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Posttranscriptional Modulation of Cytokine Production in T Cells for the Regulation of Excessive Inflammation by TFL

Kentaro Minagawa,* Kanako Wakahashi,* Hiroki Kawano,* Shinichiro Nishikawa,* Chie Fukui,* Yuko Kawano,* Noboru Asada,* Mari Sato,* Akiko Sada,* Yoshio Katayama,*, and Toshimitsu Matsui†

Posttranscriptional machinery regulates inflammation and is associated with autoimmunity as well as tumorigenesis in collaboration with transcription factors. We previously identified the tumor suppressor gene transformed follicular lymphoma (TFL) on 6q25 in a patient with follicular lymphoma, which transformed into diffuse large B cell lymphoma. TFL families have a common RNase domain that governs macrophage-mediated inflammation. In human peripheral blood, TFL is dominantly expressed at the glycine- and tryptophan-rich cytoplasmic processing bodies of T lymphocytes, and it is persistently upregulated in activated T cells. To address its physiological role, we established TFL-/-/ mice in which TFL-/- lymphocytes proliferated more rapidly than TFL+/- upon stimulation with inappropriate cytokine secretion, including IL-2, IL-6, and IL-10. Moreover, TFL inhibited the synthesis of cytokines such as IL-2, IL-6, IL-10, TNF-α, and IL-17a by 3' untranslated region RNA degradation. Experimental autoimmune encephalitis induced in TFL-/- mice demonstrated persistent severe paralysis. CNS-infiltrated CD4+ T cells in TFL-/- mice contained a higher proportion of Th17 cells than did those in TFL+/- mice during the resolution phase, and IL-17a mRNA levels were markedly increased in TFL-/- cells. These results suggest that TFL may play an important role in attenuating local inflammation by suppressing the infiltration of Th17 cells in the CNS during the resolution phase of experimental autoimmune encephalitis. TFL is a novel gradual and persistent posttranscriptional regulator, and the TFL-driven attenuation of excessive inflammation could contribute to recovery from T cell-mediated autoimmune diseases. The Journal of Immunology, 2014, 192: 000–000.

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Abbreviations used in this article: CCCH, Cys-Cys-Cys-His; DCP1a, DCP1(decapping enzyme homolog a; EAE, experimental autoimmune encephalomyelitis; ES cell, embryonic stem cell; FL, follicular lymphoma; GW/P bodies, glycine- and tryptophan-rich cytoplasmic processing bodies; mRNA, microRNA; MOG, myelin oligodendrocyte glycoprotein; PARP, poly(ADP-ribose) polymerase; p21, peroxisome proliferator-activated receptor; qPCR, quantitative RT-PCR; SNP, single nucleotide polymorphism; TFL, transformed follicular lymphoma; TTP, tristetraprolin; UTR, untranslated region; Zc3h12, zinc finger Cys-Cys-His type containing 12.

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to stress granules (20). These results suggest that the induced TFL assembled in G/W/P bodies appears to play a crucial role in post-transcriptional regulation, including cancer development.

Zc3h12a has been well documented among members of the Zc3h12 family. A recent study showed that most Zc3h12a-/- mice died within 12 wk of birth, and also that the production of IL-6 and IL-12p40 in response to LPS was increased in macrophages from these mice (21). Zc3h12a was also shown to contain Nedd4-binding protein 1 and bacterial YacP-like proteins, and this domain acts as an RNase that is destabilized with 3'UTR of target mRNA (21, 22). Zc3h12a also possess deubiquitination activity, and this regulates the suppression of NF-κB activation through TNFR-associated factor proteins (23). These families were shown to be inducible proteins based on cell stimulation and function as negative feedback regulators that control the inflammatory response similar to TTP, which is induced by LPS (24). However, these concepts have mostly been developed in macrophage studies. Regarding lymphocytes, little is known about how the RNA-binding protein associates with diseases. Furthermore, although posttranscriptional factors have been studied in detail in terms of initiating LPS- or TNF-α-induced inflammation (25, 26), little is currently known about the resolution of inflammation (27). Thus, the physiological function of TFL in immune responses needs to be elucidated.

In the present study, we established TFL-/- mice and focused on the physiological functions of TFL in T cells. The gradual and persistent expression of TFL suppressed lymphocytic proliferation and the inappropriate secretion of cytokines that can affect the resolution of inflammation.

Materials and Methods

Generation of TFL-/- mice

All animal studies, including the generation of TFL-/- mice, were approved by the Animal Protection Committee of Kobe University. A TFL gene fragment from a mouse 129sv genomic library was subcloned into pBluescript SK-/-. Exon 2 was replaced by a PGK-neo cassette. This replacement deleted the methionine of the translation initiation that would have resulted in a total deficit in the TFL protein. The targeting vector included a diphtheria toxin A fragment cassette at the 5' end of the short homologous sequence, as described previously (28). Embryonic stem (ES) cells were electroporated with the linearized targeting vector and selected with Geneticin on embryonic fibroblast feeder cells. ES cell clones resistant to Geneticin and devoid of the 5' homologous sequence were microinjected into blastocysts with poly(ADP-ribose) polymerase (PARP), cyclin D3, and proliferating cell nuclear Ag were purchased from Cell Signaling Technology. LPS and Abs for cyclin D2 and actin were purchased from Sigma-Aldrich. Abs for CDK4 and CDK6 were purchased from MBL International.

Immunofluorescence assay

Human T cells were permeabilized with 0.1% saponin and 5% mouse serum and incubated with DyLight-488 (Pierce)-conjugated anti-TFL or control Ab for TFL staining. Cells were fixed and permeabilized followed by blockage with goat serum and incubated with mouse anti-human DCPI, decapping enzyme homolog a (DCP1a) (Abnova; IgG2a) and TFL (IgG2b) for multilabeling. Cells were labeled with goat anti-mouse IgG2a Alexa Fluor 555 and IgG2b Alexa Fluor 488 (Molecular Probes). Images were captured with an LSM700 confocal laser scanning microscope system with ZEN software (Carl Zeiss).

Cell proliferation and apoptosis assay

The T cell proliferation assay used was described previously (32). Plate-coated anti-CD3 (145-2C11, UCHT-1) was cultured in PBS at 37˚C for 2 h. The liquid phase anti-CD28 (37.51) was used for CD28 costimulation. LPS was used for B cell proliferation. Cells (2 × 10^5/ml) were cultured for 24–72 h. Cell proliferation was determined by adding 1 μCi [3H]thymidine per well 16 h before the end of the incubation. LPS. Phospho-Rb and Bmi1 assays were described previously (20). Pan T cells were harvested 48 h after anti-CD3 stimulation, fixed with 70% ethanol, and then stained with propidium iodide (20 μg/ml; Sigma-Aldrich) and RNase (100 μg/ml; Sigma-Aldrich). The stained cells were subjected to flow cytometric analysis. The cell cycle phase was estimated with FlowJo software (Digital Biology). Annexin V+ (BD International) and propidium iodide – fractions were defined as apoptotic cells.

Cytokine assays

Cytokines were quantified in the supernatant of T and B cell proliferation cultures by the BD cytometric bead array (mouse Th1/Th2/Th17 cytokine kit for mouse T cells and mouse inflammation kit for mouse B cells; BD Biosciences) following the manufacturer’s instructions. The supernatant was obtained by centrifugation at 24, 48, and 72 h after the culture was initiated.

RNA isolation and quantitative RT-PCR

Samples were mixed with 1.5 ml TRIzol (Life Technologies) and stored at −80˚C. RNA extraction and quantitative RT-PCR (qPCR) were performed as described previously (33). The primers used are available upon request.

Cytokine and luciferase assay

Cytokines were quantified in the supernatant of cultures by a cytometric bead array (BD Biosciences). The luciferase assay was performed as described previously (33). HeLa cells were transfected with psiCheck2 3'UTR plasmids together with the TFL expression plasmid. Luciferase activity was determined using the Dual-Luciferase reporter assay system (Promega). Luciferase activity was measured 72 h after small interfering RNA (Cell Signaling Technology) transfection for dicer knockdown.

mRNA stability assay

To analyze endogenous IL-2 mRNA stability, MACS-sorted pan T cells were activated by anti-CD3 stimulation for 48 h. Actinomycin D (2 μg/ml; Sigma-Aldrich) was then added and total RNA was harvested after 0, 30, 60, and 120 min. IL-2 mRNA levels were measured by qPCR and normalized to β-actin.

Experimental autoimmune encephalitis

Mice (at 11–12 wk of age) were immunized s.c. with 100 μg myelin oligodendrocyte glycoprotein (MOG)35-55 emulsified in CFA (Hooke Laboratories). The pertussis toxin was administered i.p. 2 d after immunization to enhance neuroinflammation. Mice were examined for clinical symptoms daily, and signs were translated into a clinical score as follows: 0, no detectable signs of EAE; 1, decreased tail tone; 2, complete tail paralysis; 3, partial hindlimb paralysis; 4, complete bilateral hindlimb paralysis; 5, total paralysis of the forelimbs and hindlimbs; 6, death. CD4+ T cells were collected at approximately day 20 and day 40 after immunization with MOG. CNS tissues were centrifuged on a 30/37/70% Percoll gradient (34).

Intracellular staining and flow cytometry

We performed intracellular staining for IL-17a (eBioTC11-18H10.1) and IFN-γ (XMG1.2) after stimulation with PMA (50 ng/ml) and ionomycin.
(1 μg/ml) for 4 h in the presence of brefeldin A. We used the anti-mouse Fosp3 Ab (FJK-16s) for Fosp3 staining.

**Gene expression analysis**

Gene expression profiling data were obtained from the National Center for Biotechnology Information Gene Expression Omnibus database under the accession number GSE38010 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE38010). Data were analyzed using GeneSpring GX12 (Agilent Technologies).

**Statistics**

All data are presented as the means ± SEM. All statistics were performed using GraphPad Prism.

**Results**

**TFL upregulation in activated T cells**

We generated anti-TFL mAbs, which specifically detected intracellular TFL in immunoblot analysis as well as flow cytometric and immune fluorescence analyses (Fig. 1A, 1B, 1E). We performed immunoblot analysis for human peripheral B, T, and macrophage cells to determine the expression profile of TFL in the peripheral blood. Whereas TFL was expressed in peripheral B and T cells, macr

**TFL localized in GW/P bodies with a C terminus proline-rich domain**

We performed dual intracellular staining of endogenous TFL and DCP1α as a GW/P body marker in THP-1 and stimulated human peripheral T cells. TFL colocalized with DCP1α (Fig. 2A). Another domain for TFL was the proline-rich domain in the C terminus of TFL (Fig. 2B, black-filled box). We established five types of GFP-fused TFL mutant vectors deleted from the C terminus of TFL by 50 aa (TFL-500 aa to TFL-300 aa; Fig. 2B) and transfected these vectors into HeLa cells. Discrete cytoplasmic granules were found in TFL-, TFL-500 aa–, TFL-450 aa–, and TFL-400 aa–transfected HeLa cells (Fig. 2Ci–iv). Alternatively, TFL-350 aa and TFL-300 aa, which do not have a proline-rich domain, lost their characteristic localization, and the expression of GFP expanded diffusely through the nucleus (Fig. 2Ci–iv). Moreover, diffuse GFP expression was observed in the cytoplasm of the proline-rich domain deletion mutant (TFL-ΔPro; Fig. 2Cvi). The zinc finger deletion mutant of TFL (TFL-ΔZn; Fig. 2B) did not affect the appearance of these discrete cytoplasmic granules (Fig. 2Cvi). These results suggest that the proline-rich domain in the C terminus of TFL may be associated with the localization of cytoplasmic granules and may affect the binding of mRNAs to 3’UTR.

**Noninterference of TFL with the static state of immunity**

To elucidate the pathophysiological function of TFL, we established TFL−/− mice (Fig. 3A). Two ES cell clones were obtained and were established TFL−/− mice (Fig. 3B). TFL expression in the thymus and spleen was undetectable in TFL−/− mice and decreased by half in TFL+/- mice (Fig. 3C). Whereas splenic T and B cells expressed TFL, its expression in splenic macrophages was less, and it was similar to that in human peripheral macrophages (Figs. 1C, 3D). TFL−/− mice were born in the expected Mendelian ratios, developed without problems, and lived for ~2 y (median survival, 119 wk; 95% CI, 100.9–131.6; n = 10) in contrast to Zc3h12a−/− mice (21, 23). We backcrossed C57BL/6J for at least eight generations and peripheral blood counts were assessed to demonstrate almost comparable data to TFL−/− mice (Fig. 3E). Regarding the lymphocyte profile including regulatory T cells (CD4+CD25+Foot3+) in the spleen, each lymphocyte cell number in the splenocytes of TFL−/− mice was similar to that of TFL+/− mice in the steady-state (Fig. 3F). Moreover, we examined CD4 and CD8 populations in the inguinal and mesenteric lymph nodes. No significant differences were observed between TFL+/+ and TFL−/− mice (Fig. 3G). Taken together, these results indicate that a TFL deficiency did not appear to interfere with steady-state lymphopoiesis.

**Importance of the RNA-binding zinc finger motif in inhibiting lymphocyte proliferation via the TFL negative feedback loop**

If induced TFL functioned as a negative feedback regulator to prevent excess inflammation, the proliferation of TFL−/− splenic T cells activated by the anti-CD3 Ab with or without anti-CD28 costimulation would be higher than that of TFL+/+ T cells. No significant differences were observed in the expression levels of several cell cycle regulator proteins, such as cyclin D2/D3 and CDK4/6, between wild and TFL-deficient splenic T cells within 24 h of stimulation. However, Rb phosphorylation was enhanced in TFL-deficient T cells even at 24 h (Fig. 4A). Moreover, the incorporation of [3H]thymidine was stronger in TFL−/− T cells than in the TFL+/+ control 48 h after stimulation (Fig. 4B). Cell cycle analysis by propidium iodide staining also revealed that the synthesis of DNA was markedly higher in TFL−/− T cells than in TFL+/+ T cells (Fig. 4C). Immunoblot analysis also demonstrated that TFL was persistently upregulated during 72 h, and also that phospho-Rb was more intense in TFL−/− splenic T cells than in TFL+/+ mice 48 and 72 h after stimulation (Fig. 4D). Similar results were also obtained in splenic B cells (Supplemental Fig. 1). Apoptosis in TFL−/− T cells occurred in a time-dependent manner similar to that in TFL+/+ cells (Fig. 4E). Collectively, these results indicated that TFL could suppress late cell overgrowth in splenic lymphocytes.

TFL has a conserved RNA-binding CCCH-type zinc finger domain and an A/G nonsynonymous SNP at codon 106, which has been shown to exert tumor suppressor function (19). Therefore, these mutants could affect cell proliferation. To clarify this, we generated the mutant vectors described above and transfected them into the mouse normal pro-B cell line Ba/F3. The zinc finger deletion mutant canceled the suppression of Rb phosphorylation and cell growth inhibition in Ba/F3 cells, whereas the A106G SNP mutant did not affect the suppression of cell growth by TFL (Fig. 4F, 4G).

**Cytokine regulation by TFL via the posttranscriptional pathway**

We measured several cytokines in the supernatant of cultured T cells activated by the anti-CD3 Ab with or without anti-CD28 costimulation. The amounts of IL-2, IL-4, IL-6, IFN-γ, TNF-α, IL-10, and IL-17a (Supplemental Fig. 2A). However, in case of anti-CD3 and anti-CD28 costimulation, the secretion of IL-2 in T cells was largely upregulated in both TFL−/− and TFL+/+ mice, which canceled the difference between them (Supplemental Fig. 2A). We then examined cytokine levels in the supernatant of LPS-stimulated splenic B cells and performed qPCR on these cells. The mRNA...
FIGURE 1. Generation of mAb for human TFL and increased expression of TFL by activated human T cells. (A) Human leukemic cell lines were subjected to immunoblotting with the anti-TFL mAb. Whereas THP-1, Daudi, and Nalm-6 were positive for TFL, Jurkat, MOLT-14, and RPMI-8602 cells were negative. Data representative of three independent experiments are shown. (B) Flow cytometric analysis of each cell line. Consistent with the results of the immunoblot analysis (A), the TFL mAb demonstrated a quantitative shift (open histogram) for each cell line. Gray-filled histogram indicates the nonimmune control. (C) Immunoblot analysis of human B, T, and macrophage cells. Each cell was sorted by the MoFlo XDP cell sorter. Positive fractions of anti-CD19, -CD2, and -CD14 were sorted for B, T, and macrophage cells, respectively. Each experiment was performed three times, and representative data are shown. (D) Human purified pan T cells (n = 3) were activated with the anti-CD3 Ab (5 μg/ml). Immunoblot analyses of TFL, phospho-Rb, cleaved PARP, and actin as the loading control are shown. Representative data are shown. (E) Flow cytometric and immunocytochemistry analyses of TFL when pan T cells were activated as in (D). Expression intensity of TFL (open histogram) peaked from 48 to 72 h after stimulation. Gray-filled histogram indicates the nonimmune IgG control. TFL had a cytoplasmic granular expression pattern (green). The blue color indicates DAPI as nuclear staining. Scale bars, 10 μm. Representative data of three independent experiments are shown. (F) Quantification of induced TFL with flow cytometric analysis. Human purified pan T cells were activated with the anti-CD3 Ab (5 μg/ml). The Δ geometric mean (geometric mean of TFL minus that of isotype control) was calculated with a flow cytometer in each time course and was demonstrated in a bar graph as the relative ratio to prestimulation (0 h) (n = 5). Collective data were analyzed with the Mann–Whitney U test. **p < 0.01.
and protein expression levels of IL-6 and IL-10 in B cells were significantly higher in $TFL^{-/-}$ than in $TFL^{+/+}$ (Supplemental Fig. 2B, 2C). Collectively, these results demonstrated that $TFL^{-/-}$ lymphocytes proliferated with inappropriate cytokine secretion.

To elucidate whether posttranscriptional gene regulation by TFL was dependent on mRNA stability, we examined mRNA stability using actinomycin D. We found that the stability of IL-2 mRNA was significantly higher in $TFL^{-/-}$ T cells than in $TFL^{+/+}$ T cells.

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**FIGURE 2.** Partially colocalized TFL with GW/P bodies and the importance of C terminus proline-rich domains for the localization of cytoplasmic granules. (A) Endogenous expression of TFL (green) and GW/P body marker DCP1a (red) in THP-1 (left) and CD3-activated peripheral T (right) cells. Each protein was partially colocalized in the cytoplasm (yellow: arrows). Scale bars, 10 μm. Each experiment was performed three times and representative data are shown. (B) Vector constructions of TFL. TFL is a protein with a molecular mass 58 kDa and 521 aa (TFL), which contains unique types of the zinc finger domain (gray-filled box) and proline-rich domain (black-filled box). TFL-500 aa was formed by deleting 21 aa from the C terminus of TFL. TFL-450 aa, TFL-400 aa, TFL-350 aa, and TFL-300 aa were formed by deleting 50 aa from the C terminus of TFL-500 aa, TFL-450 aa, TFL-400 aa, and TFL-350 aa, respectively. TFL-ΔPro and TFL-ΔZn indicate the proline-rich and zinc finger domain deleted from TFL, respectively. (C) Localization of the TFL protein in HeLa cells expressing GFP tagged with each TFL vector as shown in (B). (i) TFL, (ii) TFL-500 aa, (iii) TFL-450 aa, (iv) TFL-400 aa, (v) TFL-350 aa, (vi) TFL-300 aa, (vii) TFL-ΔPro, and (viii) TFL-ΔZn. Whereas TFL, TFL-500 aa, TFL-450 aa, TFL-400 aa, and TFL-ΔZn retained cytoplasmic granules, a diffuse pattern of localization was observed in TFL-350 aa, TFL-300 aa, and TFL-ΔPro. Scale bars, 10 μm. Each experiment was performed three times and representative data are shown.
These results suggest that TFL is involved in the posttranscriptional gene regulation of IL-2.

To clarify whether TFL could degrade several cytokines via the posttranscriptional pathway, we generated Dual-Luciferase reporter vectors with the 3'UTR of several cytokines. These vectors were transfected into the T cell line Jurkat with various test vectors, including TFL. As expected, IL-2, IL-6, and IL-10 were degraded by TFL, whereas IFN-γ remained unaffected. Zc3h12a degraded

FIGURE 3. Establishment of TFL−/− mice. (A) Vector construction for TFL−/− mice. A neomycin cassette was inserted into exon 2 including the mouse ATG transcription initiation codon. Each asterisk indicates the EcoRI enzyme restriction site. DTA, diphtheria toxin A. (B) Generated ES cell clones and second federation (F2) TFL−/− mice. Mice were backcrossed with C57BL/6J mice. EcoRI-digested DNA blot analysis was performed with probe A or probe B. The 7.3-kb band indicates the wild allele and the 5.2-kb band indicates the mutant allele. (C) RNA blot analysis (left) and immunoblot analysis (right) for the brain, thymus, and spleen obtained from TFL+/+, TFL+/-, and TFL−/− mice. TFL was not expressed in the brain. Each experiment was performed three times and representative data are shown. (D) Mouse TFL expression in splenic T cells (T), B cells (B), and macrophages (M) in TFL+/+, TFL+/-, and TFL−/− mice. Each cell was sorted with a cell sorter by gating CD90+ (for T cells), B220+ (for B cells), and F4/80+ (for macrophages) fractions of splenocytes. Actin indicates the loading control. Each experiment was performed three times and representative data are shown. (E) Peripheral blood counts of TFL+/+ (n = 7) and TFL−/− mice (n = 6). Each histogram demonstrates the means ± SEM. (F) Total spleen cell counts and each subset of splenic lymphocytes in TFL+/+ (n = 7) and TFL−/− mice (n = 6). Each histogram indicates the means ± SEM. (G) Total cell counts for the CD4/8 subset of inguinal and mesenteric lymph nodes in TFL+/+ (n = 3) and TFL−/− mice (n = 3). Each histogram indicates the means ± SEM. No significant differences were observed in (E)–(G) with the unpaired t test.
FIGURE 4. Inhibition of splenic T cell proliferation in vitro by mouse TFL. (A) Non-alteration of the early phase of the cell cycle by TFL. MACS-sorted pan T cells derived from the splenocytes of TFL+/+ and TFL−/− mice were stimulated with the plate-coated anti-CD3 Ab (2.5 μg/ml). Cells were harvested every 4 h until 24 h after stimulation and subjected to immunoblotting for phospho-Rb, cyclin D3, cyclin D2, CDK6, CDK4, proliferating cell nuclear Ag, mouse TFL, and actin as the loading control. Each experiment was performed three times and representative data are shown. (B) Tritium thymidine assay. Purified pan T cells were stimulated for 48 h with the plate-coated anti-mouse CD3 Ab with (right; CD3/CD28) or without (left; CD3) anti-mouse CD28 (1 μg/ml). The incorporation of tritium thymidine in TFL−/− mice (filled bar) is shown in proportion to that in TFL+/+ mice (open bar). Data were shown as the means ± SEM bars (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 (unpaired t test). (C) Cell cycle analysis using propidium iodide staining. MACS-sorted pan T cells derived from the splenocytes of TFL+/+ and TFL−/− mice were stimulated with the plate-coated anti-CD3 Ab (5 μg/ml) with or without the anti-CD28 Ab (1 μg/ml). Cells were analyzed 48 h after stimulation. Each histogram indicates the means ± SEM. Each experiment was performed five times and collective data were analyzed with the Mann–Whitney U test. **p < 0.01. (D) Immunoblot analysis of splenic pan T cells obtained from TFL+/+ and TFL−/− mice. Each pan T cell was stimulated as shown in (A). Each cell was stimulated with the plate-coated anti-mouse CD3 Ab (2.5 μg/ml). Each experiment was performed three times and representative data are shown. (E) Annexin binding assay. The proportion of apoptotic cells (propidium iodide− and annexin+ fractions) is shown. Each pan T cell was stimulated as shown in (A). In these experiments, the concentration of the plate-coated anti-mouse CD3 Ab was 5 μg/ml. Collective data from each independent experiment (n = 5) are shown. (F) Importance of the zinc finger (Figure legend continues).
IL-2 and IL-6, as reported previously (21, 35, 36). Additionally, it also affected IL-10 and TNF-α. IL-17a was degraded by TFL, but not by Zc3h12α (Fig. 5C). The deletion mutant of the zinc finger domain lost its ability to degrade the 3’UTR mRNA of IL-2, IL-6, IL-10, and IL-17a (Fig. 5C). Similar results were obtained from the experiment using HeLa cells to determine cytokine regulation by TFL, although TNF-α was significantly suppressed by the zinc finger deletion mutant of TFL in Jurkat, but not HeLa, transfectants. In contrast to Jurkat, Zc3h12α only suppressed IL-2 and IL-6 and not IL-10 or TNF-α in HeLa cells (Supplemental Fig. 3A). Moreover, whereas TFL-500 aa, TFL-450 aa, and TFL-400 aa, which localize to the GW/P bodies, retained the ability to degrade cytokines, human TFL-350 aa, TFL-300 aa, and TFL-ΔPro, which do not localize to the GW/P bodies, lost their function (Figs. 2B, 2C, 5D).

We further confirmed that the mRNA stabilities of IL-2 and IL-17a were also altered by TFL using HeLa transfectants (Supplemental Fig. 3B). We investigated whether the increase in IL-2 played a role in the increased proliferation of TFL−/− cells. Therefore, we performed cell cycle analysis by adding IL-2. After 48 h stimulation, the proliferation of TFL−/− cells was still higher than that in TFL+/+ cells in both anti-CD3 only– and anti-CD3/anti-CD28–stimulated cells (Fig. 5E). Therefore, the increase in IL-2 could not explain the increased proliferation of TFL−/− cells. Zc3h12α was shown to preferentially recognize the IL-6 conserved element over the AU-rich element, and these sequences resemble the precursor microRNA (miRNA) hairpin loop (21, 37). To establish whether TFL recognized the IL-6 conserved element, we established the IL-6 mutant luciferase vector and assessed its incorporation with flow cytometry. To divide the BrdU fraction into the G0/G1 phase and G2/M phase, DNA was stained with 7-aminoactinomycin D. Data were analyzed in triplicate. SE bars are shown.

Resolution of EAE by TFL through mRNA decay of IL-17a

If TFL is a negative feedback regulator that suppresses excess inflammation, TFL−/− mice may not survive under inflammatory conditions. Because the deregulation of IL-17a by TFL may partly affect Th17-related diseases, such as multiple sclerosis, we adopted EAE, which was previously thought to be caused by Th1 but was recently shown to be caused by Th17 (41). TFL−/− mice did not show any difference at the onset of paralysis. However, during the peak of EAE (~20 d after MOG immunization), the score for TFL−/− mice was significantly higher than that for the TFL+/+ control (Fig. 6A). The severity of paralysis in TFL−/− mice continued until the resolution phase (~40 d after the MOG immunization) despite the recovery of TFL+/+ mice from paralysis (Fig. 6A). We then determined whether these results were caused by Th17 cells. Although the proportion of IL-17a–producing CD4+ T cells extracted from the CNS of TFL−/− mice was slightly higher than that in TFL+/+ mice during the peak of the disease (p = 0.19; Fig. 6B, 6C), the number of TFL−/− Th17 cells in the CNS was significantly increased during the resolution phase (p < 0.001; Fig. 6B, 6D). Alternatively, the population of IFN-γ–producing CD4+ Th1 cells was similar in both groups at both phases (Fig. 6B, 6D). The absolute numbers of IL-17a– as well as IFN-γ–producing CD4+ T cells in both phases were not significantly changed (Supplemental Fig. 4A). However, these numbers were slightly higher in TFL−/− Th17 cells during the resolution phase (p = 0.11). Moreover, qPCR analysis of CD4+ T cells sorted from the CNS during the resolution phase demonstrated that IL-17a, IL-23Rα, IL-12Rβ1, IL-7Rα, and IL-13Rα1 were significantly increased, IL-21R was slightly higher, and no difference was observed in IFN-γ or Foxp3 in TFL−/− T cells (Fig. 6E, 6F). MOG-induced TFL expression in CD4+ T cells in the CNS, spleen, and lymph nodes was significantly higher than that in nonimmune CD4+ splenocytes (Fig. 6G). Moreover, gene expression analysis derived from the Gene Expression Omnibus database (GSE38010) (42) indicated the persistent upregulated expression of TFL over control samples from the histologically characterized acute to chronic plaques through chronic active plaques of multiple sclerosis brains, whereas the expression of Zc3h12α was lower than that in the control (Fig. 6H).

We also analyzed IL-17a−, IFN-γ−, and Foxp3−producing CD4+ T cell populations in the spleen and lymph nodes. Cytokine-producing cell populations were almost the same in both groups during both phases of the disease (Supplemental Fig. 4B). Furthermore, we investigated whether TFL−/− splenic CD4+ T cells were prone to shift Th17 under Th17 cell-skewing conditions. No significant difference was observed in their ability to produce IL-17a after 3 d of culture (Supplemental Fig. 4C).

Collectively, these results indicate that the persistence of induced TFL in CD4+ T cells in the CNS only plays an important role in the resolution of inflammation, but not the induction of EAE.

Discussion

We demonstrated that activated T cells persistently expressed TFL and caused the assembly of GW/P bodies due to the activation of posttranscriptional machinery. Despite the regulation of cytokines by TFL, which could inhibit cellular proliferation, the global expression of these proteins has been demonstrated in peripheral lymphoid organs. However, once these activated T cells migrate into inflammatory sites, such as the CNS in EAE, they degrade IL-17a mRNA and decrease the number of Th17 cells during the resolution phase (Fig. 6I).

We found that TFL was more dominantly expressed in lymphocytes than in macrophages. TFL expression was induced slowly and persistently upon stimulation. This expression pattern is very different
FIGURE 5. TFL regulation of several cytokines through the 3’UTR of mRNA. (A) MACS-sorted pan T cells were stimulated with plate-coated anti-mouse CD3 at indicated concentrations, and the production of IL-2 was measured in the supernatant of each well. Cells were also harvested and IL-2 mRNA was measured by qPCR analysis at several time points after anti-mouse CD3 stimulation (5 μg/ml). Collective data (n = 5) were analyzed with the Mann–Whitney U test. *p < 0.05, **p < 0.01. (B) mRNA stability assay for IL-2 in T cells. MACS-sorted pan T cells were (Figure legend continues)
from other members of the Zc3h12 family, Zc3h12a in CD4+ T cells was recently shown to be indispensable for TCR-induced immune activation. Designated as Regnase-1, Zc3h12a in T cells regulates the mRNAs of c-Rel, Ovx40, and IL-2 through the cleavage of their 3′UTR (36). TTP was also induced by stimulation in human peripheral T cells and was reported to regulate T cell–mediated cytokines, such as IL-2 (6, 43). TTP levels peaked 3 h after T cell activation, and Zc3h12a was also induced in T cells by 12 h (35, 43). However, its expression returned to baseline at 24 h. Thus, these molecules may have important functions as regulators for initial inflammatory activation, but not for the resolution of inflammation. Alternatively, the induction of TFL in T cells was gradual and persistent for several days after stimulation (Figs. 1D, 3A, 3D). TFL was also associated with the mRNA decay of several cytokines, including IL-17a. Prolonged severe paralysis was observed in TFL−/− mice during the resolution phase in EAE. However, no advantage was noted for the induction of EAE. Thus, TFL affects the chronic inflammatory response rather than the acute setting.

Transcriptional mechanisms have also been implicated in the resolution of inflammation. For example, the peroxisome proliferator–activated receptors (PPARs) expressed on several types of cells including T cells were found to affect the chronic inflammatory response rather than during the acute setting (44). Consistent with our study, heterozygous mice with a deficiency in PPARγ, one of the PPAR subfamilies, developed exacerbated EAE with prolonged paralysis and impaired remission despite only a marginal difference in the day of onset (45). Transcriptional control is an essential step in the resolution of inflammation; nevertheless, factors that promote mRNA degradation play a dominant role in coordinating the overall immune response. Therefore, both transcriptional and posttranscriptional regulation could collaborate to resolve chronic inflammation, such as that in EAE.

We confirmed that endogenous TFL was partially localized to GW/P bodies (Fig. 2A). Because TFL acts as an mRNA regulatory molecule similar to Zc3h12a, it is reasonable to assume that TFL is one of the components of GW/P bodies. According to cell stimulation, TFL-containing cytoplasmic granules assembled as if posttranscriptional machinery had been activated (Figs. 1E, 6I). We also found that the C terminus proline-rich domain of TFL was indispensable for localization to GW/P bodies and posttranscriptional cytokine regulation. Because TFL has endonuclease in the N terminus, which can sometimes be harmful for the cell itself, it is reasonable that the C terminus proline-rich domain keeps it within GW/P bodies and enables the restricted function of TFL.

In terms of cytokine regulations, we observed very interesting results in the suppression by zinc finger deletion mutant of TFL (Fig. 5C, Suplemental Fig. 3A). The suppression of 3′UTR of several cytokines, including IL-2, IL-6, IL-10, and TNF-α, was not significant in HeLa (Suplemental Fig. 3A), but the deletion mutant suppresses TNF-α significantly in Jurkat. Notably, the deletion mutant still localized in P bodies, which enable interaction with mRNAs (Fig. 2C). These results indicate that this domain could not exclusively interact with target cytokine mRNAs. Moreover, Zc3h12a suppressed not only IL-2 and IL-6, but also IL-10 and TNF-α in Jurkat transfectants (Fig. 5C, Supplemental Fig. 3A). Overexpression of Zc3h12a was reported to suppress TNF-α mRNA expression in mouse macrophage cell line Raw264.7 cells (24). Whether different posttranscriptional regulation may occur in a cell type–specific manner, even though the target 3′UTR is the same, warrants further study.

Alternatively, no significant differences were noted in the secretion of TNF-α and IL-17a between TFL+/+ and TFL−/− T cells (Supplemental Fig. 2A), although the overexpression of TFL could modulate these mRNAs (Fig. 5C). These differential effects may be caused by other cytokine regulators such as the RNA binding protein and/or transcriptional factors. For example, Roquin was recently shown to regulate the TNF-α message via degradation of the stem loop structure in 3′UTR (39). Other induced RNA regulators may compensate for the effects of TFL in stimulated T cells. The costimulation of CD28 is known to enhance mRNA IL-2 stability as well as its promoter activity through the JNK and Akt signaling pathway (46, 47). In the experiment on anti-CD3 and anti-CD28 costimulation, the overproduction of IL-2 induced by a TFL-deficiency might be canceled by these mRNA regulators (Supplemental Fig. 2A). Because cytokine regulation is critical for the development and resolution of inflammation, regulators including TFL may co-operate with each other to maintain T cell integrity.

Although the oversecretion of IL-2, IL-6, and IL-10 was noted in stimulated TFL−/− splenocytes in vitro, these cytokines were not altered under the EAE condition regardless of the induced expression of TFL (Fig. 6G and data not shown). Thus, excessive cytokine suppression by TFL could be restricted in specific organs in which inflammation is activated. This finding may indicate that expression of the TFL protein is not equivalent to functional expression and

stimulated with anti-CD3 (2.5 μg/ml) for 48 h. Cells were then incubated for the indicated hours in the presence of actinomycin D (2 μg/ml). Cells were subjected to qPCR analysis. Data are shown as a proportion of 0 h. Collective data (n = 5) were analyzed with the Mann–Whitney U test. **p < 0.01. (C) Luciferase assay for mRNA 3′UTR degradation in Jurkat cells. The full length of mRNA 3′UTR for mouse IL-2, IL-6, IL-10, IL-17a, TNF-α, and IFN-γ was inserted into the multiloning site of the psiCheck2 vector. Both the luciferase vector and control, TFL, or Zc3h12a vector were transfected into Jurkat cells with Lipofectamine. Cells were harvested and lysed with lysis buffer 24 h after transfection, and firefly and Renilla luciferase activities were measured using a luminometer. The open bar indicates the control luciferase vector, and each filled bar indicates the cytokine vector. hTFL, human TFL expression vector; hTFL-Δ2z, zinc finger–deleted human TFL expression vector; mTFL, mouse TFL expression vector; Zc3h12a, mouse Zc3h12a expression vector. Each bar is shown in proportion to the control vector. Collective data (n = 5) were analyzed with the Mann–Whitney U test. *p < 0.05, **p < 0.01. (D) The IL-2 3′UTR luciferase vector and control, C terminus truncated human TFL, or proline-rich domain deletion mutant TFL vector shown in Fig. 2B was cotransfected into HeLa cells and luciferase activity was assessed, as described in (C). Data are shown in proportion to the control vector. Data (n = 5) were analyzed with the Mann–Whitney U test. *p < 0.05, **p < 0.01. (E) Cell cycle analysis by the addition of IL-2. MACS-sorted pan T cells were stimulated with the plate-coated anti-CD3 Ab (5 μg/ml) and IL-2 (20 ng/ml; R&D Systems) with or without the anti-CD28 Ab (1 μg/ml). Cells were analyzed using propidium iodide 48 h after stimulation. Each histogram indicates the means ± SEM. Each experiment was performed five times, and collective data were analyzed with the Mann–Whitney U test. *p < 0.05, **p < 0.01. (F) The control (open bar), IL-6 3′UTR minimal conserved element (30 nucleotides) deleted (gray bar), IL-6 Δ conserved element, or IL-6 3′UTR (black bar) luciferase vector, and control, mouse Zc3h12a, or mouse TFL vector were transfected into HeLa cells and luciferase activity was assessed, as described in (C). Data are shown in proportion to the control vector. Collective data (n = 5) were analyzed with the Mann–Whitney U test. ***p < 0.01. (G) Immunoblot analysis shows the level of dicer knockdown in HeLa cells 72 h after small interfering RNA transfection (upper panel). Both the control (open bar) or IL-2 3′UTR (filled bar) luciferase vector and control or mouse TFL vector were transfected into HeLa cells 48 h after small interfering RNA transfection and luciferase activity was assessed, as described in (C) (lower panel). Immunoblot analyses were performed three times, and representative data are shown. Each bar of luciferase assay is shown in proportion to the control vector. Collective data (n = 5) were analyzed with the Mann–Whitney U test. *p < 0.05, ***p < 0.001.
FIGURE 6. Progression of paralysis in EAE-induced TFL−/− mice. (A) TFL+/+ (black box) mice and TFL−/− (red triangle) mice were immunized with MOG33–55/CFA and injected with pertussis toxin on day 0 and day 1. Paralysis (clinical score of EAE) was assessed every day after immunization. Data are shown as the mean clinical score ± SEM of 10 sex-matched mice for each group. (B) Intracellular cytokine staining of CD4+ T cells isolated from the CNS of TFL+/+ and TFL−/− mice on day 20 (peak of disease) and day 40 (resolution phase) after the MOG immunization. (C and D) Percentages of IL-17a (C) and IFN-γ (D) were determined by intracellular flow cytometry during the peak of the disease (TFL+/+, n = 9; TFL−/−, n = 12) and the resolution phase (TFL+/+, n = 6; TFL−/−, n = 9). (E) Expression of mRNA for Th17-related genes relative to control actin for CD4+ T cells isolated from the CNS of TFL+/+ (n = 8) and TFL−/− (n = 12) mice during the resolution phase after the MOG immunization. (F) The percentage of Foxp3+ cells in CD4+ T cells in the CNS was determined by intracellular flow cytometry during the resolution phase (TFL+/+, n = 5; TFL−/−, n = 5). These results were not significant. (G) Expression of TFL mRNA relative to control actin for CD4+ T cells isolated from the CNS, spleen, and lymph nodes of TFL−/− mice during the resolution phase after the MOG immunization (EAE+, n = 9) and spleen of TFL+/+ nonimmune mice (EAE−, n = 6). (H) Gene expression analysis of human multiple sclerosis brains. Analyzed data were normalized by the means of control samples. AP, acute plaque; CAP, chronic and acute plaque; CP, chronic plaque. The expression of TFL expression was higher in each section than in the control. (I) Critical role of TFL expression in CD4+ (Figure legend continues)
is spatiotemporally regulated during chronic inflammation. The mechanism for how the functional expression of TFL is regulated has not yet been determined. However, TFL may have more than one mechanism of action that is regulated by kinases and phosphatases similar to TTP (48) and Zc3h12a (49) and may have specific function in inflammatory sites.

We previously reported that TFL inhibited cellular growth in the mouse normal pro-B cell line as well as human leukemia cells by suppressing phospho-Rb (20) (Fig. 4F). The overexpression of TFL also blocked G1/S checkpoint entry in those cell lines (20) (Fig. 4G). In contrast to the findings of the transfectant studies, we demonstrated that TFL deficiencies facilitated the proliferation of stimulated lymphocytes (Fig. 4B, Supplemental Fig. 1A). Phospho-Rb also appears to be the most affected protein involved in the cell cycle pathway in activated **TFL**−/− T cells (Fig. 4A, 4D, Supplemental Fig. 1B). TFL-deficient lymphocytes also facilitate entry into the S phase more than that of wild-type cells (Fig. 4C). Taken together with our previous studies, TFL could influence T cell proliferation through the Rb pathway and may suppress excessive cellular growth. Cytokine regulation by TFL could be one of the possible reasons why induced TFL inhibits tumor cellular overgrowth. However, the simple addition of IL-2, which TFL modulates, could not adequately compensate for T cell proliferation (Fig. 5E). Therefore, other mechanisms may be involved in the proliferation of **TFL**−/− T cells. In addition to cytokine regulation, TFL could regulate other molecules, such as receptors and transcription factors similar to Zc3h12a (36). Post-transcriptional RNA modification affects some cyclins and oncogene myc, which are upstream of Rb and involve the G1/S checkpoint (50, 51). This deregulation eventually contributes to undesired inflammation and tumorigenesis. We have not yet observed the significant overproduction of cyclin D2 or D3 in **TFL**−/− stimulated lymphocytes (Fig. 4A, 4D, Supplemental Fig. 1B). Nevertheless, whether the posttranscriptional regulator TFL modulates Rb pathway-related transcripts via 3′ UTR cleavage is of interest.

Moreover, TFL could be associated with miRNA machinery because both Zc3h12a and TFL localize to GW/P bodies, in which the RNA-induced silencing complex exists, and recognize the hairpin loop structure of precursor miRNA. Although we found that TFL-induced cytokine degradation was dicer-independent, **TFL**−/− T cells downregulated several miRNAs depending on the stimulation (data not shown). Alternatively, members of the Zc3h12 family were reported to possess deubiquitination activity, which modulates NF-κB cellular signaling (23, 31). We evaluated the generalized ubiquitination status in the CNS, spleen, and lymph nodes during the resolution phase of EAE and IL-17a–induced NF-κB signaling in TFL-transfected HeLa cells; however, no significant difference was observed in deubiquitination activity (data not shown). Although members of the Zc3h12 family have conserved regions and similar functions, the N and C terminus domains and each expression pattern in human tissues differ from one another (Supplemental Fig. 4D). Therefore, the Zc3h12 family could use these functions according to each allocated tissue and then orchestrate each other’s immune reaction.

T cells play a pivotal role in intrinsic B cell lymphomagenesis in the microenvironment. For example, the ablation of CD4+ T cells in Vav-P-Bcl2 transgenic mice, which developed FL, caused a 10-fold reduction in lymphoma cells in the germinal centers (52). Moreover, follicular helper T cells, a specialized subset of CD4+ T cells in the germinal center, form a FL cell niche because they interact with other FL cells via the CXCL13/CXCR5 axis and secrete IL-4, which activates lymphoma cellular signaling, such as ERK and STAT6 (53, 54). These findings suggest that B cell lymphomagenesis is somewhat dependent on CD4+ T cells. The inducible T cell costimulator regulated by Roquin is also important for the formation of the germinal center (55). Roquin paralogs and Zc3h12a control follicular helper T cell differentiation through the regulation of Ox-40 (12, 36). Thus, posttranscriptional regulators could be associated with the development of a lymphoma cell niche. Because we originally identified TFL from a B cell lymphoma, it would be of interest to examine whether TFL contributes to B cell lymphomagenesis in a lymphoma cell–intrinsic manner or via other cells in the microenvironment, such as persistently activated TFL-deficient T cells, as shown in the present study.

To our knowledge, this is the first study to investigate the pathophysiological function of TFL in vivo. Whether TFL is associated with other T cell–mediated autoimmune disorders, such as rheumatoid arthritis, psoriasis, Crohn’s disease, and systemic lupus erythematosus, as well as chronic graft-versus-host disease is of interest (56–60). Another Zc3h12 family gene, Zc3h12c, was also recently reported to be one of the psoriasis susceptibility genes (61). The findings for TFL in lymphocytes reported in the present study could provide important guidance for the better understanding of how posttranscriptional regulation functions in autoimmune diseases and potentially how lymphomagenesis occurs.

**Disclosures**

The authors have no financial conflicts of interest.

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