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Journal of Cellular Biochemistry 116:149–157 (2015)

Journal of Cellular Biochemistry

Zfat-Deficient CD4$^+$CD8$^+$ Double-Positive Thymocytes Are Susceptible to Apoptosis With Deregulated Activation of p38 and JNK

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ABSTRACT

Zfat, which is a nuclear protein harboring an AT-hook domain and 18-repeats of C2H2 zinc-finger motif, is highly expressed in immune-related tissues, including the thymus and spleen. T cell specific deletion of the *Zfat* gene by crossing *Zfat*$^{f/f}$ mice with *LckCre* mice yields a significant reduction in the number of CD4$^+$CD8$^+$ double-positive (DP) thymocytes. However, physiological role for Zfat in T cell development in the thymus remains unknown. Here, we found that *Zfat*-deficient DP thymocytes in *Zfat*$^{f/f}$-*LckCre* mice were susceptible to apoptosis both at an unstimulated state and in response to T cell receptor (TCR)-stimulation. The phosphorylation levels of p38 and JNK were elevated in *Zfat*-deficient thymocytes at an unstimulated state with an enhanced phosphorylation of ATF2 and with an over-expression of Gadd45α. On the other hand, the activation of JNK in the *Zfat*-deficient thymocytes, but not p38, was strengthened and prolonged in response to TCR-stimulation. All these results demonstrate that Zfat critically participates in the development of DP thymocytes through regulating the activities of p38 and JNK. J. Cell. Biochem. 116: 149–157, 2015.

KEY WORDS: ZFAT; THYMOCYTE; APOPTOSIS; p38; JNK

Zfat (zinc-finger protein in autoimmune thyroid disease susceptibility region/zinc-finger protein with AT-hook) is a nuclear protein harboring an AT-hook domain and 18-repeats of C2H2 zinc-finger motif [Shirasawa et al., 2004]. Zfat is highly conserved from fish through higher primates [Koyanagi et al., 2008] and thus is considered to be an essential molecule in development and cellular differentiation. Indeed, *Zfat* gene ablation in mice yields an embryonic lethality by embryonic day 8.5 and an impaired primitive hematopoiesis in yolk sac blood islands [Tsunoda et al., 2010; Tsunoda and Shirasawa, 2013].

Zfat was originally identified as a candidate susceptibility gene for autoimmune thyroid disease [Shirasawa et al., 2004]. In fact, Zfat is highly expressed in immune-related tissues in adult mice, including the thymus and spleen [Koyanagi et al., 2008]. T cell specific deletion of *Zfat* in mice by crossing *Zfat*$^{f/f}$ mice with *CD4Cre* mice yields a drastic reduction in the number of peripheral T cells [Doi et al., 2012]. *Zfat*-deficient peripheral T cells exhibit a decreased expression of interleukin-7 receptor α and an impaired induction of interleukin-2 receptor α in response to T cell receptor (TCR)-stimulation [Doi et al., 2012]. Furthermore, we generated *Zfat*$^{f/f}$-*LckCre* mice to elucidate a role for Zfat in T cell development in the thymus and reported a significant reduction in the number of CD4$^+$CD8$^+$double-positive (DP) thymocytes with an impaired positive selection in *Zfat*$^{f/f}$-*LckCre* mice [Ogawa et al., 2013]. *Zfat*-deficient DP thymocytes

Conflict of interest: The authors have declared that no competing interests exist.
Shuhei Ishikura and Masahiro Ogawa contributed equally to this work.
Grant sponsor: Japanese Society for the Promotion of Science (JSPS) ; Grant sponsor: Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan; Grant sponsor: Clinical Research Promotion Foundation.
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Manuscript Received: 28 April 2014; Manuscript Accepted: 22 August 2014
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 28 August 2014
DOI 10.1002/jcb.24954 • © 2014 Wiley Periodicals, Inc.

show a decreased CD3ζ phosphorylation followed by an attenuation of ERK activation in response to TCR-stimulation [Ogawa et al., 2013]. Taken together, Zfat is an essential molecule in the homeostasis at periphery and development in the thymus of T cells.

We previously found that siRNA-mediated Zfat knockdown resulted in apoptosis in human acute T lymphoblastic leukemia cells and mouse embryonic fibroblasts, indicating that Zfat is a critical molecule for cell survival [Fujimoto et al., 2009; Doi et al., 2011]. The survival and death of thymocytes in the thymus are individually regulated by different mitogen-activated protein kinases (MAPK), including extracellular signal-related kinases (ERK), c-Jun N-terminal kinases (JNK), and p38-MAPK (p38). ERK1/2-deficient mice had defects in the positive selection, indicating that ERK is required for the thymocyte survival [Fischer et al., 2005]. By contrast, p38 and JNK are known to be involved in the thymocyte death. Thymocytes from JNK2 deficient mice were resistant to anti-CD3 antibody-mediated apoptosis and thymocytes expressing dominant negative JNK1 transgene showed a decreased level of apoptosis after treatment with anti-CD3 antibody [Rincon et al., 1998; Sabapathy et al., 1999]. In addition, inhibition of p38 in fetal thymic organ cultures resulted in the impaired apoptosis induced by anti-CD3 antibody [Sugawara et al., 1998]. Thus, it is established that p38 and JNK are critical in apoptotic cell death of thymocytes. However, a physiological role for Zfat in thymocyte death remains unknown.

In this study, we found that *Zfat*-deficient thymocytes were susceptible to apoptosis both at an unstimulated state and in response to TCR-stimulation. Furthermore, we demonstrated that the phosphorylation levels of both p38 and JNK at an unstimulated state were elevated in *Zfat*-deficient thymocytes with an enhanced phosphorylation of ATF2 and with an increased expression of growth arrest and DNA damage-inducible gene 45α (Gadd45α). By contrast, JNK in the *Zfat*-deficient thymocytes, but not p38, showed a strengthened and prolonged activation upon TCR-stimulation in vitro, suggesting that the deregulated activation of JNK is involved in a facilitation of apoptosis in response to TCR-stimulation in vitro. These data imply that Zfat is required for the proper activation of particular MAPK signaling cascades, which distinctively regulate survival and death of thymocytes.

MATERIALS AND METHODS

MICE

Zfat$^{f/f}$*-LckCre* mice were originally generated as described previously [Ogawa et al., 2013]. All animal experiments were approved by the Animal Care and Use Committee of the NCGM Research Institute, and the experiments on mice were carried out under the guidelines of the Institutional Animal Care and Use Committee of Fukuoka University.

FLOW CYTOMETRY

Cell sorting and flow cytometry analysis were performed using FACSAria II (BD Biosciences) as described previously [Ogawa et al., 2013]. Annexin V Apoptosis Detection Kit (BD Pharmingen) was used for the detection of apoptotic cells. The fluorophore-conjugated antibodies were as follows: CD4 (RM4–5, Biolegend), CD8 (53–6.7, BioLegend), Vα2 TCR (B20.1; BD Biosciences), Fas (Jo2, BD Bioscience), and FasL (MFL3, BioLegend).

IMMUNOBLOTTING

Cells were lysed in the RIPA buffer and subjected to immunoblotting as described previously [Ogawa et al., 2013]. The antibodies used for immunoblotting analysis were as follows: Cleaved Caspase-3, Phospho-p38 (Thr180/Tyr182), p38, Phospho-MKK3/6 (Ser189/207), MKK3, Phospho-ATF2 (Thr71), Phospho-JNK (Thr183/Tyr185), JNK, Phospho-MKK4 (Ser257), MKK4, Gadd45α (all from Cell Signaling Technology), Gadd45β (Abcam), ATF2 (New England Biolabs), and Actin (Sigma). Anti-Zfat antibody was prepared as described previously [Koyanagi et al., 2008]. The quantitative analysis of the immunoblotting was performed using the ImageJ software (National Institute of Health).

QUANTITATIVE RT-PCR

cDNA was synthesized from total RNA extracted by TRIZol reagent (Life Technologies) using superscript VILO cDNA synthesis kit (Life Technologies). Quantitative-PCR was performed by using Thunderbird SYBR qPCR Mix (Toyobo) with ABI PRISM 7900HT (Applied Biosystems). The primer sequences used for mRNA quantification were; *Gadd45α,* 5′-TGAGCTGCTGCTACTGGAGA-3′ and 5′-TCCC GGCAAAAACAAATAAG-3′; *Actin,* 5′-CATCCGTAAAGACCTCTAT GCCAAC-3′ and 5′-ATGGAGCCACCGATCCACA.

TCR-STIMULATION IN VIVO AND IN VITRO

For in vivo stimulation, *Zfat*$^{f/f}$ and *Zfat*$^{f/f}$*-LckCre* mice were intraperitoneally administered 200 μl of PBS with or without 30 μg of anti-CD3ε antibody (145–2C11; BioLegend). OT-II *Zfat*$^{f/f}$ and OT-II *Zfat*$^{f/f}$*-LckCre* mice [Barnden et al., 1998] were intraperitoneally administered 200 μl of PBS with or without 1 mg of OVA peptide (Bex). For in vitro experiments, isolated thymocytes were stimulated with cross-linked antibody of 10 μg/ml anti-CD3ε antibody with 80 μg/ml goat anti-hamster IgG antibody (Southern Biothech) at 37°C. Tissue culture plates were incubated with PBS containing anti-CD3ε and anti-CD28 (37.51; BioLegend) antibodies at 4°C overnight for the stimulation with plate-bound antibodies.

STATISTICAL ANALYSIS

Data are presented as the mean ± s.d. and statistical analyses were performed using an unpaired Student's *t*-test when comparing the means of two groups. Differences of $P < 0.05$ were considered to be statistically significant ($^{*}P < 0.05$; $^{**}P < 0.01$).

RESULTS

ZFAT-DEFICIENCY IN DP THYMOCYTES INCREASED A SUSCEPTIBILITY TO APOPTOSIS

As the number of thymocytes was significantly decreased in the *Zfat*$^{f/f}$*-LckCre* thymus [Ogawa et al., 2013], we first addressed proportions of apoptotic cells in the CD4^{-}CD8^{-}double-negative (DN) and DP thymocytes by detecting Annexin V-positive cells. Flow cytometry analysis showed very few Annexin V-positive

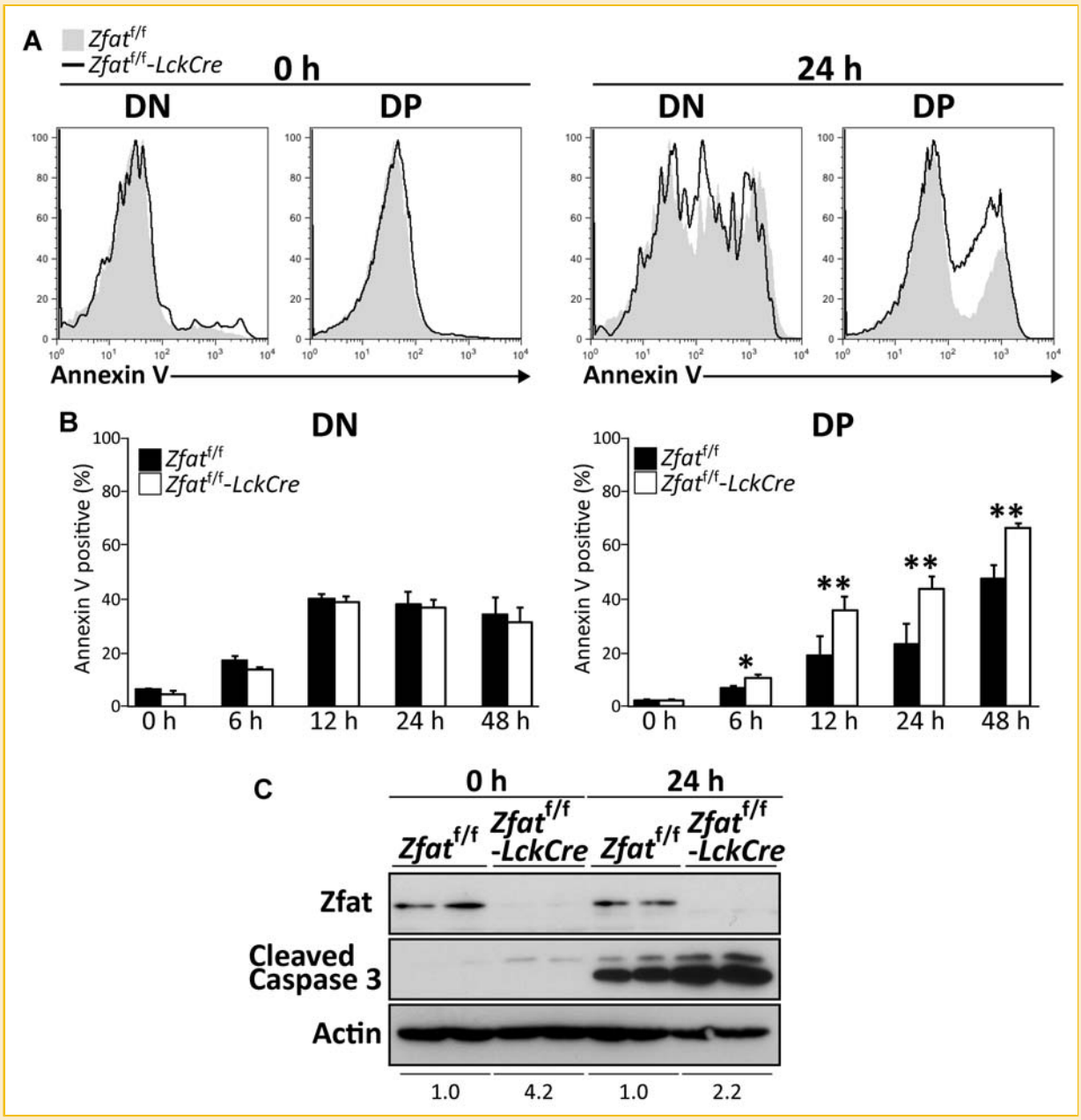

Fig. 1. *Zfat*-deficiency increased apoptosis of DP thymocytes. A, B: Percentages of Annexin V-positive cells at the indicated time points in DN and DP subpopulations. Total thymocytes isolated from *Zfat*$^{f/f}$ and *Zfat*$^{f/f}$*-LckCre* mice were cultured for the indicated time points. Cells were stained with anti-CD4 and -CD8 antibodies, and Annexin V for flow cytometry. The data are the mean ± s.d.; n = 3; **P* < 0.05; ***P* < 0.01. C: Expressions of Zfat and cleaved caspase 3. Total thymocytes isolated from the indicated genotype mice were cultured for 24 h and then lysed. Expression of the indicated proteins in the lysates were examined by immunoblotting. Actin was used as a loading control. Levels of cleaved caspase 3 were quantified by densitometry and normalized to actin levels. The values represent the relative ratio to levels of *Zfat*$^{f/f}$ thymocytes at each condition. (A–C) Data are representative of three independent experiments.

cells in freshly isolated thymocytes (0 h) from both *Zfat*$^{f/f}$ and *Zfat*$^{f/f}$*-LckCre* mice and that there were no differences in a proportion of Annexin-V positive cells between genotypes, irrespective of DN and DP subpopulations (Figs. 1A and B). This result will be explained by a report showing that the majority of apoptotic thymocytes in mice are rapidly deleted by the thymic macrophages [Surh and Sprent, 1994].

To examine whether *Zfat*$^{f/f}$*-LckCre* thymocytes are more sensitive to apoptosis, thymocytes were cultured in a medium without particular cytokines, mitogens, or antibodies used for stimulation. There were no differences in a proportion of Annexin V-positive cells in DN subpopulation until 48 h, between *Zfat*$^{f/f}$ and *Zfat*$^{f/f}$*-LckCre* mice (Figs. 1A and B). By contrast, Annexin V-positive cells in the DP subpopulation in *Zfat*$^{f/f}$*-LckCre* mice were increased

compared to those in *Zfat*$^{f/f}$ mice (Figs. 1A and B). Significant differences in a proportion of Annexin V-positive cells between *Zfat*$^{f/f}$ and *Zfat*$^{f/f}$-*LckCre* mice were observed during the time course from 6 to 48 h (Fig. 1B). Considering that Zfat expression in *Zfat*$^{f/f}$-*LckCre* thymocytes was apparently decreased in the DP subpopulation [Ogawa et al., 2013], an increase in a proportion of apoptotic cells in *Zfat*$^{f/f}$-*LckCre* DP cells will result from a loss of Zfat expression in the DP subpopulation. Furthermore, amounts of cleaved caspase 3 were dramatically augmented by *Zfat*-deficiency in the thymocytes cultured for 24 h in vitro (Fig. 1C), indicating that proapoptotic phenotype caused by *Zfat*-deficiency in the DP thymocytes occurs, in part, through a caspase-dependent pathway.

DEREGULATED CONSTITUTIVE ACTIVATION OF P38 AND JNK IN THE *ZFAT*-DEFICIENT THYMOCYTES

As we previously reported that ERK activation in response to TCR-stimulation was attenuated in the *Zfat*-deficient thymocytes [Ogawa et al., 2013], phosphorylation status of other MAPK, including p38 and JNK were examined by immunoblotting. Surprisingly, *Zfat*$^{f/f}$-*LckCre* thymocytes showed increased phosphorylation levels of both p38 and JNK compared to those of *Zfat*$^{f/f}$ thymocytes (Fig. 2A), suggesting that aberrant activation of p38 and JNK will be involved in the facilitated apoptosis of *Zfat*-deficient thymocytes.

JNK-mediated apoptosis induces the expression of FasL in T cells [Faris et al., 1998; Kasibhatla et al., 1998] and high expression of FasL in DP thymocytes causes massive cell death in *Fasl* transgenic mice [Cheng et al., 1997]. Therefore, we investigated surface expression of FasL and Fas on the DP thymocytes by flow cytometry analysis. However, there were no differences in the surface expression of FasL or Fas between *Zfat*$^{f/f}$ and *Zfat*$^{f/f}$-*LckCre* mice (Fig. 2B).

Activation of p38 and JNK promote phosphorylation of numerous apoptosis-related molecules, including ATF2 [Dodeller and Schulze-Koops, 2006; Dhanasekaran and Reddy, 2008]. The phosphorylation level of ATF2 was drastically elevated in the *Zfat*$^{f/f}$-*LckCre* thymocytes in comparison with that of *Zfat*$^{f/f}$ thymocytes (Fig. 2A). These results, together, suggested that the loss of Zfat augments phosphorylation of ATF2 through the constitutive activation of p38 and JNK, leading to the facilitated apoptosis in the *Zfat*-deficient DP thymocytes.

MAPK signal transduction is typically executed through cascades of three kinases, namely, MAPK kinase kinase (MKKK), MAPK kinase (MKK), and MAPK. We, then, investigated phosphorylation status of MKK3/6 and MKK4, which are upstream MKKs for p38 and JNK, respectively [Rincon and Davis, 2009]. Consistent with the increased phosphorylation levels of p38 and JNK, phosphorylation levels of both MKK3/6 and MKK4 were elevated in the *Zfat*-deficient thymocytes (Fig. 2C). The Gadd45 is known to be a direct upstream activator of MEKK4, which is an upstream MKKK for MKK3/6 and MKK4 [Takekawa and Saito, 1998]. Interestingly, the protein level of Gadd45α, but not Gadd45β, was increased in the *Zfat*$^{f/f}$-*LckCre* thymocytes in comparison with those of the *Zfat*$^{f/f}$ thymocytes (Fig. 2C). However, expression level of mRNA for *Gadd45*α was unaffected by *Zfat*-deficiency (Fig. 2D), indicating that Zfat was not implicated in the transcriptional regulation of *Gadd45*α mRNA. Taken together, these results indicate that a loss of Zfat in the DP thymocytes results in the deregulated constitutive activation of p38 and JNK, which is accompanied by the hyper-activation of MKK3/6 and MKK4, and by the increased expression of Gadd45α.

ZFAT-DEFICIENCY INCREASED A SUSCEPTIBILITY TO TCR-STIMULATION-INDUCED APOPTOSIS OF THYMOCYTES IN VIVO

Next, we addressed whether *Zfat*-deficiency in the thymocytes affects a susceptibility to cell death induced by TCR-stimulation in vivo. Injection of anti-CD3 antibody into mice results in the apoptotic death of DP cells through TCR-stimulation within 24 to 48 h [Rincon et al., 1998]. We injected anti-CD3 antibody intraperitoneally into *Zfat*$^{f/f}$ and *Zfat*$^{f/f}$-*LckCre* mice and analyzed the thymus for apoptosis of DN and DP cells at 24 h after injection. *Zfat*$^{f/f}$ control mice injected with anti-CD3 antibody exhibited a reduction in the number of DP cells, but not DN cells, compared to the mice treated with vehicle alone (Fig. 3A). On the other hand, DP cells in *Zfat*$^{f/f}$-*LckCre* mice were more markedly decreased by injection of anti-CD3 antibody compared to those in *Zfat*$^{f/f}$ mice, namely 30% of *Zfat*$^{f/f}$ DP cells survived after anti-CD3 antibody injection, whereas only 10% of *Zfat*$^{f/f}$-*LckCre* DP cells survived after the treatment (Figs. 3A and B). Furthermore, a proportion of apoptotic cells in DP subpopulation treated with anti-CD3 antibody was higher in *Zfat*$^{f/f}$-*LckCre* mice than in *Zfat*$^{f/f}$ mice (Fig. 3C). All these results suggested that *Zfat*-deficient DP cells are susceptible to apoptosis induced by TCR-stimulation in vivo.

To confirm the relevance of *Zfat*-deficiency to increased apoptosis induced by TCR-stimulation, we used the OT-II TCR transgenic mice having transgenic Vα2Vβ5 TCR specific for OVA peptide [Barnden et al., 1998]. The OT-II mice are known to show massive cell death of DP thymocytes through the TCR-stimulation by administration of the OVA peptide. OT-II *Zfat*$^{f/f}$ and OT-II *Zfat*$^{f/f}$-*LckCre* mice were intraperitoneally administered the OVA peptide and a proportion of apoptotic cells in the thymus was examined. In the Vα2- negative population, which does not recognize the OVA peptide, apoptotic cell death was not induced by the OVA peptide injection, despite the presence or absence of Zfat (Fig. 3D). By contrast, injection of the OVA peptide increased a proportion of apoptotic cells in the Vα2-positive population, which recognizes the OVA peptide, in both OT-II *Zfat*$^{f/f}$ and OT-II *Zfat*$^{f/f}$-*LckCre* mice (Fig. 3D). Furthermore, a considerable increase in a proportion of apoptotic cells was observed by the OVA peptide injection in Vα2-positive OT-II *Zfat*$^{f/f}$-*LckCre* thymocytes in comparison with Vα2-positive OT-II *Zfat*$^{f/f}$ thymocytes (30.6% vs. 12.7%, respectively) (Fig. 3D). All these results, together, indicate that *Zfat*-deficiency in the thymocytes leads to the increased susceptibility to apoptosis induced by peptide-MHC-stimulation in vivo.

As JNK activation precedes the depletion of DP cells induced by anti-CD3 antibody treatment [Rincon et al., 1998], the phosphorylation status of p38 and JNK were examined in the thymocytes from *Zfat*$^{f/f}$ and *Zfat*$^{f/f}$-*LckCre* mice treated with anti-CD3 antibody for 6 h. In *Zfat*$^{f/f}$ control mice, the phosphorylation levels of both p38 and JNK were increased by anti-CD3 antibody treatment (Fig. 3E). By contrast, the phosphorylation levels of p38 and JNK in the *Zfat*$^{f/f}$-*LckCre* mice were not augmented in response to anti-CD3 antibody (Fig. 3E), indicating that anti-CD3 antibody treatment could not further activate the hyper-phosphorylated p38 and JNK in the *Zfat*-deficient thymocytes in vivo. However, the phosphorylation levels of

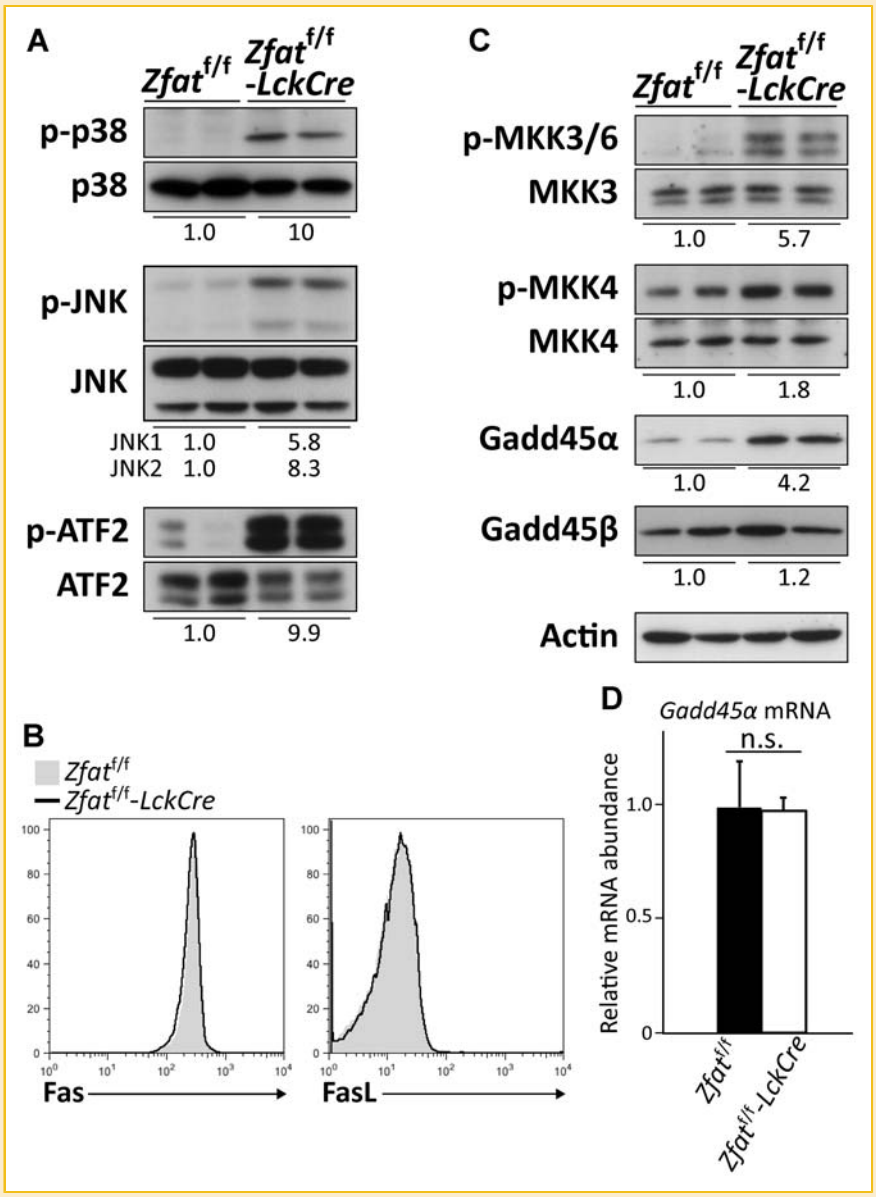

Fig. 2. Deregulated constitutive activation of p38 and JNK in *Zfat*-deficient thymocytes. A: Expression and phosphorylation of p38, JNK, and ATF2. Total thymocytes were isolated from *Zfat*$^{f/f}$ and *Zfat*$^{f/f}$*-LckCre* mice and lysed. Expression and phosphorylation of the indicated proteins in the lysates were examined by immunoblotting using specific antibodies. B: Flow cytometry analysis of surface expression of FasL and Fas on DP thymocytes. Total thymocytes isolated from *Zfat*$^{f/f}$ and *Zfat*$^{f/f}$*-LckCre* mice were stained with anti-CD4, -CD8, -FasL, -Fas antibodies for flow cytometry. C: Expression and phosphorylation of MKK3/6, MKK4, Gadd45α, and Gadd45β. Expression and phosphorylation of the indicated proteins in total thymocyte lysates were examined by immunoblotting. Actin was used as a loading control. (A, C) Levels of phosphorylated proteins were quantified by densitometry and normalized to total protein levels. Levels of Gadd45α and Gadd45β were normalized to actin levels. The values represent the relative ratio to levels of *Zfat*$^{f/f}$ thymocytes. D: Quantitative RT-PCR analysis of *Gadd45α* mRNA expression in DP thymocytes; n.s., not significant. (A–D) Data are representative of three independent experiments.

both p38 and JNK in the *Zfat*$^{f/f}$*-LckCre* thymocytes in response to anti-CD3 antibody were still higher than those in *Zfat*$^{f/f}$ thymocytes (Fig. 3E).

HIGH SUSCEPTIBILITY TO APOPTOSIS AND PROLONGED JNK ACTIVATION UPON TCR-STIMULATION IN *ZFAT*-DEFICIENT THYMOCYTES IN VITRO

To determine whether the elevated phosphorylation of p38 and JNK is involved in the increased susceptibility to apoptosis induced by TCR-stimulation in *Zfat*-deficient DP cells, we examined the effect of *Zfat*-deficiency on the apoptosis of thymocytes induced by TCR-stimulation in vitro. As TCR-stimulation with a combination of anti-CD3 and -CD28 antibodies in vitro induces the cell death of thymocytes [Punt et al., 1997], we analyzed DP cells cultured for 24 h with or without TCR-stimulation. Proportions of apoptotic *Zfat*$^{f/f}$*-LckCre* DP cells were higher compared to those of apoptotic *Zfat*$^{f/f}$ DP cells, irrespective of antibody concentration (Fig. 4A). Notably, the differences in a proportion of apoptotic cells between *Zfat*$^{f/f}$*-LckCre*

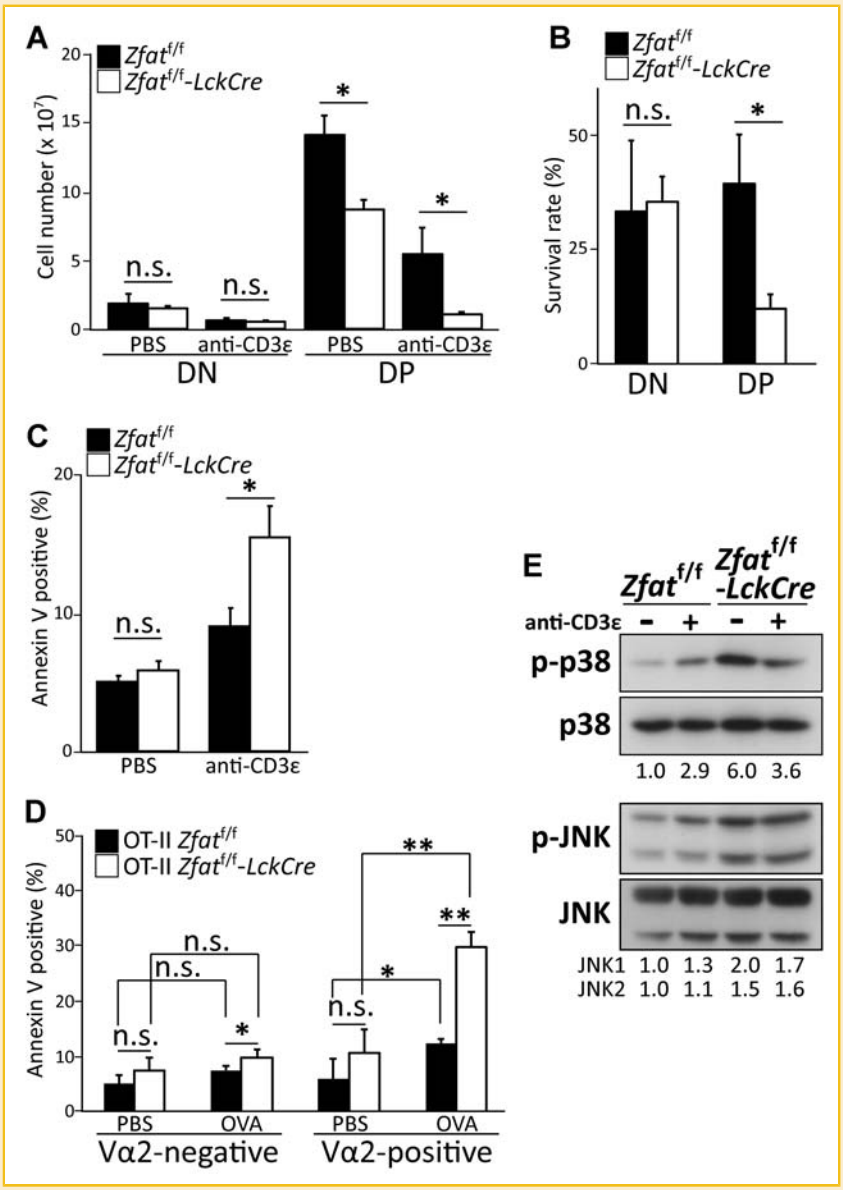

Fig. 3. *Zfat*-deficiency increased susceptibility to TCR-stimulation-induced apoptosis of DP thymocytes in vivo. A: Number of DN and DP cells in the thymus. B: The proportion of surviving cells adjusted by the numbers of TCR-stimulated cells with PBS-treated cells. C: Percentages of Annexin V positive cells in DP cells. (A–C) Total thymocytes were isolated from *Zfat*$^{f/f}$ and *Zfat*$^{f/f}$*-LckCre* mice administered with 30 μg of anti-CD3ε antibody or vehicle alone at 24 h after the administration and stained with anti-CD4 and -CD8 antibodies, and Annexin V for flow cytometry. The data are the mean ± s.d., $*P < 0.05$; n.s., not significant. D: Percentages of Annexin V positive cells in Vα2 TCR-negative and -positive populations in OT-II *Zfat*$^{f/f}$ and OT-II *Zfat*$^{f/f}$*-LckCre* mice. Total thymocytes were isolated from mice administered with 1 mg of the OVA peptide or vehicle at 48 h after the administration and stained with Annexin V and anti-Vα2 TCR antibody for flow cytometry. The data are the mean ± s.d.; $*P < 0.05$; $**P < 0.01$; n.s., not significant. E: Expression and phosphorylation of p38 and JNK. Total thymocytes were isolated from the indicated genotypes mice administered with 30 μg of anti-CD3ε antibody or vehicle alone at 6 h after the administration and lysed. Expression and phosphorylation of the indicated proteins were detected by immunoblotting. Levels of phosphorylated proteins were quantified by densitometry and normalized to total protein levels. The values represent the relative ratio to levels of *Zfat*$^{f/f}$ thymocytes administered with vehicle alone. (A–E) Data are representative of two or three independent experiments.

DP cells and *Zfat*$^{f/f}$ DP cells were more prominent at higher concentrations of 1 and 3 μg/ml of anti-CD3 antibody, indicating that *Zfat*$^{f/f}$*-LckCre* DP cells were more susceptible to apoptosis at a stronger TCR-stimulation compared to *Zfat*$^{f/f}$ DP cells in vitro.

We, next, examined phosphorylation status of p38 and JNK in the thymocytes upon TCR-stimulation in vitro. Activation of p38 was not observed in response to TCR-stimulation in vitro in both *Zfat*$^{f/f}$ and *Zfat*$^{f/f}$*-LckCre* thymocytes, indicating that p38 is not involved in the apoptosis induced by TCR-stimulation in vitro (Fig. 4B). By contrast, the phosphorylation of JNK was increased upon TCR-stimulation in vitro in both *Zfat*$^{f/f}$ and *Zfat*$^{f/f}$*-LckCre* thymocytes (Fig. 4B). Furthermore, *Zfat*$^{f/f}$*-LckCre* thymocytes exhibited a strengthened and prolonged activation of JNK at least until 60 min after TCR-stimulation in comparison with *Zfat*$^{f/f}$ thymocytes

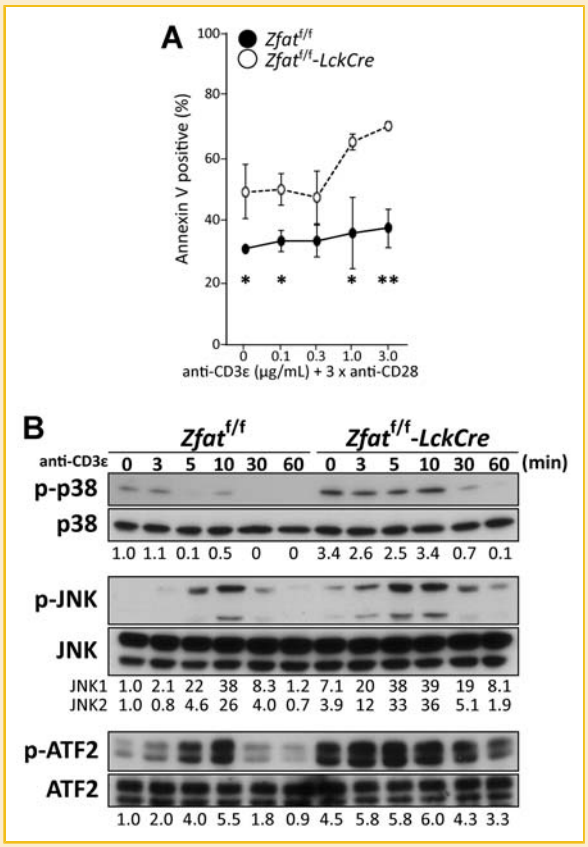

Fig. 4. Increased susceptibility to apoptosis and prolonged JNK activation induced by TCR stimulation in *Zfat*-deficient thymocytes in vitro. A: Percentages of Annexin V-positive cells in the DP cells cultured for 24 h in vitro. DP cells were cultured for 24 h in the plates coated with anti-CD3 and anti-CD28 antibodies at the indicated concentrations and stained with Annexin V for flow cytometry. Data are representative of three independent experiments. The data are the mean ± s.d.; n = 3; $^{*}P < 0.05$; $^{**}P < 0.01$. B: Expression and phosphorylation of p38, JNK, and ATF2. Total thymocytes were stimulated with 10 μg/ml of anti-CD3ε antibody cross-linked with goat anti-hamster IgG for the indicated time points in vitro and then lysed. Expression and phosphorylation of the indicated proteins in the lysates were detected by immunoblotting. Levels of phosphorylated proteins were quantified by densitometry and normalized to total protein levels. The values represent the relative ratio to levels of *Zfat*$^{f/f}$ thymocytes at time 0.

(Fig. 4B). These results suggested that the strengthened and prolonged activation of JNK results in the increased susceptibility to apoptosis induced by TCR-stimulation in the *Zfat*-deficient DP thymocytes in vitro.

DISCUSSION

In this study, we demonstrated that *Zfat*-deficient DP thymocytes showed increased susceptibility to apoptosis with deregulated activation of p38 and JNK. Considering that the positive selection was impaired in *Zfat*-deficient thymocytes [Ogawa et al., 2013], the significant reduction in the number of DP thymocytes in the *Zfat*$^{f/f}$-*LckCre* thymus would be partly attributed to the acceleration of apoptotic cell death. Furthermore, *Zfat*-deficiency enhanced the phosphorylation of p38 and JNK in thymocytes, suggesting that *Zfat*- deficiency in the DP thymocytes culminates in the deregulated constitutive activation of p38 and JNK, thus leading to the increased susceptibility to apoptosis. Taken together, all these results suggest that Zfat is a critical regulator for apoptotic cell death and is required for the proper activation of p38 and JNK in the DP thymocytes.

The facilitation of apoptotic cell death was evidently observed in DP cells, but not in DN cells of the *Zfat*$^{f/f}$-*LckCre* thymocytes. This result is consistent with the fact that Zfat expression in DP cells is higher than that of DN cells and that Zfat is barely expressed in the *Zfat*$^{f/f}$-*LckCre* DP cells, but Zfat expression is not diminished in the *Zfat*$^{f/f}$-*LckCre* DN cells [Ogawa et al., 2013]. Thus, the possibility that Zfat plays critical roles in cell survival and/or differentiation in DN thymocytes still does exist.

p38 and JNK are activated through phosphorylation by MKK3/6 and MKK4, respectively, which are activated by MEKK4-mediated phosphorylation [Rincon and Davis, 2009]. Gadd45 protein can bind the autoinhibitory domain of MEKK4 and relieve the autoinhibition of MEKK4 [Mita et al., 2002], leading to the activation of the p38/JNK MAPK cascades. The results presented here suggest that the increase in Gadd45α expression caused by *Zfat*- deficiency in DP thymocytes results in the deregulated activation of p38 and JNK through hyper-activation of MKK3/6 and MKK4, indicating that Zfat is involved in the proper activation of Gadd45α-MEKK4-MKK3/4/6-p38/JNK signaling cascades (Fig. 5).

We previously showed that phosphorylations of CD3ζ and Zap70, which are required for the activation of p38 and JNK upon TCR-stimulation, were decreased in *Zfat*-deficient DP thymocytes [Ogawa et al., 2013]. Therefore, deregulated activation of p38 and JNK via Gadd45α in the *Zfat*-deficient DP thymocytes will be unrelated to Zap70-PLC-γ-Raf-MEK-ERK pathway (Fig. 5). Considering that ERK activation upon TCR-stimulation was attenuated in the *Zfat*-deficient thymocytes [Ogawa et al., 2013], Zfat will be required for a proper regulation of particular MAPK signaling cascade distinctively, which regulates survival and death of the thymocytes (Fig. 5).

In this study, we have not elucidated how exactly Zfat regulates the expression of Gadd45α protein. Zfat is expected to be a transcriptional regulator in the nucleus. However, the mRNA level of *Gadd45*α was not affected by *Zfat*-deficiency, indicating that Zfat is not involved in the transcriptional regulation of Gadd45α. Loss of Zfat may affect stability and/or degradation of Gadd45α protein in the DP thymocytes. Explicit molecular mechanisms of Zfat function in the Gadd45α expression should await further studies.

The *Zfat*$^{f/f}$-*LckCre* DP thymocytes were susceptible to anti-CD3 antibody-mediated apoptotic cell death in vivo. Although DP thymocyte death induced by TCR-stimulation in vivo has been used as a model for studying negative selection, Brewer et al. [2002] reported that the DP cells depletion induced by anti-CD3 antibody injection results from glucocorticoids secretion stimulated by polyclonal T cell activation rather than the negative selection. Therefore, injection of anti-CD3 antibody into mice might affect their physical constitution. Furthermore, H-Y *Zfat*$^{f/f}$-*LckCre* male mice did not exhibit obvious defects in the negative selection [Ogawa et al., 2013]. Thus, the facilitation of apoptotic cell death upon TCR-

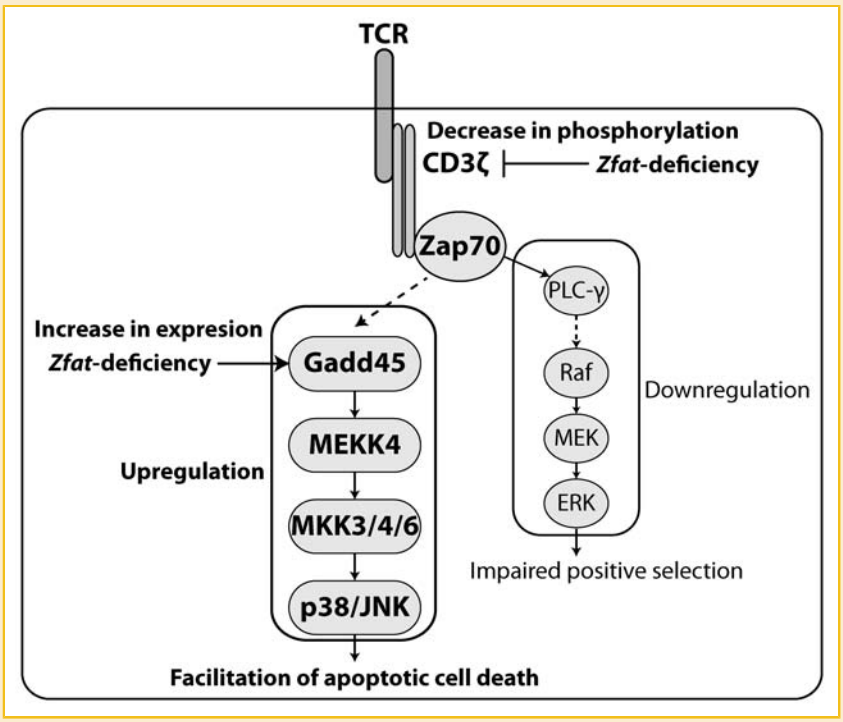

Fig. 5. Deregulated MAPK activation in *Zfat*-deficient DP thymocytes. Decreased phosphorylation of CD3ζ in *Zfat*-deficient DP thymocytes results in the downregulation of Raf-MEK-ERK pathway, leading to the impaired positive selection [Ogawa et al., 2013]. By contrast, *Zfat*-deficiency increases Gadd45α expression in DP thymocytes, which in turn upregulates MEKK4-MKK3/4/6-p38/JNK pathway, leading to facilitation of apoptotic cell death.

stimulation in *Zfat*-deficient DP thymocytes may result from the deregulated activation of p38 and JNK, independent of the negative selection.

In conclusion, we demonstrated that *Zfat*-deficient DP thymocytes are susceptible to apoptosis with deregulated activation of p38 and JNK. As Zfat is required for a proper regulation of particular MAPK signaling cascade, elucidation of the molecular mechanisms of Zfat function will provide deeper insights into the thymocyte differentiation.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid for Scientific Research (B to S.S. and C to K.D.) and for Young Scientists (B to S.I.) from the JSPS, a Grant-in-Aid for the FCAM from MEXT-Supported Program for the Strategic Research Foundation at Private Universities, and a Grant from The Clinical Research Promotion Foundation (to S.I.).

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