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Suppression of Th17 cell differentiation by misshapen/NIK-related kinase MINK1

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T helper type 17 cells (Th17 cells) are major contributors to many autoimmune diseases. In this study, we demonstrate that the germinal center kinase family member MINK1 (misshapen/NIK-related kinase 1) negatively regulates Th17 cell differentiation. The suppressive effect of MINK1 on induction of Th17 cells is mediated by the inhibition of SMAD2 activation through direct phosphorylation of SMAD2 at the T324 residue. The importance of MINK1 to Th17 cell differentiation was strengthened in the animal model of experimental autoimmune encephalomyelitis (EAE). Moreover, we show that the reactive oxygen species (ROS) scavenger N-acetyl cysteine boosts Th17 cell differentiation in a MINK1-dependent manner and exacerbates the severity of EAE. Thus, we have not only established MINK1 as a critical regulator of Th17 cell differentiation, but also clarified that accumulation of ROS may limit the generation of Th17 cells. The contribution of MINK1 to ROS-regulated Th17 cell differentiation may suggest an important mechanism for the development of autoimmune diseases influenced by antioxidant dietary supplements.

INTRODUCTION

T helper type 17 cells (Th17 cells) have been identified and characterized as a distinct T cell lineage that maintains mucosal tissue homeostasis and contributes to the host defense against extracellular pathogens (Harrington et al., 2005; Park et al., 2005; Korn et al., 2009; Song et al., 2016). Importantly, dysregulated Th17 cell activity has been linked to a predisposition to the pathogenesis of human autoimmune diseases and relevant mouse models (Langrish et al., 2005; Reynolds et al., 2010; Wang et al., 2015). Transforming growth factor β (TGF-β), in combination with IL-6, has been well established as the most important instructive stimulus for the differentiation of Th17 cells (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006a). Ablation of TGF-β signaling in T cells prevents the induction of Th17 cells and the initiation of experimental autoimmune encephalomyelitis (EAE; Veldhoen et al., 2006b). However, it has recently been reported that stimuli other than cytokines, including high salt, lipid metabolites, and redox status, can also influence Th17 cell differentiation (Kleinemietfeld et al., 2013; Wang et al., 2015; Ravindran et al., 2016). It is currently unknown whether these stimuli work through signal cross talk with cytokine-induced pathways, including the TGF-β–receptor-regulated SMAD (TGF-β–R–SMAD) axis.

Misshapen (Msn)/NIK-related kinase 1 (MINK1) is a serine-threonine kinase that belongs to the germinal center kinase IV family (Dan et al., 2000). MINK1 has been reported to mediate oncogene-induced ovarian epithelial cell growth arrest via a mechanism involving reactive oxygen species (ROS) and MAPK p38 activation (Nicke et al., 2005). It has also been suggested that MINK1 may mediate thymocyte selection in bone marrow chimeras through activation of JNK and Bim phosphorylation (McCarty et al., 2005). Recent studies have demonstrated that increased oxidative metabolism and the production of ROS inhibit the survival and proliferation of Th17 cells (Won et al., 2013; Gerriets et al., 2015). Thus, MINK1 may act as an intermediate to bridge the ROS effect to Th17 induction. Intriguingly, besides functioning as an upstream kinase for MAPK pathways, MINK1 can also serve as an Msn kinase phosphorylating R-SMADs (Kaneko et al., 2011). Therefore, it is of interest to determine whether MINK1 is a vital player in ROS/redox balance—

Abbreviations used: CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; GST, glutathione S-transferase; LP, lamina propria; MAD, mother against decapentaplegic; MINK1, Msn/NIK-related kinase 1; MOG, myelin oligodendrocyte glycoprotein peptide; Msn, misshapen; NAC, N-acetyl cysteine; R-SMAD, receptor-regulated SMAD; ROS, reactive oxygen species.
flavored Th17 cell induction and to elucidate the signaling pathways associated with MINK1.

Here, we evaluated the role of MINK1 in Th17 cell differentiation in a mouse EAE model. Upon induction of EAE, Mink1<sup>−/−</sup> mice exhibited increased Th17 cell differentiation and disease severity. Direct phosphorylation at residue T324 in the α-helix 1 region and inactivation of SMAD2 were found to be the major cellular mechanism by which MINK1 modulated Th17 cell differentiation. Moreover, the ROS scavenger N-acetyl cysteine (NAC) markedly boosted the induction of Th17 cells in a MINK1-dependent manner both in vitro and in vivo. In sum, MINK1 is a suppressor in the redox status-regulated differentiation of Th17 cells.

RESULTS

Increased numbers of Th17 cells in the periphery of Mink1<sup>−/−</sup> mice

First, we analyzed the T cell compartment in Mink1<sup>−/−</sup> mice. The numbers and ratios of major thymocyte subsets were comparable with those in Mink1<sup>+/+</sup> littersmates (WT), suggesting a minimal role of MINK1 in thymocyte development (Fig. 1A and not depicted). Although in vitro studies have documented a Ras-dependent apoptotic pathway that is mediated by MINK1 (Nicke et al., 2005), additional analysis has suggested a functional redundancy in Ras-dependent negative selection (Kortum et al., 2012) that may be independent of MINK1. In the periphery, the numbers of splenocytes and CD4<sup>+</sup> T cells in Mink1<sup>−/−</sup> mice were normal, except for a slightly reduced number of CD8<sup>+</sup> T cells (Fig. 1B and not depicted). However, we found a marked increase in memory-like (CD45<sup>+</sup>CD62L<sup>+</sup>) T cells as well as a reduction of naive T cells in Mink1<sup>−/−</sup> CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Fig. 1C and not depicted). Then, we enumerated the effector T cell subsets in the periphery of both Mink1<sup>−/−</sup> and WT mice. Upon stimulation with PMA and ionomycin, MINK1 deficiency led to comparable with those in Mink1<sup>+/+</sup> littermates (WT), suggesting a minimal role of MINK1 in thymocyte development (Fig. 1A and not depicted). Although in vitro studies have documented a Ras-dependent apoptotic pathway that is mediated by MINK1 (Nicke et al., 2005), additional analysis has suggested a functional redundancy in Ras-dependent negative selection (Kortum et al., 2012) that may be independent of MINK1. In the periphery, the numbers of splenocytes and CD4<sup>+</sup> T cells in Mink1<sup>−/−</sup> mice were normal, except for a slightly reduced number of CD8<sup>+</sup> T cells (Fig. 1B and not depicted). However, we found a marked increase in memory-like (CD45<sup>+</sup>CD62L<sup>+</sup>) T cells as well as a reduction of naive T cells in Mink1<sup>−/−</sup> CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Fig. 1C and not depicted). Then, we enumerated the effector T cell subsets in the periphery of both Mink1<sup>−/−</sup> and WT mice. Upon stimulation with PMA and ionomycin, MINK1 deficiency led to a two-to-three-times increase of Th17 (IL-17A<sup>+</sup>CD4<sup>+</sup>) and Th1 (IFN-γ<sup>+</sup>CD4<sup>+</sup>) cells, compared with WT cells (Fig. 1D and E). Meanwhile, the percentage of Th2 (IL-4<sup>+</sup>CD4<sup>+</sup>) cells was not markedly changed (not depicted and Fig. 1E).

Intriguingly, the frequency and number of Foxp3<sup>+</sup> regulatory T cells (T reg cells) did not change in Mink1<sup>−/−</sup> mice in both spleen and LN T cells (Fig. 1D and E; and not depicted). Mink1<sup>−/−</sup> T reg cells were able to suppress CD4<sup>+</sup> T cell proliferation in vitro with similar efficiency as WT T reg cells (Fig. 1F). We also compared the expression of Th cell signature genes in CD4<sup>+</sup> T cells from Mink1<sup>−/−</sup> and WT mice. We found that the expression of Th17 lineage–specific genes (Il17a, Il17f, and Rorc) and Th1 signature Tbx21 was significantly increased in the Mink1<sup>−/−</sup> T cells, whereas Foxp3 and Gata3 gene expression were unchanged (Fig. 1G). Together, these data suggest that MINK1 deficiency may favor Th1 cell differentiation toward Th17 and Th1 cell lineages.

Changes of Th17, Th1, and T reg cells by MINK1 deficiency were further checked in the CD4<sup>+</sup>CD44<sup>+</sup> T cell population. When Mink1<sup>−/−</sup> cells were compared with WT cells, the frequency of Th17 cells was three to four times higher (Fig. 2A), whereas the frequencies of Th1 and T reg cells were comparable (Fig. 2A). The data indicate that the increased frequency of Th17 cells in the Mink1<sup>−/−</sup> mice was not caused by the changed ratio of effector/memory T cells and suggest a specific regulatory role of MINK1 in Th17 cell differentiation. This is consistent with the observations in the lamina propria (LP) where an increased frequency of IL-17A<sup>+</sup>CD4<sup>+</sup> but not Foxp3<sup>+</sup>CD4<sup>+</sup> and IFN-γ<sup>+</sup>CD4<sup>+</sup> T cells was detected in the small intestine of Mink1<sup>−/−</sup> mice (Fig. 2B). Because γδ T cells are another major source of IL-17 production (Martin et al., 2009), we checked IL-17A production in γδ T cells from Mink1<sup>−/−</sup> mice. IL-17A–producing γδT cells were more prevalent in Mink1<sup>−/−</sup> mice than in WT controls (Fig. 2C). Currently, we do not know whether it is a γδT-intrinsic effect.

MINK1 suppresses in vivo Th17 cell differentiation through a cell-intrinsic mechanism

To test whether the defects in Th17 cell differentiation in the Mink1<sup>−/−</sup> mice were intrinsic to T cells, we reconstituted irradiated Rag1<sup>−/−</sup> mice with Mink1<sup>−/−</sup> (CD45.2<sup>+</sup>) and WT congenic (CD45.1<sup>+</sup>) bone marrow cells mixed at a 1:1 ratio and analyzed the phenotypes of donor T cells 8 wk later. The frequency of the Mink1<sup>−/−</sup> IL-17A<sup>+</sup> CD4<sup>+</sup> T cells in the spleen was consistently higher than their WT counterparts derived from the cotransferred congenic WT bone marrow cells in the same host (Fig. 2D). Once more, the numbers of Foxp3-expressing T reg cells were comparable with those in controls (Fig. 2D). The peripheral Mink1<sup>−/−</sup> T cells from those Rag1<sup>−/−</sup> mice also recapitulated the enhanced memory phenotypes of CD4<sup>+</sup> T cells from the parental Mink1<sup>−/−</sup> mice, suggesting an increased spontaneous activation of these cells caused by dysregulation of Th17 cell differentiation (Fig. 2E). Together, these data demonstrate that MINK1 suppresses Th17 cell lineage differentiation in vivo through a T cell–intrinsic mechanism.

MINK1 specifically suppresses Th17 cell differentiation in vitro

To further elucidate the contribution of MINK1 to Th17 cell differentiation, we polarized-sorted naive CD4<sup>+</sup> T cells into Th17 cells through the use of TGF-β1 and IL-6 and compared the efficiency of Mink1<sup>−/−</sup> and WT naive T cells. The frequency of IL-17–producing cells generated from Mink1<sup>−/−</sup> T cell cultures was much greater than that of WT T cells (Fig. 3A), and this was correlated with enhanced IL-17A secretion into culture supernatants (Fig. 3B). Th1 and Th2 cell differentiation was not notably affected upon MINK1 deficiency (Fig. 3, C–F). The enhancement of Th17 cells was not accompanied by induced T reg cell (iT reg cell) dysfunction in Mink1<sup>−/−</sup> T cells, as naive T cells from Mink1<sup>−/−</sup> and WT mice expressed comparable levels of Foxp3 and IL-10 when activated under neutral (Th0) or iT reg cell–polarizing conditions (Fig. 3, G and H). We also found that ex-
pression of the key signatures of Th17 cells, such as Il17a, Il17f, Rorc, and Il23r, but not Il21 and Rora, were elevated in Mink1−/− T cells under the Th17 condition (TGF-β1 + IL-6; Fig. 3 I). Furthermore, CFSE dilution showed parallel proliferation capacity of Mink1−/− and WT T cells cultured under TCR stimulation or Th17 conditions (Fig. 3 J). In sum, these results indicate that MINK1 plays a negative role in Th17 cell differentiation.

Cytokine combinations of IL-1β + IL-6 + IL-23 or TGF-β3 + IL-6 have recently been implicated in inducing pathogenic Th17 cells (Lee et al., 2012; Zhou et al., 2015). Thus, we examined the in vitro differentiation of naive Mink1−/− CD4+ T cells through the use of these two combinations. We found that the Th17 cell differentiation from naive Mink1−/− T cells was augmented in both conditions when compared with WT T cells (Fig. 3 K). A neutralizing antibody against TGF-β could block this Th17 cell differentiation in both Mink1−/− and WT T cells (Fig. 3 K).

MINK1 deficiency leads to increased severity of EAE

To determine whether the altered number of Th17 cells caused by MINK1 deficiency impacts the development of Th17-dependent inflammatory diseases, we immunized Mink1−/− and WT mice with myelin oligodendrocyte glycoprotein peptide 35–55 (MOG35–55) to induce EAE. Mink1−/− mice exhibited significantly increased disease severity...
Figure 2. MINK1 suppresses Th17 cell differentiation through a cell-intrinsic mechanism. (A, left) Splenocytes from Mink1−/− and WT mice were stimulated with PMA + ionomycin for 5 h, and the CD44+CD4+ population was gated and then analyzed for IL-17A+, IFN-γ+, and Foxp3+ cells as indicated. Numbers in quadrants indicate the percentages of cells in each throughout. (Right) Summary of IL-17A+, IFN-γ+, and Foxp3+ cells from the CD44+CD4+ population of Mink1−/− and WT mice. (B, left) FACS analysis of LP CD4+ T cells in the small intestine for IL-17A, Foxp3, and IFN-γ expression. (Right) Summary of CD4+IL-17A+, CD4+Foxp3+ T, and CD4+IFN-γ+ cells in Mink1−/− and WT small intestine. (C) Splenic γδ T cells derived from Mink1−/− or WT animals were stimulated with PMA + ionomycin for 5 h and stained for IL-17A production. (D) Rag1−/− mice were reconstituted with mixed bone marrow cells from B6.SJL (CD45.1+) and Mink1−/− (CD45.2+) or from B6.SJL (CD45.1+) and WT (CD45.2+) mice at a 1:1 ratio. Total splenocytes in the recipient mice were analyzed 8 wk later for IL-17A+CD4+ and Foxp3+CD4+ T cells. The data shown were gated on CD4+CD62L+ or CD4+CD44+ (Mink1−/− or WT) populations. (E, top) Total splenocytes in the recipient mice as in Fig. 2 D were analyzed for CD62L and CD44 expression. (Bottom) Percentages of naive (CD4+CD62L+) and memory (CD4+CD44+) T cells in the spleen of host mice. The numbers in the flow cytometry graphs show the percentages of the gated populations. Error bars show mean ± SD. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001. n = 3–6 in each group; Student’s t test. Data are representative of three (A and B) or two (C–E) experiments.
Histopathological analysis revealed more prominent demyelination in both the central nervous system (CNS) and spinal cord of MOG35–55-immunized Mink1−/− mice, as well as more numerous inflammatory foci (not depicted). Consistently, CD4+ T cells that infiltrated the CNS produced more IL-17A in Mink1−/− mice (Fig. 4 B). When we restimulated T cells isolated from mice immunized with MOG35–55 in vitro, the production of IL-17A by Mink1−/− T cells was considerably greater than that by WT T cells, whereas IFN-γ production was unaffected (not depicted). To delineate the intrinsic role of MINK1 in T cells, we sorted naive T cells (CD4+CD62LhiCD44loCD25−) from Mink1−/− and WT mice, transferred these cells into Rag1−/− mice, and then monitored them for the development of EAE after MOG 35–55 immunization. In contrast to the transfer of WT T cells, which resulted in mild disease, transfer of Mink1−/−-naive T cells resulted in EAE with high disease incidence, severity, and mortality (Fig. 4 C and not depicted). Histological examination showed significantly more mononuclear cell infiltration and demyelination in the spinal cord of hosts with Mink1−/− cells (Fig. 4, D).
MINK1 inhibits SMAD2 activation through direct phosphorylation of SMAD2-T324 residue

Then, we examined the pathways whereby MINK1 regulates Th17 cell differentiation. IL-6 signaling is required for the differentiation of Th17 cells, and the activation of STAT3 is a vital component of the Th17 cell-inducing mechanism. However, Western blot analysis showed no difference in STAT3 activation between Mink1−/− and WT CD4+ T cells stimulated with IL-6 (Fig. 5 A), suggesting that MINK1 does not play a major role in the modulation of early signaling events downstream of the IL-6 receptor. A previous study demonstrated a negative regulatory role of the Msn family kinase in MAD (mother against decapentaplegic; Drosophila SMAD) activation through a built-in phosphorylation of MAD in Drosophila. The Msn phosphorylated site of MAD (T312) is conserved in mouse SMAD2 (T324; Kaneko et al., 2011), expression of WT SMAD2 enhanced Th17 cell differentiation compared with the expression of the empty vector (Fig. 6, A and B). More importantly, SMAD2-T324V mutants were much more potent in inducing Th17 cell differentiation than WT SMAD2 (38 vs. 23%), whereas T324D mutants had the opposite effect (19 vs. 23%; Fig. 6, A and B), indicating that phosphorylation of SMAD2 at α-helix 1 inhibits Th17 cell differentiation. In Mink1−/− T cells, overexpression of WT SMAD2 by retrovirus also induced more IL-17A–expressing cells than the vector, and this was higher than in infected WT T cells (Fig. 6, A and B). However, SMAD2−/− T cells, which could not be phosphorylated by MINK1, induced similar proportions of IL-17A–expressing cells from both Mink1−/− and WT T cells (Fig. 6, A and B). Together with the results from the biochemical studies described above, our data suggest that MINK1 negatively controls SMAD2-dependent Th17 cell differentiation through phosphorylation of SMAD2 at T324.

NAC potentiates Th17 cell differentiation in vitro in a MINK1–dependent manner

MINK1 has been reported to be activated by ROS (Nicke et al., 2005), and ROS have recently been implicated in regulating Th17 cell differentiation, although their effect is quite controversial (Zhi et al., 2012; Lu et al., 2013; Won et al., 2013; Gerriets et al., 2015). Considering the increased Th17 phenotype in Mink1−/− mice, we raised the question of whether MINK1 mediates the suppression of Th17 cell differentiation. In Mink1−/− T cells, overexpression of WT SMAD2 by retrovirus also induced more IL-17A–expressing cells than the vector, and this was higher than in infected WT T cells (Fig. 6, A and B). However, SMAD2−/− T cells, which could not be phosphorylated by MINK1, induced similar proportions of IL-17A–expressing cells from both Mink1−/− and WT T cells (Fig. 6, A and B). Together with the results from the biochemical studies described above, our data suggest that MINK1 negatively controls SMAD2-dependent Th17 cell differentiation through phosphorylation of SMAD2 at T324.
Figure 4. T cell–intrinsic effects of MINK1 deficiency in EAE induction and Th17 cell accumulation. (A) Mean clinical scores for EAE in Mink1\(^{-/-}\) and WT mice after being immunized with MOG\(_{35-55}\) + CFA plus pertussis toxin. (B, left) 18 d after EAE induction (at the peak of disease), CD4\(^{+}\) T cells were gated from the isolated leukocytes in the CNS and further analyzed for the frequencies of IL-17A\(^{+}\) and Foxp3\(^{+}\) cells. (Right) Summary of CNS IL-17A\(^{+}\)CD4\(^{+}\) and Foxp3\(^{+}\)CD4\(^{+}\) cells in Mink1\(^{-/-}\) and WT EAE mice. (C) Mean clinical scores for EAE in Rag1\(^{-/-}\) recipients of Mink1\(^{-/-}\) and WT naive CD4\(^{+}\) T cells. (D and E) Representative histology of the spinal cord (hematoxylin and eosin in D and Luxol fast blue [F&B] in E) of Rag1\(^{-/-}\) mice after EAE induction (day 32). (D) Arrowheads indicate inflammatory infiltration. (E) Outlines indicate demyelination by loss of normal blue staining of myelin. (F) On day 18 after EAE induction of Rag1\(^{-/-}\) hosts, CD4\(^{+}\) T cells were gated from leukocytes isolated from the CNS and analyzed for the frequencies of IL-17A\(^{+}\) and Foxp3\(^{+}\) cells. (G, left) On day 18 after EAE induction of Rag1\(^{-/-}\) hosts, spleen CD4\(^{+}\) T cells were analyzed for IL-17A\(^{+}\) and Foxp3\(^{+}\) expression. (Right) Summary of CNS and splenic IL-17A\(^{+}\)CD4\(^{+}\) and Foxp3\(^{+}\)CD4\(^{+}\) cells in Rag1\(^{-/-}\) EAE mice. (H) Splenocytes isolated from EAE Rag1\(^{-/-}\) mice (day 20) were restimulated in vitro with 5 µg/ml MOG peptide for 3 d, and then, the indicated cytokine production in the culture supernatants was assessed by ELISA. The numbers in the flow cytometry graphs show the percentages of the gated populations. Error bars show mean ± SD. *, P \(\leq 0.05\); **, P \(\leq 0.01\); Student's t test. Data are representative six pairs of mice from two independent experiments.
the antioxidant NAC (Fig. 7 C). Also, the kinase activity of MINK1 was profoundly reduced in Th17 conditions by NAC treatment (Fig. 7 D). Furthermore, we investigated the differentiation of Th17 cells under Th17 cell–polarizing conditions in the presence of NAC and confirmed increased numbers of IL-17A+ cells with additional 0.5-mM NAC (Fig. 7 E). Notably, NAC-induced increase in Th17 cell development did not occur in MINK1-deficient T cells (Fig. 7 E). In contrast, NAC treatment did not affect iTreg cell differentiation or Treg cell suppressive activity (Fig. 7, F and G). Consistently, treatment of CD4+ T cells with TGF-β plus NAC resulted in stronger C-terminal phosphorylation and weaker T324 phosphorylation of SMAD2 than TGF-β alone (Fig. 7 H). Importantly, the phosphorylation levels of both were not affected by
NAC treatment in Mink1−/− CD4+ T cells (Fig. 7 H). Thus, our data indicated that NAC boosts Th17 cell differentiation in vitro in a MINK1-dependent manner.

NAC induces Th17 cells in vivo and exacerbates EAE through a T cell–intrinsic mechanism

To determine whether increased Th17 cells upon NAC treatment could result in an enhanced pathogenicity in autoimmune disease, we differentiated MOG35–55-immunized CD4+ T cells (day 8) cells into Th17 cells with or without NAC treatment in vitro and then transferred these cells to Rag1−/− mice and induced EAE. Mice that received NAC-treated cells developed more severe disease compared with those that received control cells (Fig. 8, A and B). We observed a dramatically higher frequency of Th17 cells in the CNS, spleen, and mesenteric LN of Rag1−/− mice that received NAC-treated cells than in those of controls (Fig. 8, A and B). These observations suggest that NAC treatment directly affected the pathogenicity of antigen-specific T cells through promoting Th17 cell differentiation.

Previous studies have shown that different components of common dietary supplements and the gut microbiota can overtly affect the percentage of effector T cells in the gut (Berer et al., 2011; Atarashi et al., 2015). In addition, recent data indicate that molecules associated with ROS homeostasis can influence Th17 cell responses and inflammation (Gerriets et al., 2015; Ravindran et al., 2016). To further understand the effect of NAC on Th17 cell generation in vivo, we added NAC into the drinking water of Mink1−/− and WT mice. After 2 mo on NAC, nonimmunized WT mice showed a marked increase in the frequency of Th17 cells in the LP and mesenteric LNs (Fig. 8, E, F, and I), whereas there was no change in IFN-γ+ and Foxp3+ cells (Fig. 8, G–I). Importantly, the Th17 cell frequency was not affected in the gut of MINK1-deficient mice that had received NAC (Fig. 8, E and I).

Finally, to understand better whether NAC supplementation would affect the development of EAE in vivo, we immunized NAC-treated mice with MOG35–55 to induce EAE. To our surprise, NAC supplementation did not significantly affect the severity of EAE as measured by clinical scores during the course of disease (not depicted). We suspect NAC may affect cells other than T cells. It is well known that NAC could reduce tissue injury during inflammation (Sayin et al., 2014; Schieber and Chandel, 2014). To focus the effect of NAC on T cells, we purified CD4+ T cells from these mice, transferred them into Rag1−/− hosts, and then examined their ability...
to induce EAE (Fig. 9 A). Mice that received T cells from NAC-treated mice developed more severe disease than those that received T cells from control mice. Again, the severity of disease was increased in mice receiving MINK1-deficient T cells, which, however, was not further elevated by NAC treatment (Fig. 9, B–D). Consistently, Th17 cell numbers in the CNS of Rag1$^{−/−}$ mice that received NAC-treated T cells were notably higher than in controls (Fig. 9, E–G). We also found a higher frequency of Th17 cells in the lymphoid organs of mice with T cell transfer from NAC-treated mice at the peak of disease (Fig. 9, E–G). The data presented here indicate that dietary supplementation with the antioxidant NAC poten-

tiates Th17 cell generation in vivo in a MINK1-dependent manner and therefore has the potential to increase the risk of promoting autoimmune inflammatory diseases.

**DISCUSSION**

Here, we reveal that MINK1 suppresses Th17 cell differentiation. Activated downstream of ROS, MINK1 phosphorylated SMAD2 at T324 and inhibited the activation of SMAD2. This regulation was further demonstrated in vivo as more severe EAE disease in Mink1$^{−/−}$ mice. These findings suggest cross talk between redox status and cytokine-induced signals during Th17 cell differentiation.
Although MINK1 had previously been established as a regulator in thymocyte development, this study is the first to unveil its contribution to Th17 cell differentiation. MINK1 (MAP4K6) is a kinase that belongs to the MAP4K family, several members of which have been implicated in T cell activation and differentiation. For instance, loss of HGK (HPK1/GCK-like kinase; MAP4K4) in T cells enhances Th17 cell differentiation in adipose tissue (Chuang et al., 2014). GLK (GCK-like kinase; MAP4K3) deficiency impairs T cell–mediated immune responses in mice (Chuang et al., 2011). HPK1 (MAP4K1) has been identified as a negative regulator of TCR signaling (Shui et al., 2007). Interestingly, despite their original identification as regulators of MAPK pathways, these MAP4Ks modulate T cell development and differentiation in both a MAPK-dependent (HPK1) and a MAPK-independent (HGK and GLK) manner (Shui et al., 2007; Chuang et al., 2011, 2014). The suppression of Th17 cell differentiation by MINK1 is clearly independent of MAPKs, as indicated by unaltered TCR-induced activation of Erk, p38, and JNK in Mink1−/− CD4+ T cells (unpublished data).

Figure 8. Dietary supplementation of NAC potentiates Th17 cell differentiation in vivo. (A) On day 8 after immunized with MOG35–55 + CFA, WT CD4+ T cells were sorted and differentiated with 10 ng/ml IL-6 and 3 ng/ml TGF-β1 in the presence or absence of 0.5 mM NAC and transferred into Rag1−/− mice. 1 d later, the recipient mice were immunized with MOG35–55 plus pertussis toxin to induce EAE. Clinical scores were recorded and calculated each day for the indicated times. (B) Linear regression curves of Fig. 8 A. Dashed lines indicate the 95% confidence intervals of the regression lines. (C) IL-17A and IFN-γ production by CD4+ T cells isolated from the indicated organs of EAE animals at the peak of disease in Fig. 8 A (E) Representative intracellular staining of IL-17A+ CD4+ T cells from the small intestine of Mink1−/− and WT mice treated with NAC or regular drinking water for 2 mo. (F) Mesenteric LN IL-17A+CD4+ and IFN-γ+CD4+ cell frequencies of Mink1−/− and WT mice treated with either NAC or regular drinking water. (G and H) Small intestine LP IFN-γ+CD4+ (G) or Foxp3+CD4+ (H) cell frequencies of unimmunized WT mice either untreated or treated with NAC. (I) Percentages of small intestine LP IL-17A+CD4+, IFN-γ+CD4+, and Foxp3+CD4+ cell frequencies of NAC-treated or untreated mice. Ctrl, control. The numbers in the flow cytometry graphs show the percentages of the gated populations. Error bars show mean ± SD. *, P ≤ 0.05; **, P ≤ 0.01; Student’s t test. Data are representative of six (A–D) or three (E–I) mice in each group from two independent experiments.
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To specifically target the differentiation of Th17 cells, but not iT reg cells, via selective phosphorylation of SMAD2, which dampened its activity. The cell-type preference of MINK1 is in keeping with findings that SMAD2 is dominant in Th17 cell differentiation (Malhotra et al., 2010; Martinez et al., 2010; Yoon et al., 2015). Despite bearing almost homologous amino acid sequences, SMAD2 and SMAD3 do differ in certain residues, including those that can be specifically
modified (Wrighton et al., 2009; Kaneko et al., 2011; Bruce and Sapkota, 2012). This feature was exemplified in our experiments that showed that SMAD3 was not phosphorylated by MINK1 and that the activity of SMAD3 was not changed by MINK1 deficiency. The unaffected iT reg differentiation in MINK1–deficient cells may largely reflect the unaltered SMAD3 activity. Serine residue phosphorylation is critical for the activation of SMAD proteins, and this is best indicated by studies on regulation of Th17 and T reg cell differentiation by phosphorylation of C-terminal SXS motifs and the linker region on SMAD2 and SMAD3 (Chang et al., 2011; Yoon et al., 2015). In accord with a previous study in Drosophila showing that MAD activation can be regulated by the conserved threonine residue T324 in the α-helix 1 region (Kaneko et al., 2011), we have provided evidence that MINK1 regulates the activation of SMAD2 through direct phosphorylation of this residue during Th17 cell differentiation. To the best of our knowledge, this is the first report of the physiological/pathophysiological significance of this regulation, which is involved in the differentiation of a crucially important T cell subtype in the context of autoimmune disease. The T324 residue of SMAD2 also exists in SMAD1 (T312), which can be activated by TGF-β3 (Lee et al., 2012). Considering the augmented in vitro Th17 cell differentiation in Mink1−/− T cells under the TGF-β3 + IL-6 condition (Fig. 3 K), our supposition does not exclude the possibility that the EAE phenotype (Fig. 4) of Mink1−/− mice is a functional redundancy of both SMADs’ activation.

It is well known that intracellular ROS are elevated during T cell activation as a result of increased glycolytic flux after TCR engagement (Sena et al., 2013). Because of their destructive roles in cells and tissues upon release, ROS are traditionally viewed as strong mediators of inflammation (Sena et al., 2013; Schieber and Chandel, 2014). However, ROS were recently identified as possible negative regulators of the T cell–mediated inflammatory responses (Hultqvist et al., 2007). ROS are elevated in the context of autoimmune disease. The T324 residue of SMAD2 also exists in SMAD1 (T312), which can be activated by TGF-β3 (Lee et al., 2012). Considering the augmented in vitro Th17 cell differentiation in Mink1−/− T cells under the TGF-β3 + IL-6 condition (Fig. 3 K), our supposition does not exclude the possibility that the EAE phenotype (Fig. 4) of Mink1−/− mice is a functional redundancy of both SMADs’ activation.

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MATERIALS AND METHODS

Mice, EAE induction, and adoptive transfer

Mink1−/− mice were generated by homologous recombination–mediated gene targeting as previously described on a mixed B6 × 129 background and backcrossed to B6 for at least eight times (Yue et al., 2016). C57BL/6 (B6) and Rag1−/− mice were purchased from the Model Animal Research Center of Nanjing University, and CD45.1 (B6.SJL) mice were purchased from The Jackson Laboratory. All mice were housed in Zhejiang University Laboratory Animal Center. The experimental protocols were approved by the Review Committee of Zhejiang University School of Medicine and were in compliance with institutional guidelines.

EAE was induced as described previously (Lu et al., 2007). In brief, male mice age 6–8 wk were immunized with 200 μg MOG35–55 (MEGWYRSFPRVHYLGK; Sangon) in an equal amount of complete Freund’s adjuvant (Chondrex, Inc.) and received 200 μg pertussis toxin (List Biochemicals) intravenously on days 0 and 2 after induction. Clinical evaluation was performed daily using a five-point scale: 0, no clinical signs; 1, limp tail; 2, limp tail, impaired righting reflex, and paresis of one limb; 3, hind-limb paralysis; 4, hind-limb and fore-limb paralysis; and 5, moribund. NAC (616-91-1; ≥99% purity; Sigma–Aldrich) was administered in the drinking water at 1 g/L. The NAC water was replaced every 3 d.

Bone marrow cells were transplanted as described previously (Wang et al., 2012). In brief, bone marrow cells were isolated from Mink1−/−, WT, or CD45.1 mice, and 5 × 106 cells were injected intravenously into Rag1−/− mice, which had received sublethal irradiation (400 rad) 1 d before. Chimeric mice were analyzed 6–8 wk after transplantation.

For EAE induction in Rag1−/− mice, naïve T cells or CD4+ T cells from WT and Mink1−/− mice were sorted by an AutoMacs separator (STEMCELL Technologies) and intravenously transferred into Rag1−/− mice at 7 × 106 per mouse for naïve T cell transfer, 1 × 106 per mouse for CD4+ T cell transfer, and 2 × 106 per mouse for differentiated Th17 cell transfer. 2 d later, the recipient mice were immunized with MOG35–55 as described in the second paragraph of this section.

Antibodies, recombinant cytokines, and reagents

The following monoclonal antibodies and reagents were used: for surface staining, anti–CD4 (GK1.5), anti–CD8a (53–6.7), anti–CD25 (PC61), anti–CD44 (IM7), anti–CD62L (MEL-14), anti–CD45.1 (A20), anti–CD45.2 (104), and anti–TCR–β chain (H57-597; all from BioLegend); for intracellular (cytoplasmic and nuclear) staining, anti–IFN-γ (XMG1.2; from BioLegend), anti–Foxp3 (FJK–16B), and anti–RORγt (B2D; from eBioscience); and for T cell stimulation, anti–CD3 (145–2c11) and anti–CD28 (37.51; from BioLegend). Recombinant mouse TGF–β1, IL–6, IL–4, IL–12, IL–1β, and IL–23 were from R&D Systems and recombinant mouse IL–2

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was from PeproTech. Neutralizing anti–IFN-γ (XMG1.2), anti–IL-4 (11B11), and anti–IL-12 (C17.8) were from Bio-Legend. CFSE was from Invitrogen.

In vitro T cell differentiation
Sorted naive CD4+ T cells (CD4+CD62L−CD44−) were cultured on irradiated (30 Gy) C90D.29 splenocytes with 1 µg/ml anti–CD3 and 2 µg/ml anti–CD28 at a ratio of 1:5. The naive cells were cultured at 10⁵/ml in RPMI 1640 medium supplemented with 10% FBS, sodium pyruvate, Hepes, penicillin/streptomycin, gentamicin sulfate, and 2-mercaptoethanol. The following cytokines were added to generate each subset: Th1, 10 ng/ml IL-12 and 10 µg/ml anti–IL-4; Th2, 50 ng/ml IL-4 and 10 µg/ml anti–IFN-γ; and Th17, 10 ng/ml IL-6, 3 ng/ml TGF-β1, 3 ng/ml TGF-β3, 20 ng/ml IL-1β, 20 ng/ml IL-23, 10 µg/ml anti–IFN-γ, and 10 µg/ml anti–IL-4. When indicated, 5 µg/ml TGF-β antibody (ID11; R&D Systems) was added into the culture. For T reg cells, 3 ng/ml TGF-β, 10 µg/ml anti–IFN-γ, and 10 µg/ml anti–IL-4 were used. On day 3 after stimulation, cells were split 1:2 and replated with 50 U/ml IL-2 alone for an additional 2 d before analysis.

In vitro suppression assays
Mouse CD4+ T cells were negatively selected from the spleen and LNs of mice by using an AutoMacs separator (STEMCELL Technologies). Sorted CD4+CD25+ T reg cells were added to CD4+ T cells at a final concentration of 3 × 10⁵ cells per 500 µl and stimulated for 72 h with anti–CD3.

Isolation of LP lymphocytes
Mice were sacrificed, and the intestines were removed and placed in ice-cold PBS. After removal of residual mesenteric fat tissue, Peyer’s patches were carefully excised, and the intestine was opened longitudinally. Then, the intestine was thoroughly washed in ice-cold PBS and cut into 1–2-cm pieces. The pieces were incubated in 15 ml of 5 mM EDTA in 10% FBS/RPMI medium for 15–20 min at 37°C with slow rotation at 100 rpm. After incubation, the epithelial cell layer, containing the intraepithelial lymphocytes, was removed by intensive vortexing and passed through a 100-µm cell strainer. Then, the pieces were washed in RPMI medium and placed in 10 ml of digestion solution containing 4% fetal calf serum, 1 mg/ml collagenase II (Sigma–Aldrich), and 50 µg/ml DNase I (Sigma–Aldrich). Digestion was performed by incubating the pieces at 37°C in a water bath for 20 min with rotation at 120 rpm. After the initial 20 min, the solution was vortexed intensely and passed through a 100-µm cell strainer. The pieces were collected and placed into fresh digestion solution, and the procedure was repeated. Supernatants from both digestions (or from the EDTA treatment for intraepithelial lymphocyte isolation) from a single small intestine were combined, washed once in cold FACS buffer, resuspended in 5 ml of the 40% fraction of a 40:80 Percoll gradient, and overlaid on 2.5 ml of the 80% fraction in a 15-ml Falcon tube. Percoll gradient separation was performed by centrifugation for 20 min at 2,500 rpm at room temperature. LP lymphocytes were collected at the interphase of the Percoll gradient, washed once, and resuspended in FACS buffer or T cell medium. The cells were used immediately for experiments.

Retroviral infection
Retroviruses were produced by transfecting Plat-E cells with pMX-IRES-GFP, pMX-WT SMAD2 IRES-GFP, and pMX-SMAD2-T324D/V IRES-GFP. Fresh viruses from the supernatants were collected 3 d later and used to infect T cells. To infect T cells, naive T cells were first stimulated with anti–CD3 and anti–CD28 antibodies. At the 24- and 36-h time points, activated T cells (0.5 × 10⁶) were spun infected for 1 h at 2,000 rpm with 500 µl of viral supernatant in the presence of 10 µg/ml polybrene and incubated at 37°C for one additional hour before being removed from the viral supernatant and resuspended in the indicated T cell differentiation medium.

Flow cytometry and cytokine detection
Mice were perfused with PBS before brains were harvested. Brains were pretreated with 2 µg/ml collagenase D (Roche) and 1 µg/ml DNase I (Sigma–Aldrich), and total cells were isolated by cell straining (70 µm for spleen and mesenteric LN and 100 µm for brain). Brain homogenates were separated into neuronal and leukocyte populations by discontinuous density gradient centrifugation using Percoll (Pharmacia). For surface staining, cells were stained with the respective antibodies for 20 min in PBS containing 2% FBS before analysis. For intracellular staining, cells were stimulated for 4–5 h with 50 ng/ml PMA and 500 ng/ml ionomycin (both from Beyotime) in the presence of brefeldin A (Bio-Legend), fixed, made permeable (Fix/Perm; eBioscience) according to the manufacturer’s instructions, and stained with the respective antibodies for 20–30 min for intracellular cytokine detection. Before fixation, cells were stained with Fixable Viability Dye (eBioscience) to exclude dead cells. Data were acquired on a FACSCalibur cell analyzer (BD) and analyzed with FlowJo software (Tree Star). Culture supernatants were measured by ELISA for secretion of IL-17A, IL-6, IL-2, TNF, and IFN-γ (eBioscience) according to the manufacturer’s instructions.

Quantitative RT-PCR
Cells were stimulated as indicated, and RNA was extracted using RNeasy–Plus (9109; Takara Bio Inc.). The following primers were used in the study. Il17a: forward, 5′-TCCAGAAGGCCTCTGACTA-3′ and reverse, 5′-TCAGGACCAAGATCTTTTGAC-3′; Il17f: forward, 5′-CCCCATGGGATTACAACATCAC-3′ and reverse, 5′-CATTGATGCAGCCTAGTGTCTCT-3′; Il23r: forward, 5′-AACATGACAGGCACCTGGAA-3′ and reverse, 5′-TCCATGCTCTGGAATTTGAC-3′; Cxcl3: forward, 5′-GTTGTGCGCCAGTGAGCTG-3′ and reverse, 5′-CAAGCTCTGGATGTTCCTCAA-3′; Tbx21: forward, 5′-GCCAGGAACCGGTT
β-actin expression.

For real-time PCR, and data were analyzed with 7500 software (v2.0.1; Applied Biosystems). Results were normalized to β-actin expression.

**Western blotting and immunoprecipitation**

2.5 × 10^6 cells were lysed in whole-cell extract buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.5% NP-40 [NDB0385; Life-Biotech], 1 mM EDTA, 1 mM Na3VO4, 10% glycerol, 1 mM PMSE, and protease inhibitors) to obtain whole-cell lysates. Proteins were separated by SDS-PAGE (6–10%) followed by transfer to nitrocellulose membrane. To block nonspecific binding, membranes were incubated with 5% BSA in TBST (0.5 M NaCl, Tris-HCl, pH 7.5, and 0.05% [vol/vol] Tween 20) for 60 min and washed twice with TBST. Proteins of interest were detected by incubating the membranes overnight at 4°C in 10% BSA/TBST with the indicated antibodies (listed in this section), washed three times with TBST for 10 min, and incubated with horseradish peroxidase–conjugated anti–rabbit or anti–mouse antibody (A0208 and A0216; Beyotime). Bound antibody was detected using Immobilon Western chemiluminescent HRP substrate (Cyanogen). Antibodies were used for Western blot analysis as follows. The pT324-SMAD2 antibody was raised against the peptide NRNA [pT] TVEMTRRRHIC, and the sera were first absorbed by beads conjugated with the nonphosphopeptide and then affinity purified using the phosphopeptide. Anti-MINK1 (NBP1-22990; Novus Biologicals), anti–p–C–SMAD2 (3101; Cell Signaling Technology), anti–SMAD2 (3103; Cell Signaling Technology), anti–SMAD3 (9523; Cell Signaling Technology), anti–p–C–SMAD3 (9520; Cell Signaling Technology), anti–lamin A (613501; BioLegend), anti–β–actin (LK2002; LKtag), anti–GAPDH (EM1101; HuaAn Biotechnology), anti–Flag (MA191878; Thermo Fisher Scientific), and anti–HA (0906–1; HuaAn Biotechnology) were used.

For immunoprecipitation, cell lysates were prepared as described in the previous paragraph, and proteins were immunoprecipitated by incubation of lysates with 1–10 µg antibody overnight at 4°C and pulldown of antibody–protein precipitates with SureBeads Protein A (or protein G) magnetic beads (Bio–Rad Laboratories). The presence of immunocomplexed proteins was determined by Western blot analysis.

**Cell fractionation and in vitro kinase activity assay**

3 × 10^7 mice CD4+ T cells were treated with TGF-β at the time periods indicated in Fig. 5, harvested with fractionation buffer (BSP001; Sangon Biotech) for 20 min on ice, and then fractionated by centrifugation. The fractions were analyzed on SDS–PAGE and by subsequent Western blot analysis.

Cell lysates were prepared, and MINK1 was immunoprecipitated from the lysates with anti-MINK1 antibody as described in the Mice, EAE induction, and adoptive transfer section of Materials and Methods. The immunoprecipitate was washed extensively, and MINK1 kinase activity was assessed with a luminescence–based assay (MINK1 kinase ADP–Glo kinase assay; V8001; Promega) and an anti–thio–phosphate antibody (ab92570; Abcam) according to the manufacturers’ instructions. Purified GST–MINK1 kinase domain (Sigma–Aldrich) was used for in vitro kinase reactions at 10 µg/ml in a final volume of 25 µl.

**Plasmids**

Mouse MINK1 cDNA was cloned into the BamHI and XhoI sites of the pcDNA 3.1 expression vector and SMAD2 at the EcoRI and XhoI sites.GST-tagged SMAD2 or SMAD3 was constructed by subcloning of the appropriate cDNA into the pGEX–4T–1 GST expression vector (GE Healthcare).

**Statistical analysis**

Statistical analysis was performed using Prism (GraphPad Software). The data were analyzed by the paired sign Student’s t test or Student’s t test. All p-values ≤0.05 were considered statistically significant.

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