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_J Immunol_ 2014; 192:1547-1557; Prepublished online 8 January 2014;
doi: 10.4049/jimmunol.1300989
http://www.jimmunol.org/content/192/4/1547

Supplementary Material
http://www.jimmunol.org/content/suppl/2014/01/07/jimmunol.1300989.DCSupplemental

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Dual-Specificity Phosphatase 14 (DUSP14/MKP6) Negatively Regulates TCR Signaling by Inhibiting TAB1 Activation

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T cell activation is dependent upon phosphorylation of MAPks, which play a critical role in the regulation of immune responses. Dual-specificity phosphatase 14 (DUSP14; also known as MKP6) is classified as a MAPK phosphatase. The in vivo functions of DUSP14 remain unclear. Thus, we generated DUSP14-deficient mice and characterized the roles of DUSP14 in T cell activation and immune responses. DUSP14 deficiency in T cells resulted in enhanced T cell proliferation and increased cytokine production upon T cell activation. DUSP14 directly interacted with TGF-β–activated kinase 1 (TAK1)–binding protein 1 (TAB1) and dephosphorylated TAB1 at Ser438, leading to TAB1–TAK1 complex inactivation in T cells. The phosphorylation levels of the TAB1–TAK1 complex and its downstream molecules, including JNK and IκB kinase, were enhanced in DUSP14-deficient T cells upon stimulation. The enhanced JNK and IκB kinase activation in DUSP14-deficient T cells was attenuated by TAB1 short hairpin RNA knockdown. Consistent with that, DUSP14-deficient mice exhibited enhanced immune responses and were more susceptible to experimental autoimmune encephalomyelitis induction. Thus, DUSP14 negatively regulates TCR signaling and immune responses by inhibiting TAB1 activation. The Journal of Immunology, 2014, 192: 1547–1557.

The serine/threonine MAPK family includes ERK, p38, and JNK (1). MAPks regulate various cellular responses, such as proliferation, stress response, differentiation, and immune defense (2). MAPks are activated through a phosphorylation cascade, beginning with the phosphorylation of MAPK kinases (MAPKKs) by MAPKK kinases. Activated MAPKKs then phosphorylate MAPks at the adjacent threonine and tyrosine residues in the conserved Thr-X-Tyr motif within the activation loop of the kinase. The biological outcome of MAPK signaling is dictated by the duration and magnitude of kinase activation. Dual-specificity phosphatases (DUSPs) are responsible for dephosphorylating both threonine and tyrosine residues within the Thr-X-Tyr motif in MAPks (3). DUSPs can be categorized into typical DUSPs (also known as MAPK phosphatases [MKPs]), which contain an N-terminal region composed of two CDC25 homology 2 (CH2) domains and a C-terminal catalytic domain, and atypical DUSPs, which lack the N-terminal CH2 domain (4). DUSP14 (also known as MKP6) is an atypical DUSP; DUSP14 contains the consensus C-terminal catalytic domain but lacks the N-terminal CH2 domain. DUSP14 dephosphorylates JNK, ERK, and p38 in vitro (5); however, dominant-negative DUSP14 mutant enhances the activation of JNK and ERK, but not of p38, in the overexpressing system (5). DUSP14 dominant-negative mutant enhances pancreatic β cell proliferation by increasing ERK activation in the overexpressing system (6). Overexpression of DUSP14 inhibits TNF- and IL-1–induced NF-κB activation (7). The functions of DUSP14 in vivo are still unclear.

T lymphocytes play a critical role in the regulation of immune responses. Engagement of TCR triggers LCK activation, which leads to phosphorylation of the ITAMs of CD3, resulting in ZAP70 recruitment and activation. ZAP70 activation induces the assembly of the proximal signaling complex, which includes LAT, GRB2, GADS, phospholipase C-γ1 (PLC-γ1), VAV, and SLP-76 (8). SLP-76 is negatively feedback regulated by hematopoietic progenitor kinase 1 (HPK1; MAP4K1) (9). The activated PLC-γ1 hydrolyzes inositol phospholipids into inositol triphosphates and diacylglycerols. The production of inositol triphosphates leads to the release of intracellular Ca2+. TCR-induced production of diacylglycerols results in the activation of two major pathways involving Ras and protein kinase C-0 (PKC-0) (10). GCK-like kinase (GLK; MAP4K3) can directly activate PKC-0 during TCR signaling (11). PKC-0 can phosphorylate CARMA1, which leads to the formation of a trimeric complex comprising CARMA1, BCL-10, and MALT1 (12). This complex can lead to the activation of IκB kinase (IKK) and downstream transcriptional factor NF-κB (12). Also, PKC-0 regulates TGF-β–activated kinase 1 (TAK1), a member of the MAPKK kinase family, leading to activation of IKK and NF-κB in a CARMA1-independent manner in the TCR pathway (12). TAK1 can also activate MKK4 and MKK3/6, resulting in the activation of JNK and p38, respectively. The major pathway involved in ERK activation is mediated by the sequential activation of Ras, Raf, and MEK1/2 (8).

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Received for publication April 11, 2013. Accepted for publication December 8, 2013.

This work was supported by grants from the National Health Research Institutes, Taiwan (98A1-IMPP01-014 to T.-H.T.) and the Taichung Veterans General Hospital, Taichung 40705, Taiwan; ‡Division of Allergy, Immunology, and Rheumatology, Taichung Veterans General Hospital, Taichung 40402, Taiwan; †Division of Immunology and Rheumatology, China Medical University Hospital, Taichung 40402, Taiwan; †Department of Pathology and Immunology, Baylor College of Medicine, Houston, TX 77030

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The online version of this article contains supplemental material.

Abbreviations used in this article: CH2, CDC25 homology 2; DUSP, dual-specificity phosphatase; EAE, experimental autoimmune encephalomyelitis; GLK, GCK-like kinase; IKK, IκB kinase; KLH, keyhole limpet hemocyanin; MAPKK, MAPK kinase; MAPK, MAPK phosphatase; MOG, myelin oligodendrocyte protein; NP, 4-hydroxy-3-nitrophenylacetyl; PKC-0, phospholipase C-0; PLC-γ1, phospholipase C-γ1; siRNA, short hairpin RNA; TAB1, TGF-β–activated kinase 1–binding protein 1; TAK1, TGF-β–activated kinase 1; WT, wild-type.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1300989
TAK1 kinase activity is controlled by TAK1-binding protein 1 (TAB1) (13, 14). The activation of TAK1 is impaired in TAB1-deficient mouse embryonic fibroblasts during IL-1 or TNF-α signaling (15). The association between TAB1 and TAK1 causes a conformational change of the catalytic domain, inducing the kinase activity of TAB1 (16). TAB1 is phosphorylated at Ser423, Thr431, and Ser438 in response to LPS, proinflammatory cytokines, and cellular stresses (17). p38 negatively regulates TAK1 kinase activity by phosphorylating TAB1 at Ser42 and Thr431 during IL-1 or TNF-α signaling (17). TAB1 is essential for JNK and NF-κB activation during TCR signaling in primary T cells (18); however, the role of TAB1 in TCR signaling is still unknown.

To study the in vivo function of DUSP14 in TCR signaling and immune responses, we generated DUSP14-deficient mice. In this article, we report genetic evidence that T cell activation and proliferation are enhanced in DUSP14-deficient T cells. DUSP14-deficient mice showed enhanced immune responses in vivo. Furthermore, our results indicate that DUSP14 negatively regulates TCR signaling and immune responses by inhibiting TAB1 activation.

Materials and Methods

Generation of DUSP14-deficient mice

A 129 mouse embryonic stem cell clone with DUSP14 gene deficiency from the International Gene Trap Consortium was injected into C57BL/6 blastocysts to generate chimeric mice at the Transgenic Mouse Model Core, National Research Program for Genomic Medicine (Taipei, Taiwan). The heterozygous DUSP14 progeny from the crossing of the chimeras with C57BL/6 mice were used to generate DUSP14-deficient mice. For genotyping by PCR, common 3′ primer, wild-type (WT)-specific 5′ primer, and knockout (KO)-specific 5′ primer were mixed together in the same reaction, which generated 900-bp and 1300-bp DNA fragments for the WT and KO genotypes. Mouse used in each experiment were 6–12 wk old and were backcrossed to C57BL/6 for at least five generations. Animals were housed under specific pathogen–free conditions under institutional guidelines, and all animal protocols were approved by the Institutional Animal Care and Use Committee at the National Health Research Institutes.

Flow cytometry analysis and purification of T cells

Thymocytes, splenocytes, and lymph node cells were dissected and crushed in RPMI 1640 medium containing 10% FBS. Brains was removed by ACK lysis buffer. Cells were stained with different fluorescently labeled Abs at 4°C for 15–20 min, washed twice, and analyzed on a FACSCanto II (BD Biosciences). Flow cytometry data were analyzed with FlowJo software (TreeStar). Fluorescence-conjugated Abs to the following mouse Ags were used for flow cytometry (all from BioLegend): CD4 (RM4-5), CD8 (53-6.7), CD3 (145-2C11), CD122 (IL-4Rα), CD11b (M1/70), CD49d (2B7), CD25 (PC61), CD25 (PC61), CD44 (H129.19), CD45 (30-F11), CD69 (H1.2F3), IFN-γ (XMG1.2), and IL-17A (TC11-18H10). Abs to JNK phosphorylated at Thr183/Tyr185 (9053) was from Cell Signaling. Abs to ERK phosphorylated at Thr202/Tyr204 (4695) was from BD Biosciences. For intracellular staining, cells were permeabilized for 1 h in 100 μl Cytofix/Cytperm buffer (BD Biosciences) and then stained for intracellular cytokines at 4°C for 1 h. To purify T cells, RBC-depleted splenocytes and lymph node cells were isolated and negatively selected using a mixture of anti-B220, anti-CD11b, and anti-CD49d Abs (BioLegend) on a MACS column at 4°C for 1 h. To stain for CD4 and CD8, followed by flow cytometry analysis. For measurement of activation-induced cell death in vitro, purified T cells were stimulated for 24 h with PMA (10 ng/ml) and ionomycin (1 μg/ml; both from Sigma). Cells were washed and then cultured in IL-2 for an additional 3 d. Cells were restimulated for 48 h with plate-bound anti-CD3 (145-2C11; ebiosis) or anti-Fas (BD Bioscience). Cell viability was measured by staining with annexin V (BD Pharmingen).

Keyhole limpet hemocyanin immunization

Mice used in each experiment were 8–12 wk-old sex-matched littersmates. Mice (five/group) were immunized by i.p. injection with 200 μg 4-hydroxy-3-nitrophenylacetyl (NP)-coupled keyhole limpet hemocyanin (KLH; Biosearch Technologies) emulsified in alun (Sigma). Sera were collected at day 14 after immunization. The titers of anti-NP-specific IgM, IgG1, IgG2a, IgG2b, and IgG3 in the sera were measured by ELISAs (ebiosis) using NP-BSA (Biosearch Technologies) as the coating Ag, followed by incubation with anti-IgM, anti-IgG1, anti-IgG2a, anti-IgG2b, and anti-IgG3 goat anti-mouse Abs (all from Bethyl Laboratories) (19). To determine the activation of Ag-specific T cells, mice were immunized s.c. at the tail base with KLH emulsified in CFA (Chondrex). Seven days later, cells from lymph nodes of the immunized mice were cultured for 3 d in the presence of 0–5 μg/ml KLH, and cytokine production was analyzed by ELISA. Cell proliferation was measured at 72 h, with [3H]thymidine added during the last 16 h of culture.

Experimental autoimmune encephalomyelitis induction

Mice used in each experiment were 8–12 wk-old female littersmates. Experimental autoimmune encephalomyelitis (EAE) was induced by s.c. injection of mice (five per group) with 100 μg myelin oligodendrocyte protein (MOG) peptides emulsified in CFA (Chondrex). Mice were also injected i.p. with 200 μg pertussis toxin (List Biological Laboratories) on days 0, 1, and 2. All mice were monitored for clinical signs and were assigned scores on a scale of 0–5 as follows: 0, no overt signs of disease; 1, limp tail; 2, limp tail and partial hindlimb paralysis; 3, complete hindlimb paralysis; 4, complete hindlimb and partial forelimb paralysis; and 5, moribund state or death (9). The brains and spinal cords were collected from MOG-immunized mice at day 14 after immunization. Mononuclear cells were isolated using Percoll gradient (GE Healthcare), followed by surface staining for CD4 and CD11b and intracellular staining for IL-17A. To determine the activation of Ag-specific T cells, cells from lymph nodes of the immunized mice were cultured in the presence of 0–100 μg/ml MOG, and cytokine production was analyzed by ELISA for 72 h. Cell proliferation was measured at 72 h, with [3H]thymidine added during the last 16 h of culture.

In vitro T cell differentiation

CD4+CD25− T cells were purified from the spleen of mice. Cells were activated with 2 μg/ml anti-CD3 (145-2C11) plus 2 μg/ml anti-CD28 (37.51; both from BD Biosciences) Abs, for Th17 differentiation, cells were cultured in RPMI 1640 plus 10% FBS medium containing IL-6 (20 ng/ml; R&D Systems), TGF-β (10 ng/ml; Sigma), IL-23 (50 ng/ml; R&D Systems), anti-IL-4 (2.5 μg/ml; BioLegend), and anti–INF-γ (2.5 μg/ml; BioLegend) for 4 d. For Th1 differentiation, cells were cultured in RPMI 1640 plus 10% FBS medium containing IL-12 (2.5 μg/ml; R&D Systems) and IL-2 (2.5 μg/ml; R&D Systems), and anti–IL-4 (0.625 μg/ml; BioLegend) for 5 d. Cells were restimulated with PMA plus ionomycin in the presence of GolgiStop (BD Biosciences) for 3 h. Cells were then permeabilized and stained with the appropriate Abs.

Liquid chromatography–mass spectrometry

The sample preparation and liquid chromatography–mass spectrometry analysis were as described (20).

Plasmids, Abs, and purified proteins

Ab to PKC-θ phosphorylated at Thr538 (07-885) was from Upstate Biotechnology. Abs to PLC-γ phosphorylated at Tyr383 (2821), TAK1 phosphorylated at Thr183 (4508), ZAP70 phosphorylated at Tyr197 (2717), MKK4 phosphorylated at Ser223/Thr226 (9156), IKK phosphorylated at Ser76/116 (2694), TAB1 (3226), and TAK1 (4505) were from Cell Signaling. Abs to JNK phosphorylated at Thr183 (2155-1) and SLP-76 phosphorylated at Tyr421 (2419-1) were from Epitomics. Abs to TAB1 phosphorylated at Ser538 (06-1333) and TAB1 phosphorylated at Thr531 (06-1334) were from Millipore. Abs to DUSP14 and to ERK phosphorylated at Thr202/Tyr204 were generated by immunization of rabbits with individual peptides. Anti-DUSP14 Ab (H00011702-M02) was from Abnova. Anti-Flag (M2), anti-Myc (9E10), anti-tubulin (T4026), and anti-actin (A5441) Abs were from Sigma. Flag-tagged DUSP14 WT and Myc-tagged DUSP14 WT
plasmids were constructed by subcloning DUSP14 cDNA into the vector pCMV6-AC-Flag (OriGene Technologies) or pcDNA4-Myc-His (Invitrogen). The pCMV6-DUSP14 (C111S) mutant plasmid was generated by PCR mutagenesis. For in vitro binding assays, purified TAB1 proteins were isolated from HEK293T cells transfected with Flag–TAB1, followed by Flag-peptide elution. Purified TAB1 proteins were incubated with either GST only or rGST-DUSP14 proteins at 4°C for 2 h, followed by GST pull-down assays. rGST-TAK1 proteins were incubated with either purified DUSP14 or TAB1 proteins at 4°C for 2 h, followed by GST pull-down assays. The lysates were washed, boiled in 2× sample buffer, and analyzed by immunoblotting.

**Transient transfection and T cell activation**

For transient transfection assays, cells were transfected with a Neon Transfection System (Invitrogen). Electroporation settings were as follows: 1440 V for a duration of 40 ms and one pulse for Jurkat (J-TAg clone) T cells and 2200 V for a duration of 20 ms and one pulse for primary T cells. For induction of T cell activation, Jurkat (J-TAg clone) T cells were stimulated with anti-CD3 Ab (3 μg/ml; OKT3; eBioscience) for the indicated times at 37°C. Primary T cells were stimulated with biotin-conjugated anti-CD3 (3 μg/ml) and anti-CD28 (3 μg/ml; 37.51; eBioscience) Abs plus streptavidin (3 μg/ml) or PMA (0.02 μg/ml; Sigma) plus ionomycin (0.2 μg/ml; Sigma) for the indicated times at 37°C. Murine primary T cells were transfected with a mixture of four unique 29-mer GFP-tagged TAB1 short hairpin RNAs (shRNAs) or a scrambled GFP-tagged shRNA (both from OriGene Technologies) for 36 h. Cells were stimulated with biotin-conjugated anti-CD3 (3 μg/ml; 500A2; eBioscience) Ab plus streptavidin (3 μg/ml; Sigma) for 15 min, followed by intracellular staining for p-JNK (Cell Signaling), p-IKK (Santa Cruz), or p-ERK (Cell Signaling). The GFP-tagged shRNA-transfected cells were GFP gated for flow cytometry analysis.

**Alpha technology/protein–protein interaction assay**

Alpha technology experiments were performed according to the manufacturer’s protocol (PerkinElmer Life Sciences). HEK293T cells were cotransfected with Myc-DUSP14 plus either Flag–TAK1 or Flag–TAB1 for 24 h. The cells were lysed in lysis buffer (50 mM Tris-HCl, 125 mM NaCl, 5% glycerol, 0.2% Nonider P-40, 1.5 mM MgCl2, 25 mM NaF, 1 mM Na3VO4). The cell lysates were incubated with anti-Myc beads for 60 min and then incubated with anti-Flag beads (both from PerkinElmer) for another 60 min. The Alpha signaling was determined by an EnVision 2104 Multilabel Reader (PerkinElmer).

**Statistical methods**

The p values were determined using the Student t test. Values < 0.05 were considered statistically significant.

**Results**

**Normal lymphoid organ development in DUSP14-deficient mice**

To study the function of DUSP14 in TCR signaling, we generated DUSP14-deficient mice. An insertion vector containing a splice

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**FIGURE 1.** Normal lymphoid organ development in DUSP14-deficient (DUSP14-KO) mice. (A) Flow cytometry of thymocyte cellularity and phenotype. (B) The expression of CD25 and CD44 on CD4−CD8− double-negative (DN) thymocytes was analyzed by flow cytometry. (C) Flow cytometry of surface marker expression on splenocytes for CD3+ T cells, CD4+ T cells, CD8+ T cells, and B220+ B cells. (D) Surface expression of CD44 and CD62L on gated CD4+ T cells. (E) Flow cytometry of regulatory T (CD4+Foxp3+) cells in the thymus and spleen. Percentage of cells within each quadrant is shown on the plots. Five mice were analyzed for each genotype. Data are mean ± SEM. DN1, CD25−CD44+; DN2, CD25−CD44−; DN3, CD25+CD44−; DN4, CD25+CD44+; DP, double positive; SP, single positive.
acceptor, β-geo (a fusion of β-galactosidase and neomycin resistance genes), and a polyadenylation sequence was inserted in the intron between exons 2 and 3 of the targeted DUSP14 allele (Supplemental Fig. 1A). Successful targeting was confirmed by PCR analysis of genomic DNA from mice tails (Supplemental Fig. 1B). Western blot analysis confirmed the loss of DUSP14 expression in the thymus of the DUSP14-deficient mice (Supplemental Fig. 1C). DUSP14-deficient mice were born at the expected Mendelian ratio and survived to adulthood without any obvious physical abnormalities. We next examined whether loss of DUSP14 expression might show any defect in the lymphocyte development in DUSP14-deficient mice. Total thymic cellularity was similar in WT and DUSP14-deficient mice. Flow cytometric analysis showed that DUSP14 deficiency did not alter the development of CD^4^-CD^8^- double-negative, CD^4^-CD^8^+ double-positive, CD^4^-single-positive, or CD^8^- single-positive thymocytes (Fig. 1A). The expression pattern of CD44 or CD25 on CD^4^-CD^8^- double-negative thymocytes was also unaffected by DUSP14 deficiency (Fig. 1B). In the spleen and lymph nodes, there were similar numbers of CD3^+ T cells, CD4^+ T cells, CD8^+ T cells, and B220^+ B cells between WT and DUSP14-deficient mice (Fig. 1C, Supplemental Fig. 1D). The frequencies of naive (CD44^-CD62L^+), effector memory (CD44^+CD62L^-), and memory (CD44^+CD62L^+) T cells in the spleen were also unaffected in DUSP14-deficient mice (Fig. 1D). Cell numbers of regulatory T cells (CD4^+Foxp3^+) in thymus, spleen, and lymph nodes were also unaffected in DUSP14-deficient mice (Fig. 1E, Supplemental Fig. 1E). These data suggest that DUSP14 is not essential for lymphocyte development.

**T cell activation and proliferation are enhanced in DUSP14-deficient T cells**

After TCR ligation, T cells undergo a series of signaling events, including upregulation of cell surface activation markers, proliferation, and cytokine production. To examine the role of DUSP14 in TCR signaling, we purified naïve T cells from WT and DUSP14-deficient mice and determined T cell activation upon anti-CD3 Ab stimulation. The percentages of activated CD69^+ T cells (Fig. 2A) or CD25^+ T cells (Fig. 2B) were increased in DUSP14-deficient T cells upon anti-CD3 Ab stimulation. We further studied the effect of DUSP14 on T cell proliferation using [3H]thymidine-incorporation assays. DUSP14 deficiency significantly enhanced T cell proliferation induced by stimulation with anti-CD3 Ab
Next, we studied whether the hyperproliferation of DUSP14-deficient T cells is due to a reduction in apoptosis. To study activation-induced cell death in vivo, we examined CD4+CD8+ thymocyte death induced by injection of anti-CD3 Ab. We found that anti-CD3–induced thymocyte deletion was similar between WT and DUSP14-deficient mice (Fig. 2D). Furthermore, WT and DUSP14-deficient T cells showed similar percentages of cell death induced by anti-CD3 Ab or anti-Fas Ab restimulation in vitro (Fig. 2E). These results indicate that the hyperproliferation of DUSP14-deficient T cells is not due to a reduction in apoptosis.

To examine the effect of DUSP14 deficiency on T cell cytokine production, we purified naive T cells from WT and DUSP14-deficient mice and determined cytokine production upon T cell activation using ELISA assays. Upon anti-CD3 Ab stimulation, DUSP14-deficient T cells also produced more IL-2, IFN-γ, and IL-4 than did WT T cells (Fig. 2F). CD28 provides a costimulatory signal for T cell activation, and the expression of CD28 is significantly reduced in DUSP14-deficient T cells, as shown by reduced surface expression of CD28 in these cells (Fig. 2G). These results suggest that the reduced CD28 expression may contribute to the altered cytokine production in DUSP14-deficient T cells.

**FIGURE 3.** DUSP14 interacts with TAB1 in T cells. (A) Sequence of TAB1. Five TAB1 peptides (underlined) were identified by mass spectrometry. (B) DUSP14 interacts with TAB1 in HEK293T cells. Coimmunoprecipitation and immunoblot analysis of DUSP14 and TAB1 in lysates of HEK293T cells transfected with empty vector or plasmid encoding Flag–TAB1, with or without plasmid encoding Myc–DUSP14. (C) DUSP14 interacts with TAB1 in Jurkat T cells. Coimmunoprecipitation and immunoblot analysis of DUSP14 and TAB1 in lysates of Jurkat (J-TAg clone) T cells transfected with plasmid encoding Flag–DUSP14. (D) Interaction of endogenous DUSP14 and TAB1 in murine primary T cells. Coimmunoprecipitation and immunoblot analysis of DUSP14 and TAB1 in lysates of primary T cells.

**FIGURE 4.** DUSP14 interacts directly with TAB1 and dephosphorylates TAB1 at Ser438. (A) DUSP14 interacts directly with TAB1 in vitro. In vitro binding assays of purified Flag–TAB1 and GST–DUSP14 proteins. (B) DUSP14 does not interact directly with TAK1 in vitro. In vitro binding assays of GST–TAK1 proteins with either purified Flag–DUSP14 proteins or purified Flag–TAB1 proteins. (C) DUSP14–TAB1 interaction was determined by Alpha technology protein–protein interaction assay. Data are representative of at least three independent experiments. (D) DUSP14–TAB1 interaction was determined by Alpha technology protein–protein interaction assay. Data are representative of at least three independent experiments. (E) In vitro phosphatase assays of purified DUSP14 WT or phosphatase-dead mutant C111S (CS), using purified Flag–TAB1 WT or Flag–TAB1 mutant (S438A) as substrates. (F) Immunoblotting of lysates of Jurkat (J-TAg clone) T cells stimulated with anti-CD3 Ab at the indicated times. The lysates were immunoblotted with Abs to p-TAB1 at Ser438 and Thr431. (G) Coimmunoprecipitation and immunoblot analysis of phosphatase-dead DUSP14 mutant (C111S) and Ser438-phosphorylated TAB1 in lysates of Jurkat (J-TAg clone) T cells transfected with plasmid encoding DUSP14 mutant (C111S).
tory signal by enhancing TCR-regulated proliferation, cytokine production, and immune responses. T cell proliferation was also enhanced in DUSP14-deficient T cells upon anti-CD3 plus anti-CD28 Ab costimulation using [3H]thymidine-incorporation assays and CFSE dye–dilution assays (data not shown). Upon anti-CD3 plus anti-CD28 Ab costimulation, DUSP14-deficient T cells also produced more IL-2, IFN-γ, and IL-4 than did WT T cells (data not shown). Also, T cell proliferation induced by PMA plus ionomycin, which bypasses proximal TCR-signaling events and directly activates PKC-θ, was enhanced in DUSP14-deficient T cells (Fig. 2G). Cytokine production, including IL-2, IFN-γ, and IL-4, was increased in DUSP14-deficient T cells upon stimulation with PMA plus ionomycin (Fig. 2H). These results demonstrate that DUSP14 negatively regulates T cell proliferation and cytokine production upon T cell activation.

To further study the molecular mechanism of DUSP14 in TCR signaling, we analyzed TCR-induced activation of both proximal and distal signaling molecules. Jurkat (J-TAg clone) T cells were transfected with DUSP14 WT or DUSP14 mutant (C111S), and these cells were stimulated with anti-CD3 Ab or PMA plus ionomycin. In DUSP14 mutant (C111S) cells, the Cys to Ser substitution resulted in the loss of its phosphatase activity (Supplemental Fig. 2A). Overexpression of DUSP14 in Jurkat (J-TAg clone) T cells did not alter the activation of LCK, ZAP70, or SLP-76 during TCR stimulation. The relative phosphorylation levels were determined by densitometry analysis.

FIGURE 5. DUSP14 negatively regulates activation of the TAB1–TAK1 complex and its downstream molecules. Purified T cells were stimulated with anti-CD3 (A and C) or PMA plus ionomycin (C) at the indicated times. The cells were lysed; the lysates were immunoblotted with Abs to p-TAB1 at Ser438 or Thr431, p-TAK1, p-IKK, p-JNK, p-ERK, p-p38, and β-actin. (B and D) The relative phosphorylation levels were determined by densitometry analysis.
TCR signaling (Supplemental Fig. 2B). Consistently, TCR-induced activation of LCK, ZAP70, and SLP-76 was not affected by DUSP14 deficiency (Supplemental Fig. 2C). The activation of IKK, JNK, and ERK was significantly reduced in DUSP14 WT, but not in DUSP14 mutant (C111S), overexpressed cells upon TCR stimulation (Supplemental Fig. 2D). Furthermore, compared with vector control, DUSP14 WT overexpression inhibited the activation of IKK, MKK4, JNK, and ERK upon PMA plus ionomycin stimulation (Supplemental Fig. 2E), suggesting that ERK may be a direct target of DUSP14 upon T cell activation. In addition, the activation of MKK4 and IKK is inhibited by DUSP14; thus, DUSP14 may function upstream of both MKK4 and IKK upon T cell activation.

**DUSP14 directly dephosphorylates and inactivates TAB1 in TCR signaling**

Because the activation of MKK4 and IKK is blocked by DUSP14, DUSP14 may target a signaling molecule upstream of both MKK4 and IKK in the TCR-signaling pathway. Thus, we searched for the target(s) of DUSP14 in TCR signaling using mass spectrometry. Jurkat (J-TAg clone) T cells were transfected with Flag–DUSP14 and then stimulated with anti-CD3 Ab. DUSP14-associated proteins were coimmunoprecipitated with anti-Flag Ab and then fractionated by SDS-PAGE. The excised protein bands were analyzed by liquid chromatography–mass spectrometry. We found that a protein with an M₉ ∼ 60 kDa coimmunoprecipitated with DUSP14; mass spectrometry identified this as TAB1 (Fig. 3A, Supplemental Fig. 3). An interaction of DUSP14 with TAB1 was detected using a coimmunoprecipitation assay in HEK293T cells (Fig. 3B). To determine whether DUSP14 regulates TAB1 activation during TCR signaling, we investigated the potential interaction between DUSP14 and TAB1 during TCR signaling by coimmunoprecipitation assays. DUSP14 constitutively interacted with TAB1 in Jurkat (J-TAg clone) T cells (Fig. 3C) during TCR signaling. Coimmunoprecipitation of DUSP14 also pulled down TAK1, possibly through TAB1 because TAB1 is a TAK1-binding protein (Fig. 3C). The interaction between endogenous DUSP14 and TAB1 proteins in murine primary T cells was further confirmed using coimmunoprecipitation assays (Fig. 3D). We further studied whether DUSP14 interacts directly with TAB1 in vitro. We found a direct interaction between DUSP14 and TAB1 in vitro using purified GST-DUSP14 and Flag–TAB1 proteins (Fig. 4A). In contrast, DUSP14 did not interact directly with TAK1 in vitro using purified proteins, whereas a direct interaction between TAK1 and TAB1 was detectable as expected (Fig. 4B). Alpha technology is a bead-based assay used to study protein–protein interaction up to 200 nm in solution. HEK293T cells were transfected with Myc–DUSP14 plus either Flag–TAK1 or Flag–TAB1. The cells were lysed; the lysates were incubated with anti-Myc beads and then anti-Flag beads. A direct interaction between DUSP14 and TAB1, but not DUSP14 and TAK1, was detected (Fig. 4C).

Tab1 is phosphorylated at Ser⁴³⁸, Thr⁴³¹, and Ser⁴²³ in several inflammatory signaling pathways (13). Next, we studied whether DUSP14 dephosphorylates TAB1 in vivo and in vitro. HEK293T cells were cotransfected with DUSP14 WT or DUSP14 mutant (C111S) plus either TAB1 WT or TAB1 mutant (S438A). The in vivo phosphatase assay showed that DUSP14 WT dephosphorylated TAB1 at Ser⁴³⁸, but not Thr⁴³¹, whereas the phosphatase-dead DUSP14 mutant (C111S) did not dephosphorylate TAB1.
with Ser438-phosphorylated TAB1 during TCR signaling. Because Thr431 (Fig. 4D, 4E). Thus, we studied whether DUSP14 interacts investigated TAB1 phosphorylation at Ser438 during TCR signaling. First, we showed that DUSP14 dephosphorylated TAB1 at Ser438 but not at Thr431 (Fig. 4D, 4E). Thus, we studied whether DUSP14 interacts with Ser438-phosphorylated TAB1 during TCR signaling. Because it may be difficult to detect the interaction between DUSP14 and phosphorylated TAB1, because phosphatases may quickly dissociate from their substrates after dephosphorylation, we used the phosphatase-dead DUSP14 mutant (C111S) to study the interaction. Jurkat (J-TAg clone) T cells were transfected with phosphatase-dead DUSP14 mutant (C111S) and then stimulated with anti-CD3 Ab. Coimmunoprecipitation assays showed that phosphatase-dead DUSP14 mutant (C111S) inducibly interacted with Ser438-phosphorylated TAB1 during TCR signaling (Fig. 4G). Our results suggest that DUSP14 interacts with nonphosphorylated TAB1 in resting T cells and that DUSP14 inducibly interacts with Ser438-phosphorylated TAB1 during TCR signaling. We then studied the effect of DUSP14 deficiency on TAB1 phosphorylation in primary T cells. We found that the phosphorylation level of TAB1 at Ser438, but not at Thr431, was enhanced in DUSP14-deficient T cells upon anti-CD3 plus anti-CD28 costimulation (Fig. 5A, 5B). Also, the phosphorylation levels of TAK1, IKK, JNK, and ERK were enhanced in DUSP14-deficient T cells (Fig. 5A, 5B). In addition, the phosphorylation levels of TCR-proximal signaling, including PLC-γ1 and PKC-θ, were unaffected in the absence of DUSP14 (Fig. 5A). Using PMA plus ionomycin treatment, we also observed that the phosphorylation levels of TAB1 at Ser438, TAK1, IKK, JNK, and ERK were enhanced in DUSP14-deficient T cells (Fig. 5C, 5D). Next, we studied whether TAB1 knockout attenuates the hyperactivation of JNK and IKK in DUSP14-deficient T cells upon TCR stimulation. Murine primary T cells were transfected with GFP-tagged TAB1 shRNAs or a scrambled GFP-tagged shRNA for 36 h and then stimulated with anti-CD3, followed by flow cytometry analysis. The knockdown efficiency of GFP-tagged shRNA for 36 h and then stimulated with anti-CD3 Abs were determined by ELISA. Results are presented relative to those of normal serum from a WT mouse. Five mice were analyzed for each genotype. Data are mean ± SEM. *p < 0.05, two-tailed t test.

FIGURE 7. Enhanced in vivo immune responses in DUSP14-deficient (DUSP14-KO) mice. WT and DUSP14-KO mice were immunized with KLH emulsified in alum. Seven days later, enlarged lymph nodes were isolated and restimulated with KLH for 72 h. (A) Cell proliferation was measured using [3H]thymidine incorporation. (B) IL-2, IL-4, and IFN-γ levels in supernatants were measured using ELISA. Three mice were analyzed for each genotype. Data are mean ± SEM. (C) WT and DUSP14-KO mice were immunized as before. Sera were collected at 14 d after the primary immunization. NP-specific IgM, IgG1, IgG2a, IgG2b, and IgG3 Abs were determined by ELISA. Results are presented relative to those of normal serum from a WT mouse. Five mice were analyzed for each genotype. Data are mean ± SEM. *p < 0.05, two-tailed t test.

To determine whether DUSP14-deficient mice are more susceptible to autoimmune disease, we immunized mice with MOG in CFA to induce EAE. DUSP14-deficient mice were more susceptible to EAE induction and displayed severe paralysis of forelimbs and hindlimbs (Fig. 8A). Serum IL-17A and IFN-γ levels were higher in DUSP14-deficient mice than in WT mice (Fig. 8B). More CD4+ T cells infiltrated the brain tissues of diseased DUSP14-deficient mice, whereas similar numbers of CD11b+ cells infiltrated the brain tissues of diseased DUSP14-deficient mice (Fig. 8C). The percentage of Th17 cells in the infiltrating CD4+ T cells in the brain and spinal cords of diseased mice at day 14 was significantly higher in DUSP14-deficient mouse than in WT mice (Fig. 8D).

Restimulated DUSP14-deficient lymph node T cells obtained from MOG-immunized mice showed enhanced proliferation (Fig. 8E) and increased production of cytokines, including IFN-γ and IL-17A (Fig. 8F). To investigate whether the enhanced Th17 and Th1 responses in DUSP14-deficient mice are T cell intrinsic, we determined the in vitro Th17 and Th1 differentiation of DUSP14-deficient T cells. Consistent with the results obtained above, the in vitro differentiation of Th17 and Th1 were enhanced in DUSP14-deficient cells (Fig. 8G). Thus, DUSP14 deficiency promotes the proliferation and the activation of autoactive T cells, leading to enhanced EAE induction in DUSP14-deficient mice (Fig. 9). In addition to T cells, other immune cells may also contribute to the exacerbated EAE symptoms in DUSP14-deficient mice.
Discussion

At least 25 members of the DUSP family have been identified. Only two DUSPs (DUSP4 and DUSP10) are reported to be involved in T cell activation and T cell–mediated immune responses using KO mice (21–23). DUSP10 (also named MKP5) is a positive regulator in T cell proliferation and T cell–mediated immune responses (22). DUSP4 (also named MKP2) suppresses CD4+ T cell proliferation but induces normal Th1/Th2 responses following KLH immunization (23), whereas DUSP4 enhances Th1 responses following *Leishmania mexicana* infection (21). To our knowledge, our study demonstrates for the first time a novel immune function for DUSP14 in T cells in vivo. A key finding of our study is the identification of DUSP14 as a negative regulator upon T cell activation and immune responses using DUSP14-deficient mice. DUSP14-deficient T cells proliferate more and produce excess IL-2, IFN-γ, and IL-4 upon stimulation with anti-CD3, anti-CD3 plus anti-CD28, or PMA plus ionomycin. Also, DUSP14-deficient mice are more susceptible to EAE induction. A previous report (5) showed that DUSP14 interacts with CD28 in overexpressing systems. Primary human peripheral blood T cells transduced with a dominant-negative DUSP14 mutant secrete more IL-2 in response to anti-CD3 plus anti-CD28 costimulation but not anti-CD3 alone or PMA plus ionomycin. Dominant-negative mutants may show artifacts; it is plausible that ectopically expressed DUSP14-deficient (DUSP14-KO) mice to EAE induction. WT and DUSP14-KO female mice were immunized with MOG peptide emulsified in CFA, followed by injection of pertussis toxin at days 0, 1, and 2. (A) All mice were monitored daily for clinical signs and were assigned disease scores. Five mice were analyzed for each genotype. (B) ELISA of IL-17A and IFN-γ in sera from EAE-diseased mice. Five mice were analyzed for each genotype. (C) Flow cytometry of infiltrated mononuclear cells from the brains and spinal cords of MOG-immunized mice. Numbers indicate percentages of CD4+ cells (upper panels) and CD11b+ cells (lower panels). Three mice were analyzed for each genotype. (D) Flow cytometry of infiltrated Th17 cells from the brains and spinal cords of MOG-immunized mice. Three mice were analyzed for each genotype. (E) Proliferation of splenic T cells obtained from MOG-immunized mice, restimulated with MOG peptide and analyzed by [3H]thyminde incorporation. Three mice were analyzed for each genotype. (F) ELISA of the production of IFN-γ and IL-17A by splenocytes obtained from MOG-immunized mice and restimulated with MOG peptide. Three mice were analyzed for each genotype. (G) Flow cytometry of IL-17A–producing CD4+ T cells and IFN-γ–producing CD4+ T cells among in vitro–differentiated Th17 cells and Th1 cells, respectively. Data are representative of at least three independent experiments. Data are mean ± SEM. *p < 0.05, two-tailed *t* test.

FIGURE 8. Enhanced susceptibility of DUSP14-deficient (DUSP14-KO) mice to EAE induction. WT and DUSP14-KO female mice were immunized with MOG peptide emulsified in CFA, followed by injection of pertussis toxin at days 0, 1, and 2. (A) All mice were monitored daily for clinical signs and were assigned disease scores. Five mice were analyzed for each genotype. (B) ELISA of IL-17A and IFN-γ in sera from EAE-diseased mice. Five mice were analyzed for each genotype. (C) Flow cytometry of infiltrated mononuclear cells from the brains and spinal cords of MOG-immunized mice. Numbers indicate percentages of CD4+ cells (upper panels) and CD11b+ cells (lower panels). Three mice were analyzed for each genotype. (D) Flow cytometry of infiltrated Th17 cells from the brains and spinal cords of MOG-immunized mice. Three mice were analyzed for each genotype. (E) Proliferation of splenic T cells obtained from MOG-immunized mice, restimulated with MOG peptide and analyzed by [3H]thyminde incorporation. Three mice were analyzed for each genotype. (F) ELISA of the production of IFN-γ and IL-17A by splenocytes obtained from MOG-immunized mice and restimulated with MOG peptide. Three mice were analyzed for each genotype. (G) Flow cytometry of IL-17A–producing CD4+ T cells and IFN-γ–producing CD4+ T cells among in vitro–differentiated Th17 cells and Th1 cells, respectively. Data are representative of at least three independent experiments. Data are mean ± SEM. *p < 0.05, two-tailed *t* test.
TAK1-deficient CD4+ thymocytes show impaired IL-2 production, which may be due to the hyperactivation of both TAK1 and ERK2. The hyperactivation of TAK1 and ERK2, respectively, results in the enhanced IL-2 and IFN-γ production in T cells, as well as ERK2 in IL-2 regulation in T cells, needs to be addressed. ERK2-deficient CD4 T cells show impaired Th1, but not Th17, differentiation in vitro (28). Thus, the enhanced Th1 differentiation in vivo in DUSP14-deficient T cells may be due to the effect of ERK2 hyperactivation. The results from TAB1–small interfering RNA knockdown in myeloid cells and TAK1-conditional KO in glia cells suggest that TAK1 is required for Th1 and Th17 differentiation and in vivo induction of autoimmunity (29, 30). ERK1-deficient mice in the 129 Sv, but not the C57BL/6, genetic background are more susceptible to EAE induction (31). Thus, the enhanced Th1/Th17 differentiation in vivo and exacerbated EAE symptoms in DUSP14-deficient mice are likely due to the effect of TAB1 hyperactivation. In summary, DUSP14 attenuates TCR signaling and immune responses by inhibiting both TAB1–TAK1 and ERK activation.

Acknowledgments

We thank Academia Sinica’s Institute of Biological Chemistry (Taipei, Taiwan) for use of its LTQ-Orbitrap XL hybrid mass spectrometer to analyze the proteomics data.

Disclosures

The authors have no financial conflicts of interest.

References


