International Immunology, Vol. 21, No. 4, pp. 393–401
doi:10.1093/intimm/dxp007

Regulation of NF-κB-dependent T cell activation and development by MEKK3

Hisaaki Shinohara[1], Sho Yamasaki[2], Shiori Maeda[1], Takashi Saito[2] and **Tomohiro Kurosaki[1,3]**

[1]Laboratory for Lymphocyte Differentiation, and [2]Laboratory for Cell Signaling, RIKEN Research Center for Allergy and Immunology, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan
[3]Laboratory for Lymphocyte Differentiation, World Premier International Immunology Frontier Research Center, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan

Keywords: development, MEKK3, NF-κB, signal, T-cell

Abstract

The serine/threonine kinase MEKK3, also known as mitogen-activated protein kinase kinase kinase 3, is a critical activator of the transcription factor NF-κB in innate immunity. However, the physiological function of MEKK3 in adaptive immunity is unclear. Here we report that following TCR signaling, MEKK3 positively regulated the kinase, IκB kinase, leading to NF-κB activation. T cells lacking MEKK3 were defective in TCR-induced and cytokine-induced responses. Furthermore, T cell-specific deletion of MEKK3 resulted in reduced numbers of thymocytes and peripheral T cells. Thus, our results provide genetic evidence that MEKK3 plays a crucial role in adaptive immunity.

Introduction

Mitogen-activated protein kinases (MAPKs) such as ERK, JNK and p38 are ubiquitously expressed and regulate a wide variety of functions in mammals, including immune regulation and activation (1). MAPKs are activated by conserved cascades through MAPK kinase (MAPKK) and MAPKK kinase (MAP3K). Many members of the MAP3K family, including MEKK1, MEKK2, MEKK3, MEKK4, ASK1, TAK1 and Tpl2, have been identified (2). However, their specific functions in particular signaling pathways and physiological processes are not fully understood.

Some MAP3Ks are known to control the responses of innate immune cells to Toll-like receptor (TLR) stimulation and to inflammatory cytokines by regulating IκB kinase (IKK) and JNK activity (3, 4). For instance, TAK1 has been reported to activate IKK and JNK following tumor necrosis factor (TNF)-α, IL-1 and LPS stimulation (5–7). However, the activation of NF-κB and JNK by TLR8 was TAK1 independent (8), and IKK activation by IL-1 was not completely blocked in TAK1-deficient cells (6, 7), suggesting the existence of TAK1-independent mechanisms of IKK and JNK activation. This could be mediated by other MAP3Ks such as MEKK2 or MEKK3. Indeed, in MEKK3-deficient MEF cells, there is a delay in the activation of NF-κB and JNK by TNF-α, IL-1 and LPS stimulation (9, 10). Moreover, MEKK2 knock-down MEFs could not induce the late phase of NF-κB activation following TNF-α and IL-1 stimulation (11). In contrast to such an established role of MEKK2 and MEKK3 in the inflammatory and innate immune responses, their roles in adaptive immunity are less clear and somewhat controversial. Initial studies using the Jurkat T cell line showed that a dominant-negative form of MEKK2 blocked TCR-mediated activation of JNK (12). Subsequent gene-targeting studies, however, demonstrated that this enzyme did not play a significant positive role in TCR-mediated JNK activation (13). Together, these data suggest that depending on the cell type and cellular contexts, distinct types of MAP3Ks could be utilized, thereby raising the possibility that TCR might use MEKK3, rather than MEKK2, for JNK and IKK activation in primary T cells. Hence, in this study, we focus upon whether MEKK3 participates in TCR-mediated IKK activation in primary T cells, possibly regulating the development and function of T cells.

Here, we show that deletion of MEKK3 in a T cell-specific manner resulted in reduced development of thymocytes. In mature thymocytes, MEKK3 was required for TCR-dependent activation of NF-κB. In peripheral T cells, MEKK3 was also required for IL-2-mediated proliferative responses. Together, our results suggest that MEKK3 participates in TCR and cytokine receptor signals to regulate the development and function of T cells.

Materials and methods

Mice

The targeting vector was electroporated into Bruce-4 ES cells of C57BL/6 origin (provided by K. Rajewsky, the CBR

Correspondence to: T. Kurosaki; E-mail: kurosaki@rcai.riken.jp

Received 26 November 2008, *accepted* 16 January 2009

Transmitting editor: T. Takai

Advance Access publication 17 February 2009

Institute for Biomedical Research, Harvard Medical School). Neomycin-resistant clones were screened for homologous recombination by Southern blot using probe as shown in Fig. 1. Correctly targeted clones were transiently transfected with pMC1-Cre (gift from K. Rajewsky) to delete the *loxP*-flanked neomycin-resistant gene cassette. Progeny clones that became sensitive to neomycin were subjected to Southern blot analysis to detect floxed allele. Targeted clones were microinjected into C57BL/6 blastocysts to generate chimeras. Mating of chimeric male mice to C57BL/6 female mice resulted in transmission of the floxed allele in the germline. Mice with the floxed genotype were bred with a transgenic mouse line carrying the *Cre* transgene under control of the Lck promoter (C57BL6/J background) (14), which were then intercrossed to generate T cell-specific MEKK3-deficient [*Lck-Cre-MEKK3*$^{flox/flox}$ (T-KO)] mice. Mice were kept in specific pathogen-free conditions and all experiments were done in compliance with the guidelines by the animal research committee at RIKEN.

Reagents

Antibodies for ERK, JNK, p38, IκBα and Bcl10 were purchased from Santa Cruz; anti-phospho-ERK, anti-phospho-JNK, anti-phospho-p38, anti-phospho-IKKβ, anti-phospho-IκBα, anti-phospho-threonine, anti-phospho-ATF2 and anti-TAK1 antibodies from Cell Signaling Technology; anti-GAPDH, anti-CARMA1 and anti-IKKβ antibodies from Abcam; anti-MEKK3 mAb from BD and anti-HisX6 mAb was obtained from QIAGEN. Anti-phospho-serine was purchased from Zymed. Anti-CARMA1 antibody for immunoprecipitation was prepared as previously described (15). Antibodies used for TCR stimulation of Jurkat cells were C305 (anti-TCR mAb) and OKT3 (anti-CD3ε mAb) as described previously (16).

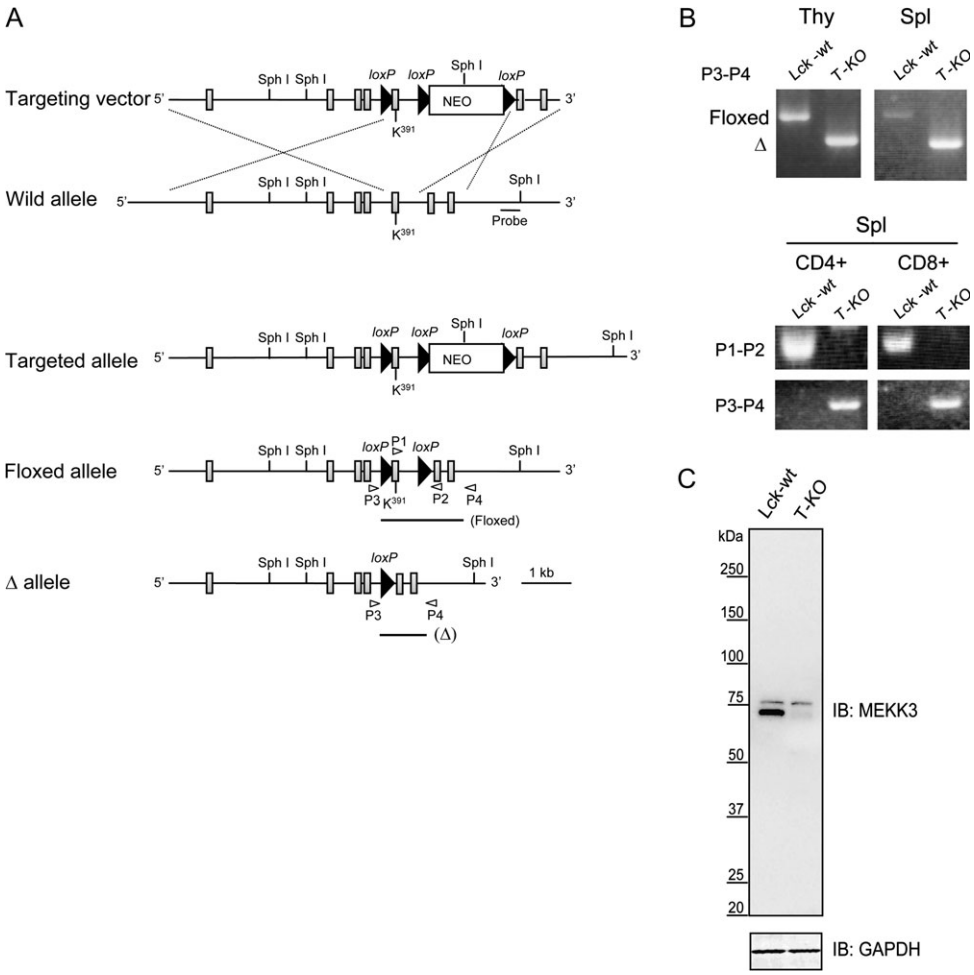

Fig. 1. T cell-specific gene targeting of MEKK3. (A) The conditional targeting vector was constructed to delete a genomic fragment containing exons 11 and 12 of the *MEKK3* gene. Exon 11 encodes the ATP-binding site (K391) essential for its kinase activity. One *loxP* site was introduced into intron 10 and two *loxP* sites which flanked the neomycin-resistant gene cassette in intron 11. The expected DNA lengths (using P3–P4 primers) to detect the floxed and D allele are depicted. (B) The MEKK3 genotype was analyzed by PCR using the following primer pairs: P1-CTCCTGGGTCAAGGTGCCTTCGGCAGGGTCTACTTGTGCT and P2-AACTGGATCTCACACTCCAGAGCACTCACCTCCTAGAGA (for the floxed allele) or P3-TCAGAATGATCTAATGTTTGTGAGCAGCTT and P4-TCACTATGCTGACCAGCTGGCCTCACAGTGCACAGA (for the Δ allele; Δ, for floxed allele; floxed). Genomic DNA was isolated from whole thymocytes (Thy), CD5-positive spleen cells (Spl), CD4-positive (CD4$^+$) or CD8-positive (CD8$^+$) spleen cells from *Lck-Cre/WT* (Lck-WT) and *Lck-Cre/MEKK3*$^{flox/flox}$ (T-KO). (C) Western blot analysis of MEKK3 proteins from CD4 SP thymocytes of *Lck-Cre/WT* (Lck-WT) and *Lck-Cre/MEKK3*$^{flox/flox}$ (T-KO) mice.

Flow cytometry

Lymphocytes were isolated from the thymi, spleens and peripheral blood of mice 5–8 weeks old. Antibodies for flow cytometry were purchased from eBioscience. Stained cells were analyzed on a FACSCalibur (BD). Cell survival was assessed by propidium iodide staining.

Cell culture

T cells were cultured in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. CD4 single-positive (SP) thymocytes were purified by anti-CD4 magnetic beads following depleting $CD8^+$ cells with magnetic beads using AutoMACS (Miltenyi Biotec). Isolated T cells were activated with 10 μg ml^{-1} of plate-bound anti-CD3 (2C11) and 2 μg ml^{-1} of anti-CD28 (37.51) from BD Biosciences, with 100 ng ml^{-1} of phorbol myristate acetate (PMA) (Sigma) and 2 μg ml^{-1} of anti-CD28 or with 20 ng ml^{-1} of recombinant mouse (rm) IL-2 or rmIL-7 (Peprotech). T cell proliferation was determined by Cell Counting Kit-8 (Dojindo Labs) as described by the manufacturer and the absorbance at 450 nm was measured in a microplate reader (Bio-Rad). IL-2 and IFN-γ production were analyzed by ELISA according to the manufacturer's protocols (BD PharMingen).

Retroviral transinfection

The gene encoding Cre recombinase was cloned into the pMX-IRES-EGFP retroviral vector (a gift from T. Kitamura, University of Tokyo). The packaging cells EcoPack2 (BD Biosciences) were transfected by FuGENE6 according to the manufacturer's protocols (Roche). The culture soup containing retroviruses was harvested 48 h after transfection. After 24 h of stimulation with 10 μg ml^{-1} of 2C11 (anti-CD3 mAb) and 5 μg ml^{-1} of anti-CD28 antibody, isolated $CD4^+$ splenocytes were infected with retrovirus by spin infection at 3000 r.p.m. for 2 h in a centrifuge. The infected GFP^+ cells were sorted after cultured for additional 2–3 days with media containing rmIL-2 using as effector T cells (17). For Jurkat cell transfectants, MEKK3 wild-type (WT) and kinase-dead genes were cloned into pMXpuro. Retroviruses were produced by transfection to pantropic packaging cell GP2 (Clontech). The spin-infected Jurkat cells without stimulation were selected with puromycine (2 μg ml^{-1}) for a week.

Biochemical analyses

Immunoblotting and kinase assay were performed by using the standard protocol as previously described (15). Briefly, cells were incubated with 10 μg ml^{-1} of 2C11 for 20 min on ice and then stimulated with 50 μg ml^{-1} goat anti-hamster IgG (Cappel) for 5 min at 37°C. After stimulation, the cells were lysed in 1% NP-40 lysis. For MEKK3 kinase assays, 2×10^7 $CD4^+$ splenocytes were lysed in 1% NP-40 lysis buffer. Pre-cleared lysates were immunoprecipitated by 1 μg anti-MEKK3 mAb, followed by incubation with 40 μl of protein G–sepharose. The beads were washed three times with lysis buffer and two times with kinase buffer. The immunoprecipitates were re-suspended in kinase buffer containing 100 μM ATP. His-tagged MKK6 was purified as per the manufacturer's instruction (QIAGEN) and added to the kinase reaction mixture as a substrate. Glutathione-*S*-transferase-fused IKKβ as substrates were prepared as previously described (15, 18). After 30 min incubation at 30°C, the reaction was terminated by addition of SDS sample buffer followed by boiling for 5 min.

Statistical analysis

Data are presented as average ± SD. Statistical analysis was performed with Student's *t*-test and analyzed using Microsoft Excel software.

Results

MEKK3 is essential for normal T cell development

Null mutation of MEKK3 results in death at approximately embryonic day 11 (19). Thus, to investigate the intrinsic function of MEKK3 in T cells, we constructed a targeting vector that was designed to delete the ATP-binding site (K391) by Cre recombinase as shown in Fig. 1(A). To delete the *MEKK3* allele specifically in T cells, we crossed *MEKK3*$^{flox/flox}$ mice with the *Lck-Cre* transgenic mice that express the Cre recombinase under the control of the T cell-specific Lck promoter (*Lck-Cre*). *Lck-Cre-MEKK3*$^{flox/flox}$ mice (called T-KO here) were born alive and appeared healthy. Genomic PCR showed that in thymocytes and splenic T cells, the floxed alleles containing exons 11 and 12 of *MEKK3* flanked by the *loxP* sites had been efficiently deleted (Fig. 1B). The deletion of *MEKK3* was further confirmed by western blotting using a mAb against its N-terminal; the short form of the MEKK3 protein was undetectable (Fig. 1C).

We initially conducted FACS analysis of T cell populations in T-KO mice. Because there was no phenotypical difference between T cells from *Lck-Cre-MEKK3*$^{+/+}$ and *Lck-Cre-MEKK3*$^{flox/+}$ mice (data not shown), we used *Lck-Cre-MEKK3*$^{+/+}$ mice as WT control mice. The T-KO mice showed an increased percentage of CD4 and CD8 double-negative (DN) thymocytes and decreased percentage of double-positive (DP) thymocytes as WT control mice (Fig. 2A). The numbers of total thymocytes, DP, CD4 SP and CD8 SP thymocytes in T-KO were significantly reduced by 40–60% of WT counterparts (Fig. 2B). However, the expression of thymocyte maturation markers, including CD5, CD69 and CD24, was not fundamentally altered in DP or CD4 SP thymocytes from T-KO mice (data not shown). In the spleen of T-KO mice, the numbers of $CD4^+$ and $CD8^+$ T cells were reduced by ~50 and 60%, compared with control mice (Fig. 2A and B).

Since a survival defect in these mutant T cells was one of the possibilities for the reduction of the number of SP thymocytes and peripheral T cells, we examined the survival of these T cells. However, the capacity of these T cells to survive in culture was not significantly different between WT and MEKK3-deficient backgrounds (Supplementary Figure S1A, available at *International Immunology* Online). Moreover, activation-induced cell death in $CD4^+$ splenic T cells from T-KO mice did not differ significantly from WT mice (Supplementary Figure S1B, available at *International Immunology* Online).

MEKK3 positively regulates TCR-mediated cellular responses

To investigate the mechanisms underlying the reduction of SP thymocytes in T-KO mice, we then examined the response to

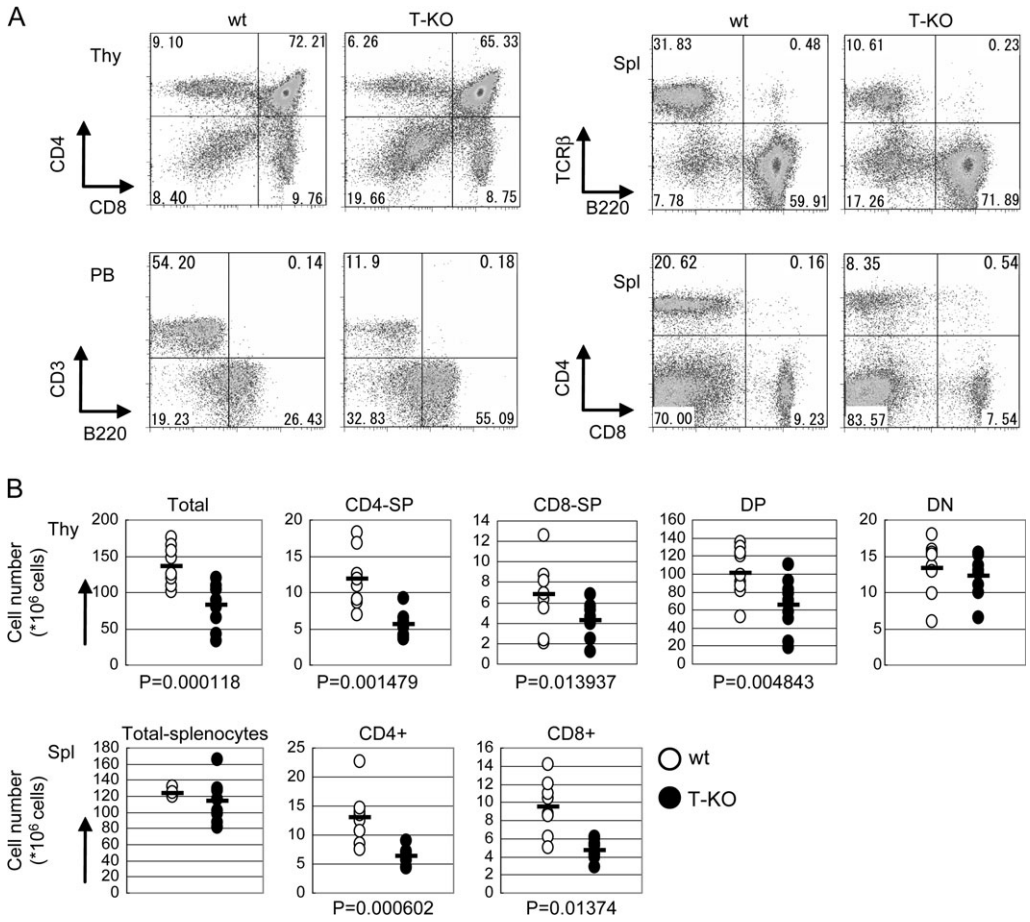

Fig. 2. MEKK3 is essential for normal T cell development. (A) Flow cytometry of lymphocytes from thymi (Thy), spleens (Spl) and PB of *Lck-Cre/WT* (WT) and *Lck-Cre/MEKK3*$^{flox/flox}$ (T-KO). Numbers in each box indicate the representative percentages of population. (B) Cellularity of thymocytes (Thy) or spleen cells (Spl). *P* values (*P* =) in the bottom of graph represent significance for differences of cell numbers (n = 8).

TCR stimulation in mature CD4 SP thymocytes. T-KO cells exhibited decreased cell proliferation and cytokine production (IL-2 and IFN-γ) in response to anti-CD3 antibody, with or without anti-CD28 antibody stimulation (Fig. 3A and B). Furthermore, CD4 SP thymocytes from T-KO mice were less capable than their WT counterparts of inducing expression of the activation marker CD25 after CD3/CD28 stimulation (Fig. 3C). Next, to examine the function of MEKK3 in effector T cells, we deleted MEKK3 in activated peripheral T cells from *MEKK3*$^{flox/flox}$ mice by retrovirus-mediated Cre expression. Cell proliferation and cytokine production after CD3/CD28 stimulation were significantly reduced in Cre-expressing effector CD4$^+$ T cells (Fig. 4A and B). Thus, we conclude that MEKK3 positively regulates TCR-mediated proliferation and production of IL-2 and IFN-γ.

MEKK3 is required for T cell cytokine responses

Cytokines such as IL-2 can promote T cell proliferation and survival of effector T cells (20). To evaluate the requirement for MEKK3 in the cytokine responses of peripheral T cells, we examined T cell proliferation in response to IL-2 or IL-7. The proliferation of Cre-expressing cells from *MEKK3*$^{flox/flox}$ mice in response to IL-2 or IL-7 was reduced as compared with mock-infected effector CD4$^+$ T cells (Fig. 4C). The phosphorylation of p38 in response to IL-2 or IL-7 was not sufficiently induced in Cre-expressing effector CD4$^+$ T cells (Fig. 4D). These results suggest that MEKK3 contributes to not only TCR-mediated responses but also cytokine-induced responses in peripheral CD4$^+$ T cells. The expression of IL-2 and IL-7 receptors was not changed and similar phosphorylation of STAT5 was induced by IL-2 and IL-7 stimulation in Cre-expressing cells from *MEKK3*$^{flox/flox}$ mice (data not shown).

MEKK3 positively regulates NF-κB activation in TCR signaling

Having demonstrated that MEKK3 is required for optimal TCR-mediated cellular responses, we further investigated the effects of MEKK3 ablation on TCR signaling events. As reported (21), NF-κB was active in CD4 SP thymocytes from WT mice by measuring DNA-binding activity. In contrast, this activity was greatly diminished in CD4 SP thymocytes from T-KO mice (Supplementary Figure S2A, available at *International Immunology* Online). Moreover, anti-CD3/CD28 antibody or PMA stimulation induced IκBα phosphorylation, whereas in T-KO cells, induced phospho-IκBα was significantly reduced (Fig. 5A). Thus, MEKK3 regulates TCR signaling activating NF-κB in CD4 SP thymocytes. Next, we

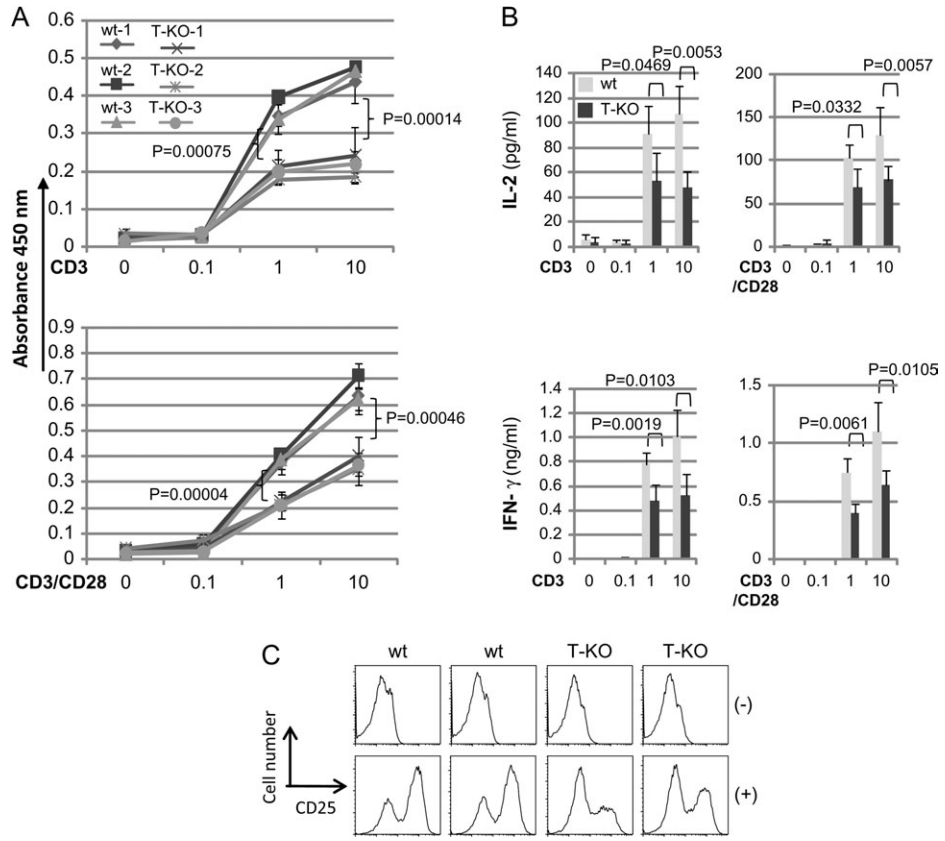

Fig. 3. MEKK3 positively regulates TCR-mediated cellular responses. (A and B) Cell proliferation (A) and cytokine production (B) induced by TCR stimulation. CD4 SP thymocyte cells from *Lck-Cre/WT* (WT) and *Lck-Cre/MEKK3*$^{flox/flox}$ (T-KO) mice were cultured in indicated concentration (μg ml^{-1}) of anti-CD3-coated plates with or without 2 μg ml^{-1} of anti-CD28 antibody for 48 h. Levels of IL-2 or IFN-γ were determined by ELISA using 24-h culture supernatants. Data of cell proliferation are presented as average ± SD for triplicate cultures of three experiments (1, 2 and 3). Data of cytokine production are shown as mean ± SD of the results from triplicate samples of four experiments. Significant differences are shown as *P* value. (C) Flow cytometric analysis of CD25 expression as activation marker. Purified CD4 SP thymocyte cells were untreated (−) or stimulated (+) with anti-CD3 antibody (10 μg ml^{-1}) plus anti-CD28 antibody (2 μg ml^{-1}) for 48 h and stained with antibody against CD25.

examined the activation status of ERK, JNK and p38, assessed by their phosphorylation status in CD4 SP thymocytes. After anti-CD3 or PMA stimulation, MAPKs activation was induced, but slightly, as previously reported (22). ERK status did not differ significantly between WT and T-KO. In contrast, stimulation-induced status of JNK, p38 or ATF2 (23) was reduced, albeit a small extent, in CD4 SP thymocytes from T-KO mice (Fig. 5B). Since in MEKK4-deficient mice, a partial reduction of TCR-mediated p38 activation has been reported (24), it is possible that MEKK4 might play a compensatory role particularly in the p38 pathway.

Given the participation of MEKK3 in TCR signaling, we designed experiments for testing whether endogenous MEKK3 is indeed activated in primary T cells by using biochemical analysis. MEKK3 was immunoprecipitated and we measured its *in vitro* kinase activity. As a substrate, MKK6 or MEKK3 by itself was used. As shown in Fig. 5(C), MEKK3 was activated in response to anti-CD3/CD28 antibody stimulation. We obtained similar results by PMA stimulation (Supplementary Figure S3A and B, available at *International Immunology* Online).

Because PMA directly induces activation of protein kinase C (PKC), the defective PMA-mediated IκBα phosphorylation of T cells from T-KO mice suggests that MEKK3 may act downstream of PKCs (Fig. 5A). The adaptor protein CARMA1 is known to be a direct downstream target of PKCs in the TCR-mediated NF-κB activation pathway (25). CARMA1 is thought to function as an organizer of a macromolecular complex that permits activation of IKK following TCR stimulation (26). Thus, we examined whether MEKK3 is associated with this macromolecular complex. As shown in Fig. 5(D), efficient interaction between MEKK3 and CARMA1 in primary T cells was observed after anti-CD3/CD28 antibody stimulation. Given the evidence that IKKβ binds to this macromolecular complex, undergoes phosphorylation and induces subsequent IκB phosphorylation in TCR signaling context (26, 27), these data suggest that MEKK3 might participate in phosphorylating IKKβ after its recruitment to the macromolecular complex. In support of this possibility, the immunoprecipitated MEKK3 after TCR stimulation was able to phosphorylate IKKβ *in vitro* (P-IKKβ; Fig. 5C). Since Jurkat T cells also demonstrated requirement for MEKK3 in TCR-mediated NF-κB activation (Supplementary Figure S4A and B, available at *International Immunology* Online), we tested necessity of its kinase activity for IKKβ phosphorylation by using this system. For this purpose, we obtained Jurkat

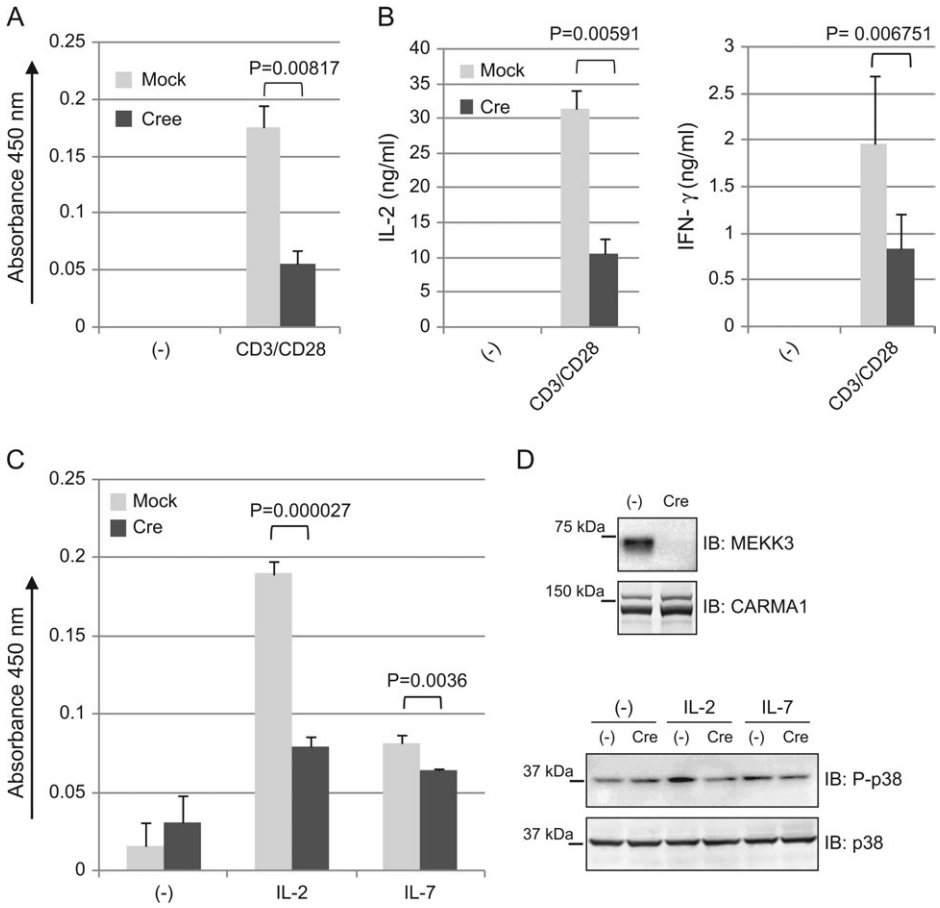

Fig. 4. MEKK3 regulates cellular responses induced by TCR or cytokine stimulation in the effector T cells. (A and B) Analyses of the effector T cells. The splenocytes from *MEKK3*$^{flox/flox}$ mice were transinfected with retrovirus encoding Cre with IRES-EGFP (Cre) or solely IRES-EGFP (mock) as described in Materials and methods. Cells were cultured in 10 μg ml^{-1} of anti-CD3-coated plates plus 2 μg ml^{-1} of anti-CD28 antibody or 100 ng ml^{-1} of PMA with 2 μg ml^{-1} of anti-CD28 antibody for 48 h. Level of IL-2 or IFN-γ was determined by ELISA using 24-h culture supernatants. Data are the results from triplicate culture from one representative of two experiments. (C) Cytokine response of effector T cells. Cells were prepared as in (A and B). All cytokines as a stimulant were used at concentration of 20 ng ml^{-1}. Data are shown as the results from triplicate culture from one representative of two experiments. (D) Activation of p38. Activation of p38 in response to cytokines at concentration of 20 ng ml^{-1} for 30 min. Cell lysates from the effector cells as in (A, B and C) were analyzed by western blotting.

T cells expressing WT T7-tagged MEKK3 or its kinase mutant (Supplementary Figure S5A, available at *International Immunology* Online). As shown in Fig. 5(E), WT MEKK3, but not its kinase mutant, was able to P-IKKβ. Together, these data suggest that MEKK3 or a kinase that may be associated with the MEKK3 complex is responsible for phosphorylating IKKβ.

Discussion

Previous studies have shown that MEKK3 is required for NF-κB activation in innate immune responses. Here, we have shown that MEKK3 is needed for optimal T cell development and TCR-mediated cellular responses. Our data also suggest that following TCR stimulation, MEKK3 interacts with the macromolecular complex that includes CARMA1 and P-IKKβ directly or indirectly, thereby participating in NF-κB activation.

The defective thymocyte development observed in T-KO mice is similar to that seen in mice lacking NEMO (alternatively named IKKγ) or expressing a kinase-dead mutant of IKKβ in T cells (28), suggesting the link between MEKK3 and IKK activation. However, the deletion of TCR signal components such as CARMA1, Bcl10 and MALT1, which are involved in IKK activation in mature T cells, do not severely affect T cell development in the thymus (26). Two alternative, which are not necessarily mutually exclusive, possibilities could be envisaged to explain the relatively mild phenotype of CARMA1-, Bcl10- and MALT1-deficient mice. First, the NF-κB signal needed for T cell development can be provided by other NF-κB-activating receptors when TCR-mediated NF-κB activation is absent (in the case of CARMA1, Bcl10 or MALT1 knockout mice). According to this scenario, MEKK3 in thymocytes might integrate signaling from the two NF-κB-activating receptors, thereby participating in thymocyte development. Second, rather than upstream receptors, multiple downstream targets of MEKK3 might explain why the thymocyte development is more affected in T-KO than in CARMA1, Bcl10 or MALT1 knockout mice. For instance, MEKK3 participates in not only IKK but

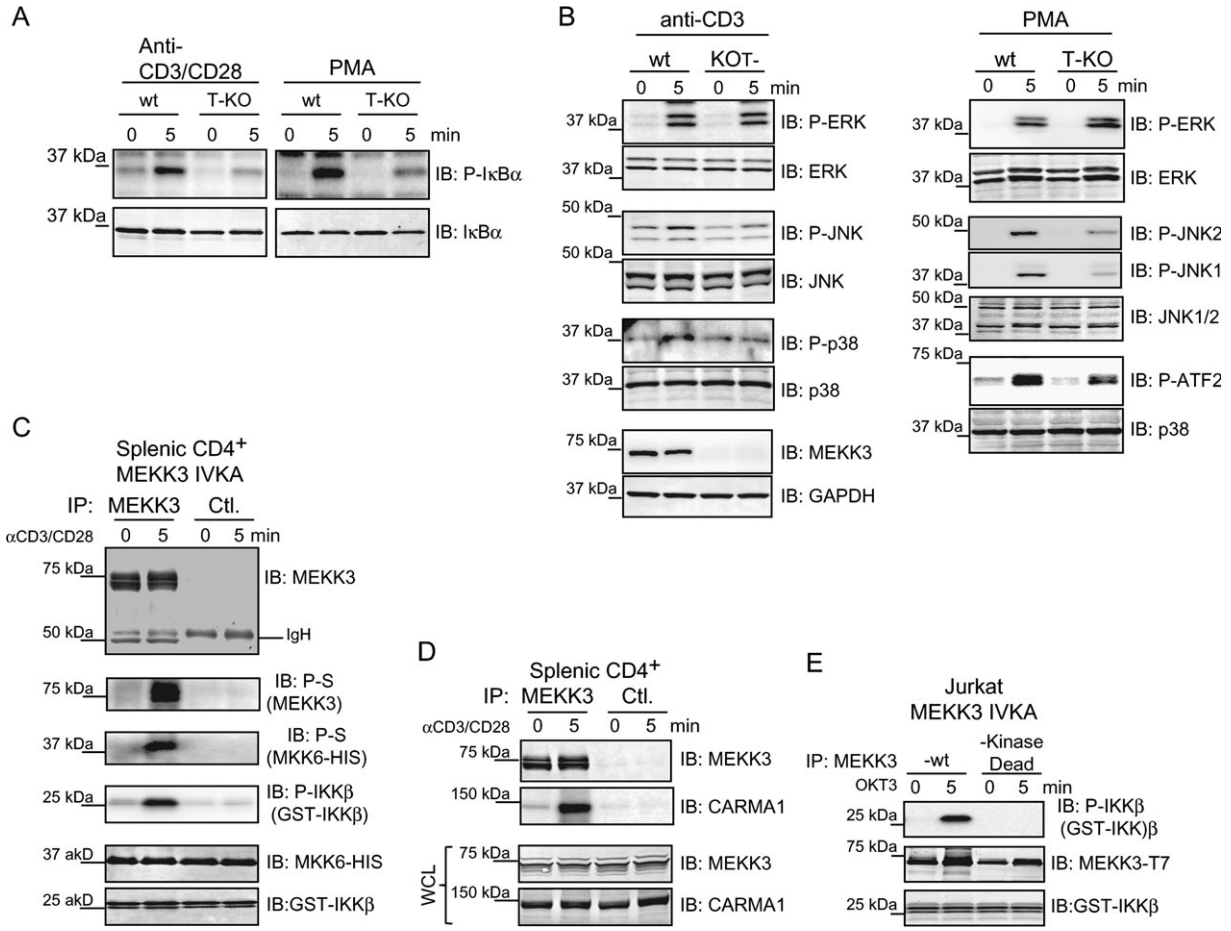

Fig. 5. MEKK3 positively regulates NF-κB activation in TCR signaling. (A) The IκBα phosphorylation of CD4 SP thymocyte in response to anti-CD3/CD28 (10 μg ml^{-1}) or PMA (100 ng ml^{-1}) stimulation for 5 min. (B) Immunoblot analysis of MAPKs. The purified CD4 SP thymocytes from *Lck-Cre/WT* (WT) and *Lck-Cre/MEKK3*$^{flox/flox}$ (T-KO) mice were stimulated with anti-CD3 (10 μg ml^{-1}) or PMA (100 ng ml^{-1}) for 5 min. (C) *In vitro* MEKK3 kinase assay (IVKA). Cell lysates (4×10^7) were immunoprecipitated by anti-MEKK3 mAb (MEKK3) or non-immune mouse IgG (Ctl.) prepared from WT $CD4^+$ spleen T cells stimulated by anti-CD3 with anti-CD28 (10 μg ml^{-1}). Phosphorylation status was detected by anti-phospho-serine (P-S) or anti-phospho-IKKβ antibody (P-IKKβ). As a substrate, MKK6, IKKβ-glutathione-*S*-transferase or MEKK3 by itself was used. (D) Inducible interaction of MEKK3 with CARMA1. Cell lysates as same in (C) were immunoprecipitated by anti-MEKK3 mAb non-immune mouse IgG (Ctl.) and detected by anti-CARMA1 antibody. (E) *In vitro* kinase activity of MEKK3 in Jurkat cells. MEKK3 kinase activity was determined by its *in vitro* phosphorylation activity toward IKKβ-glutathione-*S*-transferase and detected by anti-phospho-IKKβ antibody. Lysates from Jurkat cells expressing MEKK3-WT-T7 (WT) or its kinase-dead-T7 (Kinase Dead) were immunoprecipitated by anti-T7 mAb.

also p38 activation, even partial, in the TCR signaling context, while CARMA1/Bcl10/MALT1 molecules appear to participate in IKK but not p38 activation (29). However, the role of p38 MAPK pathway, by itself, in thymocyte development has been controversial (30–32). Thus, it is also possible that an as yet unidentified target might exist downstream of MEKK3, contributing to thymocyte development together with the NF-κB.

The decrease in numbers of T-KO thymocytes, relative to WT mice (Fig. 2), suggests a problem with thymocyte survival, differentiation and/or proliferation (33). We examined these possibilities. First, the survival capacity of thymocytes in culture was not significantly different between WT and MEKK3-deficient backgrounds in our experimental conditions (Supplementary Figure S1, available at *International Immunology* Online). Second, the development of DN thymocytes in T-KO mice, assessed by expression of CD25 and CD44, was also not significantly changed (data not shown), and a massive repertoire change appears not to occur in T-KO mice because CD4 SP thymocytes from the mutant mice had no significant differences in Vβ usage (determined by anti-TCR Vβ 2-, 3-, 4-, 5-, 6-, 7-, 8-, 9-, 10-, 11-, 12-, 13-, 14- and 17-specific mAb) (data not shown). Finally, the proliferation of T-KO thymocytes in response to TCR stimulation was defective as shown in Fig. 3. Together, our data suggest that decreased proliferation capability of MEKK3-deficient thymocytes could contribute to the reduced numbers of T-KO thymocytes, at least partly. However, survival and differentiation problems cannot be completely excluded by our data, simply because our assay systems might not have sufficed enough to detect small changes in these aspects.

MEKK3 was initially isolated as an activation molecule to the ERK pathway (34). However, recent genetic analysis has revealed that MEKK3 contributes to NF-κB activation but not to ERK in TNF receptor (TNFR) and Toll/IL-1 receptor

signaling (9, 10). As shown in our data, MEKK3 is also not involved in TCR-mediated ERK activation. Our results shed new light on the mechanism of TCR-mediated NF-κB activation. In the Jurkat T cell line, the signaling complex including CARMA1, Bcl10, MALT1 and TNFR-associated factor (TRAF)6 has been shown to be essential for TCR-mediated NF-κB activation (5). MEKK3 is recruited to this macromolecular complex; the inducible interaction between MEKK3 and CARMA1 was observed in primary T cells. This interaction appears to be kinase dependent, since the TCR-mediated interaction was significantly reduced in Jurkat cells expressing the kinase-dead mutant of MEKK3 (Supplementary Figure S5B, available at *International Immunology* Online). Although this study has not addressed the mechanism by which MEKK3 interacts with the macromolecular complex including CARMA1, it is possible that MEKK3 interacts directly with CARMA1 or with TRAF2 via receptor-interacting protein 1, as has been observed in the case of TNFR signaling (3). Importance of phosphorylation of the activation loop serine residues of IKKβ has been demonstrated by *in vivo* as well as *in vitro* experiments (3). Our data highly suggest that MEKK3 participates in linking the signaling complex including CARMA1 to IKKβ phosphorylation. First, MEKK3 was activated upon TCR stimulation. Second, MEKK3 was demonstrated to play a critical role in TCR-mediated NF-κB activation in primary thymocytes, the effector T cells and Jurkat T cells (Fig. 5A, Supplementary Figure S2B, available at *International Immunology* Online). Third, immunoprecipitated MEKK3 was able to P-IKKβ in *in vitro* conditions. Fourth, the inducible association of Bcl10 with CARMA1 was substantially decreased in MEKK3 knock-down cells, suggesting that MEKK3 participates in the formation of the macromolecular complex, directly or indirectly (Supplementary Figure S4C, available at *International Immunology* Online). Given that the IKK is also recruited to the signaling complex that includes CARMA1 (26, 27), recruitment of MEKK3 to this complex also allows the activated MEKK3 to gain access to its substrate IKKβ in *in vivo* contexts. As discussed above, direct phosphorylation of IKKβ by MEKK3 is a potential mechanism. But, considering that TAK1 is associated with MEKK3 in some conditions (35) and is also capable of phosphorylating IKKβ and recruited to TRAF6 in TCR signaling context (5), another explanation for our data is that MEKK3 might regulate such other IKK kinases, thereby promoting phosphorylation of IKKβ.

Our results demonstrate that not only TCR but also cytokines such as IL-2 utilize MEKK3 for exerting their cellular responses. In fact, MEKK3-deficient effector T cells showed the significantly reduced activation of p38 in response to IL-2. The activity of p38 is shown to be essential for cytokine-driven T cell proliferation (36, 37). Thus, it is reasonable to anticipate that MEKK3 is involved in IL-2-mediated p38 activation, which in turn contributes to proliferation of effector T cells. Further work is needed to dissect the role of MEKK3 in TCR versus cytokine signaling in the development and activation of T cells.

Supplementary data

Supplementary figures are available at *International Immunology* Online.

Funding

KAKENHI (20590496); Grant-in-Aid for Scientific Research (C) to H.S.; Ministry of Education, Science, Sports and Culture in Japan to T.K.

Acknowledgements

We would like to thank our colleague. H.S. designed and performed research and analyzed data; S.Y. and S.M. performed research; T.S. contributed analytic tools; T.K. wrote the paper. Conflict-of-interest disclosure: The authors have no conflicting financial interests.

Abbreviations

DN	double-negative
DP	double-positive
IKK	IκB kinase
MAPK	mitogen-activated protein kinase
MAPKK	MAPK kinase
MAP3K	MAPKK kinase
PKC	protein kinase C
PMA	phorbol myristate acetate
rm	recombinant mouse
SP	single-positive
T-KO	*Lck-Cre-MEKK3*$^{flox/flox}$
TLR	Toll-like receptor
TNF	tumor necrosis factor
TNFR	TNF receptor
TRAF	TNFR-associated factor
WT	wild type

References

1 Dong, C., Davis, R. J. and Flavell, R. A. 2002. MAP kinases in the immune response. *Annu. Rev. Immunol.* 20:55.
2 Cuevas, B. D., Abell, A. N. and Johnson, G. L. 2007. Role of mitogen-activated protein kinase kinase kinases in signal integration. *Oncogene* 26:3159.
3 Hacker, H. and Karin, M. 2006. Regulation and function of IKK and IKK-related kinases. *Sci. STKE:* re13.
4 Schulze-Luehrmann, J. and Ghosh, S. 2006. Antigen-receptor signaling to nuclear factor kappa B. *Immunity* 25:701.
5 Adhikari, A., Xu, M. and Chen, Z. J. 2007. Ubiquitin-mediated activation of TAK1 and IKK. *Oncogene* 26:3214.
6 Shim, J. H., Xiao, C., Paschal, A. E. *et al.* 2005. TAK1, but not TAB1 or TAB2, plays an essential role in multiple signaling pathways *in vivo*. *Genes. Dev.* 19:2668.
7 Sato, S., Sanjo, H., Takeda, K. *et al.* 2005. Essential function for the kinase TAK1 in innate and adaptive immune responses. *Nat. Immunol.* 6:1087.
8 Qin, J., Yao, J., Cui, G. *et al.* 2006. TLR8-mediated NF-kappaB and JNK activation are TAK1-independent and MEKK3-dependent. *J. Biol. Chem.* 281:21013.
9 Huang, Q., Yang, J., Lin, Y. *et al.* 2004. Differential regulation of interleukin 1 receptor and Toll-like receptor signaling by MEKK3. *Nat. Immunol.* 5:98.
10 Yang, J., Lin, Y., Guo, Z. *et al.* 2001. The essential role of MEKK3 in TNF-induced NF-kappaB activation. *Nat. Immunol.* 2:620.
11 Schmidt, C., Peng, B., Li, Z. *et al.* 2003. Mechanisms of proinflammatory cytokine-induced biphasic NF-kappaB activation. *Mol. Cell.* 12:1287.
12 Su, B., Cheng, J., Yang, J. and Guo, Z. 2001. MEKK2 is required for T-cell receptor signals in JNK activation and interleukin-2 gene expression. *J. Biol. Chem.* 276:14784.
13 Guo, Z., Clydesdale, G., Cheng, J. *et al.* 2002. Disruption of Mekk2 in mice reveals an unexpected role for MEKK2 in modulating T-cell receptor signal transduction. *Mol. Cell. Biol.* 22:5761.
14 Takahama, Y., Ohishi, K., Tokoro, Y. *et al.* 1998. Functional competence of T cells in the absence of glycosylphosphatidylinositol-anchored proteins caused by T cell-specific disruption of the Pig-a gene. *Eur. J. Immunol.* 28:2159.

15 Shinohara, H., Yasuda, T., Aiba, Y. *et al.* 2005. PKC beta regulates BCR-mediated IKK activation by facilitating the interaction between TAK1 and CARMA1. *J. Exp. Med.* 202:1423.
16 Yamasaki, S., Nishida, K., Hibi, M. *et al.* 2001. Docking protein Gab2 is phosphorylated by ZAP-70 and negatively regulates T cell receptor signaling by recruitment of inhibitory molecules. *J. Biol. Chem.* 276:45175.
17 Wan, Y. Y., Chi, H., Xie, M., Schneider, M. D. and Flavell, R. A. 2006. The kinase TAK1 integrates antigen and cytokine receptor signaling for T cell development, survival and function. *Nat. Immunol.* 7:851.
18 Shinohara, H., Maeda, S., Watarai, H. and Kurosaki, T. 2007. IkappaB kinase beta-induced phosphorylation of CARMA1 contributes to CARMA1 Bcl10 MALT1 complex formation in B cells. *J. Exp. Med.* 204:3285.
19 Yang, J., Boerm, M., McCarty, M. *et al.* 2000. Mekk3 is essential for early embryonic cardiovascular development. *Nat. Genet.* 24:309.
20 Vella, A. T., Dow, S., Potter, T. A., Kappler, J. and Marrack, P. 1998. Cytokine-induced survival of activated T cells *in vitro* and *in vivo*. *Proc. Natl Acad. Sci. USA.* 95:3810.
21 Liu, H. H., Xie, M., Schneider, M. D. and Chen, Z. J. 2006. Essential role of TAK1 in thymocyte development and activation. *Proc. Natl Acad. Sci. USA.* 103:11677.
22 Sun, Z., Arendt, C. W., Ellmeier, W. *et al.* 2000. PKC-theta is required for TCR-induced NF-kappaB activation in mature but not immature T lymphocytes. *Nature* 404:402.
23 Zhang, J., Salojin, K. V., Gao, J. X., Cameron, M. J., Bergerot, I. and Delovitch, T. L. 1999. p38 mitogen-activated protein kinase mediates signal integration of TCR/CD28 costimulation in primary murine T cells. *J. Immunol.* 162:3819.
24 Chi, H., Lu, B., Takekawa, M., Davis, R. J. and Flavell, R. A. 2004. GADD45beta/GADD45gamma and MEKK4 comprise a genetic pathway mediating STAT4-independent IFNgamma production in T cells. *Embo. J.* 23:1576.
25 Rueda, D. and Thome, M. 2005. Phosphorylation of CARMA1: the link(er) to NF-kappaB activation. *Immunity* 23:551.
26 Thome, M. 2004. CARMA1, BCL-10 and MALT1 in lymphocyte development and activation. *Nat. Rev. Immunol.* 4:348.
27 Hara, H., Bakal, C., Wada, T. *et al.* 2004. The molecular adapter Carma1 controls entry of IkappaB kinase into the central immune synapse. *J. Exp. Med.* 200:1167.
28 Schmidt-Supprian, M., Courtois, G., Tian, J. *et al.* 2003. Mature T cells depend on signaling through the IKK complex. *Immunity* 19:377.
29 Hara, H., Wada, T., Bakal, C. *et al.* 2003. The MAGUK family protein CARD11 is essential for lymphocyte activation. *Immunity* 18:763.
30 Tamura, K., Sudo, T., Senftleben, U., Dadak, A. M., Johnson, R. and Karin, M. 2000. Requirement for p38alpha in erythropoietin expression: a role for stress kinases in erythropoiesis. *Cell* 102:221.
31 Diehl, N. L., Enslen, H., Fortner, K. A. *et al.* 2000. Activation of the p38 mitogen-activated protein kinase pathway arrests cell cycle progression and differentiation of immature thymocytes *in vivo*. *J. Exp. Med.* 191:321.
32 Mulroy, T. and Sen, J. 2001. p38 MAP kinase activity modulates alpha beta T cell development. *Eur. J. Immunol.* 31:3056.
33 Werlen, G., Hausmann, B., Naeher, D. and Palmer, E. 2003. Signaling life and death in the thymus: timing is everything. *Science* 299:1859.
34 Ellinger-Ziegelbauer, H., Brown, K., Kelly, K. and Siebenlist, U. 1997. Direct activation of the stress-activated protein kinase (SAPK) and extracellular signal-regulated protein kinase (ERK) pathways by an inducible mitogen-activated protein Kinase/ERK kinase kinase 3 (MEKK) derivative. *J. Biol. Chem.* 272:2668.
35 Di, Y., Li, S., Wang, L., Zhang, Y. and Dorf, M. E. 2008. Homeostatic interactions between MEKK3 and TAK1 involved in NF-kappaB signaling. *Cell. Signal.* 20:705.
36 Crawley, J. B., Rawlinson, L., Lali, F. V., Page, T. H., Saklatvala, J. and Foxwell, B. M. 1997. T cell proliferation in response to interleukins 2 and 7 requires p38MAP kinase activation. *J. Biol. Chem.* 272:15023.
37 Geginat, J., Sallusto, F. and Lanzavecchia, A. 2001. Cytokine-driven proliferation and differentiation of human naive, central memory, and effector memory CD4(+) T cells. *J. Exp. Med.* 194:1711.