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Mnk1 and 2 Are dispensable for T cell development and activation but important for the pathogenesis of experimental autoimmune encephalomyelitis

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T cell development and activation are usually accompanied by expansion and production of numerous proteins that require active translation. The eukaryotic translation initiation factor 4E (eIF4E) binds to the 5' cap structure of mRNA and is critical for cap-dependent translational initiation. It has been hypothesized that MAPK-interacting kinases 1 and 2 (Mnk1/2) promote cap-dependent translation by phosphorylating eIF4E at serine 209 (S209). Pharmacologic studies using inhibitors have suggested that Mnk1/2 have important roles in T cells. However, genetic evidence supporting such conclusions is lacking. Moreover, the signaling pathways that regulate Mnk1/2 in T cells remain unclear. We demonstrate that TCR engagement activates Mnk1/2 in primary T cells. Such activation is dependent on Ras-Erk1/2 signaling and is inhibited by diacylglycerol kinases α and ζ. Mnk1/2 double deficiency in mice abolishes TCR-induced eIF4E S209 phosphorylation, indicating their absolute requirement for eIF4E S209 phosphorylation. However, Mnk1/2 double deficiency does not affect the development of conventional αβ T cells, regulatory T cells, or NKT cells. Furthermore, T cell activation, in vivo primary and memory CD8 T cell responses to microbial infection, and NKT cell cytokine production were not obviously altered by Mnk1/2 deficiency. Although Mnk1/2 deficiency causes decreased IL-17 and IFN-γ production by CD4 T cells following immunization of mice with myelin oligodendrocyte glycoprotein peptide in complete Freund’s adjuvant, correlating with milder experimental autoimmune encephalomyelitis scores, it does not affect Th cell differentiation in vitro. Together, these data suggest that Mnk1/2 has a minimal role in T cell development and activation but may regulate non–T cell lineages to control Th1 and Th17 differentiation in vivo. The Journal of Immunology, 2013, 190: 1026–1037.

T cells have a critical role in adaptive immune responses. Activation of T cells is critical for mounting immune responses against foreign Ags and to protect the host from infection (1). However, tight regulation of this process is important for the maintenance of self-tolerance (2). The signal from the TCR, via multiple intracellular signaling pathways such as the RasGRP1-Ras-Erk1/2-AP1, PKCθ-NF-κB, PI3K-Akt, and Ca2+/calcinurin-NFAT pathways, has critical roles in T cell maturation and activation (3–5). Orchestrated actions of these signaling cascades ensure proper T cell maturation and T cell activation.

In addition to TCR engagement, various other extracellular stimuli such as growth factors, cytokines, and stress can induce activation of MAPKs. Based on the signals that trigger their activation, MAPKs are categorized as ERKs (Erk1/2), p38 kinases, and C-Jun N-terminal kinase/stress-activated protein kinases (JNK) (6). MAPKs control a wide range of functions including proliferation, differentiation, survival, and apoptosis through direct phosphorylation and activation of substrates (7). These substrates, called MAPK-activated protein kinases (MAPKAPKs), are divided into four families based on the type of phosphorylating MAPK (8, 9). For example, the p90 ribosomal S6 kinase (Rsk) family includes Rsk1, Rsk2, and Rsk3 that are specifically phosphorylated and activated by ERKs (10); MAPKAPKs such as MK2/3 and MK5 are activated by JNKs (11); and mitogen- and stress-activated kinases (MSKs) including MSK1 and MSK2 are phosphorylated by p38 MAPKs (12). Different from these MAPKAPKs, MAPK-interacting kinases 1 and 2 (Mnk1/2) are serine/threonine kinases and are phosphorylated by both ERKs and p38 kinases (13). Murine Mnk1/2 are phosphorylated at threonine 197 and 202 (T197 and T202) or T244 and T249 respectively, which leads to their activation (14–18). Activated Mnk1/2 directly phosphorylates the eukaryotic translation initiation factor 4E (eIF4E) at S209 downstream of growth factor receptors (18). eIF4E binds to 5’ methylguanosine (m7GpppN) cap structure found in all eukaryotic mRNAs, and this binding is obligatory for the initiation of cap-dependent translation (19, 20). Cap-dependent translation is the primary

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B.K.G. and S.K. were involved in experimental design and execution, data analysis, and preparation of the manuscript. J.S. designed and performed experiments. M.I. and M.L.S. were involved in the experimental autoimmune encephalomyelitis experiments. J.M.G. and R.F. provided essential reagents. X.-P.Z. conceived the project and was involved in experimental design, data analysis, and manuscript preparation.

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Abbreviations used in this article: DAG, diacylglyceroi; DGK, DAG kinase; EAE, experimental autoimmune encephalomyelitis; eIF, eukaryotic translation initiation factor; α-GalCer, α-galactosylceramide; iNKT, invariant NKT; LCMV, lymphocytic choriomeningitis virus; LM-OVA, Listeria monocytogenes-expressing recombinant OVA; LN, lymph node; MAPKAPK, MAP-activated protein kinase; Mnk1/2, MAPK-interacting kinase 1 and 2; MOG, myelin oligodendrocyte glycoprotein; mTOR, mammalian target of rapamycin; Rsk, ribosomal S6 kinase; WT, wild-type.

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mode of eukaryotic translation by which 95% of total cellular mRNAs are translated (21). It has been hypothesized that Mnk1/2 are key protein kinases that can promote cap-dependent translation through eIF4E phosphorylation (22).

The roles of Mnk1/2 were originally studied in Drosophila, whose Mnk ortholog is called Lk6. Deficiency of this gene was found to impair growth and development, leading to a shortened life span (23). However, in mice, Mnk1/2 double deficiency did not grossly affect development and growth, although eIF4E phosphorylation at S209 was abolished (18). While dispensable for murine development, Mnk1 and Mnk2 have been demonstrated to play an oncogenic role in mice, and their deficiency delays tumor development in a murine tumor model (24). Studies using pharmacologic inhibitors and eIF4E phosphorylation mutants have shown that eIF4E phosphorylation has an important role in cell survival and cancer progression (25).

Using chemical inhibitors, several previous studies have reported that Mnk1/2 could have an important role in immune cells. For example, chemical inhibition of Mnk1/2 was found to decrease the translation of IL-17 in CD4+ T cells (26), IFN-γ and IL-4 in invariant NKT (iNKT) cells (27), and inflammatory cytokines in macrophages (28). Although these studies provide preliminary evidence that Mnk1/2 activity could have a critical role in immune cell function, possible off-target effects of the chemical inhibitors used cannot be overlooked. In this report, we demonstrate that TCR engagement induces activation of Mnk1/2 and phosphorylation of eIF4E, which is enhanced by Ras signaling, and inhibited by diacetylplcyoglycerol (DAG) kinases α and β that terminate DAG-mediated signaling (29). By using mice deficient in Mnk1/2 (Mnk1/2DKO), we show that Mnk1 and Mnk2 are essential for TCR-induced phosphorylation of eIF4E. However, deficiency of both Mnk1 and Mnk2 does not affect gross T cell development, activation, proliferation, or cytokine production. Furthermore, Mnk1/2 activities are dispensable during CD8 T cell–mediated immune responses against Listeria monocytogenes and lymphocytic choriomeningitis virus (LCMV) and for iNKT cell development and cytokine production.

Materials and Methods

Mice

C57BL/6J mice and TCR-OT1 transgenic mice were purchased from the Jackson Laboratory. Mice expressing a conditional constitutively active form of Ras in a T cell–specific manner (caKRas-CD4Cre) and Mnk1/2DKO mice on a C57BL/6J background were described previously (18, 30–32). DAG kinase (DGK) α and β double-knockout mice were reported previously (33, 34). All mice were used according to a protocol approved by the Duke University Institutional Animal Care and Use Committee.

Flow cytometry

Thymocytes, splenocytes, and lymph node (LN) cells were prepared following standard procedures. Cells were stained with fluorochrome-conjugated Abs for CD4, CD8, CD62L, CD44, CD25, TCRβ, CD24, NK1.1, and CD69 (BioLegend) as well as CD1d-Tetramer (provided by the National Institutes of Health Tetramer Facility) in 2% FBS-PBS at 4°C for 30 min. In addition, Live/Dead Fixable Violet Dead Cell Stain (Invitrogen) was used to identify the viable cells. The stained cells were collected using a BD FACS Canto II flow cytometer. The collected data were analyzed using FlowJo software. Isolation of liver mononuclear cells and staining of iNKT cells were performed as described previously (32, 35).

Activation, anergy, and proliferation assays

Splenocytes from wild type (WT) or Mnk1/2DKO mice were left unstimulated or stimulated with anti-CD3 (1 μg/ml; 2C-11) overnight in the presence or absence of either anti-CD28 (0.5 μg/ml) or CTLA4-Ig (10 μg/ml; BioXcell) to assess the upregulation of early activation markers by FACS. For proliferation assays, splenocytes were labeled with CFSE as described previously (36), left unstimulated or stimulated with anti-CD3 for 72 h. After staining for CD4 and CD8, cells were subjected to FACS analysis. In some experiments, CPG57380 (Tocris Bioscience, a Mnk1/2 inhibitor) was added in the culture at the indicated concentrations. To examine T cell anergy, WT and Mnk1/2DKO splenocytes were stimulated with anti-CD3 in the presence of either anti-CD28 (0.5 μg/ml) or CTLA4-Ig (10 μg/ml) at 37°C for 48 h. Cells were then washed three times and rest in IMDM at 37°C for 24 h. During the last 5 h of stimulation, PMA, PHA (12-μg/ml h-3-acetate, 50 ng/ml), ionomycin (500 ng/ml Sigma) and GolgiPlug were added. After surface staining with anti-TCR-β Ab and the PBS-57-loaded mouse CD1d tetramer (CD1d-Tet), cells were intracellularly stained for IFN-γ and IL-17 followed by FACS analysis. iNKT cell proliferation was similarly assessed except that thymocytes were labeled with CFSE and PMA, and ionomycin stimulation was not added.

Immunoblot

Immunoblots were prepared as described previously (31). Thymocytes or splenocytes were washed with PBS. Cells were suspended in PBS with calcium and left unstimulated or stimulated with 5 μg/ml of anti-CD3ε (500A2; BD Pharmingen) for different times. After stimulation, cells were centrifuged and lysed in 1% Nonidet P-40 buffer (1% Nonidet-40, 150 mM NaCl, and 50 mM Tris, pH 7.4) supplemented with protease and phosphatase inhibitor mixture (Sigma). Total proteins were separated by SDS-PAGE and transferred to a Trans-Blot Nitrocellulose Membrane (Bio-Rad). To examine protein phosphorylation, the membranes were incubated overnight with Abs specific for phospho Erk1/2, phospho p38, phospho 4E-BP1 (T37/46), phospho-eIF4E (S209), phospho-Mnk1 (T197/202), and phospho-eIF4E (S223/225) (Santa Cruz Biotechnology). Later, the membranes were incubated with the appropriate secondary, peroxidase-conjugated Abs. The blots were developed using the ECL System from Perkin-Elmer. The same blots were stripped and reprobed using control Abs.

T helper differentiation assay

Naive CD4+ T cells were purified from LN cells and cultured with plate-bound anti-CD3 (2C11) 5 μg/ml, soluble anti-CD28 (1 μg/ml) and indicated skewing conditions. Skewing conditions were as follows: Th1, IL-12 (5 ng/ml), IFN-γ (100 ng/ml) and anti–IL-4 (100 μg/ml), with IL-2 (100 U/ml) during the rest period; Th2, IL-4 (1 ng/ml), anti-IL-12 (100 μg/ml), and anti-IFN-γ (100 μg/ml), with IL-2 (100 U/ml) during the rest period; Th17, TGF-β (10 ng/ml), IL-6 (10 ng/ml), anti–IFN-γ (10 μg/ml) and anti-IL-4 (100 μg/ml); Th6, IL-4 (20 ng/ml) anti-IL-12 (10 μg/ml), and TGF-β (2 ng/ml) during rest period. Th0, IL-2 (100 U/ml) during the rest period. After culturing for 5 d, cells were stimulated with PMA and ionomycin in the presence of GolgiPlug at 37°C for 5 h. Following surface staining, cells were intracellularly stained for indicated cytokines, followed by FACS analysis.

Adaptive transfer and L. monocytogenes–expressing recombinant OVA infection to assess CD8+ T cell response in vivo

Naive OT1 T cells (Vwc2*CD8+ 7AAD*CD44*) were sorted from LN cells from Thy1.1 WT-OT1 and Thy1.2 Mnk1/2DKO OT1 mice. Five thousand sorted WT OT1 cells were mixed with an equal number of sorted Mnk1/2DKO OT1 cells in 200 μl of serum-free IMDM and adoptively transferred into retrovirally injected into sex-matched WT Thy1.1 WT-OT1 and Thy1.2 Mnk1/2DKO OT1 mice. After 24 h, recipient mice were injected i.v. with 1 × 107 CFUs of L. monocytogenes–expressing recombinant OVA (LM-OVA) (37). Peripheral blood samples (collected in PBS with 5 mM EDTA) and splenocytes were analyzed at 1 and 2 wk after infection. After lysing of RBCs, samples were stained with fluorochrome-conjugated Abs and analyzed with flow cytometry. Frequencies of Thy1.1 WT-OT1 and Thy1.2 Mnk DKO populations were corrected to account for the deviation of the input ratio from 50:50.
LCMV infection

LCMV Armstrong stocks were propagated on BHK-21 cells and quantitated as described previously (38). LCMV infection and assessment of viral-specific CD8 T cell responses were performed as described previously (39). Mice were infected with 2 × 10⁶ PFU of virus i.p. and monitored by serial bleeding and tetramer staining. For memory experiments, viable CD8^+CD44^+ memory cells were sorted from donor mice 8 wk after LCMV infection. While donor mice were Thy1.1^+Thy1.2^+, recipients were Thy1.1^+Thy1.2^+. Frequency of H-2D^b tetramer loaded with LCMV gp33–41 (TetG)-positive cells in the sorted population was determined by flow cytometry, and an appropriate number of total memory cells was transferred such that each recipient mouse received 5000 TetG^+ memory cells. Recipients were infected with 2 × 10⁶ PFU of LCMV i.p. the next day and taken 7 d later to assess the memory response.

Induction and scoring of experimental autoimmune encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) was induced in 6–10-wk-old female mice by s.c. injection of myelin oligodendrocyte glycoprotein (MOG35–55) peptide (100 μg/mouse) emulsified in CFA containing 2 mg/ml Mycobacterium tuberculosis (100 μl/mouse). Mice were also injected i.p. with 200 ng of pertussis toxin on day 0 (day of immunization) and day 2. Mice were monitored for ∼40 d to assess the development of a clinical score based on the following criteria: 1 = tail limpness; 2 = impaired righting reflex; 3 = hind limb paralysis; 4 = complete paralysis; 5 = death. Some mice were sacrificed on day 7 to assess CD4 T cell impairment righting reflex; 3 = hind limb paralysis; 4 = complete paralysis; 5 = death. Some mice were sacrificed on day 7 to assess CD4 T cell differentiation in response to the immunization. Draining LN cells were stimulated with MOG35–55 peptide for 3 d to expand the pool of Ag-specific CD4 T cells, followed by stimulation for 5 h with PMA (50 ng/ml) and ionomycin (500 ng/ml) in the presence of GolgiPlug. After stimulation, cells were stained for cell surface CD4 and intracellularly stained for IL-17A and IFN-γ.

Statistical analysis

Statistical significance was determined using the ANOVA or Student test. The p values are defined as follows: p < 0.05, p < 0.01, p < 0.001.

Results

Regulation of Mnk1/2 activation and expression in T cells

TCR signaling has been shown to trigger several intracellular signaling pathways leading to phosphorylation and activation of p38 and Erk1/2. We assessed anti-CD3–induced Mnk1 activation in freshly isolated thymocytes or splenic T cells from WT mice. As shown in Fig. 1A, TCR engagement induced Mnk1 and eIF4E phosphorylation in both thymocytes and splenic T cells, correlated with Erk1/2 and p38 activation. In T cells, DAG binds to and activates RasGRP1, which in turn activates the Ras-Mek1/2-Erk1/2 pathway. In the presence of a constitutively active form of Ras (kRas), TCR-induced Erk1/2, Mnk1, and eIF4E phosphorylation were significantly increased, indicating that Ras signaling promotes Mnk1/2 activation in T cells (Fig. 1B). We have previously demonstrated that DGKα and ζ inhibit the activation of the Ras-Erk1/2 signaling (33, 34, 40, 41). In DGKα and ζ double-deficient thymocytes (α/ζDKO), both Erk1/2 and eIF4E phosphorylation were enhanced (Fig. 1C). Furthermore, this phosphorylation was greatly inhibited by U0126 (U0), a MEK1/2 inhibitor. Together, these observations indicate that TCR engagement induces Mnk1/2 activation, and that such activation is mediated by the Ras-Erk1/2 pathway and is inhibited by DGK activity.

Although Mnk1 and Mnk2 are ubiquitously expressed, their expression is varied in different tissues (17). Using real-time quantitative PCR, we assessed Mnk1/2 mRNA levels in naive and activated CD4^+ and CD8^+ T cells. Both Mnk1 and 2 mRNA levels were expressed at higher levels in naive T cells than in activated T cells. Mnk1 was decreased to 45% and 30%, whereas Mnk2 expression was reduced to 50% and 20% in activated CD4 and CD8 T cells, respectively, compared with naive T cells (Fig. 1D). The decreased expression of Mnk1/2 proteins in activated T cells was further confirmed by immunoblotting analysis (Fig. 1E).

Naive and activated T cells are drastically different in metabolism

![FIGURE 1](http://www.jimmunol.org/) Regulation of Mnk1/2 activation in T cells. Thymocytes and splenocytes of indicated genotypes were rested in PBS at 37°C for 30 min and were then left unstimulated or stimulated with 5 μg/ml anti-CD3 (500A2) for the indicated times. Lysates were subjected to immunoblot analysis with the indicated Abs. (A) TCR engagement activates Mnk1/2 in WT thymocytes (left) and splenocytes (right). (B) Constitutively active KRas promotes Mnk1/2 activation. Thymocytes from WT and ca.Kras–CD4Cre mice were subjected to similar analysis as in (A). (C) DGKα and ζ inhibit TCR-induced Mnk1/2 activation in a MEK1/2–dependent manner. WT and in DGKαζDKO (αζDKO) thymocytes were examined as in (A) with the addition of groups treated with the MEK1/2 inhibitor U0126 (10 μM). (D) and (E) Differential expression of Mnk1/2 in naive and activated T cells. Mnk1 and Mnk2 mRNA (D) and protein (E) levels in sorted WT naive and in vitro–activated CD4 and CD8 T cells were examined by real-time quantitative PCR and Western blotting analysis, respectively. *p < 0.05, **p < 0.01, ***p < 0.001.
and in protein synthesis. Given the proposed role of Mnk1/2 in cytokine production, it is intriguing that Mnk1/2 expression is decreased in activated T cells, in which proteins including cytokines are actively translated.

**Effect of combined Mnk1/2 deficiency on T cell development**

To investigate the role of Mnk1/2 in T cells, we analyzed mice with germline deletion of these two genes. Because individual deficiency of Mnk1 or Mnk2 did not affect T cell development and activation (data not shown), we examined Mnk1/2DKO mice. The percentages and absolute numbers of CD4 and CD8 subsets in the thymus and spleen from Mnk1/2DKO mice were similar to those from WT control mice (Fig. 2A–D). The overall thymic and splenic cellularity in Mnk1/2DKO mice was also comparable to WT mice (Fig. 2E). To examine the role of Mnk1/2 in T cell development, we generated Mnk1/2DKO mice carrying the OT1 TCR transgene, which directs CD8 T cell development. As shown in Figure 2F, thymocyte numbers were not obviously different between Mnk1/2DKO-OT1 mice and WT OT1, supporting a minimal role of Mnk1/2 in intrathymic T cell development.

Mnk1/2DKO mice did not display an obvious alteration in natural regulatory T cell numbers as compared with WT controls (Fig. 2G). Furthermore, CD44 and CD62L staining showed similar naive and effector T cell populations in WT and Mnk1/2DKO mice (Fig. 2H, 2I). These observations indicate that Mnk1/2 double deficiency does not cause obvious defects in T cell development or homeostasis.

**Mnk1/2 are required for TCR induced eIF4E phosphorylation**

As mentioned above, TCR engagement induced eIF4E phosphorylation at S209. To determine whether such phosphorylation is dependent on Mnk1/2, we compared TCR-induced eIF4E phosphorylation in WT and Mnk1/2DKO T cells. Although eIF4E total protein was similar between WT and Mnk1/2DKO T cells, TCR-induced eIF4E phosphorylation was virtually abolished in Mnk1/2DKO T cells (Fig. 3A). On the contrary, TCR-induced phosphorylation of Erk1/2, Rsk1 (Erk1/2 substrate), and p38 was not affected by Mnk1/2 deficiency (Fig. 3B), suggesting that Mnk1/2 deficiency does not cause global signaling defects, and that there is no obvious negative feedback regulation of Erk1/2 and p38 by Mnk1/2 in T cells. Binding of eIF4E to mRNA is inhibited by its association with 4E-BP1. Mammalian target of rapamycin (mTOR) phosphorylates 4E-BP1, leading to the release of eIF4E from 4E-BP1 to initiate translation (42). Neither 4E-BP1 protein levels nor its phosphorylation was altered in Mnk1/2-deficient T cells compared with WT T cells (Fig. 3C), suggesting that Mnk1/2-mediated phosphatidylinositol 3-kinase (PI3K) and phosphoinositide 3-kinase (PI3K) pathways are involved in the regulation of 4E-BP1 phosphorylation.

![Figure 2](http://www.jimmunol.org/)  
**FIGURE 2.** T cell development in Mnk1/2DKO mice. (A and B) CD4 and CD8 expression in WT and Mnk1/2DKO (DKO) thymocytes (A) and splenocytes (B). Representative dot-plots of CD4 and CD8 staining are shown. (C and D) Absolute numbers of thymic (C) and splenic (D) T cell populations in WT and Mnk1/2DKO mice (n = 6). (E) Total thymic and splenic cellularity in WT and Mnk1/2DKO mice. (F) Absolute numbers of thymic T cell populations in WT-OT-I and Mnk1/2DKO-OT-I mice (n = 3). (G) Regulatory T cell staining in the thymus and spleen. CD25 and Foxp3 staining in CD4+ T cells are shown. (H) CD44 and CD62L staining of gated WT and Mnk1/2DKO CD4+ and CD8+ T cells. (I) Mean ± SEM presentation of cell numbers of indicated T cell populations (n = 5). Data shown are representative of at least three experiments.

![Figure 3](http://www.jimmunol.org/)  
**FIGURE 3.** Effect of Mnk1/2 deficiency on TCR-induced signaling. WT and Mnk1/2DKO thymocytes and splenocytes were similarly stimulated and analyzed by immunoblot as in Figure 1A. (A) Mnk1/2 are critical for TCR-induced eIF4E phosphorylation at S209. Neither 4E-BP1 protein levels nor its phosphorylation was altered in Mnk1/2-deficient T cells compared with WT T cells (Fig. 3C), suggesting that Mnk1/2-mediated 4E-BP1 phosphorylation occurs downstream of PI3K and PI3K pathways.
eIF4E phosphorylation does not affect 4E-BP1-mediated suppression of eIF4E or mTOR activity. These results suggest that TCR-induced eIF4E phosphorylation is mediated by Mnk1/2.

Normal in vitro T cell activation in the absence of Mnk1/2
To investigate whether Mnk1/2 deficiency affects T cell activation, we first examined the upregulation of early activation markers CD69 and CD25 following overnight anti-CD3 stimulation in the presence or absence of CD28-mediated costimulation. Mnk1/2DKO T cells upregulated CD69 and CD25 similarly to WT controls in response to a wide range of anti-CD3 stimulation in the presence of anti-CD28 (Fig. 4A). The presence of CTLA4-Ig to block CD28-mediated costimulation decreased CD25 and CD69 upregulation in WT T cells. However, the absence of Mnk1/2 did not cause further reduction of CD25 and CD69 expression in T cells.

Mnk1/2DKO T cells also showed comparable proliferation to WT T cells following anti-CD3 stimulation for 72 h as demonstrated by a CFSE dilution assay (Fig. 4B). Moreover, Mnk1/2DKO CD4 and CD8 T cells produced similar levels of IFN-γ and TNF-α after stimulation for 48 h, compared with WT controls.

**FIGURE 4.** Mnk1 and Mnk2 are not essential for in vitro T cell activation. (A) Upregulation of early activation markers in Mnk1/2DKO T cells. WT and Mnk1/2DKO splenocytes were left unstimulated or stimulated overnight with an anti-CD3 Ab (2C11) at the indicated concentrations in the presence or absence of an anti-CD28 Ab (37.51, 0.5 μg/ml) or CTLA4-Ig (10 μg/ml). Overlaid histograms show CD69 and CD25 expression on gated CD4+ and CD8+ cells. (B) Mnk1/2 deficiency does not affect T cell proliferation. CFSE-labeled WT and Mnk1/2DKO splenocytes were unstimulated or stimulated with an anti-CD3 Ab for 72 h. Cultured cells were stained for CD4 and CD8 and analyzed by flow cytometry. Histograms show CFSE intensity on CD4+ and CD8+ cells. (C) Effect of Mnk1/2 deficiency on cytokine production by T cells. Splenocytes from WT or Mnk1/2DKO mice were left unstimulated (top) or stimulated with an anti-CD3 Ab (bottom) for 48 h, followed by PMA (50 ng/ml) and ionomycin (500 ng/ml) stimulation in the presence of a GolgiPlug for 5 h. Cells were stained for surface CD4 and CD8 and intracellular cytokines followed by FACS analysis. IFN-γ and TNF-α expression in gated CD4+ and CD8+ T cells are shown. (D) Mnk1/2 deficiency does not affect OT1 T cell proliferation. Splenocytes from WT OT1 and Mnk1/2DKO OT1 mice were either labeled or not labeled with CFSE, and then treated with SIINFEKL peptide at indicated concentrations for 18 or 72 h to assess early T cell activation and proliferation, respectively. Overlaid histograms show CD25 and CD69, and CFSE intensity on live-gated CD8+Vα2+ T cells. (E and F) WT and Mnk1/2DKO splenocytes were stimulated with anti-CD3 in the presence or either anti-CD28 (0.5 μg/ml) or CTLA4-Ig (10 μg/ml) at 37°C for 48 h. After resting for 24 h, live T cells were restimulated with plate-bound anti-CD3 (1 μg/ml) and soluble anti-CD28 (0.5 μg/ml) in the presence of 5 μM GolgiPlug at 37°C for 24 h. Cells were surface stained for CD4 and CD8 and intracellularly stained for IFN-γ. FACS plots show IFN-γ expression in live gated CD4 and CD8 T cells (E). Bar graph is mean ± SEM presentation of percentages of IFN-γ+ cells in the indicated populations of cells (n = 3). Data shown are representative of three experiments.
based on intracellular staining (Fig. 4C). To study the effect of Mnk1/2 deficiency on Ag-specific T cell activation, we used OT1 T cells, which express the Vα2ββ5 TCR and recognize the OVA257–264 (SIINFEKL) epitope of OVA presented on H-2Kb. When stimulated with different concentrations of OVA257–264 peptide, *Mnk1*/2*KO* OT1 T cells upregulated CD25 and CD69, and proliferated similarly to WT OT1 T cells (Fig. 4D). These data indicate that Mnk1/2 double deficiency does not obviously affect T cell activation in vitro.

As mentioned earlier, mTORC1 phosphorylates 4E-BP1 to promote eIF4E-mediated translation initiation. Decreased mTORC1 activity causes T cell anergy while enhanced mTORC1 activity leads to resistance to anergy (43–45). We asked further whether Mnk1/2-mediated phosphorylation of eIF4E has a role in T cell anergy. We stimulated WT and *Mnk1*/2*KO* splenocytes with anti-CD3 in the presence of CTLA4-Ig to block CD28-mediated costimulation for 48 h. After an additional 24 h of resting, live T cells were restimulated by plate bound anti-CD3 and soluble anti-CD28 overnight, followed by intracellular staining for IFN-γ. As shown in Figure 4E and 4F, similar IFN-γ levels were detected in WT and *Mnk1*/2*KO* T cells under anergic conditions, suggesting that Mnk1/2 deficiency does not obviously affect T cell sensitivity to anergy induction in vitro.

**Minimal effect of Mnk1/2 deficiency on Th differentiation in vitro**

Using a Mnk1/2 inhibitor, a recent study has implicated Mnk1/2 in IL-17 production by T cells (26). We examined whether deficiency of Mnk1/2 affected Th differentiation. Sorted naive WT and *Mnk1*/2*KO* CD4 T cells were subjected to in vitro Th1, Th2, Th17, and Th9 differentiation. Intracellular staining was used to assess the production of IFN-γ, IL-17, and IL-9 under different skewing conditions, while IL-4 levels were measured by ELISA. As shown in Figure 5, no obvious difference was observed between WT and *Mnk1*/2*KO* T cells in the production of these cytokines. These observations suggest that Mnk1/2 and Mnk1/2-mediated eIF4E phosphorylation are dispensable for Th differentiation in vitro.

**Mnk1/2 deficiency may impair Th1 and Th17 differentiation in vivo in the EAE model**

To determine whether Mnk1/2 deficiency could affect CD4 cell differentiation in vivo, we used the EAE model because the differentiation of CD4 cells into Th1 and particularly into Th17 lineage is known to have an important role in the pathogenesis of the disease (46). Spleens and draining lymph nodes from WT and *Mnk1*/2*KO* mice immunized with MOG35–55 peptide emulsified in CFA showed comparable total cellularity and frequency of CD4 cells 1 wk after immunization (Fig. 6A). When Ag-specific cells were expanded by ex vivo stimulation of lymph node cells with MOG35–55 for 3 d, stimulation with PMA and ionomycin revealed a marked reduction in the frequency of *Mnk1*/2*KO* CD4 T cells that were able to produce IL-17A or IFN-γ (Fig. 6B). Bearing in mind our previous results that Mnk1/2-deficient T cells survive and proliferate similar to WT counterparts (Fig. 4), the reduction in the IFN-γ–producing and IL-17A–producing pools of Ag-specific cells suggests that the absence of Mnk1/2 might somehow impair the differentiation of CD4 T cells into Th1 and Th17 cells in response to Ag stimulation in vivo. Correlating with the smaller pool of IFN-γ–producing and IL-17A–producing cells, mice deficient in Mnk1/2 developed milder EAE disease than WT counterparts (Fig. 6C). These results suggest that Mnk1/2 deficiency impairs Th1 and Th17 differentiation in vivo to perturb disease development in the EAE model.

**Combined Mnk1/2 deficiency does not affect CD8 T cell response to *L. monocytogenes* infection in vivo**

The data described above have revealed that Mnk1 and Mnk2 are dispensable for T cell activation in vitro. We used the *L. monocytogenes* infection model to determine whether Mnk1/2 is required for T cell responses in vivo. Equal numbers of sorted naive WT (Thy1.1++) and *Mnk1*/2*KO* (Thy1.2+) Vα2βCD8+ OT1 cells were mixed and coinjected i.v. into WT Thy1.1++Thy1.2+ recipient mice. Recipients were subsequently infected with LM-OVA (Fig. 7A, 7B). Expansion of OVA-specific OT1 T cells was monitored in the peripheral blood and spleen on days 7 and 14 after LM-OVA infection. No significant difference was observed in the frequency of WT (Thy1.1++) and *Mnk1*/2*KO* (Thy1.2++) OT1 cells in the recipient mice 7 and 14 d after infection (Fig. 7C, 7D). These results suggest that Mnk1/2 might not have a critical role in the expansion and early contraction phases of Ag-specific CD8 T cell responses, at least in the LM-OVA model.

**Mnk1/2-deficient mice mount normal primary and memory CD8 responses to LCMV infection**

We next sought to better understand the effects of Mnk1/2 deficiency on polyclonal primary and memory CD8 T cell responses to viral infection. To this end, we infected WT and *Mnk1*/2*KO* mice with the Armstrong strain of LCMV that causes acute infection (Fig. 8A). We then examined by flow cytometry at 1, 2, and 4 wk after infection.
after infection the frequency of CD8 cells in the peripheral blood that could recognize the LCMV GP33–41 peptide presented on H-2Db tetramers (TetG+ cells). Our results showed that the frequencies of TetG+ cells were comparable in WT and Mnk1/2DKO mice at these time points (Fig. 8B, 8C). Similar results were obtained when we determined the frequency of CD8 T cells that

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** Mnk1/2 deficiency can impair Th1 and Th17 differentiation in vivo. (A) Total cell numbers in the spleen and LNs and percentages of CD4 T cells in these organs in WT and Mnk1/2DKO mice 7 d after immunization with MOG and CFA. (B) IL-17A and IFN-γ–producing cells within CD4 T cells following MOG peptide stimulation for 3 d ex vivo. Dot plots show IFN-γ and IL-17A expression in gated CD4 T cells. Bar graph represents mean ± SEM of IFN-γ and IL-17A producing cells within CD4 T cells from multiple mice (n = 6). (C) EAE score of immunized WT and Mnk1/2DKO mice monitored at indicated time points and scored as indicated in Materials and Methods (n = 5). Mean ± SEM are calculated for the indicated number of mice per group. Data shown are representative of two or more independent experiments. *p < 0.05.

![Figure 7](http://www.jimmunol.org/)

**FIGURE 7.** Combined Mnk1/2 deficiency does not affect Ag-specific primary CD8 T cell responses in vivo. (A) Schematic representation of experimental design showing competitive adoptive transfer of WT Thy1.1+ and Mnk1/2DKO Thy1.2+ OT1 T cells, and the LM-OVA infection model. (B) Thy1.1 and Thy1.2 staining of mixture of sorted naive WT and Mnk1/2DKO OT1 cells before injection. (C) Representative FACS analysis of peripheral blood and splenocytes detecting Vα2+CD8 T cells (top panels), and Thy1.2/Thy1.2 congenic markers within the gated Vα2+CD8+ T cell population at indicated time points after infection. (D) Percentages of WT OT1 (Thy1.1+) and Mnk1/2DKO OT1 (Thy1.2+) cells among total Vα2+CD8+ cells in the peripheral blood and spleen. Mean ± SEM was calculated after correcting for the input ratio. Each dot represents one mouse. Data shown are representative of three independent experiments.
Mnk1/2 deficiency does not affect primary or memory antiviral CD8 responses in vivo. (A) Schematic representation of experimental design showing primary infection with LCMV Armstrong, adoptive transfer of memory cells, and rechallenge. (B and C) Primary response. (B) Representative FACS plots of peripheral blood samples obtained at indicated time points and stained with anti-CD8 Ab and TetG. (C) Mean ± SEM presentation of percentages of CD8+TetG+ cells in the peripheral blood at the indicated time points (n = 6 WT, n = 7 Mnk1/2DKO). (D and E) Recall response. (D) Representative FACS plots of peripheral blood and spleen samples from recipient mice that received WT or Mnk1/2DKO memory cells. Top panels show CD8 and TetG staining of peripheral blood and splenocytes. Bottom panels show Thy1.1 expression in the gated CD8+ TetG+ population. (E) Mean ± SEM presentation of percentages of CD8+TetG+Thy1.1+ cells in peripheral blood and spleen samples from recipient mice (n = 5). Data shown are representative of two independent experiments.

Effects of Mnk1/2 deficiency on iNKT cell development and function

The iNKT cells are a rare subset of T cells with the ability to bridge innate and adaptive immunity by rapidly producing and secreting copious amounts of cytokines. The mechanisms regulating cytokine production in iNKT cells are not well understood. We have recently demonstrated that proper iNKT cell development requires tight regulation of DAG-mediated signaling. Deficiency of RasGRP1 or enhanced activation of DAG-mediated signaling owing to DGKα and ζ deficiency can lead to defects in iNKT cell development (32, 35). Because Mnk1 and Mnk2 are downstream effectors molecules of the DAG-RasGRP1-Ras-Erk1/2 pathway, we investigated whether Mnk1 or Mnk2 have a role in regulating iNKT cell development. Like conventional T cells, individual or combined deficiency of Mnk1/2 did not affect the development of iNKT cells in the thymus, spleen, and liver (Fig. 9A). iNKT cell percentages and absolute numbers were similar in WT and Mnk1/2DKO mice (Fig. 9B). Further analysis of iNKT cell developmental stages based on CD44 and NK1.1 expression did not reveal obvious differences between WT and Mnk1/2DKO iNKT cells following NKT cell development (32, 35). Because Mnk1 and Mnk2 are downstream effectors molecules of the DAG-RasGRP1-Ras-Erk1/2 pathway, we investigated whether Mnk1 or Mnk2 have a role in regulating iNKT cell development. Like conventional T cells, individual or combined deficiency of Mnk1/2 did not affect the development of iNKT cells in the absence of Mnk1/2.

It has been reported that inhibition of Mnk1/2 by CGP57380 decreased cytokine production from iNKT cells following α-GalCer stimulation (27). We stimulated WT and Mnk1/2DKO iNKT cells with α-GalCer in vitro for 72 h and intracellularly stained for IFN-γ and IL-17 production. The percentages of IFN-γ and IL-17–positive Mnk1/2DKO iNKT cells were similar to those of WT iNKT cells (Fig. 9C). Using a CFSE dilution assay, we also examined iNKT cell proliferation following α-GalCer stimulation for 72 h. Mnk1/2DKO iNKT cells appeared to proliferate slightly better than WT iNKT cells (Fig. 9D). These observations indicate that Mnk1 and...
Mnk2 are dispensable for NKT cell development, production of cytokines, and proliferation.

Effects of Mnk1/2 inhibitor CGP57380 on activation of Mnk1/2 double-deficient T cells

The discrepancies between our data from Mnk1/2 double-deficient mice and those generated by chemical inhibition of Mnk1/2 with CGP57380 raise concerns about the selectivity of CGP57380 for Mnk1/2 and about the conclusions drawn from studies based on this inhibitor. To determine whether CGP57380 contains activities beyond inhibiting Mnk1/2, we examined the effects of CGP57380 on Erk1/2, Mnk1/2, and eIF4E phosphorylation following TCR engagement. As shown in Figure 10A, CGP57380 inhibited not only eIF4E phosphorylation but also Mnk1/2 phosphorylation following TCR engagement. Moreover, it inhibited both WT and Mnk1/2 DKO T cell proliferation in similar magnitudes (Fig. 10B), and reduced IFN-γ but not IL-17 production in both WT and Mnk1/2 DKO NKT cells (Fig. 10C). These observations are consistent with the findings that CGP57380 is able to inhibit other protein kinases such as MAPK kinase-1, casein kinase 1, and brain-specific kinase 2 (47). Thus, the effects of CGP57380 on T cells might not solely be attributed to Mnk1/2.

Discussion

Mnk1 and Mnk2 are downstream substrates for the Ras-Mek1/2-Erk1/2 and MKK3/MKK6-p38 kinase pathways. The importance of these pathways in T cells, the ability of Mnk1/2 to phosphorylate eIF4E, and the extremely dynamic nature of T cells during development and immune responses raise the possibility that Mnk1 and Mnk2 could have important roles in T cells by promoting protein translation via eIF4E regulation. Several recent studies lend credence to the idea that Mnk1/2 may be important regulators of the immune system. Inhibition of Mnk1/2 by CGP57380 has been found to reduce the translation of proinflammatory cytokines in keratinocytes (48), macrophages (28), and dendritic cells (49). CGP57380 has also been shown to decrease cytokine production by NKT cells and IL-17 production in CD4+ T cells during Th17 differentiation (26, 27). In this report, we have demonstrated that TCR-induced Mnk1/2 activation is promoted by Ras-Erk1/2 signaling and is negatively controlled by DGKα and ζ. In addition, both Mnk1 and Mnk2 are expressed at high levels in naive T cells, but are downregulated in activated T cells. Using Mnk1/2 double-deficient mice, we have demonstrated that Mnk1 and Mnk2 are dispensable for the development of conventional αβ T cells, natural regulatory T cells, and NKT cells. Moreover, Mnk1/2 double deficiency does not obviously affect activation of conventional T cells and NKT cells, or Th differentiation in vitro. The conclusion of a minimal role for Mnk1/2 in T cell activation is further strengthened by the observation that Mnk1/2 deficiency does not impair in vivo CD8 T cell responses in a bacterial model and a viral model of infection. Furthermore, our data also raise concerns over CGP57380 as a Mnk1/2–specific inhibitor because CGP57380 reduces eIF4E and Mnk1/2 phosphorylation following TCR engagement, inhibits both WT and Mnk1/2 DKO CD4 T cell proliferation, and decreases both WT and Mnk1/2 NKT cell production of IFN-γ.

Mnk1/2 deficiency does not affect Th1 or Th17 differentiation in vitro, suggesting that there might be no obvious intrinsic defect of Mnk1/2 DKO CD4 T cells in Th differentiation. However, Th1 and Th17 differentiation is diminished in Mnk1/2 DKO mice in the...
EAE model, suggesting the possibility that Mnk1/2 deficiency affects in vivo Th lineage differentiation in a T cell–extrinsic manner. Additional experiments are required to explore these extrinsic mechanisms, including the possibility that Mnk1/2 functions in APCs to shape Th differentiation. This notion is supported by a recent report demonstrating that Mnk1 and Mnk2 regulate innate immune responses by modulating NF-kB activity (50).

Signals generated from the TCR can trigger the activation of T cells from naive or resting conditions, resulting in a significant increase in transcription, protein synthesis, and DNA synthesis (51, 52). Translation is one of the early events in activated T cells that increase in transcription, protein synthesis, and DNA synthesis (51, 52). Translation is one of the early events in activated T cells that can contribute to protein synthesis. Translational control is one of the key processes by which cells can generate crucial gene products quickly from preexisting mRNA without delay that results from mRNA transcription and RNA processing (53, 54). Translation is a complex process and involves at least 10 translation initiation factors called eukaryotic translational initiation factors (eIFs), a complex process and involves at least 10 translation initiation factors (eIFs), scaffolding or adaptor proteins, and 40S ribosomes. Binding of eIF4E to the mRNA is the foremost regulatory step in the formation of a preinitiation complex that further leads to the formation of complete translation machinery (19). However, how eIF4E is regulated is poorly understood. It has been proposed that recruitment of eIF4E to the 5′ cap region can be controlled by at least two intracellular signaling pathways such as the PI3K-mTOR pathway (55) and the Ras-Mek–Erk1/2-Mnk1/2 and MKK3/6-p38-Mnk1/2 pathways (56, 57). 4E-BP1 binds to eIF4E, preventing its association with the 5′ cap. Activation of mTOR leads to hyperphosphorylation of eIF4E at S209, which results in the dissociation of eIF4E from 4E-BP1 to allow eIF4E binding to the 5′ cap of mRNA to drive translation. Inhibition of mTOR results in T cell anergy, whereas deregulation of mTOR renders T cells resistant to anergy induction (43–45), suggesting that mTOR-mediated release of eIF4E from suppression by 4E-BP1 is critical for T cell activation. Several studies have put forth a notion that Mnk1/2 signaling can regulate cap-dependent translation through phosphorylation of eIF4E at S209 (22, 58). However, the role of eIF4E phosphorylation at S209 for translation initiation has been controversial (59, 60). Similarly, the germ-line deletion of Mnk1/2 in mouse models resulted in ablation in eIF4E phosphorylation without global effects on protein translation in mouse embryonic fibroblasts. In addition, these mouse models display normal growth and development. Consistently, TCR-induced eIF4E phosphorylation is abolished in Mnk1/2DKO T cells, suggesting that eIF4E phosphorylation at S209 is not essential for T cell development, proliferation, activation, and cytokine expression, and might not globally control protein translation in T cells. It has been demonstrated that mice that carry a non-phosphorylatable form of eIF4E (S209A) allele and Mnk1/2 double-deficient mice are more resistant to tumorigenesis. This resistance was shown to be via translational control of a specific subset of genes related to tumorigenesis, such as vascular endothelial growth factor C, baculoviral IAP repeat-containing protein 2, and matrix metalloproteinase-3 (24, 61). Although we have demonstrated that Mnk1 and Mnk2 are dispensable for T cell development and activation in general, we cannot rule out the possibility that they might be selectively required for efficient translation of specific subsets of proteins that may affect specific T cell responses.

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Disclosures

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