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# ZFAT plays critical roles in peripheral T cell homeostasis and its T cell receptor-mediated response

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ABSTRACT

ZFAT, originally identified as a candidate susceptibility gene for autoimmune thyroid disease, has been reported to be involved in apoptosis, development and primitive hematopoiesis. Zfat is highly expressed in T- and B-cells in the lymphoid tissues, however, its physiological function in the immune system remains totally unknown. Here, we generated the T cell-specific *Zfat*-deficient mice and demonstrated that *Zfat*-deficiency leads to a remarkable reduction in the number of the peripheral T cells. Intriguingly, a reduced expression of IL-7Rα and the impaired responsiveness to IL-7 for the survival were observed in the *Zfat*-deficient T cells. Furthermore, a severe defect in proliferation and increased apoptosis in the *Zfat*-deficient T cells following T cell receptor (TCR) stimulation was observed with a reduced IL-2Rα expression as well as a reduced IL-2 production. Thus, our findings reveal that Zfat is a critical regulator in peripheral T cell homeostasis and its TCR-mediated response.



## 1. Introduction

We previously identified *ZFAT* (zinc-finger gene with AT-hook/zinc-finger gene in the autoimmune thyroid disease susceptibility region) as a candidate susceptibility gene for autoimmune thyroid disease [1]. *ZFAT* encodes an evolutionally conserved protein with 18 zinc-finger domains and one AT-hook domain [2], and Zfat is strongly expressed in T- and B-cells in the mouse immune-related tissues [2]. We previously reported that the *Zfat*-deficient ($Zfat^{-/-}$) mouse is embryonic lethal, and that Zfat is a critical transcriptional regulator in primitive hematopoiesis [3]. ZFAT is also involved in the regulation of apoptosis in cells of the human leukemia cell line MOLT-4 cells [4] and mouse embryonic fibroblasts [5], and in the differentiation of human umbilical vein endothelial cells [6]. Furthermore, genetic variants of *ZFAT* have been reported to be associated with adult height [7,8], with common diseases including hypertension and cancer [9,10], and with interferon-β responsiveness in multiple sclerosis [11], in which *IL-7RA* and *IL-2RA* are susceptibility genes [12–15]. Despite these advances, the function of ZFAT in the immune system remains totally unknown.

Proper regulation of peripheral T cell homeostasis is highly controlled by both cell-extrinsic and cell-intrinsic factors [16–19]. Growing evidence demonstrates that peripheral T cell homeostasis is controlled by cytokine receptor-mediated signals, especially interleukin-7 receptor (IL-7R) and interleukin-2 receptor (IL-2R) signals, as well as the interaction between TCR and major histocompatibility complex [20,21].

IL-2/IL-2R exhibits the ability to drive T cell proliferation, mediate activation-induced cell death, promote the development of regulatory T cells and modulate the expression of cytokine receptors [22,23]. The IL-2R has three chains: α, β and the common cytokine receptor γ. Resting T cells express a receptor form composed of β and γ chains that bind IL-2 with moderate affinity, whereas activation of T cells induces the α chain (a high-affinity subunit) and the formation of the high-affinity heterotrimeric receptor, which plays a critical role in T cells in the physiological context [24]. On the other hand, the IL-7R complex is composed of IL-7Rα and the γ-chain, and IL-7 signaling is mainly regulated by IL-7Rα expression in T cells [25].

Several transcriptional factors are reported to play critical roles in peripheral T cell homeostasis as cell-intrinsic factors [26–28],

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however, further studies will be needed to achieve a full understanding of the cell-intrinsic factors responsible for the integration of extrinsic signals.

Here we established and analyzed *Zfat*$^{f/f}$*-Cd4Cre* mice, revealing that Zfat is an essential molecule for peripheral T cell homeostasis and TCR-mediated cellular response.

## 2. Materials and methods

### 2.1. Mice

Zfat genomic DNA from a 129S6/SvEv$^{Tac}$ mouse strain BAC DNA library was used to create a *Zfat*-targeting construct. A 5′ loxP site and a FRT-PGKneo-FRT-LoxP cassette were inserted into a 496-bp fragment upstream and a 376-bp fragment downstream of exon 8, respectively. A total 13-kb fragment containing 7-kb of the 5′ homology arm and 1.8-kb of the 3′ homology arm were retrieved into a plasmid vector with a diphtheria toxin cassette. The linearized targeting vector was transfected into the ES cells as described previously [3] and the G418-resistant colonies were screened by PCR. Southern blot analysis of *Eco*RI-digested genomic DNA from ES clones was performed using a 5′ external probe. The targeted ES cells were microinjected into C57BL6/NCr (Japan SLC Inc., Japan) blastocysts [3], and the resulting male chimeras were bred with C57BL/6NCr females to obtain germ-line transmission. Heterozygous offspring (*Zfat*$^{f\text{-}neo/w}$) were crossed with FLPe deleter mice from the RIKEN Bioresource centre [29] and the *Zfat*$^{f/w}$ mice were established. *Zfat*$^{f/f}$ mice were crossed with CD4-Cre mice from Taconic (Germantown, NY) to generate T-cell-specific *Zfat*- knockout, *Zfat*$^{f/f}$*-Cd4Cre* mice, in the C57BL/6 background. All the 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.

### 2.2. Antibodies

The following antibodies were purchased from BD Pharmingen (Franklin Lakes): CD4 (RM4-5), CD8 (53-6.7), B220 (RA3-6B2), CD62L (MEL-14), CD44 (IM7), TCRβ (H57-597), IL-2Rα (PC61), CD69 (H1.2F3), CCR7 (4B12), IL-7Rα (A7R34), Qa-2 (695H1-9-9), CD5 (53-7.3), HSA (LG.3A10) and CD122 (TM-β1). The antibodies used were: anti-bcl-2 (10C4) from Biolegend and anti-actin (A2066) from Sigma. Anti-ZFAT was prepared as described previously [2].

### 2.3. Flow cytometry

Cells from the mouse tissues were depleted of erythrocytes by hypotonic lysis. Cells were incubated with specific antibodies in the presence of 2.4G2 antibody (anti-Fcγ receptor). Data were collected with a cytometer (FACSAria II or FACSCalibur; BD Biosciences) and were analyzed with FlowJo software (Tomy Digital Biology). CFSE staining was performed by using a CellTrace CFSE Cell Proliferation Kit (Life Technologies) according to the manufacturer's instructions. Cell separation was performed with MACS Cell Separation Reagents, CD4 MicroBeads, CD8 MicroBeads and B220 (CD45R) MicroBeads (Miltenyi Biotec) or by using the cell sorting function on FACSAria II (Becton Dickinson).

### 2.4. Cell stimulation

To analyze cell apoptosis, naive T cells were cultured in the absence or presence of IL-7 and were stained with FITC- or APC-labeled annexin V (BD Biosciences) at the indicated time points according to the manufacturer's instructions. For plate-bound CD3/CD28 stimulation, plates were coated overnight with 1 μg ml$^{-1}$ of anti-CD3 (145-2C11; BD Pharmingen) and 5 μg ml$^{-1}$ of anti-CD28 (37.51; BD Pharmingen) antibodies. The amount of IL-2 was determined by using commercial ELISA kits (Invitrogen) according to the manufacturer's instructions.

### 2.5. Quantitative RT-PCR

Quantitative RT-PCR was performed as described previously [2,3]. The PCR primers used for each gene are described in Supplementary Table 1.

### 2.6. Immunoblotting

Immunoblotting was performed as described previously [2].

### 2.7. Statistical analysis

The data are presented as the means ± standard deviation. The statistical analyses were performed using an unpaired two-tailed Student's *t*-test. Differences at $P < 0.05$ were considered to be statistically significant.

## 3. Results and discussion

### 3.1. Reduction in the number of peripheral T cells in *Zfat*$^{f/f}$*-Cd4Cre* mice

To address the physiological role of Zfat in the immune system, we herein generated *Zfat*$^{f/f}$ mice, in which exon 8 of the *Zfat*

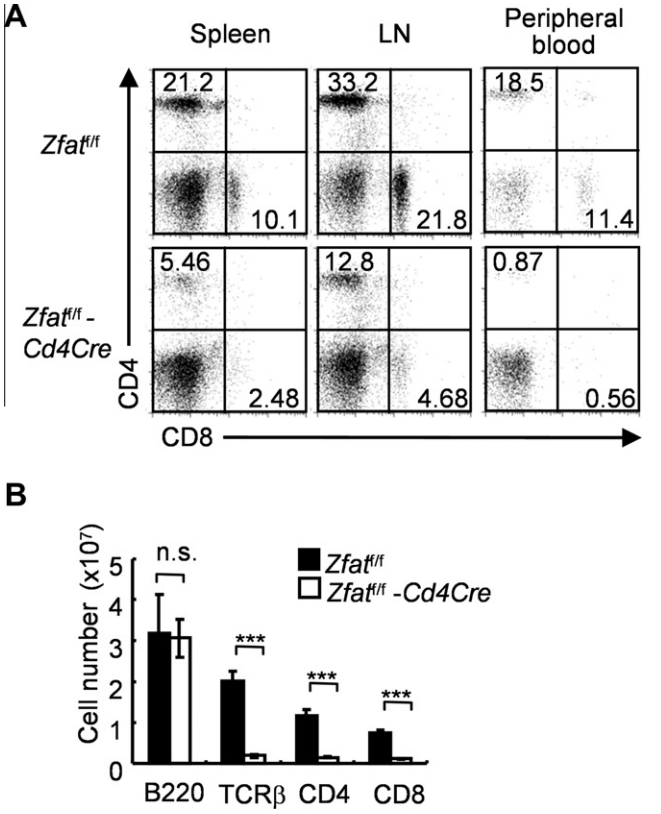


**Fig. 1.** Reduction in the number of peripheral T cells in the *Zfat*$^{f/f}$*-Cd4Cre* mice. (A) CD4$^+$ and CD8$^+$ cells in the spleen, lymph nodes (LN) and peripheral blood of the indicated genotypes at 8–12 weeks of age. Data are representative of three independent experiments. (B) Total number of B220$^+$, TCRβ$^+$, CD4$^+$ and CD8$^+$ subsets of the indicated genotypes at 6 weeks of age [mean ± standard deviation (s.d.), $n = 4–5$, $^*P < 0.0001$].

(GenBank accession No. EU221277; the same exon deleted in the *Zfat*$^{-/-}$ mice) was flanked by loxP sites (Supplementary Fig. 1A–C). We crossed *Zfat*$^{f/f}$ mice with *Cd4Cre* mice to induce a T cell-specific recombination. The efficient deletion of *Zfat* was confirmed by both PCR (Supplementary Fig. 1D) and immunoblotting of Zfat in the splenic CD4$^+$ and CD8$^+$ T cells (Supplementary Fig. 1E), thereby allowing us to study the effect of *Zfat*-deficiency in T cells *in vivo*.

We first evaluated peripheral T cells from the spleen, lymph nodes (LN) and peripheral blood. A considerable decrease in the proportions of CD4$^+$ and CD8$^+$ T cells in *Zfat*$^{f/f}$-*Cd4Cre* mice was observed in comparison to those in *Zfat*$^{f/f}$ mice (Fig. 1A). The total numbers of CD4$^+$ and CD8$^+$ T cells in the spleens of *Zfat*$^{f/f}$-*Cd4Cre* mice were significantly decreased by 7.4-fold and 6.6-fold, respectively, in comparison to those of *Zfat*$^{f/f}$ mice, whereas there were no significant differences in the number of B220$^+$ B cells (Fig. 1B), indicating that *Zfat*-deficiency results in disruption of the peripheral T cell homeostasis.

### 3.2. Impaired maturation of CD8SP thymocytes in *Zfat*$^{f/f}$-*Cd4Cre* mice

We examined whether the observed peripheral phenotype arises from a defect in T cell differentiation in the thymus. Analyses of the number and proportions of CD4$^-$CD8$^-$ double-negative (DN), CD4$^+$CD8$^+$ double-positive (DP), CD4$^+$CD8$^-$ single-positive (CD4SP) and CD4$^-$CD8$^+$ single-positive (CD8SP) thymic subsets and the phosphorylation of ERK induced by TCR-stimulation in thymocytes did not reveal any significant differences between the genotypes (Fig. 2A, Supplementary Fig. 2A), suggesting that obvious alterations in T cell development or TCR signaling do not occur in *Zfat*$^{f/f}$-*Cd4Cre* mice. Furthermore, an analysis of the expression of developmental stage-specific surface markers, including TCRβ, IL-7Rα, CD5, Qa-2 and heat-stable antigen (HSA), on DP and CD4SP cells did not reveal any differences between the genotypes (Fig. 2B and C). However, remarkable differences in the expression of CD5 and Qa-2 on CD8SP cells were observed (Fig. 2B), and the proportion of mature TCRβ$^{hi}$HSA$^{lo}$ CD8SP cells in the *Zfat*$^{f/f}$-*Cd4Cre* mice clearly decreased in comparison to that in *Zfat*$^{f/f}$ mice (Fig. 2C). These results collectively suggested that *Zfat*-deficiency affects the maturation of CD8SP cells in the thymus, whereas the CD4SP cells are normally developed in the thymus of *Zfat*$^{f/f}$-*Cd4Cre* mice, which led us to focus on analysis of the peripheral CD4$^+$ T cells of *Zfat*$^{f/f}$-*Cd4Cre* mice in this study. Precise evaluation of Zfat function in T cell development in the thymus should be examined in future studies by using more sophisticated mouse models such as Lck-Cre recombinase-induced T cell-specific deletion of Zfat.

### 3.3. Reduced expression of IL-7Rα and impaired responses to IL-7 in peripheral CD4$^+$ T cells in *Zfat*$^{f/f}$-*Cd4Cre* mice

We next characterized the Zfat functions in peripheral CD4$^+$ T cells. No difference in the proportions of the splenic and LN naive (TCRβ$^+$CD44$^{lo}$) or activated (TCRβ$^+$CD44$^{hi}$) CD4$^+$ T cells between the genotypes was observed (Fig. 3A). We then assessed the homing receptors, CCR7 and CD62L (L-selectin) [26], on naive CD4$^+$ T cells in LN. In LN naive CD4$^+$ T cells from *Zfat*$^{f/f}$-*Cd4Cre* mice, both

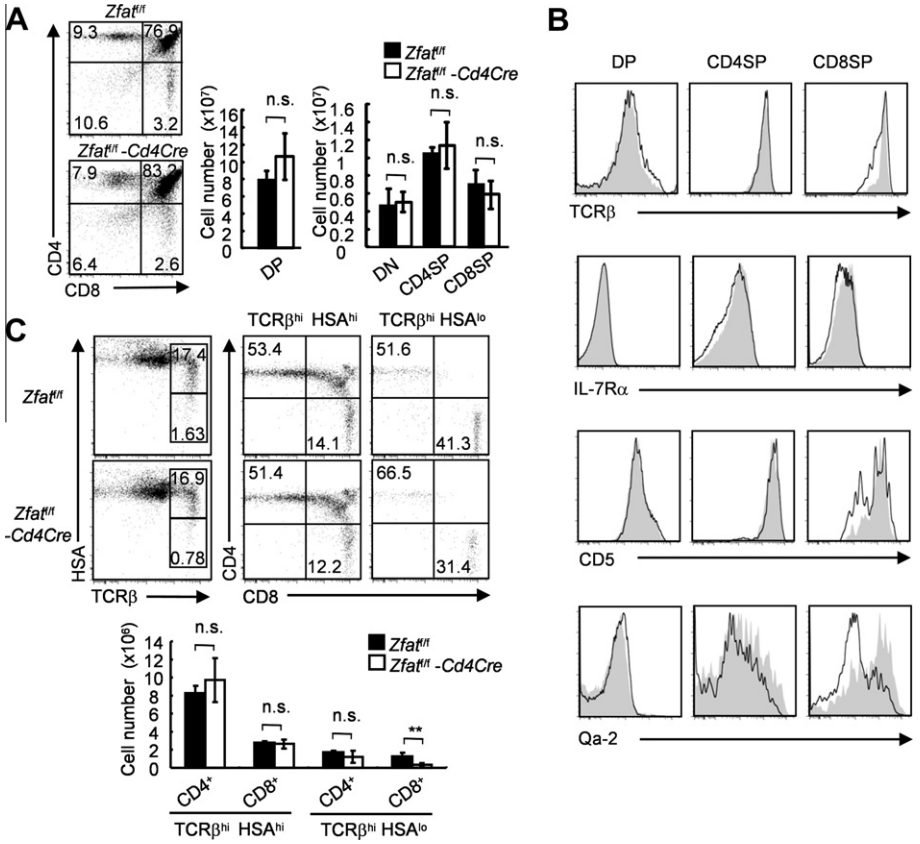


**Fig. 2.** Impaired maturation of CD8SP thymocytes in the *Zfat*$^{f/f}$-*Cd4Cre* mice. (A) Expression of CD4 and CD8 on thymocytes (left panel). Total number of thymocytes in double-positive (DP), double-negative (DN), single-positive CD4$^+$ (CD4SP) and single-positive CD8$^+$ (CD8SP) subsets at 8–12 weeks of age ($n$ = 4) (right panel). (B) Expressions of TCRβ, IL-7Rα, CD5 and Qa-2 on each thymic subset from the *Zfat*$^{f/f}$-*Cd4Cre* (black line) and *Zfat*$^{f/f}$ (gray-filled) mice at 8 weeks of age. (C) TCRβ and HSA expression by thymocytes (left panel). CD4 and CD8 expression on thymocytes gated for TCRβ$^{hi}$HSA$^{hi}$ and TCRβ$^{hi}$HSA$^{lo}$ (right panel) at 8–12 weeks of age. Data are representative of three independent experiments. Number, proportion of the cells within each gate. The absolute numbers of mature (TCRβ$^{hi}$HSA$^{lo}$) and immature (TCRβ$^{hi}$HSA$^{hi}$) thymocyte subpopulations are shown. The data are the mean ± s.d.. $^{**}P < 0.05$; n.s., not significant. ($n$ = 3).

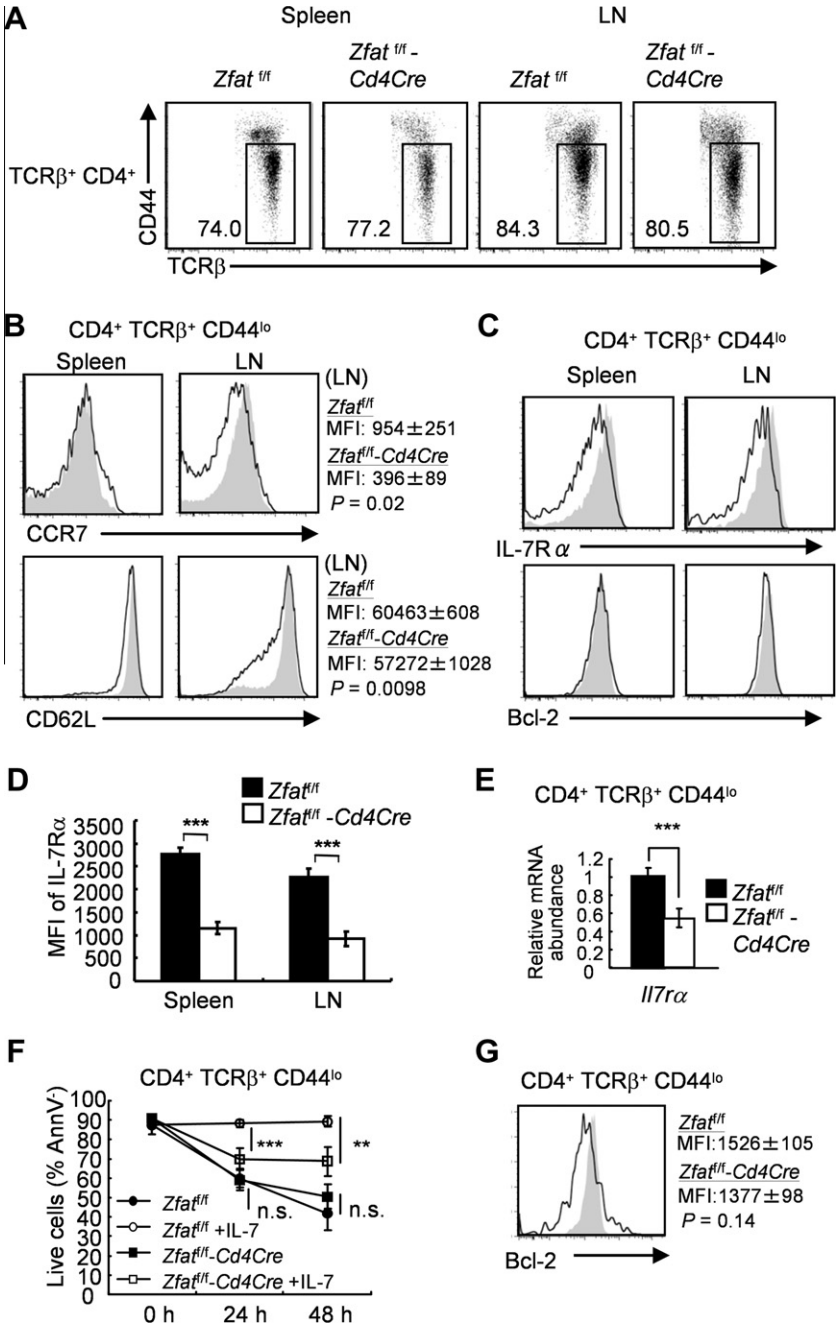


**Fig. 3.** Reduced expression of IL-7Rα and impaired responses to IL-7 in peripheral CD4$^+$ T cells from the *Zfat*$^{f/f}$*-Cd4Cre* mice. (A) Proportion of naive T cells defined as CD4$^+$TCRβ$^+$CD44$^{lo}$. Numbers indicate the proportion of the cells within each gate. (B) The expressions of surface CD62L and CCR7 on the splenic and LN CD4$^+$TCRβ$^+$CD44$^{lo}$ T cells from *Zfat*$^{f/f}$*-Cd4Cre* (black line) and *Zfat*$^{f/f}$ (gray-filled) mice. The expressions of surface CD62L and CCR7 on the LN CD4$^+$TCRβ$^+$CD44$^{lo}$ T cells were measured as the mean fluorescence intensity (MFI). The data are the mean ± s.d. of three independent experiments. (C) The expressions of surface IL-7Rα and intracellular Bcl-2 by CD4$^+$TCRβ$^+$CD44$^{lo}$ T cells in the spleen and LN. (D) The expressions of surface IL-7Rα by CD4$^+$TCRβ$^+$CD44$^{lo}$ T cells in the spleen and LN were measured as the mean fluorescence intensity (MFI). The data are the mean ± s.d. of three independent experiments. $^{***}P < 0.01$ (E) Quantitative RT-PCR analysis of IL-7Rα expression by the splenic CD4$^+$TCRβ$^+$CD44$^{lo}$ T cells from *Zfat*$^{f/f}$*-Cd4Cre* (white bars, $n = 9$) and *Zfat*$^{f/f}$ (black bars, $n = 6$) mice. The relative expression for each gene was normalized by expression of *Actb*. The data are the mean ± s.d. of three independent experiments. $^{***}P < 0.01$; n.s., not significant. (F) The proportions of live cells during *in vitro* culture with or without IL-7, and the proportion of live (annexin-V-negative, AnnV$^-$) CD4$^+$TCRβ$^+$CD44$^{lo}$ T cells measured by flow cytometry at the indicated time points. The data are mean ± s.d. of triplicate cultures. $^{**}P < 0.05$; $^{***}P < 0.01$; n.s., not significant ($n = 2$). (G) IL-7-induced Bcl-2 expression by the splenic CD4$^+$TCRβ$^+$CD44$^{lo}$ T cells from *Zfat*$^{f/f}$*-Cd4Cre* (black line) and *Zfat*$^{f/f}$ (gray-filled) mice at 24 h were measured as the mean fluorescence intensity (MFI). The data are the mean ± s.d. of three independent experiments.

CCR7 and CD62L expression were decreased in comparison to those in the *Zfat*$^{f/f}$ mice (Fig. 3B). CD62L expression in the splenic *Zfat*$^{f/f}$*-Cd4Cre* T cells was slightly decreased in comparison to that in the *Zfat*$^{f/f}$ mice (Fig. 3B). These results suggest that the reduced expression of homing receptors would partially account for the reduced peripheral T cell number in the *Zfat*$^{f/f}$ mice. However, considering the fact that reduced numbers of CD4$^+$ T cells were observed throughout the periphery, i.e., in the LN, spleen and peripheral blood (Fig. 1A), other factors rather than homing receptors seem to cause the altered T cell homeostasis in the *Zfat*$^{f/f}$ mice.

Because IL-7Rα- and TCR-mediated signals play a critical role in the survival of naive T cells [16,17], we next examined the IL-7Rα-related profiles in the peripheral CD4$^+$ T cells. IL-7Rα surface expression in the splenic and LN CD4$^+$ T cells, but not in the CD4SP

thymocytes, was significantly decreased in *Zfat*$^{f/f}$-*Cd4Cre* mice in comparison to that in *Zfat*$^{f/f}$ mice (Fig. 2B, Fig. 3C and D), but no difference in the expression of anti-apoptotic protein Bcl-2, a survival factor for peripheral T cells, was observed (Fig. 3C). Consistent with this result, the expression level of *Il7rα* mRNA in naive CD4$^+$ T cells from *Zfat*$^{f/f}$-*Cd4Cre* mouse spleen showed a significant decrease in comparison to that from *Zfat*$^{f/f}$ mouse spleen (Fig. 3E). The proportion of live cells among the CD44$^{lo}$CD4$^+$ T cells during *in vitro* culture without IL-7 was not significantly different between the genotypes (Fig. 3F), whereas the proportion of live *Zfat*$^{f/f}$-*Cd4Cre* CD44$^{lo}$CD4$^+$ T cells during the culture with IL-7 was significantly decreased in comparison with that from *Zfat*$^{f/f}$ mice (Fig. 3F). Bcl-2 expression is known to be induced by an IL-7R-mediated signal [30]. Interestingly, the IL-7-induced Bcl-2 expression in naive CD4$^+$ T cells from *Zfat*$^{f/f}$-*Cd4Cre* mice tended to be lower than that in *Zfat*$^{f/f}$ mice, although the difference was not statistically significant (Fig. 3G). These results collectively suggested that Zfat plays a critical role in the proper expression of IL-7Rα and also that the IL-7/IL-7R-mediated signal is impaired, at least in part of Bcl-2 induction, in the CD44$^{lo}$CD4$^+$ T cells from *Zfat*$^{f/f}$-*Cd4Cre* mice, resulting in a reduction in the number of CD4$^+$ T cells. As transcriptional factors including Foxo1 [26,30,31], Gabpα and Gfi-1 [32], are known to be regulators for the IL-7Rα expression in T cells, the relation between Zfat and these factors should be addressed in the future.

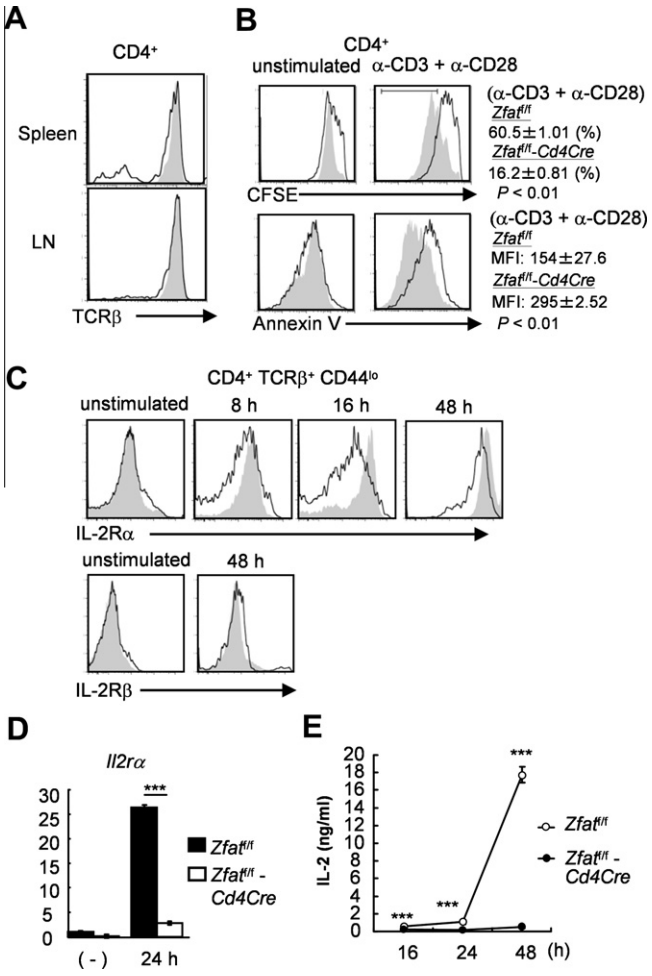


**Fig. 4.** Impaired proliferation and increased apoptosis with a reduced expression of IL-2Rα and IL-2 upon TCR-stimulation in CD4$^+$ T cells from *Zfat*$^{f/f}$-*Cd4Cre* mice. (A) Surface expression of TCRβ in the splenic and lymph node (LN) CD4$^+$ T cells from *Zfat*$^{f/f}$-*Cd4Cre* (black line) and *Zfat*$^{f/f}$ (gray-filled) mice at 8–12 weeks of age. (B) Cell division analysis using CFSE staining (upper) and apoptotic analysis using annexin-V staining (lower) in the splenic CD4$^+$ T cells from 8- to 12-week-old *Zfat*$^{f/f}$-*Cd4Cre* (black line) and *Zfat*$^{f/f}$ (gray-filled) at 48 h after stimulation with anti-CD3/CD28 antibodies. Data are representative of three independent experiments. Percentages within a panel indicate the fraction of cells that underwent proliferation. The expressions of annexin-V were measured as the mean fluorescence intensity (MFI). The data are the mean ± s.d. of three independent experiments. (C) The surface expression of IL-2Rα and IL-2Rβ on the splenic CD4$^+$TCRβ$^+$CD44$^{lo}$ T cells from *Zfat*$^{f/f}$-*Cd4Cre* (black line) and *Zfat*$^{f/f}$ (gray-filled) mice. The cells were cultured with or without anti-CD3/CD28 antibodies for 8, 16 and 48 h. The data are representative of three independent experiments. (D) Quantitative RT-PCR analysis of the *Il2rα* expression of CD4$^+$ T cells from the indicated mouse spleen before or 24 h after stimulation with anti-CD3/CD28 antibodies. The relative expression for each gene was normalized by expression of *Actb*. One representative data of three independent experiments performed in triplicate. The data are the mean ± s.d. $^{***}P < 0.01$. (E) IL-2 production by stimulation with anti-CD3/CD28 antibodies. The splenic CD4$^+$ T cells from the indicated mice were cultured with plate-coated anti-CD3 and anti-CD28 antibodies. At the indicated times, supernatants were harvested from the cultures and analyzed for IL-2 content using an ELISA. The data are the mean ± s.d. of three independent experiments. $^{***}P < 0.01$.

### *3.4. Impaired proliferation and increased apoptosis with the reduced expression of IL-2Rα and IL-2 in response to TCR-stimulation in Zfat*$^{f/f}$*-Cd4Cre T cells*

We next examined the TCR-mediated response in the peripheral CD4$^+$ T cells. The expression of TCRβ in the splenic and LN CD4$^+$ T cells, and the TCR-stimulation-induced ERK activation in the splenic CD4$^+$ T cells were comparable between the genotypes (Fig. 4A, Supplementary Fig. 2B), suggesting that the general TCR-mediated signals were not impaired in *Zfat*$^{f/f}$-*Cd4Cre* CD4$^+$ T cells. To address the functional role of Zfat in T cell response induced by TCR-stimulation, cell division and apoptosis assays were carried out, revealing a decrease in the cell division and an increase in the apoptosis in *Zfat*$^{f/f}$-*Cd4Cre* T cells in comparison to those from *Zfat*$^{f/f}$ mice (Fig. 4B, Supplementary Fig. 3A). Since the IL-2/IL-2R signal plays a critical role in peripheral T cell responses following TCR-stimulation [22], we addressed the expressions of IL-2Rα and IL-2 in *Zfat*$^{f/f}$-*Cd4Cre* T cells. The TCR-stimulation-induced IL-2Rα expression in the splenic T cells, but not the IL-2Rβ expression, was clearly decreased in the *Zfat*$^{f/f}$-*Cd4Cre* mice in comparison to that in *Zfat*$^{f/f}$ mice at 8, 16 and 48 h of TCR-stimulation (Fig. 4C, Supplementary Fig. 3B). The induction of *Il2rα* mRNA expression at 24 h of the stimulation was severely impaired in *Zfat*$^{f/f}$-*Cd4Cre* mice (Fig. 4D), and together these findings suggested that the reduced expression of IL-2Rα in the *Zfat*$^{f/f}$-*Cd4Cre* T cells was, in part, dependent on the reduced expression level of *Il2rα* mRNA. Furthermore, the production of IL-2 after the TCR-stimulation was severely impaired in the *Zfat*$^{f/f}$-*Cd4Cre* T cells (Fig. 4E). These results suggested that the impaired expressions of both IL-2Rα and IL-2 after the TCR-stimulation would account for the reduction in cell division in the *Zfat*$^{f/f}$-*Cd4Cre* T cells.

In this study, we established T cell-specific *Zfat*-deficient mice using *Cd4-Cre* mice and found that Zfat plays crucial roles as a novel cell-intrinsic factor both in peripheral T cell homeostasis and in TCR-mediated cellular response. As both the impaired IL-7Rα- and TCR-mediated signals are likely to underlie the altered T cell homeostasis and T cell survival in *Zfat*$^{f/f}$-*Cd4Cre* mice, elucidation of Zfat functions in the immune system will provide insights into the molecular mechanisms of immune-related diseases.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.07.065.

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