Arid5a controls IL-6 mRNA stability, which contributes to elevation of IL-6 level in vivo

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Posttranscriptional regulation of IL-6 has been largely uncharacterized, with the exception of the ribonuclease Regnase-1, which prevents autoimmunity by destabilizing IL-6 mRNA. Here, we identified AT-rich interactive domain-containing protein 5A (Arid5a) as a unique RNA binding protein, which stabilizes IL-6 but not TNF-α mRNA through binding to the 3′ untranslated region of IL-6 mRNA. Arid5a was enhanced in macrophages in response to LPS, IL-1β, and IL-6. Arid5a deficiency inhibited elevation of IL-6 serum level in LPS-treated mice and suppressed IL-6 levels and the development of T_H17 cells in experimental autoimmune encephalomyelitis. Importantly, Arid5a inhibited the destabilizing effect of Regnase-1 on IL-6 mRNA. These results indicate that Arid5a plays an important role in promotion of inflammatory processes and autoimmune diseases.

immune regulation | RNA-protein complex

Interleukin-6 (IL-6) is a pleiotropic cytokine with multiple functions (1). IL-6 is secreted by many cell types and is transcriptionally induced by various stimuli including the inflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) (2). Deregulated overexpression of IL-6 is associated with autoimmune inflammatory diseases and tumor growth (3). Significant therapeutic effects of anti–IL-6 receptor antibody (Tocilizumab) on various inflammatory diseases, including rheumatoid arthritis (RA), thus demonstrates that abnormal production of IL-6 is responsible for the pathogenesis of these diseases (4, 5).

Considering these previous reports, IL-6 expression must be carefully controlled at the transcriptional and posttranscriptional levels. The molecular mechanisms involved in transcriptional control of IL-6 expression have been extensively studied, and important roles for the transcription factors nuclear factor-kappa B (NF-κB) and CCAAT enhancer binding protein β (C/EBPβ), also known as NF-IL6, have been demonstrated (6, 7). However, almost nothing is known about posttranscriptional regulatory mechanisms that specifically target IL-6 mRNA, except for the identification of zinc finger CCCH-type containing protein 12A (Zc3h12a), which is also called Regnase-1 (8), an ribonuclease (RNase) controlling immune responses by selectively regulating IL-6 mRNA decay (9).

RNA-binding proteins (RNA-Bps) control the stability of a wide range of mRNA through binding to AU-rich elements (AREs) in the 3′ untranslated region (UTR) of mRNA (10). It has been reported that the activity of RNA-Bps is regulated by the activation of mitogen-activated protein kinases (MAPKs), such as p38 MAPK downstream of various stimuli, including IL-1β and lipopolysaccharide (LPS) (11, 12). The regulation of cytokine mRNA stability by RNA-Bps is particularly important because of their essential roles (such as for IL-6) in human disease.

Previously, we found that chlorpromazine (CPZ), known as an antihistaminic and antipsychotic drug, inhibits LPS-induced IL-6 production in macrophages without any inhibitory effect on other inflammatory cytokines (13). In the course of our studies on the mechanism of CPZ-induced inhibition of IL-6, we found a unique molecule, AT-rich interactive domain-containing protein 5A (Arid5a), which binds to the 3′ UTR of IL-6 mRNA, and its expression is inhibited by CPZ. Interestingly, CPZ is also known to inhibit the activity of p38 MAPK (14). It has been also shown that p38 MAPK is involved in the stabilization of IL-6 mRNA via multiple AU-rich elements in the 3′ UTR of IL-6 mRNA (15).

The AT-rich interactive domain (ARID) is a helix-turn-helix motif DNA-binding domain present in a protein family containing 15 members, with important roles in development, tissue-specific gene expression, and proliferation (16). In a previous study, the ARID family member Arid5a was demonstrated to have a role in stimulation of chondrocyte-specific transcription, in cooperation with the transcription factor SRY-box containing gene 9 (Sox9) (17).

In the current study, we have identified a previously undescribed role for Arid5a in mRNA stability. Importantly, Arid5a is a demonstration of an RNA-Bp, which stabilizes IL-6 mRNA. We also demonstrate that Arid5a contributes to elevation of IL-6 serum level in LPS-treated mice and experimental autoimmune encephalomyelitis (EAE) mice.

Results

Arid5a Is a Unique IL-6 mRNA Binding Protein. Our previous study showed that CPZ, which was used as a histamine 1 receptor antagonist, dose-dependently inhibits protein levels of LPS-induced IL-6 but not tumor necrosis factor-α (TNF-α), IL-12, and IL-10 in cultured macrophages (13). In the current study, we found that CPZ inhibits levels of IL-6 mRNA but not TNF-α in macrophages following LPS treatment (Fig. S1 *A* and *B*).

Initially, we investigated the effect of CPZ on the IL-6 promoter activity at the transcriptional level. As a result, CPZ did not significantly affect the IL-6 promoter activity (Fig. S1*C*). Recently, CPZ has been reported to inhibit p38 signaling (14). Moreover, p38 signaling has been shown to be involved in IL-6 mRNA stability (15). Therefore, we next examined the effect of CPZ on mRNA stability of IL-6 and TNF-α in macrophages treated with LPS. We found an inhibitory effect of CPZ on IL-6 mRNA stability (Fig. S1 *D* and *E*).

Because it has been reported that the *cis*-acting elements of the 3′ UTR of IL-6 mRNA (IL-6 3′ UTR) are required for IL-6 mRNA stability (15), we hypothesized that the activity of an RNA-Bp on the IL-6 3′ UTR, which is critical for stabilization of IL-6 mRNA, might be attenuated by CPZ. To test this hypothesis, we performed an RNA-protein binding assay as shown in Fig. 1*A*. After SDS/PAGE, some bands were able to be detected by coomassie brilliant blue (CBB) staining of the gel, and the bands were cut out with the three compartments separated and analyzed by LC-MS/MS (Fig. 1*B*). Mass spectral data were assessed by proteasome software (Scaffold3). The profiling data showed in one compartment (between 50 and 75 kDa) that Arid5a is one of

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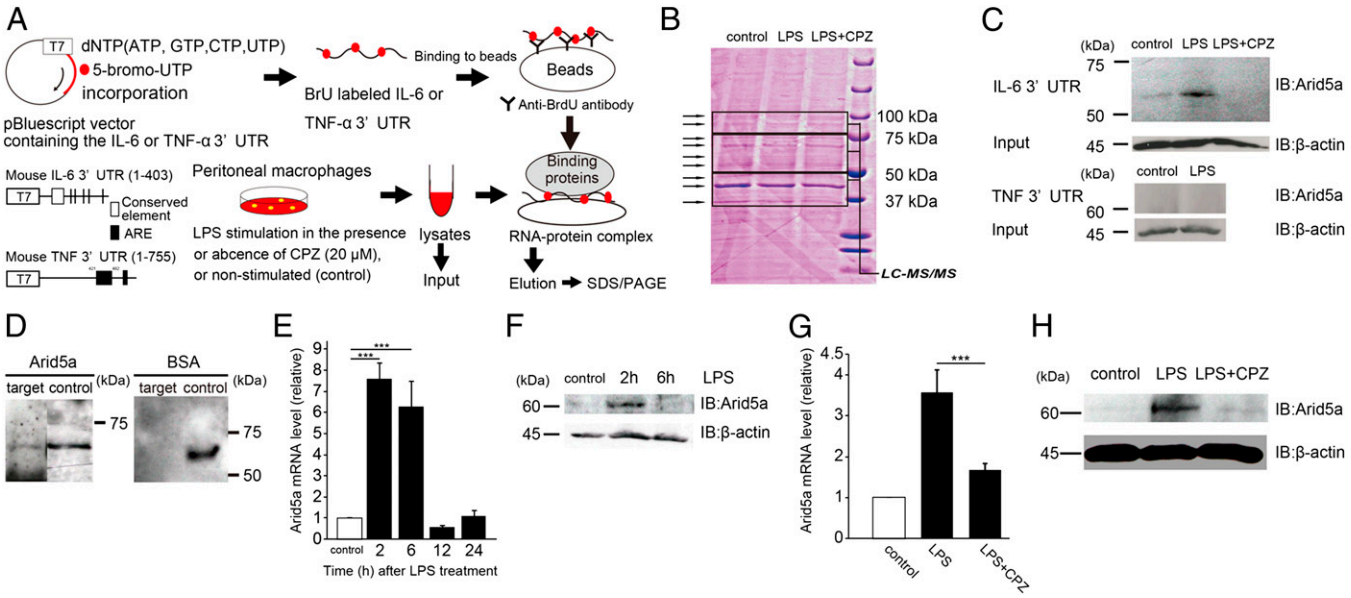

Fig. 1. Identification of a unique RNA-Bp, Arid5a, on the IL-6 3′ UTR. (*A*) Peritoneal macrophages were stimulated with LPS (1 μg/mL) for 2 h with or without CPZ (20 μM). Whole-cell lysates were mixed with the IL-6 or TNF-α 3′ UTR (containing BrU) binding protein G beads and then eluted buffer were subjected to SDS/PAGE, respectively. (*B*) The gel from the complex of the IL-6 3′ UTR and beads (as in *A*) was stained by CBB after SDS/PAGE and separated three compartments were analyzed by LC-MS/MS. The detected bands were indicated at arrows. (*C*) The samples from lysates (as in *A*) were used as input (β-actin detection by immunoblotting). After SDS/PAGE (as in *A*), endogenous Arid5a was detected with anti-Arid5a antibody by immnoblot analysis. (*D*) Arid5a recombinant protein or BSA was mixed with the IL-6 3′ UTR (containing BrU) binding protein G beads and then eluted buffer were subjected to SDS/PAGE, respectively. The samples were analyzed by immunoblotting using anti-Arid5a or anti-BSA antibody (Fig. S3). (*E*) Total RNAs at the indicated times were collected from peritoneal macrophages stimulated by LPS (1 μg/mL), and Arid5a mRNA levels were measured by qPCR. (*F*) Peritoneal macrophages were treated with LPS (1 μg/mL) at the indicated times. The cells were lysed and analyzed by immunoblotting. Endogenous Arid5a was detected with anti-Arid5a antibody. (*G*) Arid5a mRNA levels in peritoneal macrophages were compared 2 h after LPS (100 ng/mL) stimulation with or without CPZ (50 μM) pretreatment for 30 min. (*H*) Peritoneal macrophages were treated 2 h with LPS (1 μg/mL) with or without CPZ (20 μM) pretreatment. The cells were lysed and analyzed by Immunoblotting. (*C*, *D*, *F*, and *H*) Data are representative of three independent experiments. (*E* and *G*) Data show means ± SD of three independent experiments. ***$P < 0.001$ (Student's *t* test).

RNA-Bps, which is able to bind to the IL-6 3′ UTR (Fig. S2), and the binding of Arid5a increased after LPS stimulation, whereas its binding was suppressed by CPZ. In agreement with this profiling data, we confirmed that LPS treatment of peritoneal macrophages enhanced binding of endogenous Arid5a to the IL-6 3′ UTR, which was inhibited by cotreatment with CPZ (Fig. 1*C*). To examine the direct interaction of Arid5a with the IL-6 3′ UTR, we performed an RNA-protein binding assay (Fig. 1*A* and Fig. S3). We found that Arid5a associated with the IL-6 3′ UTR (Fig. 1*D*), whereas Arid5a did not bind to the TNF-α 3′ UTR (Fig. 1*C*). These results indicate that Arid5a is able to possess the specificity to sequences on the IL-6 3′ UTR.

We next performed time-course analysis of mRNA levels of Arid5a in peritoneal macrophages after LPS treatment. As a result, Arid5a mRNA levels were significantly elevated in macrophages in the early phase (2 to 6 h) after LPS stimulation (Fig. 1*E*). Interestingly, Arid5a mRNA level was rapidly degraded 12 h after LPS challenge. This result suggests that there is a mechanism underlying rapid termination of elevation of Arid5a mRNA level. We next examined levels of endogenous Arid5a protein in macrophages treated by LPS. Arid5a protein level was transiently increased 2 h, and rapidly degraded 6 h after LPS stimulation, respectively (Fig. 1*F*). LPS also enhanced Arid5a protein expression and IL-6 production in a dose-dependent manner (Fig. S4). Thus, levels of Arid5a mRNA and protein were tightly controlled under toll-like receptor (TLR) signaling. Interestingly, IL-6 and/or IL-1β, which signaling have been reported to induce IL-6 mRNA stabilization (11, 18), also induced Arid5a mRNA in macrophages and mouse embryonic fibroblasts (MEFs), respectively (Fig. S5).

Moreover, we examined whether CPZ regulates level of LPS-induced Arid5a mRNA. CPZ significantly suppressed LPS-induced Arid5a mRNA level after 2 h of the treatment and inhibited Arid5a expression (Fig. 1 *G* and *H*). Taken together, these results suggest that CPZ decreases binding of Arid5a to the IL-6 3′ UTR through inhibition of LPS-induced Arid5a expression, which might contribute to destabilization of IL-6 mRNA as showed in Fig. S1*D*.

Arid5a Enhances IL-6 mRNA Stability Through the 3′ UTR of IL-6 mRNA. Having confirmed binding of Arid5a to the IL-6 3′ UTR, we next examined whether Arid5a contributes to IL-6 mRNA stabilization. We initially introduced Flag-tagged WT Arid5a expression vector and Arid5a mutant vector lacking Arid as indicated at black (Fig. 2*A*). HEK293T cells were transfected with pGL3-luciferase vector encoding the IL-6 3′ UTR (1–403) or pGL3 empty vector, together with Arid5a expression vector or empty vector (Mock). Overexpression of Arid5a augmented the luciferase activity of pGL3 vector encoding the IL-6 3′ UTR compared with that of luciferase vector alone (Fig. 2*B*). In contrast, mutant Arid5a did not affect the luciferase activity of pGL3 vector encoding the IL-6 3′ UTR (Fig. 2*C*), suggesting that the Arid of Arid5a is essential for IL-6 mRNA stabilization. We also compared the luciferase activity of pGL3 vector encoding the TNF-α or IL-12 3′ UTR under overexpression of Arid5a with that under expression of control vector (Mock), respectively. As a result, overexpression of Arid5a did not alter the luciferase activity of both vectors (Fig. 2 *D* and *E*). Next, we sought Arid5a responsive regions on the IL-6 3′ UTR by using pGL3-luciferase vector encoding the IL-6 3′ UTR fragments (1–92, 1–142, 58–173, 122–197, 172–403), as shown in Fig. 2*F*. Overexpression of Arid5a enhanced the luciferase activity of pGL3 vector encoding a region (1–142, 58–173, 122–197) of the IL-6 3′ UTR (Fig. 2*G*). This result suggests that Arid5a responsive sites are able to compete with the sites, in which Regnase-1 acts on a region (1–142, 58–173) of the IL-6 3′ UTR (9). We next tested whether Arid5a stabilizes IL-6 mRNA through the IL-6 3′ UTR. In brief,

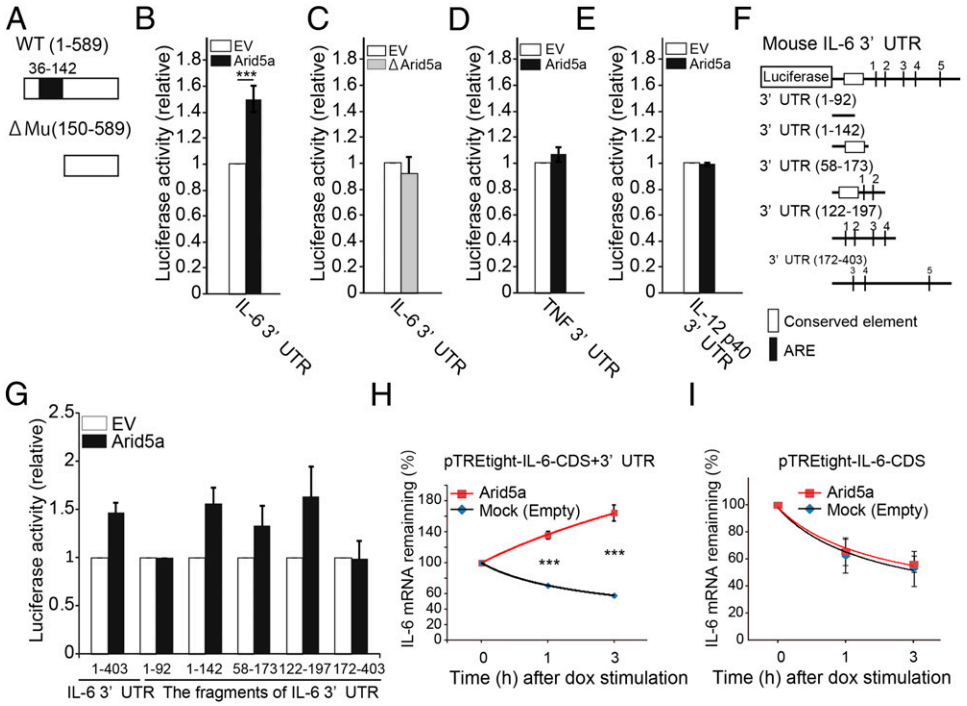

Fig. 2. Arid5a stabilizes IL-6 mRNA by recognizing the IL-6 3′ UTR. (*A*) Schematic diagram of expression constructs of Arid5a WT and mutant. The Arid is filled at black, and the amino acids present in the WT and mutant construct are indicated. (*B–E* and *G*) HEK293T cells were transfected with the pGL3-luciferase vector encoding the full length of the IL-6 3′ UTR, the fragments of the IL-6 3′ UTR (1–92, 1–142, 58–173, 122–197, 172–403), the full length of the TNF-α 3′ UTR or the full length of the IL-12 3′ UTR, or pGL3 empty vector, together with WT Arid5a expression vector (black bar; *B*, *D*, *E*, and *G*), ΔMutant Arid5a (gray bar; *C*), or empty vector. Luciferase activity was determined 24 h after transfection and normalized to that of pGL3-empty vector. The values were shown as relative to normalized after transfection with empty vector. (*F*) Schematic diagram of the full length of the IL-6 3′ UTR, and the fragments of the IL-6 3′ UTR. (*H* and *I*) HEK293 Tet-off cells were transfected with pTREtight-IL-6-CDS + 3′ UTR or pTREtight-IL-6-CDS vector, together with Arid5a expression vector or control (empty) vector. Cells were uniformly divided 3 h after transfection and incubated overnight. Total RNAs were prepared after dox (1 μg/mL) treatment, and IL-6 mRNA levels were determined by qPCR. The values were shown normalized to time 0 of dox addition (100%), respectively. (*B–E* and *G–I*) Data show means ± SD of three independent experiments. ***$P < 0.001$ (Student's *t* test).

HEK293 Tet-off cells stably expressing the enhancer (tTA) of a tetracycline-responsive promoter (TRE) were transfected with pTRE-plasmid encoding total IL-6 mRNA (CDS + 3′ UTR) or IL-6-CDS alone downstream of the TRE promoter (pTREtight-IL-6-CDS + 3′ UTR or pTREtight-IL-6-CDS vector) under coexpression of Arid5a expression vector or empty vector, respectively. Transcriptions of these pTRE vectors were terminated by doxycycline (dox) treatment. Total IL-6 mRNA levels were assessed by quantitative PCR (qPCR). As a result, overexpression of Arid5a maintained IL-6 mRNA level after dox stimulation, compared with the control transfected with empty vector (Fig. 2*H*). In contrast, overexpression of Arid5a did not affect IL-6 CDS without 3′ UTR elements (Fig. 2*I*). These results demonstrate that Arid5a stabilizes IL-6 mRNA through the function of the IL-6 3′ UTR, in which the Arid of Arid5a is required for stabilization of IL-6 mRNA.

***Arid5a*$^{-/-}$ Critically Reduces IL-6 Serum Level in LPS-Treated Mice.** Having established that Arid5a stabilizes IL-6 mRNA, we next examined whether deficiency in Arid5a suppresses protein levels of IL-6 in LPS-treated macrophages. We found that Arid5a regulates IL-6 but not TNF-α production in vitro (Fig. S6). Moreover, we investigated whether *Arid5a* deficiency (*Arid5a*$^{-/-}$) influences serum cytokine levels of IL-6, using WT and *Arid5a*-deficient mice, which were generated as shown in Fig. S7. WT and *Arid5a*$^{-/-}$ mice (6- to 8-wk-old) were i.p. injected with LPS (10 mg/kg). IL-6 serum levels in *Arid5a*$^{-/-}$ mice were critically reduced compared with those of WT mice after LPS injection (Fig. 3*A*). TNF-α serum level 2 h after LPS challenge was also slightly decreased in *Arid5a*$^{-/-}$ mice (Fig. 3*B*), suggesting that critical reduction of IL-6 level by *Arid5a* deficiency might have led to disorder of production of TNF-α in vivo. Thus, Arid5a also contributes to augmentation of IL-6 serum level in vivo.

***Arid5a*$^{-/-}$ Inhibits the Development of Experimental Autoimmune Encephalomyelitis.** To ascertain the importance of this newly identified function of Arid5a in autoimmunity, we compared the phenotype of EAE, which is reported to be an IL-6–dependent autoimmune disease (19, 20), in *Arid5a*-deficient mice with that of WT. As expected, EAE was critically suppressed in *Arid5a*$^{-/-}$ mice compared with that of WT mice (Fig. 4*A*). Consistent with the result in Fig. 3, levels of IL-6 serum significantly decreased in *Arid5a*$^{-/-}$ mice after immunization compared with those of WT mice, whereas *Arid5a* deficiency inhibited TNF-α serum level and IL-6 in vivo (Fig. 4*B*). Inhibition of induction of IL-17-producing T helper cells (T_H17 cells) by reduction of IL-6 level in vivo contributes to suppression of EAE (21). Therefore, we examined whether *Arid5a* deficiency affects the number of IL-17–producing T cells of CD4$^+$ population after EAE induction. The frequency of CD4$^+$IL-17$^+$ T cells in *Arid5a*$^{-/-}$ mice significantly decreased compared with that in WT mice (Fig. 4*C*), and the frequency of IFN-γ–producing T cells of CD4$^+$ population (T_H1 cells) increased compared with that in WT mice (Fig. 4*D*). In contrast, the frequency of CD4$^+$Foxp3$^+$ T cells (regulatory T cells, Treg) did not significantly change between WT and *Arid5a*$^{-/-}$ mice (Fig. 4*E*). In agreement with such

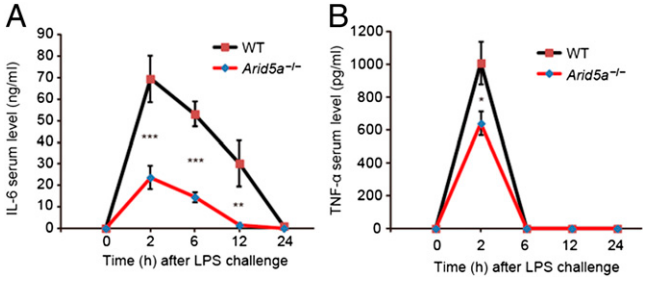

Fig. 3. *Arid5a* deficiency (*Arid5a*$^{-/-}$) reduces IL-6 and TNF serum levels in LPS-treated mice. (*A* and *B*) WT and *Arid5a*-deficient (*Arid5a*$^{-/-}$) mice (6–8 wk old) were i.p. injected with LPS (10 mg/kg). Serum levels of IL-6 (*A*) or TNF-α (*B*) in WT and *Arid5a*$^{-/-}$ mice were measured by ELISA at indicated time points after LPS challenge, respectively. Data show means ± SD of three independent experiments. ***$P < 0.0001$; **$P < 0.001$; *$P < 0.05$ (Student's *t* test).

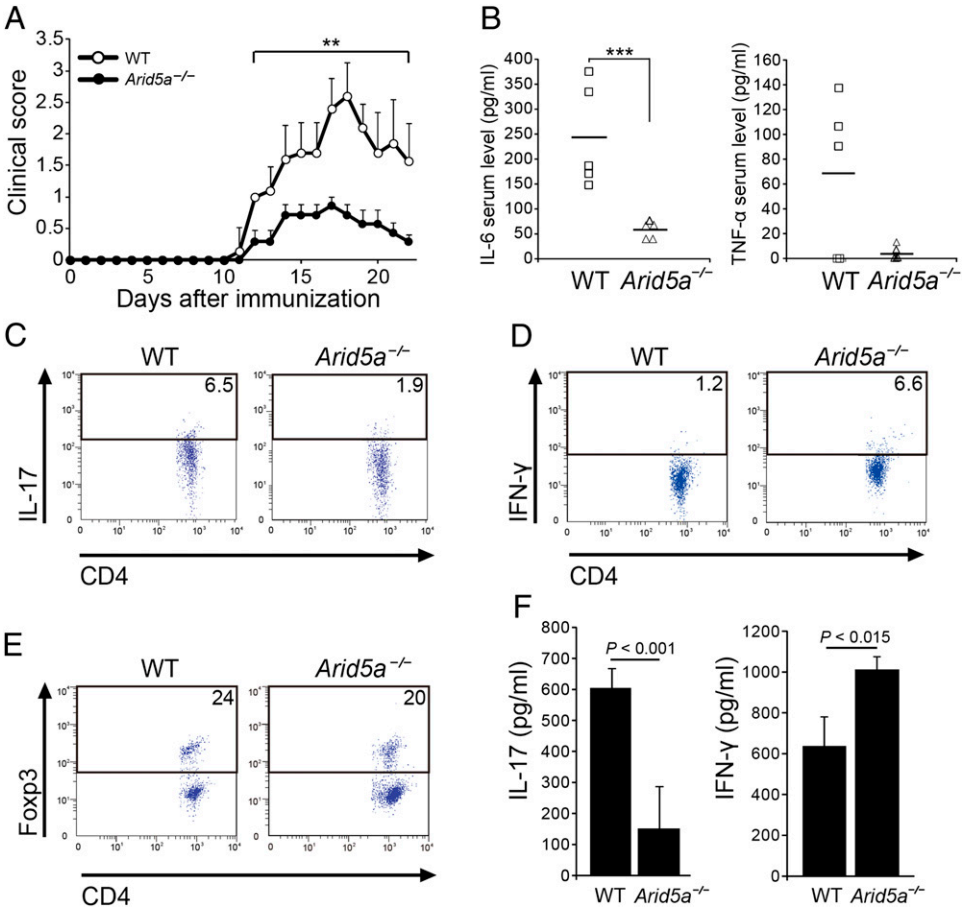

Fig. 4. *Arid5a* deficiency (*Arid5a*$^{-/-}$) inhibits the development of EAE. (*A*) EAE disease score in WT and *Arid5a*$^{-/-}$ mice (mean score ± SEM, $n = 7$, $^{**}P < 0.01$). (*B*) IL-6 and TNF-α serum samples in WT and *Arid5a*$^{-/-}$ mice were prepared at 20 d after immunization. Levels of IL-6 and TNF-α serum were measured by ELISA; $n = 5$, $^{***}P < 0.001$ (Student's *t* test). (*C*–*E*) Frequencies of IL-17, IFN-γ, and Foxp3-positive CD4$^+$ T cells in inguinal lymph node cells isolated from mice immunized with $MOG_{35–55}$. Numbers above bracketed lines indicate percent CD4$^+$IL-17$^+$, CD4$^+$IFN-γ$^+$, and CD4$^+$Foxp3$^+$ T cells. (*F*) Inguinal lymph node cells after immunization were restimulated with $MOG_{35–55}$ peptide (50 μg/mL) for 72 h. Secretions of IL-17 and IFN-γ in the supernatants were measured by ELISA. (*C*–*F*) Data are representative of two independent experiments with three mice per group (means ± SD, as in *F*).

results, compared with the draining lymph node cells from WT mice as control, cells from *Arid5a*$^{-/-}$ mice secreted a lower level of IL-17, whereas the secretion of IFN-γ in *Arid5a*$^{-/-}$ mice was significantly higher than that in WT mice (Fig. 4*F*). Thus, *Arid5a* deficiency reduced IL-6 serum levels, which led to suppression of EAE induction, possibly through reduction of the number of CD4$^+$IL-17$^+$ T cells.

Arid5a Abrogates the Destabilizing Effect of Regnase-1 on IL-6 mRNA. Because we found that Arid5a responsive sites partially overlap with Regnase-1 responsive elements on the IL-6 3′ UTR (Fig. 2*G*), we next examined whether Arid5a affects the destabilizing function of Regnase-1 on the IL-6 3′ UTR, using pGL3-luciferase vector encoding the IL-6 3′ UTR. HEK293T cells were transfected with pGL3-luciferase vector encoding the IL-6 3'UTR or pGL3 empty vector, together with empty vector, Arid5a expression vector, Regnase-1 expression vector or Arid5a and Regnase-1 expression vectors. We found that overexpression of Arid5a rescues the luciferase activity of pGL3 vector encoding the IL-6 3′ UTR under overexpression of Regnase-1 alone in a dose-dependent manner (Fig. 5*A*). Overexpression of Arid5a also dose-dependently increased the luciferase activity of pGL3 vector encoding the IL-6 3′ UTR (Fig. 5*A*). We next tested the effect of Arid5a on the destabilization function of Regnase-1 on total IL-6 mRNA (CDS + 3′ UTR). HEK Tet-off cells were transfected with pTREtight-IL-6-CDS + 3′ UTR vector, together with Arid5a and Regnase-1 expression vectors, Arid5a expression vector, Regnase-1 expression vector, or empty (control) vector. Treatment with dox terminated the transcriptions of IL-6 mRNA. Consistent with a previous report (9), we found that overexpression of Regnase-1 significantly destabilizes IL-6 mRNA compared with the control transfected with empty vector (Fig. 5*B*). Moreover, cooverexpression of Arid5a and Regnase-1 increased the stability of IL-6 mRNA compared with that under overexpression of Regnase-1 alone (Fig. 5*B*). Thus, Arid5a protects against the destabilizing function of Regnase-1 on IL-6 mRNA.

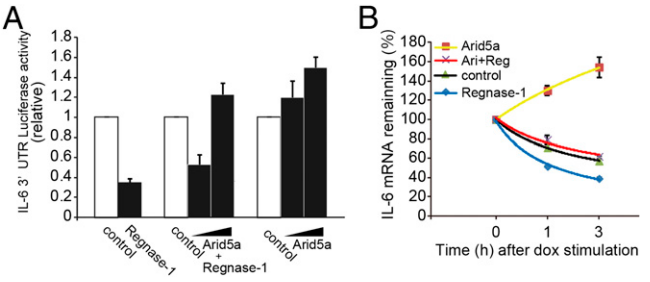

Fig. 5. Arid5a inhibits the destabilizing function of Regnase-1 on IL-6 mRNA. (*A*) HEK293T cells were transfected with the pGL3-luciferase vector encoding the IL-6 3′ UTR (1–403) or pGL3 empty vector, together with empty vector, Arid5a expression vector, Regnase-1 expression vector, or Arid5a and Regnase-1 expression vectors. Luciferase activity was determined 24 h after transfection and normalized to that of pGL3-empty vector. The values were shown as relative to normalized after transfection with empty vector. (*B*) HEK293 Tet-off cells were transfected with pTREtight-IL6-CDS + 3′ UTR vector, together with Arid5a expression vector, Regnase-1 expression vector, control (empty) vector or Arid5a and Regnase-1 expression vectors. The cells were uniformly divided 3 h after transfection, and incubated overnight. Total RNAs were prepared at the indicated times after dox (1 μg/mL) treatment, and IL-6 mRNA levels were determined by qPCR. The values were shown normalized to time 0 of dox addition (100%), respectively. All data show means ± SD of three independent experiments.

Discussion

Recently, it has been reported that *Zc3h12a* (also known as Regnase-1)-deficient mice spontaneously develop autoimmune disease, in which IL-6 mRNA decay is critically abrogated in macrophages because of the loss of its RNase activity (9). Therefore, regulation of IL-6 mRNA at the posttranscriptional level is essential for homeostasis in immune regulation. Here, we have found a unique IL-6 mRNA stability protein, Arid5a, which binds to the IL-6 3′ UTR but not TNF-α 3′ UTR. Arid5a responsive regions partially overlap with those of Regnase-1 on the IL-6 3′ UTR (Fig. 2*G*). Accordingly, a balance between levels of Arid5a and Regnase-1 expression might be important for control of IL-6–dependent autoimmune disease.

Similar to Regnase-1 mRNA level, which is controlled under TLR signaling (8), we found that LPS induces Arid5a mRNA in macrophages. IL-6 also induced Arid5a mRNA in macrophages. These findings might involve amplification of IL-6 production through positive feedback of Arid5a. In relation to such results, LPS-induced IL-6 serum levels have been critically impaired in *Arid5a*-deficient (*Arid5a*$^{-/-}$) mice compared with those of WT mice, whereas TNF-α serum levels are slightly affected. Moreover, *Arid5a*$^{-/-}$ mice are resistant to EAE, known as the experimental model of human multiple sclerosis (MS), by critical reduction of IL-6 serum level, in which IL-17–producing CD4^{+} T cells are critically decreased compared with those of WT, whereas IFN-γ–producing CD4^{+} T cells are significantly increased. In *Arid5a*$^{-/-}$ mice, both serum levels of IL-6 and TNF-α were dramatically inhibited after EAE induction. A previous report showed that anti–IL-6 antibody inhibited both IL-6 and TNF-α levels in serum after EAE induction (21). Therefore, it is conceivable that chronic elevation of IL-6 level affects TNF-α level more than acute augmentation of IL-6 level by LPS does in vivo. Taken together, expression of Arid5a contributes to elevation of IL-6 level, and augmentation of IL-17^{+}CD4^{+} T cells in vivo, which is able to promote autoimmune diseases such as MS and RA.

The *cis*-acting elements in the IL-6 3′ UTR are critical for IL-6 mRNA half-life (15). The mechanism of IL-6 mRNA decay by AU-rich element-binding proteins (ARE-bps), such as tristetraproline (TTP) and AU-rich element RNA-binding protein 1 (AUF1), on the IL-6 3′ UTR recently has been studied (22, 23). TTP bound to AREs of cytokine mRNAs through its CCCH zinc finger motif recruits deadenylases to activate the decay process (24). AUF1 has been shown to recognize mRNA stem-loop structures and AREs through its RNA recognition motif (RRM), although the mechanism of destabilization of IL-6 mRNA by AUF1 remains to be resolved (22). More recently, it has been reported that Regnase-1 is able to degrade IL-6 mRNA by recognizing the stem-loop region (its predicted site 84–103 in the IL-6 3′ UTR) via its CCCH zinc finger domain (8, 9, 25). In contrast, the role of RNA binding proteins in stabilization of IL-6 mRNA is not clearly understood. Although HuR, a member of Hu family, is known as a stability protein, which recognizes AREs of the 3′ UTR of cytokine mRNAs through RRMs (24, 26), the role of HuR on the regulation of IL-6 mRNA stability remains unresolved, as it has been recently reported that IL-6 mRNA levels are elevated in *HuR*-deficient ($^{-/-}$) nonstimulated macrophages compared with those of WT (27).

In the present study, we found that Arid5a is a unique RNA-Bp, which stabilizes IL-6 but not TNF-α or IL-12 mRNA in macrophages. Our findings contain two unique aspects. One is that Arid5a functions on the IL-6 3′ UTR, in which its Arid, but not a zinc finger motif or RRM, is essential for stabilization of IL-6 mRNA, because Arid5a mutant protein lacking Arid fails to stabilize IL-6 mRNA (Fig. 2*C*). The other is that Arid5a has specificity to IL-6 but not TNF-α or IL-12 mRNA stabilization. How Arid5a recognizes the IL-6 3′ UTR via its Arid is an intriguing study for further investigation. It will be useful in the future to determine the structure of the complex of IL-6 mRNA and Arid5a molecule by such a crystallography.

We also found that Arid5a abrogates the inhibitory effect of Regnase-1 on the IL-6 3′ UTR. Arid5a inhibits degradation of a region (1–142, 58–173) of the IL-6 3′ UTR, where Regnase-1 functions as an RNase (9). Arid5a is responsive to a region (122–197) in the IL-6 3′ UTR, which includes AREs, suggesting that Arid5a may associate with AREs of the IL-6 3′ UTR through its Arid. One possibility is that Arid5a competes with Regnase-1 on or near the stem-loop structure of the IL-6 3′ UTR, which leads to stabilization of IL-6 mRNA. Another is that the conformation change of IL-6 mRNA by the specific binding of Arid5a on the IL-6 3′ UTR might also lead to inhibition of the access of Regnase-1 to the stem-loop region of the IL-6 3′ UTR. Further study is required to clarify the precise mechanism by which Arid5a stabilizes IL-6 mRNA in relation to Regnase-1.

It has been recently shown that levels of Regnase-1 expression are regulated through TLR signaling, in which Regnase-1 is induced in peritoneal macrophages after LPS treatment (8, 9). Interestingly, levels of Arid5a protein were also controlled through TLR signaling. These data suggest that balance between Arid5a and Regnase-1 protein levels may be important for determination of IL-6 mRNA levels. Given that, it might be of interest in the future to investigate whether overproduction of IL-6 in autoimmunity is due to imbalance of Arid5a and Regnase-1 levels in vivo.

Signaling through p38 MAPK is involved in stabilization of IL-6 mRNA (15). Interestingly, we found that CPZ, which has been reported to impair p38 MAPK activity (14), selectively decreases LPS-induced IL-6 production in cultured macrophages, and also inhibits LPS-induced Arid5a expression. It has been also reported that p38 is critical for IL-6 production in MEFs under IL-1β signaling (15). We found that IL-1β induces Arid5a in MEFs and macrophages (Fig. S5). Conceivably therefore, p38 MAPK signaling might be linked to enhancement of IL-6 mRNA stability through the function of Arid5a and, in-turn, IL-6 production.

It is known that phosphorylation regulates the binding and function of RNA-Bps (12). For example, TTP phosphorylation by MK2 downstream of p38 MAPK signaling facilitates TTP to degrade mRNA through inhibition of deadenylase (28). Regnase-1 is phosphorylated by the IκB kinase (IKK) complex downstream of myeloid differentiation primary response protein 88 (Myd88) signaling, which undergoes ubiquitination and degradation (8). Accordingly, it is possible that the binding activity of Arid5a is also regulated by specific protein kinases, including p38 MAPK and IKK complex. Thus, similar to other RNA-Bps, it is conceivable that Arid5a expression and its binding activity might be tightly regulated under TLR signaling. Further study of the inhibitory mechanism of CPZ on Arid5a activation will be helpful for the understanding of the regulatory system of Arid5a expression under TLR signaling.

TLR-inducible Arid5a is a unique RNA-Bp that functions on the IL-6 3′ UTR, and in-turn, stabilizes IL-6 mRNA. Arid5a is also able to inhibit the destabilizing effect of Regnase-1 on a responsive region (1–142, 58–173) of IL-6 mRNA. Consistent with such results in vitro, IL-6 serum level was reduced in LPS-treated *Arid5a*-deficient mice compared with that of WT mice. Reduction of IL-6 level by the loss of Arid5a also contributed to inhibition of the onset of EAE disease, in which the number of IL-17^{+}CD4^{+} T cells was critically abrogated. Given that Arid5a plays an important role in autoimmunity, further study of the mechanism of control of Arid5a expression under TLR signaling (related to regulation of Regnase-1 expression) might contribute to potential therapeutics for treating IL-6–dependent diseases.

Materials and Methods

Mice and Reagents. C57BL/6 wild-type mice (6–8 wk) were obtained from CLEA Japan. *Arid5a*-deficient mice (accession no. CDB0602K: www.cdb.riken.jp/arg/mutant%20mice%20list.html) were generated as described (www.cdb.riken.jp/arg/Methods.html). All animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committees of the Graduate School of Frontier Bioscience, Osaka University. LPS (*Escherichia coli*: 055:B5), actinomycin D, Arid5a siRNA, and Arid5a scramble siRNA (control) were from Sigma-Aldrich. Chlorpromazine was from LKT Laboratories. Mouse recombinant proteins, IL-1β and TNF-α, were from Peprotech. Arid5a recombinant protein or BSA was purchased from Abnova or Pierce, respectively.

Induction of EAE. Mice were immunized s.c. with 100 μg of $MOG_{35–55}$ peptide (Peptides International) emulsified in complete Freund's adjuvant (Difco). In addition, the mice were i.p. injected with 500 ng of pertuiss toxin (List Biological Laboratories) on days 0 and 2. Mice were observed daily for clinical signs: 0, no disease; 1, limb tail; 2, hindlimb weakness; 3, hindlimb paralysis; 4, tetraplegia; 5, moribund.

Cell Culture and Transfection. Peritoneal macrophages were prepared as described (29). Thioglycolate-elicited peritoneal macrophages were cultured in RPMI medium 1640 (Sigma-Aldrich) with 10% (vol/vol) FCS, 100 μg/mL streptomycin, and 100 U/mL penicillin G (Nakalai Tesque). Primary MEFs were prepared from wild-type mouse embryos at embryonic day 13.5. MEFs, RAW264.7, and HEK293T cells were cultured in DMEM (Sigma-Aldrich) supplemented with 10% (vol/vol) FBS. Cells were transfected by using FuGENE HD (Promega) or the Neon transfection system (Invitrogen).

RNA Binding Assay. The RNA binding assay was performed according to the protocol of RiboTrap Kit (MBL International). In brief, 5-bromo-UTP (BrU) was randomly incorporated into the 3′ UTR of IL-6 or TNF-α mRNA (IL-6 or TNF-α 3′ UTR) upon transcription by using pBluescript plasmid containing the IL-6 or TNF-α 3′ UTR. Anti-BrdU antibodies were conjugated with protein G beads. Then, the IL-6 or TNF-α 3′ UTR was bound to the beads. Peritoneal macrophages were stimulated with LPS in the presence or absence of CPZ (30 min before), or nonstimulated. Then, the cell lysates were transferred to the BrU-labbeled IL-6 or TNF-α 3′ UTR on antibody conjugated beads for 2 h. Their samples were washed and eluted, and were subjected to SDS/PAGE.

qPCR Analysis. TRIzol (Invitrogen) or RNeasy (Quiagen) was used for the isolation of total RNA. Reverse transcription of mRNA was performed in a thermal cycler (Applied Biosystems). The mouse IL-6 probe (Mm00446190_m1; Applied Biosystems), mouse TNF-α probe (Mm00443258_m1), and mouse Arid5a probe (Mm00524454_m1) were used. For reference, we quantified mouse GAPDH (Applied Biosystems). The qPCR was carried out in an ABI PRISM 7900 HT (Applied Biosystems). Cycling conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. We applied the comparative ΔΔCt ($\Delta Ct_{target} - \Delta Ct_{control}$) method normalized to GAPDH for IL-6, TNF-α, and Arida mRNA quantitative analysis. The value of unstimulated cells was set at one and was used to calculate the fold change in stimulated cells.

Luciferase Assay. HEK293T cells were transfected with pGL3-luciferase plasmid encoding the IL-6 3′ UTR or pGL3-luciferase plasmid (control), together with Arid5a expression plasmid, Regnase-1 expression plasmid alone, Arid5a and Regnase-1 expression plasmids, or empty (control) plasmid, respectively. After 24 h of cultivation, these cells were lysed. RAW264.7 cells were transfected with the pGL3 luciferase plasmid encoding IL-6 promoter as used (29). Luciferase activities in lysates from treated samples were determined by using the Dual-Luciferase Reporter Assay System (Promega). The Renilla luciferase gene was simultaneously transfected as an internal control.

Knockdown of Arid5a. RAW264.7 cells were transfected with Arid5a siRNA (100 nM) or scramble Arid5a siRNA (as control) by electropolation by using the Neon Transfection system following the protocol introduced by Invitrogen.

Cytokine ELISA. Levels of mouse IL-6, TNF-α, IL-17, and IFN-γ from culture supernatants were measured by using ELISA according to the manufacturer's instructions (R&D Systems).

Intracellular Cytokine Staining and Foxp3 Staining. Draining lymph node cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL; Sigma), ionomycin (800 ng/mL; Sigma) for 4 h, and GolgiStop (BD PharMingen) for the final 4 h. After staining of surface markers, cells were fixed and permeabilized by using Cytofix/Cytoperm and Perm/Wash buffer according to the manufacturer's instructions (BD Biosciences). Cells were stained intracellularly with phycoerythrin conjugated anti–IL-17 (BD PharMingen) or FITC-labeled anti–IFN-γ (eBioscience). For Foxp3 staining, cells were fixed and permeabilized with the fixation/permeabilization buffer (eBioscience) for 3 h at 4 °C before intracellular staining with FITC-conjugated anti-Foxp3 (eBioscience). Flow cytometric analysis was performed with a Cytomics FC500 (Beckman Coulter).

Statistical Analysis. A paired Student's *t* test was used to analyze data for statistically significant differences. Values of $P < 0.05$ were regarded as statistically significant. Additional details are provided in *SI Materials and Methods*.

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1. Kishimoto T (2005) Interleukin-6: From basic science to medicine—40 years in immunology. *Annu Rev Immunol* 23:1–21.
2. Legrand-Poels S, Schoonbroodt S, Piette J (2000) Regulation of interleukin-6 gene expression by pro-inflammatory cytokines in a colon cancer cell line. *Biochem J* 349(Pt 3):765–773.
3. Neurath MF, Finotto S (2011) IL-6 signaling in autoimmunity, chronic inflammation and inflammation-associated cancer. *Cytokine Growth Factor Rev* 22(2):83–89.
4. Tanaka T, Kishimoto T (2012) Immunotherapeutic implication of IL-6 blockade. *Immunotherapy* 4(1):87–105.
5. Mima T, Nishimoto N (2009) Clinical value of blocking IL-6 receptor. *Curr Opin Rheumatol* 21(3):224–230.
6. Libermann TA, Baltimore D (1990) Activation of interleukin-6 gene expression through the NF-kappa B transcription factor. *Mol Cell Biol* 10(5):2327–2334.
7. Matsusaka T, et al. (1993) Transcription factors NF-IL6 and NF-kappa B synergistically activate transcription of the inflammatory cytokines, interleukin 6 and interleukin 8. *Proc Natl Acad Sci USA* 90(21):10193–10197.
8. Iwasaki H, et al. (2011) The IκB kinase complex regulates the stability of cytokine-encoding mRNA induced by TLR-IL-1R by controlling degradation of regnase-1. *Nat Immunol* 12(12):1167–1175.
9. Matsushita K, et al. (2009) Zc3h12a is an RNase essential for controlling immune responses by regulating mRNA decay. *Nature* 458(7242):1185–1190.
10. Gingerich TJ, Feige JJ, LaMarre J (2004) AU-rich elements and the control of gene expression through regulated mRNA stability. *Anim Health Res Rev* 5(1):49–63.
11. Patil C, Zhu X, Rossa C, Jr., Kim YJ, Kirkwood KL (2004) p38 MAPK regulates IL-1beta induced IL-6 expression through mRNA stability in osteoblasts. *Immunol Invest* 33(2):213–233.
12. Schoenberg DR, Maquat LE (2012) Regulation of cytoplasmic mRNA decay. *Nat Rev Genet* 13(4):246–259.
13. Masuda K, et al. (2011) Aryl hydrocarbon receptor negatively regulates LPS-induced IL-6 production through suppression of histamine production in macrophages. *Int Immunol* 23(10):637–645.
14. Sarkar S, Kantara C, Singh P (2012) Clathrin mediates endocytosis of progastrin and activates MAPKs: Role of cell surface annexin A2. *Am J Physiol Gastrointest Liver Physiol* 302(7):G712–G722.
15. Zhao W, Liu M, Kirkwood KL (2008) p38alpha stabilizes interleukin-6 mRNA via multiple AU-rich elements. *J Biol Chem* 283(4):1778–1785.
16. Patsialou A, Wilsker D, Moran E (2005) DNA-binding properties of ARID family proteins. *Nucleic Acids Res* 33(1):66–80.
17. Amano K, et al. (2011) Arid5a cooperates with Sox9 to stimulate chondrocyte-specific transcription. *Mol Biol Cell* 22(8):1300–1311.
18. Weigert C, et al. (2007) Upregulation of IL-6 mRNA by IL-6 in skeletal muscle cells: Role of IL-6 mRNA stabilization and Ca2+-dependent mechanisms. *Am J Physiol Cell Physiol* 293(3):C1139–C1147.
19. Okuda Y, et al. (1998) IL-6-deficient mice are resistant to the induction of experimental autoimmune encephalomyelitis provoked by myelin oligodendrocyte glycoprotein. *Int Immunol* 10(5):703–708.
20. Samoilova EB, Horton JL, Hilliard B, Liu TS, Chen Y (1998) IL-6-deficient mice are resistant to experimental autoimmune encephalomyelitis: Roles of IL-6 in the activation and differentiation of autoreactive T cells. *J Immunol* 161(12):6480–6486.
21. Serada S, et al. (2008) IL-6 blockade inhibits the induction of myelin antigen-specific Th17 cells and Th1 cells in experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci USA* 105(26):9041–9046.
22. Paschoud S, et al. (2006) Destabilization of interleukin-6 mRNA requires a putative RNA stem-loop structure, an AU-rich element, and the RNA-binding protein AUF1. *Mol Cell Biol* 26(22):8228–8241.
23. Zhao W, Liu M, D'Silva NJ, Kirkwood KL (2011) Tristetraprolin regulates interleukin-6 expression through p38 MAPK-dependent affinity changes with mRNA 3′ untranslated region. *J Interferon Cytokine Res* 31(8):629–637.
24. Anderson P (2008) Post-transcriptional control of cytokine production. *Nat Immunol* 9(4):353–359.
25. Uehata T, Akira S (March 13, 2013) mRNA degradation by the endoribonuclease Regnase-1 /ZC3H12a/MCPIP-1. *Biochim Biophys Acta*, 10.1016/j.bbagrm.2013.03.001.
26. Peng SS, Chen CY, Xu N, Shyu AB (1998) RNA stabilization by the AU-rich element binding protein, HuR, an ELAV protein. *EMBO J* 17(12):3461–3470.
27. Yiakouvaki A, et al. (2012) Myeloid cell expression of the RNA-binding protein HuR protects mice from pathologic inflammation and colorectal carcinogenesis. *J Clin Invest* 122(1):48–61.
28. Mahtani KR, et al. (2001) Mitogen-activated protein kinase p38 controls the expression and posttranslational modification of tristetraprolin, a regulator of tumor necrosis factor alpha mRNA stability. *Mol Cell Biol* 21(19):6461–6469.
29. Kimura A, et al. (2009) Aryl hydrocarbon receptor in combination with Stat1 regulates LPS-induced inflammatory responses. *J Exp Med* 206(9):2027–2035.