MAPK Phosphatase 7 Regulates T Cell Differentiation via Inhibiting ERK-Mediated IL-2 Expression

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Members of the MAPK phosphatase (MKP) protein family play critical roles in immune responses through differential regulation of MAPK activation. In this study, we show that MKP7, also known as dual-specificity phosphatase 16, was required for CD4$^+$ T cell responses in vivo. *Mkp7*$^{-/-}$ CD4$^+$ T cells exhibited enhanced ERK and JNK activation, and produced increased amount of IL-2 compared with *Mkp7*$^{+/+}$ cells upon activation. *Mkp7*$^{-/-}$ CD4$^+$ T cells were selectively defective in Th17 differentiation in vitro, which was rescued by blocking IL-2 or inhibition of ERK activation. Furthermore, mice carrying *Mkp7*$^{-/-}$ T cells were deficient in generation of Th17 and T follicular helper cells in vivo, and were resistant to autoimmune experimental encephalomyelitis. Our results thus demonstrate an essential role of MKP7 in effector T cell function. *The Journal of Immunology*, 2015, 194: 3088–3095.

Mitogen-activated protein kinase phosphatases (MKPs), also known as dual-specificity phosphatases (DUSPs), are essential regulators of MAPK signaling pathways, controlling the magnitude and duration of MAPK activation in immune responses (1, 2). For instance, MKP5 (DUSP10) has been shown to inhibit JNK activation in immune cells (1). In innate immunity, MKP5 functions as a negative regulator of inflammatory cytokine production. In adaptive immunity, MKP5 positively regulates naive CD4$^+$ T cell activation and proliferation; however, it inhibits Th1 and Th2 effector cytokine expression. It has been shown that in macrophages and dendritic cells (DCs), MKP1 (DUSP1) controls the duration of both p38 and JNK activation to inhibit cytokine production (2, 3), whereas in T cells, MKP1 controls the magnitude of JNK and maybe ERK, but not p38, to positively regulate naive T cell activation, proliferation, and Th1 and Th17 effector functions (4).

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Received for publication October 17, 2014. Accepted for publication January 20, 2015.

This work was supported by the Office of Deputy President, National University of Singapore, Ministry of Education Grant MOE2010-T2-1-079, National Medical Research Council of Singapore Grant IRG10Nov091 (all to Y.Z.), and Cancer Prevention and Research Institute of Texas Research Training Award RP101502 (to K.C.N. and MD Anderson Cancer Center).

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The online version of this article contains supplemental material.

Abbreviations used in this article: DC, dendritic cell; DUSP, dual-specificity phosphatase; EAE, experimental autoimmune encephalomyelitis; GC, germinal center; KLH, keyhole limpet hemocyanin; KO, knockout; LN, lymph node; MKP, MAPK phosphatase; MOG, myelin oligodendrocyte glycoprotein; Tfh, T follicular helper; WT, wild-type.

MKP/DUSP protein family comprises 10 typical and 16 atypical members with different subcellular localization, distinct tissue distribution, and differential regulation in their expression (5, 6), suggesting that they form a network to regulate MAPK activation and MAPK-mediated immune responses. However, there is still much to be learned on the function of individual MKPs and whether/how they work cooperatively to regulate MAPK activation and MAKP-mediated immune responses in vivo. MKP7, also known as DUSP16, binds and inactivates p38 and JNK when overexpressed in cell lines (7, 8). It has been shown that MKP7 binds to JNK scaffold proteins including JNK-interacting protein-1 and β-arrestin 2 to negatively regulate JNK activation (9, 10). It was reported that MKP7 inhibits IL-12 and TNF-α production in macrophages in response to LPS stimulation (11, 12). However, little is known on the physiological function of this protein in immune responses, especially in T cell responses in vivo.

In this study, we have generated MKP7-deficient mice and found that the deficiency of MKP7 resulted in embryonic lethality. We thus constructed fetal liver chimeras lacking MKP7 in the hematopoietic compartment to examine its function in T cell responses and found that MKP7 has an essential role in T cell activation and effector function.

Materials and Methods

Generation of Mkp7 *gene trap mice*

Targeted embryonic stem cell line with disruption of mouse *Mkp7* gene by means of a gene-trapping vector inserted into intron 4 (AE0704) obtained from the Sanger Institute Gene Trap Resource was injected into mouse blastocysts and subsequently transferred into pseudopregnant foster mothers for the generation of chimeras as described previously (13). Heterozygous ($^{+/-}$) mice were intercrossed to generate wild-type (WT) and mutant mice. The animal studies were approved by the appropriate Institutional Animal Care and utilization Committee.

Generation of MKP7$^{-/-}$ *fetal liver chimeras*

Fetal liver cell suspensions from *Mkp7*$^{+/+}$ and *Mkp7*$^{-/-}$ embryos at days 13.5–15.5 were transferred by injection into tail veins of lethally irradiated *Mkp7*$^{+/+}$ recipients. Mice were maintained for 8 wk to allow reconstitution.

Western blot analysis

To examine MKP7 protein expression in T cells, we stimulated naive CD4$^+$ T cells with plate-bound anti-CD3 for various periods, and cell lysates were prepared using Triton lysis buffer containing protease and

phosphatase inhibitors, and were subjected to Western blot analysis with anti-MKP7 Ab (Abcam). For analysis of ERK, JNK, and p38 activation, WT and knockout (KO) mouse embryonic fibroblast cells were activated with UV for 15 min, 30 min, or 1 h. Naive $CD4^+$ T cells were stimulated with 2 μg/ml anti-CD3 (2C11) and anti-CD28 (37.51), and incubated on ice for 15 min followed by cross-linking with goat anti-hamster IgG. Cell lysates were subjected to Western blot analysis with anti–p-ERK, anti–p-p38, anti–p-JNK, anti-ERK, anti-p38, or anti-JNK Abs (Cell Signaling Technology). The signal was detected with ECL reagent (Pierce Chemical).

In vitro T cell assays

Naive $CD4^+$ T cells were purified from lymph nodes (LN) and spleens of mice by FACS based on the $CD4^+CD62L^{hi}CD44^{lo}$ surface phenotypes. To analyze the regulation of MKP7 in T cell activation and proliferation, we incubated naive T cells with different concentrations of plate-bound anti-CD3 Ab or anti-CD3 plus anti-CD28 Abs. IL-2 production by T cells was measured by ELISA (BD Pharmingen, San Diego, CA) 24 h after T cell activation. Cell proliferation was determined 72 h after incubation with [^{3}H]thymidine in the last 8 h. To examine the role of MKP7 in effector function, we cultured naive $CD4^+$ T cells under Th1, Th2, or Th17 conditions as previously described (14). After 4 d of differentiation, cells were washed and treated with 3 μg/ml plate-bound anti-CD3 for cytokine measurement or treated with PMA and ionomycin in the presence of GolgiPlug (BD Biosciences) for intracellular cytokine staining, and samples were analyzed by flow cytometry.

Enzyme assay for protein phosphatases

The C-terminal His_6-tagged rat MKP3(1-381) and human MKP7(5-303) were expressed in *Escherichia coli* BL21(DE3) and purified by a nickel-NTA column (Qiagen), followed by an anion exchange Source 15Q column (Amersham Biosciences). Bisphosphorylated ERK2/pTpY was obtained by coexpressing ERK2 and constitutively active MAPK/extracellular signal-regulated kinase kinase 1 (MEK1EE**Δ**N4) in *E. coli* BL21(DE3) and was purified by a nickel-NTA column, followed by an anion exchange Source 15Q and a size exclusion Superdex 200 column. ERK2/pTpY was incubated with MKP7 or MKP3 protein in a buffer containing 50 mM MOPS, pH 7.0, 100 mM NaCl, and 10 mM Mg^{2+} at 25°C for 1 h. ERK2 phosphorylation was analyzed by Western blot analysis using anti-phosphothreonine and anti-phosphotyrosine Abs (Cell Signaling Technology).

Experimental autoimmune encephalomyelitis induction and analysis

Experimental autoimmune encephalomyelitis (EAE) was induced in $Mkp7^{+/+}$ and $Mkp7^{-/-}$ chimeras by immunization with myelin oligodendrocyte glycoprotein (MOG) peptide 35–55 ($MOG_{35–55}$) as we described previously (15). In brief, mice were immunized with the peptide in CFA on days 0 and 7 s.c. in the hind flank. Mice also received two subsequent treatments of pertussis toxin i.p., 1 d after each immunization. The mice were observed daily for clinical signs and scored on a scale of 0–5 with gradations of 0.5 for intermediate scores: 0, no clinical signs; 1, loss of tail tone; 2, wobbly gait; 3, hind-limb paralysis; 4, hind-limb and forelimb paralysis; 5, death. Preparation and stimulation of mononuclear cells from brain and spinal tissues were done as described previously (15).

Keyhole limpet hemocyanin immunization

$Mkp7^{+/+}$ and $Mkp7^{-/-}$ chimeras were s.c. immunized at the base of tail (200 μl/mouse) with keyhole limpet hemocyanin (KLH) protein emulsified in CFA. Seven days after immunization, mice were sacrificed and analyzed individually for germinal center (GC) formation and Ag-specific Ab and cytokine responses. In brief, to analyze T follicular helper (Tfh) cell population, we stained total spleen cells or LN cells from these mice with PerCP-labeled anti-CD4, biotinylated anti-CXCR5 mAb followed by allophycocyanin-labeled streptavidin, and PE-labeled anti-BCL6 mAbs (BD Biosciences). GC B cells were detected by staining with FITC-labeled anti-GL7 and PE-labeled anti-Fas mAbs, and PerCP-labeled anti-B220 mAb (BD Biosciences). Splenocytes or lymphocytes were isolated from KLH-immunized mice and stimulated with varying concentrations of KLH peptide. Cytokine concentrations in culture were determined by ELISA after 3 d of stimulation. For intracellular staining, cells were stimulated with KLH overnight followed by staining with PerCPCy5.5-labeled anti-CD4. Cells were then fixed and permeabilized (BD Cytofix/Cytoperm Kit; BD Biosciences) and stained for intracellular IFN-γ and IL-17. To measure KLH-specific Ab responses, we collected sera from immunized mice, and IgM, IgG, IgG1, and IgG2a Abs were measured by ELISA.

Statistical analysis

Statistical analysis was calculated with an unpaired Mann–Whitney *U* test and STATISTICA software (StateSoft, Tulsa, OK). The p values ≤ 0.05 were regarded as statistically significant.

Results

MKP7 expression is enhanced in T cells upon TCR activation

To address the possible role of MKP7 in T cells, we first examined the expression of MKP7 in naive $CD4^+$ T cells and effector Th cells, including Th1, Th2, and Th17 cells. MKP7 mRNA expression was greatly increased at 1 h after anti-CD3 stimulation, and the expression level was maintained at 3 h after stimulation (Supplemental Fig. 1A). MKP7 protein was weakly expressed in naive $CD4^+$ T cells (Supplemental Fig. 1B). Strong protein expression of MKP7 was observed at 3 h after anti-CD3 stimulation. MKP7 was also expressed in Th1, Th2, Th17, and Tfh effector cells at a comparable level, and anti-CD3 restimulation did not change its protein expression in Th1, Th2, and Th17 cells (Supplemental Fig. 1C, 1D). These findings suggest a possible regulatory role of MKP7 in naive $CD4^+$ T cell activation and $CD4^+$ effector cell function.

MKP7 deficiency results in embryonic lethality

To determine the function of MKP7 in immune responses in vivo, we generated mice lacking MKP7 using an embryonic stem cell line (AEF0704) containing an insertional mutation, or gene trap, in the fourth intron of the *Mkp7* gene. The gene trap vector generates a spliced fusion transcript containing *Mkp7*, the β-geo cassette encoding β-Gal, and the neomycin resistance gene (Supplemental Fig. 2A). RT-PCR was used to confirm the gene trap insertion using a forward primer from *Mkp7* exon 4 and two β-geo primers (Supplemental Fig. 2A).

Heterozygous $Mkp7^{+/-}$ mice were fertile and indistinguishable in appearance from WT littermates. Mice generated from $Mkp7^{+/-}$ intercrossing were genotyped by using DNA isolated from tail tissue. No $Mkp7^{-/-}$ mouse was found upon weaning. Timed breeding experiments were carried out to analyze $Mkp7^{-/-}$ embryos at different gestational stages. Genotypes of the embryos were determined by PCR using primers flanking the insertion in the WT allele (Supplemental Fig. 2A, 2B) and the same forward primer (P1) plus a reverse primer in the vector (P3) (Supplemental Fig. 2A, 2B). The results revealed embryonic lethality of $Mkp7^{-/-}$ mice with the majority of the KO mice dying between embryonic days 15.5 and 17.5 (Supplemental Fig. 2C), demonstrating that MKP7 is required for normal embryonic development in mice, which is similar to a recently published study (12).

MKP7-deficient T cells are hyper-responsive to TCR activation

The induced expression of MKP7 in naive $CD4^+$ T cells in response to TCR activation (Supplemental Fig. 1A, 1B) suggests its role in T cell activation. To understand the function of MKP7 in T cells, we generated fetal liver chimeras by transferring WT or MKP7 KO fetal liver cells into lethally irradiated WT recipient mice. We used RT-PCR to examine the expression of *Mkp7* in bone marrow–derived DCs to confirm the genotypes of the hematopoietic cells in the chimeras (Supplemental Fig. 2D). We examined various thymocyte subpopulations of WT and MKP7-deficient chimeras and found that WT and MKP7-deficient chimeras showed no difference in thymocytes at each developmental stage (Supplemental Fig. 3A, 3B). $CD4^+$, $CD8^+$, and regulatory T cell populations in spleens from WT and MKP7-deficeint chimeras were comparable (Supplemental Fig. 3C, 3D). These data indicate that MKP7 is not required for T cell development.

To address the regulation of naive $CD4^+$ T cell activation and proliferation by MKP7, we stimulated WT and MKP7 KO naive

$CD4^+$ T cells with plate-bound anti-CD3 Ab with or without anti-CD28 Ab. IL-2 concentration in culture supernatants was determined by ELISA. We found that upon anti-CD3 or anti-CD3 plus anti-CD28 stimulation, the production of IL-2, a hallmark of T cell activation, by MKP7 KO $CD4^+$ T cells was higher than that by WT cells (Fig. 1A). Furthermore, MKP7 KO $CD4^+$ T cells exhibited enhanced proliferation compared with WT cells in response to anti-CD3 Ab stimulation.

MKP7 is required for Th17 differentiation and effector function in vitro

To examine the regulation of MKP7 in Th cell differentiation and effector function, we cultured WT and MKP7 KO naive $CD4^+$ T cells under Th1, Th2, or Th17 conditions in vitro with exogenous IL-2 to normalize their proliferation. After 4 d of differentiation, effector Th cells were restimulated with anti-CD3 Ab to examine the production of lineage-specific cytokines. As shown in Fig. 1B, MKP7 KO Th1 cells produced a similar amount of IFN-γ and contained similar percentage of IFN-γ–producing cells compared with WT cells. Similarly, MKP7 KO Th2 cells produced a similar amount of IL-4 and IL-5, and contained comparable percentage of IL-4– and IL-5–producing cells as WT culture (Fig. 1C). Together, these data demonstrate that MKP7 is dispensable for Th1 and Th2 differentiation and function. In contrast, MKP7 KO Th17 cells produced a significantly decreased amount of IL-17A and IL-17F, and contained nearly 50% less IL-17A–producing cells compared with WT cells (Fig. 2A).

To further substantiate the earlier observations, we restimulated WT and MKP7 KO Th17 cells with anti-CD3. The expression of Th17 lineage-specific genes including IL-17A, IL-17F, IL-21 and IL-22, and RORγt, the transcription factor essential for Th17 differentiation (12, 16), were determined by quantitative RT- PCR. We found that the expression of Th17 lineage-specific genes and transcription factor was impaired in MKP7 KO Th17 cells compared with WT cells (Fig. 2B).

It has been demonstrated that IL-2 inhibits Th17 differentiation (17). To address the possible effect of increased IL-2 production by MKP7 KO T cells on Th17 differentiation (Fig. 1A), we cultured WT and MKP7 KO naive $CD4^+$ T cells under Th17 conditions in the presence of IL-2 blocking Abs. Effector Th17 cells were restimulated with anti-CD3 Ab to examine the production of IL-17A and IL-17F after 4 d of differentiation. As shown in Fig. 2C, MKP7 KO Th17 cells produced similar amounts of IL-17A and IL-17F as WT cells after blockade of IL-2. Together, these data demonstrate that MKP7 is required for Th17 differentiation through regulation of IL-2 production in response to TCR activation.

MKP7 regulation on Th17 cells acts via inhibiting ERK

To further understand the regulation of T cell activation and effector function by MKP7, we activated WT and MKP7 KO naive $CD4^+$ T cells with anti-CD3 plus anti-CD28 Abs to examine MAPK activation. MAPK activation was detected as early as 3 min after activation in both WT and KO cells (Fig. 3A). Enhanced activation of ERK and JNK, but not p38, was detected in MKP7 KO T cells at 3, 6, and 12 min after TCR activation compared with WT cells. These data demonstrate that MKP7 is a negative regulator of ERK and JNK, but not p38, in $CD4^+$ T cells.

MKP7 was previously reported to interact with JNK, p38 and ERK, but only inhibits the activation of JNK and p38 when overexpressed in COS-7 cells (8). Enhanced ERK activation in MKP7 KO T cells suggests that MKP7 is an ERK phosphatase in vivo (Fig. 3A). To test whether ERK is a substrate of MKP7, we prepared bisphosphorylated ERK2 (ERK/pTpY) to incubate with MKP7 or MKP3, a known ERK phosphatase (18, 19). Phosphorylation of Thr^{183} and Tyr^{185} of ERK was determined by Western blot analysis using anti–p-Thr and anti–p-Tyr Abs, respectively. As expected, MKP3 dephosphorylated both p-Thr and p-Tyr residues (Fig. 3B). Incubating MKP7 with bisphosphorylated ERK2 resulted in dephosphorylation of p-Tyr, but not p-Thr, residue (Fig. 3B). It has been shown that dephosphorylation of phosphotyrosine by phosphatase completely deactivates ERK (20). Therefore, MKP7 is an ERK phosphatase in vivo.

It has been shown that ERK is important for IL-2 expression in activated T cells, and ERK and IL-2 cooperate in an autoregulatory loop to constrain Th17 differentiation (21–23). To examine whether MKP7 regulates Th17 differentiation and function via ERK, we cultured WT and MKP7 KO naive $CD4^+$ T cells under Th17 conditions in the presence of different concentrations of U0126, which specially inhibits the activation of MEK1/2, therefore inhibiting ERK activation. After 4 d of differentiation, IL-17A and IFN-γ expression in the cells were analyzed by in-

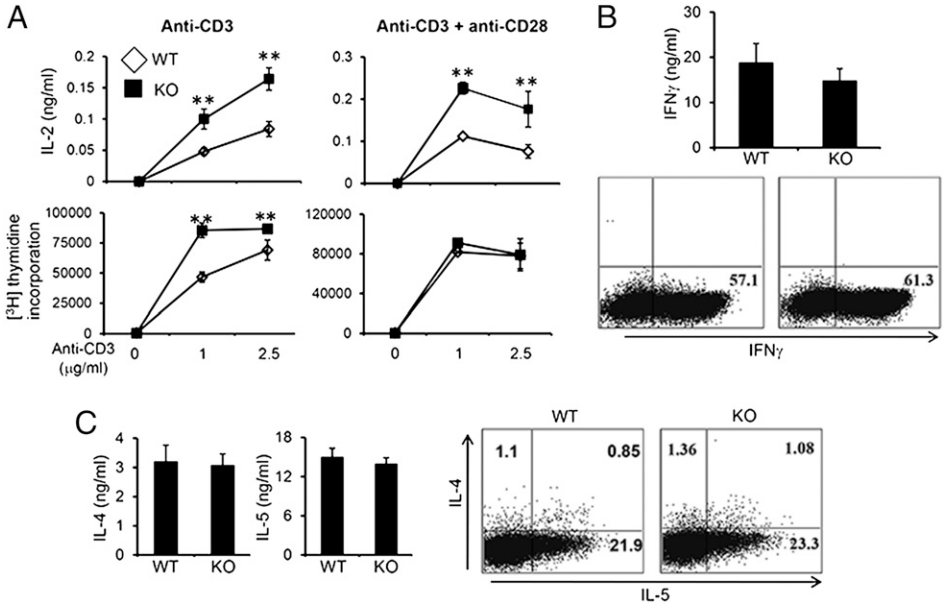

FIGURE 1. MKP7 regulates T cell activation and Th cell generation in vitro. (**A**) Naive $CD4^+$ T cells from WT and MKP7 chimeras were activated with different concentrations of anti-CD3 in the presence or absence of anti-CD28, and their IL-2 production and [^{3}H]thymidine uptake were measured. (**B** and **C**) Naive $CD4^+$ T cells from WT and KO chimeras were differentiated into Th1 (B) and Th2 (C) cells. After 4 d of differentiation, cells were stimulated with anti-CD3 for the examination of lineage-specific cytokine expression by ELISA assay and intracellular cytokine staining. Data are representative of three independent experiments. $^{**}p < 0.01$.

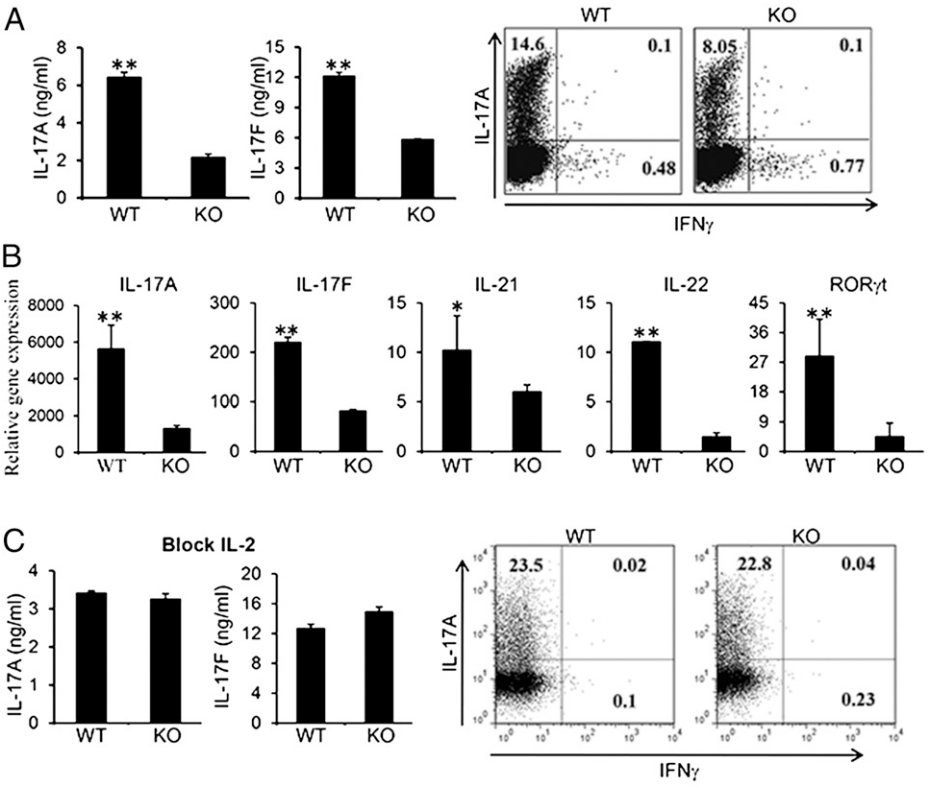

FIGURE 2. MKP7 regulates Th17 generation and effector function through IL-2. (**A**) Naive CD4$^+$ T cells from WT and MKP7 chimeras were differentiated into Th17 cells followed by anti-CD3 stimulation before ELISA assay and intracellular cytokine staining. (**B**) In vitro differentiated Th17 cells were stimulated with anti-CD3. IL-17, IL-17F, IL-21, IL-22, and RORγt gene expression were determined by quantitative RT-PCR (qPCR). (**C**). Naive CD4$^+$ T cells from WT and MKP7 chimeras were differentiated into Th17 cells in the presence of IL-2 blocking Abs followed by anti-CD3 stimulation. IL-17 and IL-17F production was measured by ELISA assay, and IL-17 expression was determined by intracellular cytokine staining. Data are representative of three independent experiments. $*p < 0.05$, $**p < 0.01$.

tracellular cytokine staining. As shown in Fig. 3C, MKP7 KO cells contained less IL-17$^+$ cells than WT cells (24.3 versus 34.8%) without ERK inhibition. ERK inhibition increased the percentage of IL-17A$^+$ cells in both WT and MKP7 KO samples. When 10 ng/ml U0126 was added to the culture, the percentage of IL-17A$^+$ cells in both WT and MKP7 KO cells became comparable. We further examined the mRNA expression of IL-17A, IL-17F, and RORγt, and protein expression of IL-17A and IL-17F in WT and MKP7 KO Th17 cells differentiated in the presence of 0 or 10 ng/ml U0126. Without U0126, the expression of IL-17A, IL-17F, and RORγt was significantly lower in MKP7 KO Th17 cells compared with WT cells in response to anti-CD3 restimulation (Fig. 3D). MKP7 KO Th17 cells also produced significantly lower amounts of IL-17 and IL-17F (Fig. 3E). In the presence of U0126, the differences in the expression of these genes between WT and KO cells were no longer observed (Fig. 3D, 3E). In contrast, inhibition of JNK activation during Th17 differentiation using a JNK-specific inhibitor, SP600125, did not rescue the deficiency of Th17 generation of MKP7 KO T cells (Supplemental Fig. 4A, 4B). Together, these data demonstrate that the regulation of Th17 differentiation and function by MKP7 is dependent on ERK.

MKP7 is required for Th17-mediated autoimmunity in vivo

Th17 cells play critical roles in various autoimmune diseases such as EAE (16, 24). To examine the regulation of Th17-mediated autoimmune responses by MKP7 in vivo, we immunized both WT and MKP7 KO chimeras with $MOG_{35–55}$ peptide to induce EAE disease as we described previously (4). We found that WT chimeras started to develop disease on day 4 after second MOG immunization (Fig. 4A). By day 8, all the WT chimeras reached a score of 3–4. Although some of the MKP7 KO chimeras started to develop disease at the same time as the WT chimeras did, only around 40% of these mice developed disease with reduced severity. By day 8, the disease score of MKP7 KO chimeras remained around 1–2. Therefore, in the absence of MKP7 in the hematopoietic compartment, mice are less susceptible to MOG-induced EAE.

Despite the markedly reduced disease incidence and severity in MKP7 KO chimeras, both CD4$^+$ T cells and CD11b$^+$ cells were present in the CNS at similar levels to those in WT chimeras (Fig. 4B). However, CD4$^+$ T cells in CNS from MKP7 chimeras contained a significantly lower percentage of IL-17A–producing cells than those from WT mice (Fig. 4C). In contrast, a higher percentage of IFN-γ–producing cells was detected in CNS from MKP7 chimeras than those from WT chimeras. Together, these results demonstrate that MKP7 is indispensable for Th17-mediated autoimmune responses in vivo.

MKP7 deficiency decreases Tfh cell generation

Tfh cells are a newly defined CD4$^+$ T cell subset that provides essential help to B cells within the GC (25). IL-2 has been shown to negatively regulate Tfh cell generation (26). To examine the possible role of MKP7 in Tfh cells, we immunized WT and MKP7 KO chimeras with KLH protein emulsified in CFA as we did previously (21). Seven days postimmunization, the mice were sacrificed and analyzed for GC formation, as well as Ag-specific Ab and cytokine responses. We found reduced percentage of GL7$^+$ Fas$^+$ GC B cells, which is correlated with decreased CXCR5$^+$ Bcl6$^+$ Tfh cells in LNs from MKP7 chimeras compared with WT chimeras (Fig. 5A). In line with reduced Tfh cells and GC B cells in the MKP7 KO chimera, we observed reduced KLH-specific Abs, including IgM, IgG, IgG1, and IgG2a, in the serum from MKP7 KO chimeras compared with those from WT chimeras (Fig. 5B). Upon KLH restimulation, splenocytes from MKP7 KO chimeras contained reduced percentage of IL-17A$^+$ T cells (Fig. 5C). Reduced IL-17A protein was detected in the culture supernatant of splenocytes from MKP7 chimeras compared with cells from WT chimeras, whereas IFN-γ$^+$ cells and protein concentrations were comparable between WT and MKP7 samples

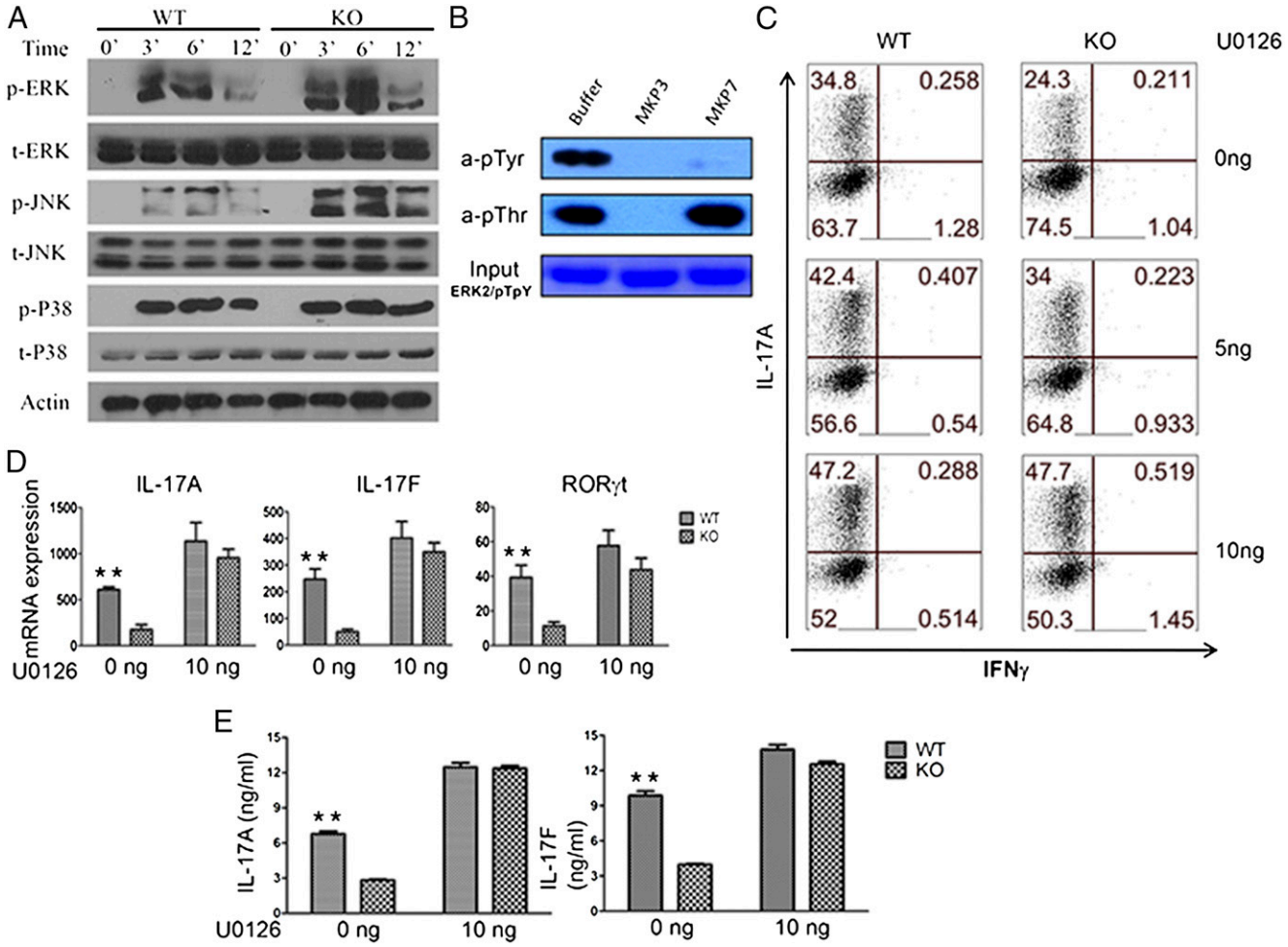

FIGURE 3. MKP7 inhibiting Th17 effector function is dependent on ERK. (**A**) Naive CD4$^+$ T cells from WT and MKP7 chimeras were activated by anti-CD3 plus anti-CD28. Cells were lysed at the indicated time points. Cell extracts were resolved on SDS-PAGE gels and transferred to nitrocellulose membranes. Blots were probed with anti–p-ERK, anti–p-JNK, anti–p-p38, anti-ERK, anti-JNK, anti-p38, or anti-actin. (**B**) ERK2/pTpY was incubated with MKP7 or MKP3 at 25°C for 1 h, followed by Western blot analysis using the indicated Abs. The *lower panel* of the SDS-polyacrylamide gel (Coomassie stained) shows the amounts of purified ERK2/pTpY used in the experiment. (**C**) WT and KO naive CD4$^+$ T cells were differentiated into Th17 cells in the presence of vehicle or indicated concentrations of U0126. Differentiated cells were activated with PMA and ionomycin for intracellular cytokine staining. WT and MKP7 KO T cells were differentiated into Th17 cells and stimulated with anti-CD3 Abs. IL-17, IL-17F, and RORγt gene expression were determined by qPCR (**D**), and IL-17 and IL-17F production were measured by ELISA assay (**E**). Data are representative of three independent experiments. $^{**}p < 0.01$.

(Fig. 5C, 5D), further confirming the role of MKP7 in regulation of Th17 responses.

Discussion

The evolutionary conserved MAPK pathways mediate various physiological processes including cell activation, differentiation, effector function, apoptosis, as well as development. Numerous components of the MAPK pathways regulating various aspects of embryonic development, including the development of embryonic vasculature, brain, liver, and erythrocytes, and the development of placenta, are well documented (27–33). The activation of MAPKs is negatively regulated by various MKPs, and loss of function of MKP proteins could lead to aberrant MAPK activities, and therefore impairs embryonic development. For instance, deficiency of MKP4 (DUSP9), a phosphatase targeting ERK and p38 (34), in mice resulted in embryonic lethality caused by defective placental development (35), which is in line with the early death of ERK2 KO and p38α KO mice caused by abnormalities of the placenta (27–30). In this study, we found that mice deficient in MKP7 are embryonic lethal (Supplemental Fig. 2). We also found that the activation of ERK, p38, and JNK was all increased in MKP7 KO MEFs compared with that in WT cells in response to UV irradiation (Supplemental Fig. 4C). Therefore, the cause of death in MKP7 KO mice could result from dysregulation of one or multiple MAPK pathways, which is currently being investigated. Interestingly, in CD4$^+$ T cells, MKP7 inhibits the activation of ERK and JNK, but not p38 (Fig. 3A, 3B). Such different substrate preferences of one MKP in different types of cells, tissues, or experimental conditions have been observed in other MKP members including MKP5, PAC-1, and MKP1 (1–3, 36, 37), and is possibly due to mechanisms such as scaffold proteins or the differential expression of MKPs with similar substrate preference in different types of cells or tissues (4). MKP7 as an ERK phosphatase was further confirmed by our dephosphorylation assay demonstrating that MKP7 dephosphorylates p-Tyr^{185} residue of ERK2 (Fig. 3B). It has been shown that protein tyrosine phosphatases including PTP-SL, STEP, and HePTP dephosphorylate phosphotyrosine to inhibit ERK activation (23, 38). Our results together with others support that phosphorylation of both Thr^{183} and Tyr^{185} is required for ERK activation (20).

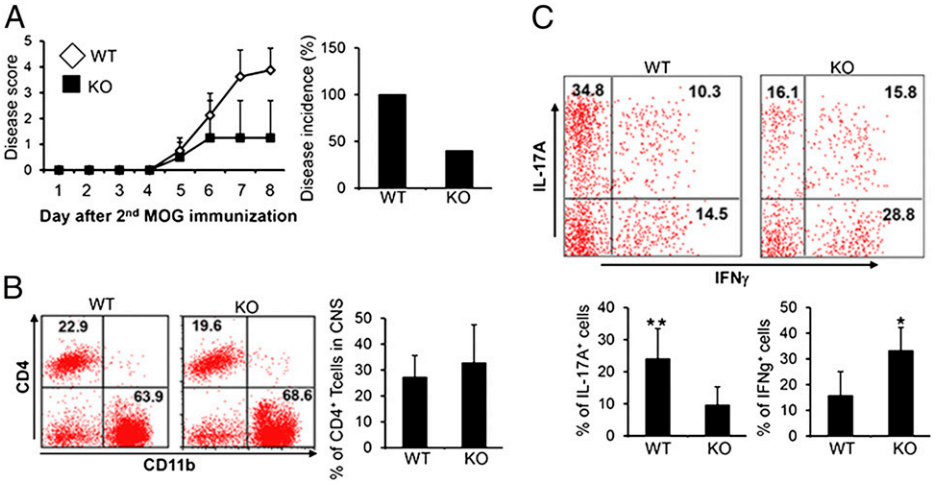

FIGURE 4. MKP7 chimeras are less susceptible to EAE disease. (**A**) WT and MKP7 chimeras (n = 5) were immunized with $MOG_{35–55}$ peptide to induce EAE. Clinical score of EAE disease was recorded daily. (**B**) Mononuclear cells from CNS were stained with anti-CD4 and anti-CD11b Abs. (**C**) CNS-infiltrated leukocytes were activated with PMA and ionomycin in the presence of GolgiPlug. IFN-γ and IL-17 expression by $CD4^+$ cells were examined be intracellular cytokine staining. Total IFN-γ– and IL-17–expressing cells were calculated and averaged. Data are representative of two independent experiments. $*p < 0.05$, $**p < 0.01$.

ERK, JNK, and p38 differentially regulate $CD4^+$ T cell activation, differentiation, and effector function (39). The functions of two MKP members, MKP5 and MKP1, in T cell activation and effector function have been examined by our group previously (1, 4). In this study, we found that MKP7 is specifically required for Th17 differentiation in vitro, and Th17 and Tfh cell responses in vivo. A possible role of MKP7 in regulating JNK activation and Th1/Th2 balance has been shown using T cell–specific transgenic mice of MKP7 with deletion of the last 242 aa in the C-terminal region ($MKP7^{WT}$ or $DUSP16^{WT}$) or with C-terminal deletion plus phosphatase inactive mutant ($MKP7^{C244S}$ or $DUSP16^{C244S}$) (40). JNK activation was found to be increased in both $MKP7^{WT}$ Th1 and Th2 cells. Interestingly, reduced IL-4 and GATA3 mRNA expression in Th2 cells and similar IFN-γ mRNA expression in

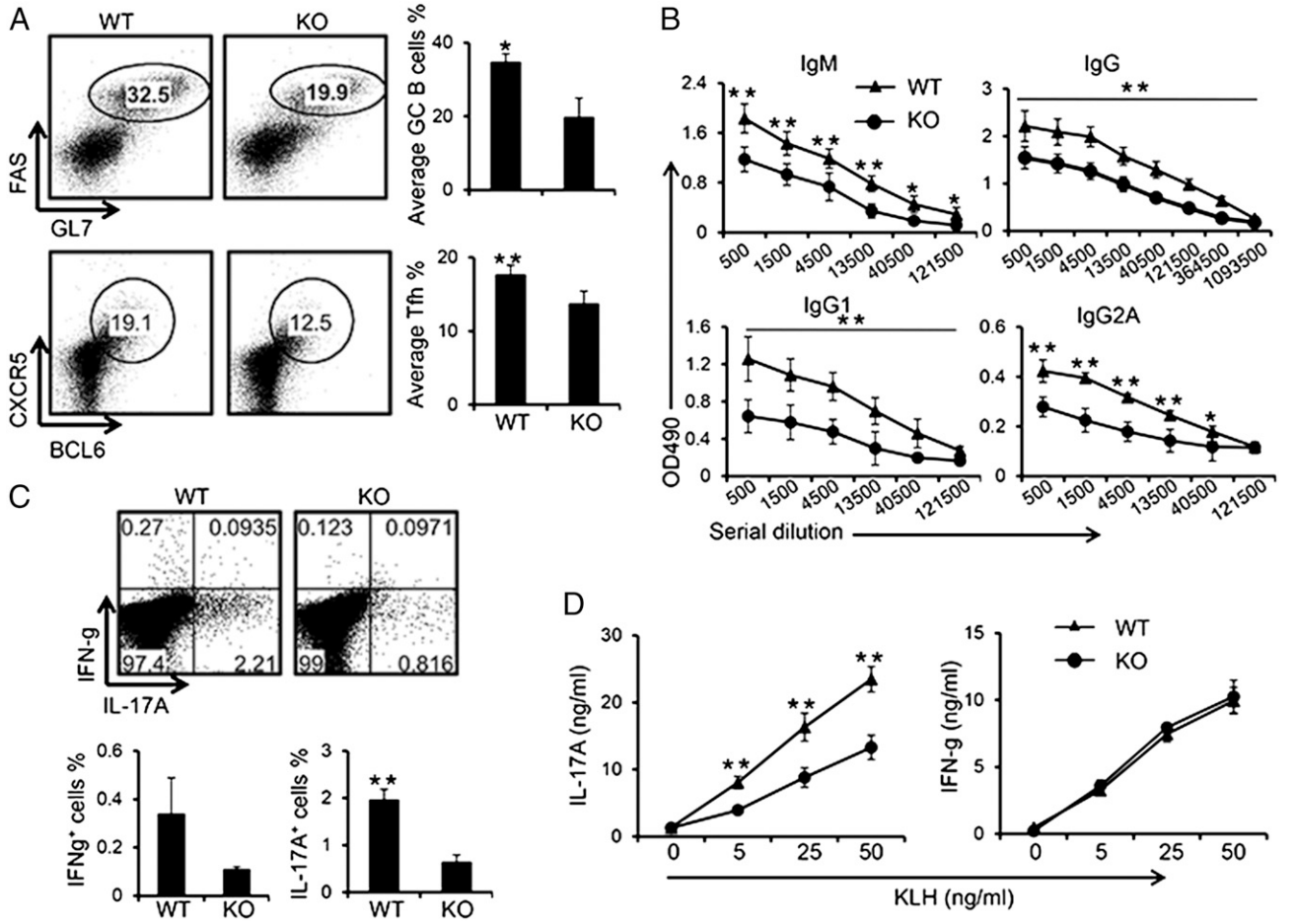

FIGURE 5. MKP7 negatively regulates Tfh and GC B cell responses. WT and MKP7 chimeras (n = 3) were s.c. immunized with KLH in CFA. (**A**) Seven days postimmunization, LNs were collected to analyze Tfh and GC B cell populations. (**B**) Sera from the mice were collected and subjected to 3-fold dilutions, and KLH-specific Ab responses (IgM, IgG, IgG1, and IgG2A) were measured by ELISA assay. (**C** and **D**) Splenocytes from KLH-immunized mice were isolated and stimulated with KLH peptide for overnight for IFN-γ and IL-17 intracellular cytokine staining (C) and for 3 d for the measurement of IFN-γ and IL-17 production by ELISA assay (D). Data are representative of three independent experiments. $*p < 0.05$, $**p < 0.01$.

Th1 cells were observed from MKP7 ^{C244s} cells compared with MKP7^{WT} cells. These observations led to a conclusion that MKP7 could enhance the course of Th2 differentiation despite that both IL-4 and GATA3 expression were not changed in MKP7^{WT} Th2 cells compared with cells from nontransgenic mice. MKP7 has been shown to interact with scaffold proteins including JNK-interacting protein and β-arrestin 2 with a domain that was preserved in the transgenic construct used in the study mentioned earlier (9, 10). The C-terminal region that was deleted in the study is unique to MKP7, containing a nuclear localization motif, a PEST sequences, and an ERK phosphorylation site that are important for its stability and subcellular localization. Therefore, such manipulation may not reveal the physiological function of a protein. In addition, data on the activation status of ERK and p38 were not available in that study.

In this study, we observed increased IL-2 production and proliferation in response to TCR activation in MKP7 KO naive CD4$^+$ T cells compared with WT cells. MKP5 and MKP1 KO naive CD4$^+$ T cells had impaired IL-2 production and proliferation in response to TCR activation (1, 4), a converse phenotype to that observed in T cells lacking both JNK1 and JNK2 (41). The different responses of MKP5-, MKP1-, and MKP7-deficient naive T cells to TCR activation are likely due to the differential regulation of MAPK activation by these proteins in T cells. Both MKP5 and MKP1 inhibit JNK activation in T cells (1, 4). MKP7, in contrast, negatively regulates ERK and JNK activation (Fig. 3B). The AP-1, the major target of MAPK signaling, plays a critical role in T cell activation–induced IL-2 production (22). Although modulation of single MAPK could lead to altered AP-1 activation, IL-2 production, and T cell activation, the compound effect of the activation of MAPK members is of the utmost importance for the regulation of IL-2 production and T cell activation. It is possible that in MKP7 KO T cells, the enhanced ERK activity overshadows the negative effect of JNK on IL-2 production and T cell activation, which result in increased IL-2 production and reduced Th17 generation. Indeed, we found that MKP7 KO naive T cells were rescued in their differentiation into Th17 cells in the presence of 10 ng/ml ERK-specific inhibitor U0126 (Fig. 3C–E). MKP7 KO T cells are impaired in Th17-specific cytokine expression and production, which resulted in reduced susceptibility to MOG-induced EAE disease (Fig. 4). MKP1 KO T cells were also defective in Th17 effector function, and MKP1 KO mice were resistant to MOG-induced EAE disease (4). However, mechanisms underlying the resistance of MKP1 and MKP7 KO mice to EAE disease are likely different. We believe that the impaired Th17 effector function in MKP1 KO cells is due to the intrinsic activation defect of the T cells, rather than the production of IL-2, because IL-2 production by MKP1 KO naive CD4$^+$ T cells was reduced compared with WT cells upon TCR activation. Therefore, MKP1 KO mice not only developed less disease, but also had delayed disease onset, which was not observed in MKP7 KO chimeric mice.

U0126 has been used in EAE and it was found that global inhibition of ERK by U0126 resulted in attenuated EAE disease (42). Mechanistically, U0126 inhibited ERK-dependent IL-23 and IL-1β expression by DCs, thereby impairing pathogenic Th17 generation and EAE development. However, in EAE, ERK1 appears to be inhibitory for the development of the disease (43). Mice deficient in ERK1 developed EAE earlier with increased severity compared with WT mice.

Inhibition of ERK in T cells in vitro using U0126 was reported to increase Th17 cell generation and cytokine production such as IL-17 (6, 44), consistent with our present data. Furthermore, Th17 cells treated with U0126 induced more rapid and severe colitis in Rag$^{-/-}$ mice compared with mice treated with T cells without ERK inhibition (6). These evidences together with our findings demonstrated that ERK activation in T cells is inhibitory for Th17 cell generation and effector function.

Tfh cells are a new subset of effector CD4$^+$ T cells whose generation is controlled by a network of transcriptional factors including Bcl6, IRF4, c-Maf, Batf, and STAT3/5 (25). The regulation of MAPKs in the generation and function of Tfh is yet to be discovered. Previous studies have shown the regulatory role of MAPKs in humoral responses. For instance, mice deficient in JNK1 had increased *Leishmania*-specific IgG1 and IgG2a Ab production compared with their control in response to *Leishmania major* infection (45). Our discovery on the regulation of Tfh responses in vivo by MKP7 suggests the possible role of ERK and JNK in Tfh cell generation and function, which requires further investigation.

In summary, this study demonstrates that MKP7 has nonredundant roles in both embryonic development and the immune system. MKP7 is the first MKP identified that plays an inhibitory role in naive CD4$^+$ T cell activation and whose deficiency selectively impairs Th17 and Tfh cell function. The specific function of MKP7 in T cell activation and effector function expands our understanding on MKP function in immune responses and provides a novel target for therapeutic intervention for T cell–mediated inflammatory and autoimmune diseases.

Disclosures

The authors have no financial conflicts of interest.

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