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Review

The Roles of ZFAT in Thymocyte Differentiation and Homeostasis of Peripheral Naive T-Cells

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Abstract. *ZFAT (zinc-finger gene in AITD susceptibility region), originally identified as a candidate susceptibility gene for autoimmune thyroid disease, has been reported to be involved in various cellular processes and several common diseases including multiple sclerosis. Recent studies revealed that mouse Zfat is a novel critical regulator for both thymocyte differentiation and peripheral T-cell homeostasis. Zfat deficiency at early thymocyte developmental stages results in the inhibition of the development of $CD4^+CD8^+$ thymocytes with an impaired positive selection. Zfat deficiency in peripheral T-cells results in a reduction in the number of T-cells with decreased expression of the interleukin-7 receptor-α (IL-7Rα) that is critical for T-cell homeostasis. In addition, T-cell antigen receptor stimulation-induced responses of Zfat-deficient T-cells are also impaired, with reduced IL-2Rα expression. This review highlights and discusses the roles of Zfat in thymocyte differentiation of T-cells and in the homeostasis of naive T-cells with recent work.*

We previously identified zinc-finger gene with AT-hook/zinc-finger gene in autoimmune thyroid disease susceptibility region (*ZFAT*) as a candidate susceptibility gene for autoimmune thyroid disease (1). *ZFAT* encodes an evolutionally-conserved protein with 18 zinc-finger motifs and one AT-hook domain (2). The ZFAT protein is strongly expressed in T- and B-cells in immune-related tissues including the thymus, the spleen, lymph nodes and peripheral blood (2). We reported that *Zfat* deficiency in the mouse ($Zfat^{-/-}$) is embryonically lethal by embryonic day 8.5 and that Zfat is a critical transcriptional regulator for *Tal1* (T-cell acute lymphocytic leukemia protein 1), *Gata1* (GATA-binding factor 1) and *Lmo2* (LIM domain only 2) expression in primitive hematopoiesis (3, 4). We also found that ZFAT is involved in the regulation of apoptosis in a human leukemia cell line (MOLT-4) (5) and mouse embryonic fibroblasts (6), and in the differentiation of human umbilical vein endothelial cells (7). Furthermore, genetic variants of *ZFAT* have been reported to be associated with the severity of Hashimoto's disease (8), with adult height in Japanese and Korean populations (9, 10), and with several common diseases including hypertension and cancer (11, 12). Of interest is that a genetic variant of *ZFAT* is reported to be most strongly associated with interferon-β responsiveness in multiple sclerosis (MS) (13), in which interleukin-7 receptor-α (*IL7RA*) and *IL2RA* are susceptibility genes (14-16). Recent studies have shown that *ZFAT* is a novel imprinted gene expressed in the human placenta (17), and was also reported to be overexpressed in unruptured aneurysms, suggesting that ZFAT could be a possible molecular marker in peripheral blood to predict a high risk of aneurysm rupture (18). The functional roles of ZFAT in biology and genetics have expanded dramatically since its identification and cloning. In our recent studies, we established and analyzed $Zfat^{f/f}$-LckCre mice and $Zfat^{f/f}$-Cd4Cre mice, and we found that Zfat is essential for both thymocyte development and peripheral T-cell homeostasis through Erk activation and the expression of Il7rα and Il2rα, respectively (19, 20). Herein, we review the function of Zfat and discuss recent research regarding the signaling factors that control positive selection in thymocyte development, and the cytokine receptors and autoimmune diseases involved in the homeostasis of naive T-cells.

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The Role of Zfat in Thymocyte Differentiation

In the thymus, signals transduced by the T-cell antigen receptor (Tcr) promote the transition of $CD4^+CD8^+$ double-positive thymocytes to $CD4^+CD8^-$ single-positive and $CD4^-CD8^+$ single-positive thymocytes, and also regulate CD4

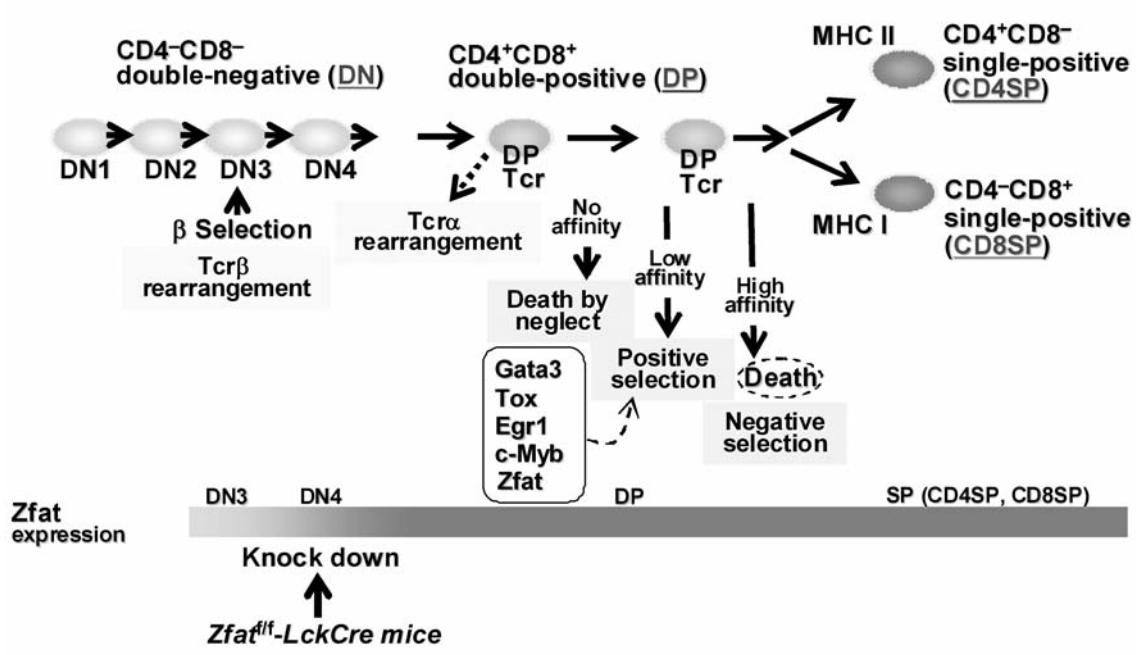

Figure 1. *Model of Zfat function in thymocyte differentiation. Thymocyte differentiation is described based on the pattern of cell surface markers expressed at each stage. Double-negative (DN) cells are divided into DN1, DN2, DN3 and DN4 stages. DN4 cells differentiate into double-positive (DP) cells expressing both CD4 and CD8. The expression of mature α/β Tcr by the re-arrangement of Tcra gene leads to the process of positive and negative selection, ensuring a diverse repertoire of T-cells. DP cells that have undergone positive selection differentiate into CD4 single-positive and CD8 single-positive cells. Zfat expression during the thymocyte development begins from the DN3 stage. Zfat deficiency in thymocyte development results in the inhibition of DP thymocyte development with impaired positive selection, indicating that Zfat is essential for the development of DP thymocytes.*

versus CD8 lineage commitment (21, 22). In the transition of DP to SP thymocytes, transcriptional factors, including Gata3 (23), Tox(Thymocyte selection-associated high mobility group box) (24), Egr1 (Early growth response protein 1) (25) and c-Myb (V-myb avian myeloblastosis viral oncogene homolog) (26), are critical for positive selection, but not for CD4 *versus* CD8 lineage commitment. However, the precise molecular mechanisms and the orchestrated gene expression programs in T-cell development are not yet fully-understood. In our study, we generated *Zfat*$^{f/f}$-LckCre mice and observed that they exhibited a loss of CD3ζ phosphorylation with dysregulation of Erk(extracellular signal-regulated kinase) and Egr activities, leading to impaired positive selection. We demonstrated that Zfat is required for the proper regulation of Tcr proximal signaling, and that Zfat is a crucial molecule for positive selection in the thymus (Figure 1).

The T-cell repertoire is whittled-down by negative selection, which abolishes T-cells with Tcrs that recognize self-peptides and major histocompatibility complex proteins (self-pMHC) with high affinity, and by positive selection, which requires T-cells with Tcrs that recognize self-pMHC with low affinity. The Tcr signaling pathway can be divided into proximal signaling, Ras(Rat sarcoma viral oncogene homolog)-mitogen activated protein kinase (Mapk) signaling and calcium-mediated signaling (Figure 2). The phosphorylation of Erk1/2 induced by TCR-stimulation through Ras-Mapk signaling was markedly decreased in *Zfat*$^{f/f}$-LckCre thymocytes. In agreement with the defects in Erk1/2 activation, the phosphorylation of both Mek1/2 and c-Raf(V-Raf-1 murine leukemia viral oncogene homolog 1), which are located upstream of the Erk signaling pathway, was also reduced in the *Zfat*$^{f/f}$-LckCre thymocytes. The phosphorylation of Zap70 (Zeta-chain-associated protein kinase) and Plcγ1 (Phospholipase C, gamma-1) was also diminished in *Zfat*$^{f/f}$-LckCre thymocytes. Finally, the Tcr stimulation-induced phosphorylation of CD3ζ, which is proximal signaling, was virtually ablated in the *Zfat*$^{f/f}$-LckCre thymocytes, and phosphorylated CD3ζ at non-stimulated status was also apparently diminished due to the Zfat deficiency (Figure 2). By responding to changes of pMHC, the Tcr signal strength provides the effects on signaling mechanisms for positive selection and negative selection during development. The protein schnurri-2 (Shn2; also known as Hivep2) has been reported to be a critical regulator of T-cell development controling the balance between death and differentiation by modulating the Tcr

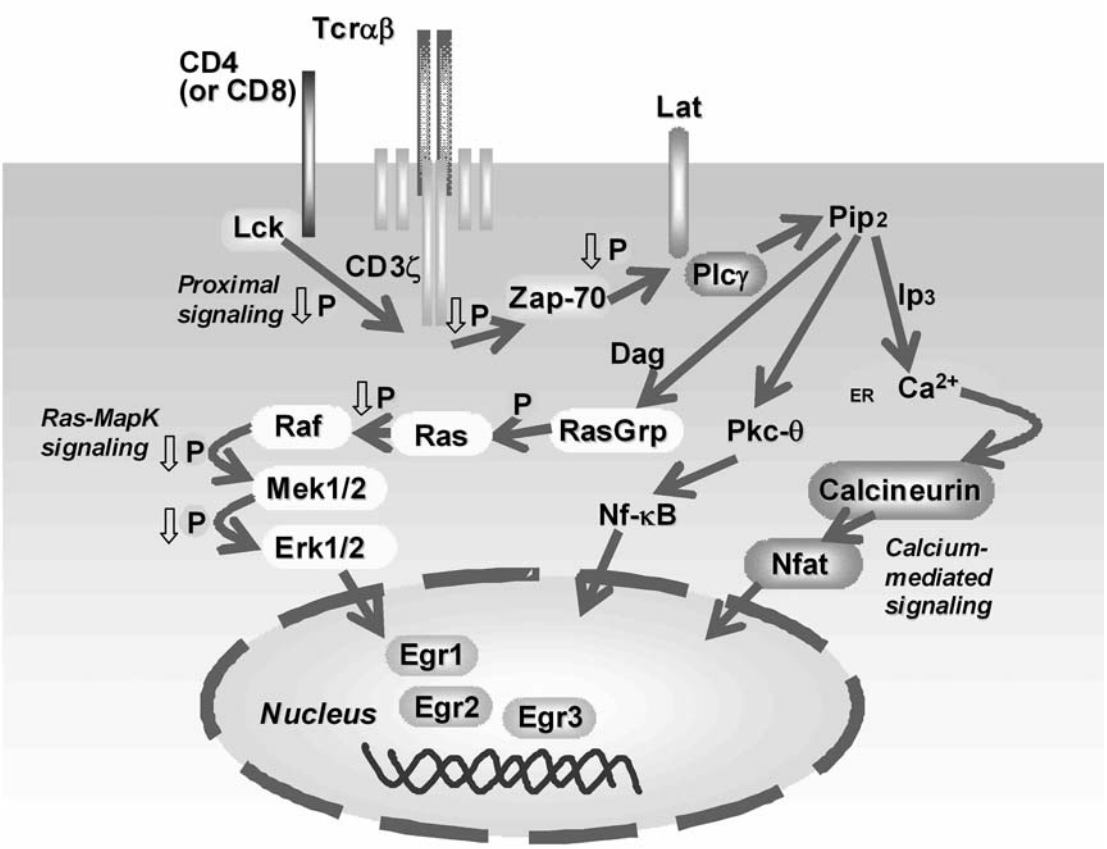

Figure 2. *Impaired Tcr signaling pathways coursed by Zfat deficiency. The Tcr signaling pathway can be divided into three pathways: proximal signaling, Ras-mitogen activated protein kinase (Mapk) signaling and calcium-mediated signaling. The recognition of pMHC ligands by Tcr leads to signaling cascades. Zfat deficiency in thymocyte development results in a loss of CD3ζ phosphorylation with dysregulation of Erk and Egr activities, leading to impaired positive selection. Deregulated phosphorylation by Zfat deficiency is indicated by open arrows.*

signal strength (27). In addition, thymocyte-expressed molecule involved in selection (Themis) was reported to set the signal threshold for positive and negative selection in T-cell development (28). Further studies are required to establish the roles of Zfat in the modulation of the Tcr signal strength for both positive and negative selection.

We further explored the relevance of *Zfat* in positive selection, but not in the lineage commitment processes during T-cell development. Gata3, Tox, Egr1 and c-Myb are critical for positive selection, and the positive selection signals are thought to result in up-regulation of *Egr1* (21). The induction of mRNA of *Egr1*, *Egr2* (29, 30) and *Egr3* (31) by Tcr stimulation was impaired in the DP thymocytes of *Zfat*$^{f/f}$-LckCre mice. Egr transcriptional factors including Egr1, Egr2 and Egr3, contain highly conserved zinc-finger DNA-binding domains that can bind a number of common target gene promoters (32). Egr proteins are central players in the development of thymocytes, and have both redundant and distinct roles in positive selection (25, 29-31). Thus, our results collectively suggest that Zfat is involved in positive selection, in part, through the regulation of the expression of Egr1, Egr2 and Egr3. However, the possibility that the decreased *Egr* expression in *Zfat*$^{f/f}$-LckCre double-positive thymocytes simply reflects the reduced number of positive-selected thymocytes cannot be excluded. A full understanding of the precise mechanisms of Zfat function in positive selection and thymocyte development awaits future studies, which will lead to a better understanding of the orchestrated gene expression programs in T-cell development.

The Role of Zfat in the Homeostasis of Naive T-Cells

In peripheral lymphoid tissues, proper regulation of T-cell homeostasis is highly controlled by both cell-extrinsic and cell-intrinsic factors (33-36). Accumulating evidence demonstrates that peripheral T-cell homeostasis is controlled by cytokine receptor-mediated signals, especially Il7r, as well as by interaction between Tcr and MHC (37, 38) (Figure 3).

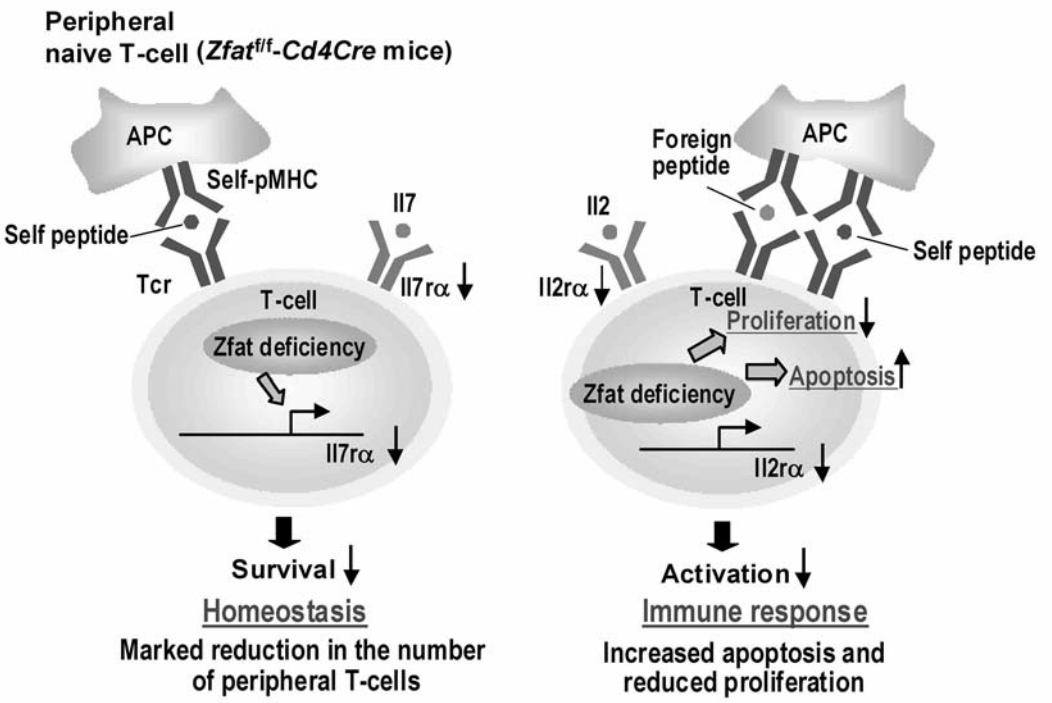

Figure 3. *Models of Zfat functions in peripheral T-cell homeostasis and immune response. Naive T-cells receive continuous Tcr signals by interaction with low-affinity self-peptide MHC ligands on antigen-presenting cells (APCs). Continuous Tcr signals together with Il7 signaling in naive T-cells leads to cell survival. In contrast, Tcr signaling induced by high-affinity foreign-peptide MHC ligands results in the activation of T-cells during immune responses. Cd4-Cre-mediated Zfat deficiency results in a reduction in the number of peripheral T-cells with decreased expression of Il7rα. In addition, the Tcr stimulation-induced responses of Zfat-deficient T-cells are also impaired, with reduced Il2rα expression. These results collectively indicate that Zfat plays important roles in peripheral T-cell homeostasis and immune response through the regulation of Il7rα and Il2rα.*

Il2/Il2r has a broad array of actions, including 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 (39, 40). Il2r 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 Il2 with moderate affinity, whereas the activation of T-cells induces the α-chain (a high-affinity subunit; CD25) and the formation of the high-affinity heterotrimeric receptor, which plays a critical role in immune response of T-cells (41). In addition, the Il7r complex is composed of Il7rα and the γ-chain, and Il7 signaling is mainly regulated by Il7rα expression in T-cells (42). Genetic variants and several haplotypes in the human *IL2RA* gene have been reported to be associated with type-1 diabetes and MS (14). Furthermore, the *IL7RA* gene is also a susceptibility gene for MS (15, 16, 43), and IL7 was recently reported to be involved in the generation of T-helper 17 (T_H17) cells, a subset of IL17-producing $CD4^+$ T-cells required for the initiation of autoimmune disorders (44, 45).

Several molecules, including forkhead box-O class transcriptional factors (Foxo) (46), Runx (47) and the Klf family (48), are reported to play critical roles in peripheral T-cell homeostasis as cell-intrinsic factors. Foxo plays important roles in various cellular processes and a wide variety of diseases (36, 49-52). In the immune system, Foxo1 and Foxo3 are predominantly expressed (49, 53), and Foxo1 critically regulates the expression of Il7rα (46, 54). However, the cell-intrinsic factors responsible for the integration of extrinsic signals have not been fully elucidated. In our study, *Cd4-Cre*-mediated *Zfat*-deficiency resulted in a remarkable reduction in the number of peripheral T-cells with a decreased expression of Il7rα, and in an impaired Tcr stimulation-induced response of T-cells with a reduced Il2rα expression (Figure 3). These findings suggest a functional association of ZFAT with the MS susceptibility genes *IL7RA* and *IL2RA*. From the viewpoint of autoimmune disorders, an IL7R-mediated signal has been reported to be involved in the generation and survival of T_H17 cells (45), a subset of IL17-producing $CD4^+$ T-cells required for the initiation of autoimmune disorders (44), together suggesting a crucial role for ZFAT in autoimmune diseases.

What is the primary event causing the reduced number of peripheral T-cells in $Zfat^{f/f}$-Cd4Cre mice? One of the

remarkable phenotypes observed in the *Zfat*$^{f/f}$-Cd4Cre mice is a decrease in the expression of Il7rα on peripheral CD4+ T-cells. The responsiveness of the *Zfat*$^{f/f}$-Cd4Cre $CD44^{lo}CD4^{+}$-naive T-cells to Il7 is indeed attenuated, which will culminate in reduced survival potency. In addition to the Il7r-mediated signal, when considering that both Il7rα- and Tcr-mediated signals play a crucial role in the survival of naive T-cells (33, 34), it seems possible that the impaired induction of Il2rα expression in response to Tcr underlies the altered T-cell homeostasis and T-cell survival in *Zfat*$^{f/f}$-Cd4Cre mice (Figure 3).

The expression of the *IL2RA* gene is tightly regulated at the transcriptional level, and the positive auto-regulatory loop of the IL2/the high-affinity receptor of IL2R system plays a major role in controlling the magnitude and duration of the T-cell immune response (39). Although positive and negative regulatory regions in the *IL2RA* gene have been characterized, other regulatory regions or elements are strongly suggested to exist based on the findings of several highly-conserved regions through the *IL2RA* locus between humans and mice, and the findings of the presence of histone acetylation islands distinct from the known regulatory regions and the autoimmune disorders-associated single-nucleotide polymorphism located in the 5' region of the *IL2RA* gene (39, 55). In addition, IL2/IL2R signaling has an essential non-redundant role in the production of $CD4^{+}CD25^{+}$ regulatory T-cells, which are critical for maintaining immune tolerance (56). Thus, both the precise mechanism of the defect in the induction of Il2rα expression by Tcr stimulation in *Zfat*-deficient T-cells and the possibility of the involvement of Zfat in the production of regulatory T-cells and in the development of autoimmune disorders should be examined in future studies.

Conclusion

The functional role of ZFAT in biology has been dramatically expanded by accumulating evidence regarding the functional consequences of Zfat deficiency in mouse models or cell lines. Our findings for the immune system show that Zfat is critical for thymocyte development and T-cell homeostasis in the periphery and that Zfat is crucial for the proper expression of Il7rα and Il2rα in peripheral T-cells. However, the detailed molecular mechanisms of action of Zfat remain to be elucidated in the thymocyte differentiation of T-cells and in the homeostasis of naive T-cells. The identification of the Zfat target genes essential for thymic development of T-cells and their homeostasis is expected to uncover novel approaches to manipulating the positive selection process in the thymus and T-cell homeostasis. The elucidation of precise molecular mechanisms of Zfat functions will provide new insights into immune regulation and a wide variety of diseases.

Disclosures

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