulation of TNF- α production (Fig. 8 C). These results suggest that TTP is required for IL-4-induced down-regulation of TNF- α production from activated mast cells.

Discussion

In this paper, we show that IL-4 inhibits TNF- α production from activated mast cells through a Stat6-dependent TTP expression. First, we found that IL-4 inhibited TNF- α production in mast cells in vitro as well as in vivo by a Stat6-dependent mechanism (Figs. 1–3). Second, we found that IL-4-Stat6 signaling down-regulated TNF- α mRNA stability in an ARE-dependent manner (Figs. 5 and 6). Third, we found that IL-4 induced the expression of TTP, which promotes ARE-dependent mRNA destabilization (16–18), in mast cells by a Stat6-dependent mechanism (Fig. 7). Finally, depletion of TTP expression by RNAi blocked IL-4-induced down-regulation of TNF- α production in mast cells (Fig. 8). These results indicate that Stat6-induced TTP expression and subsequent ARE-dependent mRNA destabilization are responsible for IL-4-induced down-regulation of TNF- α production in mast cells.

We show that IL-4-Stat6 signaling inhibits TNF- α production from mast cells that are stimulated not only with IgE engagement (Figs. 1–3) but also with LPS stimulation (not depicted). The antiinflammatory properties of IL-4 are well recognized as important negative regulators of proinflammatory gene expression, especially in monocytes and macrophages (34). Thus, our results indicate that mast cells are also targets of IL-4 to function as an antiinflammatory cytokine. Our findings are consistent with a previous finding by Matsukawa et al. (35) that TNF- α production in the peritoneal cavity in experimental peritonitis, in which mast cell-derived TNF- α plays a critical role in the protection of bacterial infection (5, 6), is enhanced in Stat6^{-/-} mice.

We have found that transcriptional activity of Stat6 is required for IL-4-induced down-regulation of TNF- α production in mast cells (Fig. 3). In addition, we have found that the expression of D685A Stat6, which exhibits a stronger transcriptional activity than WT Stat6 in mast cells (20), enhances IL-4-induced down-regulation of TNF- α production in mast cells (unpublished data). We have also found that the expression of a constitutively active Stat6VT down-regulates TNF- α production in mast cells (Fig. 2). Although, in addition to Stat6, IL-4R mediates its responses through activation of other pathways, including insulin receptor substrate 1/2 (12), our results indicate that Stat6 is essential for IL-4-induced down-regulation of TNF- α production.

We demonstrate that IL-4-induced down-regulation of TNF- α production results from the ARE-dependent mRNA destabilization (Figs. 5 and 6), but not from the inhibition of TNF- α promoter activity (Fig. 4). Increasing evidence has shown that the presence of ARE in the 3'-UTR of transcripts is associated with the regulation of mRNA stability (16–18). Indeed, in the case of TNF- α , the importance of ARE-dependent mRNA destabilization has been demonstrated *in vitro* as well as *in vivo* (27, 33), although transcription (36), splicing (37), and protein processing (38) are also involved in TNF- α production. Because ARE is found in a number of genes (17, 18), it is plausible that IL-4 may inhibit the expression of some other genes through the destabilization of mRNA. This possibility is under investigation in our laboratory.

We show that IL-4-Stat6 signaling induces the expression of TTP in mast cells through Stat6-mediated activation of TTP promoter (Fig. 7). Therefore, together with the findings of IL-4-induced TTP-dependent down-regulation of TNF- α production in mast cells (Fig. 8), our results indicate that Stat6-induced TTP expression mediates ARE-dependent destabilization of TNF- α mRNA in mast cells. The importance of TTP in the regulation of TNF- α production has been clearly demonstrated using TTP-deficient (TTP^{-/-}) mice (33, 39, 40). The phenotype of TTP^{-/-} mice, including cachexia, dermatitis, conjunctivitis, and destructive arthritis, can be largely prevented by the neutralization of TNF- α (39), implicating an excess of circulating TNF- α in the pathogenesis of TTP^{-/-} mice. In addition, it has been demonstrated that macrophages derived from TTP^{-/-} mice produce more TNF- α mRNA than macrophages from WT mice (40). Moreover, TNF- α mRNA has been shown to be markedly stabilized in TTP^{-/-} cells (33), implicating TTP as an important stimulator of decay of TNF- α mRNA. It has also been shown recently that TTP recruits the exosome to ARE-containing mRNA and thereby promotes the rapid decay of the mRNA (41). Thus, our findings that IL-4-Stat6 signaling induces the expression of TTP provide a novel insight into the ARE-dependent gene regulation in IL-4-rich environments such as allergic diseases or parasitic infection.

As aforementioned, our results indicate that IL-4 prevents TNF- α production from mast cells through Stat6-induced TTP expression (Figs. 7 and 8). IL-10, another antiinflammatory cytokine, also inhibits the production of TNF- α through an ARE-dependent mechanism (42). However, interestingly, the molecular basis for the IL-10-induced inhibition is different from that of IL-4. It has been shown that IL-10-induced down-regulation of TNF- α production does not require the presence of TTP and does not alter mRNA stability (42). Instead, IL-10-induced down-regulation of TNF- α production is exerted through the inhibition of p38 mitogen-activated protein (MAP) kinase-mediated translation of TNF- α (42). It has also been demonstrated that p38 MAP kinase phosphorylates TTP protein (43, 44), and that the phosphorylated TTP loses its activity (44). Because it has been shown that IL-10 inhibits

p38 MAP kinase (42), IL-10 may also inhibit TNF- α production by inhibiting p38 MAP kinase-mediated inactivation of TTP.

In conclusion, we have shown that IL-4-Stat6 signaling induces the expression of TTP in mast cells and, thus, down-regulates TNF- α production by destabilizing mRNA in an ARE-dependent manner. Because an excess of TNF- α is involved in many inflammatory diseases, including rheumatoid arthritis (45) and idiopathic inflammatory bowel diseases (46), the modulation of IL-4-Stat6 signaling may be useful as a therapeutic tool for rheumatoid arthritis or inflammatory bowel disease through the inhibition of TNF- α production.

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