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Erythroid differentiation regulator 1 strengthens TCR signaling in thymocytes by modulating calcium flux

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ABSTRACT

Erythroid differentiation regulator 1 (Erdr1) has been identified as a stromal survival factor released under stressful conditions. Previously, Erdr1 was reported to be expressed highly in thymus, but roles of Erdr1 in thymus were not known. Here, the effects of Erdr1 on T cell development were investigated. The expression of Erdr1 was higher in thymus than bone marrow and Erdr1 was detected in both the cortex and medulla of thymus. Erdr1 treatment significantly induced the expression of activation marker, CD69, from thymocytes in the presence of TCR stimuli *in vitro* and the induction was dependent on increased Ca^{2+} influx. In addition, *in vivo* administration of Erdr1 resulted in significant increase of total and positive selected thymocyte numbers, particularly in the number of $CD3TCR^{hi}CD69^{+}$ DP thymocytes. Taken together, our results show that Erdr1 enhances the strength of TCR signaling and cellularity of thymocytes by amplifying Ca^{2+} influx in thymocytes receiving TCR signals.

1. Introduction

T cell development occurs in the thymus through sequential differentiation stages, including $CD4^{-}CD8^{-}$ (double negative, DN), $CD4^{+}CD8^{+}$ (double positive, DP), and $CD4^{+}$ or $CD8^{+}$ (single positive, SP) stages [1]. Inparticular, DP thymocytes are the first population of cells that can express α/β heterodimic T cell receptor (TCR) on the surface [2]. Thymocyte fate is determined by the strength of TCR signaling, which is accompanied by Ca^{2+} influx [3–5]. Thus, controlled regulation of TCR signaling in thymocytes is required for proper T cell development. Based on this concept, the affinity model of thymocyte selection postulates that moderate TCR interactions promote the positive selection and survivals of the thymocytes [6]. Importantly, appropriate control of TCR signaling in thymocytes is necessary for proper immune development, as disruption of thymic selection results in severe disorders, such as autoimmune and immunodeficiency diseases [7–9].

Numerous studies have identified novel molecules which are involved in the fine tuning of TCR signaling in thymocytes. For example, the T cell protein Themis has recently been reported to regulate TCR signaling in thymocytes via Ca^{2+} and Erk pathways [10]. Another protein, known as thymocyte expressed positive selection associated 1 (Tespa1), which is also involved in the TCR-mediated Ca^{2+} and Erk pathways, was identified as a component of TCR signalosome [11]. However, despite of these efforts, the complete mechanism underlying the regulation of TCR signaling in thymocytes remains undfined.

Erythroid differentiation regulator 1 (Erdr1) was first reported as a stromal survival factor, which is secreted under stressful conditions and is thought to regulate the maintenance of growth homeostasis [12]. Previously, it was shown that Erdr1 expression is negatively regulated by interleukin (IL)-18 in melanoma cells, and Erdr1 can suppress the metastasis of melanoma and gastric cancer cells [13,14], suggesting that Erdr1 may also have immunological activities. Interestingly, expression of Erdr1 has been detected in primary immune organs, such as

Abbreviations: DN, double negative; DP, double positive; Erdr1, Erythroid differentiation regulator 1; SP, single positive

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the bone marrow and thymus [15]. However, the functions of Erdr1 in primary immune tissues, particularly in the thymus, have not been described.

In this study, we investigated the function and possible effects of Erdr1 on TCR-mediated signaling in murine thymocytes. Erdr1 was expressed in both the cortex and medulla of thymus. In addition, Erdr1 significantly enhanced expression of CD69, an activation marker, in the presence of TCR stimulus *in vitro*. Erdr1-treated DP thymocytes also showed higher levels of Ca^{2+} influx in response to TCR stimulation, and the Erdr1-induced increase in CD69 expression was inhibited by chelation of intracellular Ca^{2+}. Notably, *in vivo* administration of Erdr1 in mice increased the total numbers of thymocytes, as well as the percentage of $CD3TCR^{hi}CD69^{+}$ DP thymocytes. Collectively, these data suggest that Erdr1 affects T cell development by enhancing Ca^{2+} influx of thymocytes in the presence of TCR stimulation.

2. Materials and methods

2.1. Mice and cells

Exact eight-week or three-month old C57/BL6 mice (OrientBio, Seongnam city, Korea) were used for this study. The animals were kept in a specific pathogen-free facility and the experiments were performed according to the guidelines of Sookmyung Women's University Institutional Animal Care and Use Committee (SMWU-IACUC-1609-027). Murine primary thymocytes were prepared by homogenization and cultivated at 37 °C in 5% CO_2 using RPMI 1640 media (WelGENE Inc, Daegu, Korea) with FBS (10%, WelGENE Inc), 2-ME (50 μM, Sigma-Aldrich, St Louis, MO), penicillin (100 U/ml, Invitrogen, Carlsbad, CA), and streptomycin (0.1 mg/ml, Invitrogen).

2.2. Antibodies and recombinant Erdr1

Alexa488-conjugated anti-CD4 (GK1.5) was from BioLend (San Diego, CA). APC-conjugated anti-CD69 (H1.2F3) was from eBioscience (San Diego, CA). PerCP-Cy5.5-conjugated anti-CD4 (RM4-5), PE-conjugated anti-CD8 (35-6.7), anti-CD3ε (145-2C11), and anti-hamster IgG1 (G94-56) were purchased from BD Biosciences (San Diego, CA). Polyclonal anti-Erdr1 antibody was generated in rabbits as previously reported [13].

Recombinant Erdr1 was prepared as described previously [14]. In brief, the bacterial vector containing the CDS region of Erdr1 was constructed from the Erdr1-pCMV-SPORT6 plasmid (Open Biosystems, Huntsville, AL). Erdr1 was expressed and purified from bacteria with more than 95% of purity. The level of endotoxin was determined using the *Limulus Amebocyte* lysate method (Cape Cod, East Falmouth, MA). Lots containing low endotoxin (< 0.1 EU/ml) were used for experiments.

2.3. Real-time PCR and immunohistochemistry

For real-time PCR, red blood cells were removed from bone marrow cells and splenocytes. Total RNA was isolated from cells of bone marrow, spleen, and thymus and reverse-transcribed into cDNA. Real-time PCR was performed by LightCycler 96 (Roche, Indianapolis, IN) using SYBR Green with 45 cycles of denaturation at 95 °C for 10 s and annealing at 60 °C for 10 s. The primers used were as follows: Erdr1, 5′-CAGTGATGTCACCCACGAAA-3′ (sense) and 5′-GGCATTTCTGTACG CAGTCA-3′ (antisense); β actin, 5′-TCACCCACACTGTGCCCATCT ACG-3′ (sense) and 5′-CAGCGGAACCGCTCATTGCCAATG-3′ (antisense).

For immunohistochemistry, murine thymus was fixed in 4% paraformaldehyde and paraffin-embedded. Thymic sections were deparaffinized in xylene and rehydrated in ethanol. Antigen retrieval was performed in citrate buffer. Anti-Erdr1 antibody was incubated at a 1:100 dilution (overnight at 4 °C). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was used as a secondary antibody and Erdr1 was detected with 3,3′-diaminobenzidine (Abcam, Cambridge, UK). Nuclei were visulalizsed by hematoxylin (Dako North America, Inc., Carpinteria, CA).

2.4. Thymocytes stimulation, in vivo administration of Erdr1, and Ca^{2+} influx assay

For *in vitro* thymocytes stimulation, total thymocytes (5×10^5 cells/well) were cultivated in the presence of various concentrations of Erdr1 in anti-CD3 ε antibody (0, 0.5, 1, or 2 μg/ml)-coated 96-well plate for 18 h. For inhibition of Ca^{2+} signaling, thymocytes were pre-treated with BAPTA-AM (10 μM, Sigma-Aldrich) for 20 min, and then cultured in the anti-CD3 ε antibody-coated plate.

For *in vivo* administration, Erdr1 (10 μg/mouse) was injected i.p. 2 times with 24 h interval and 24 h later mice were sacrificed. Thymocytes from the mice were counted and analyzed using flow cytometry.

For detection of cytosolic Ca^{2+} levels, thymocytes were loaded with Fluo-4 (3 μM in PBS containing 1% FBS (MACS buffer), TEF Labs, Inc, Austin, TX) for 30 min at 37 °C and rested in the presence of anti-CD3 ε antibody (1, 2, or 5 μg/ml) for 20 min at RT. The Ca^{2+} influx was determined after the addition of anti-hamster IgG1 antibody (5 μg/ml) with or without Erdr1 (2.5 μg/ml) by flow cytometry.

2.5. Flow cytometry

Single-cell suspensions were washed and stained in 1 × FACS buffer. Antibodies were used at 1:250 dilution and non-specific staining for every antibody used in the study was monitored using fluorescent-conjugated isotype antibodies to each antibody. For the Ca^{2+} influx assay, base level of each sample was determined for 30 s, and then stimuli were added. All of flow cytometric analyses were performed with gating of live cells, using FACSCalibur with CellQuest or FlowJo software (BD Biosciences).

2.6. Statistical analyses

A non-paired Student's *t*-test (two tailed) was used to compare experimental groups and control groups. *P*-values < 0.05 were considered to be statistically significant.

3. Results

3.1. Erdr1 expression in immune organs

Previous reports have indicated that Erdr1 is expressed in thymus [15]. Here, we measured the levels of *Erdr1* mRNA in various murine immune organs, including the bone marrow, spleen, and thymus, using real time PCR. Results showed higher levels of *Erdr1* expression in the thymus, than in either the bone marrow or spleen (Fig. 1A). Immunohistochemical analysis further revealed that Erdr1 protein is expressed in both the cortex and medulla of thymus (Fig. 1B). Thus, these results suggest that Erdr1 may be involved in T cell development.

3.2. Erdr1 induces the surface expression of CD69 on DP thymocytes

Because the regulation of TCR signaling in thymocytes plays a pivotal role in T cell development [3–6], we next investigated the possible involvement of Erdr1 in TCR signaling in thymocytes. Specifically, the surface levels of CD69, a marker for lymphocyte activation and TCR signal-mediated selection of thymocytes [11], were evaluated after Erdr1 treatments. Surprisingly, Erdr1 significantly enhanced the TCR-mediated expression of CD69 on total thymocytes (Fig. 2A). Further analysis of the levels of CD69 on thymocyte subpopulations revealed that CD69 expression was dramatically induced by Erdr1 on DP

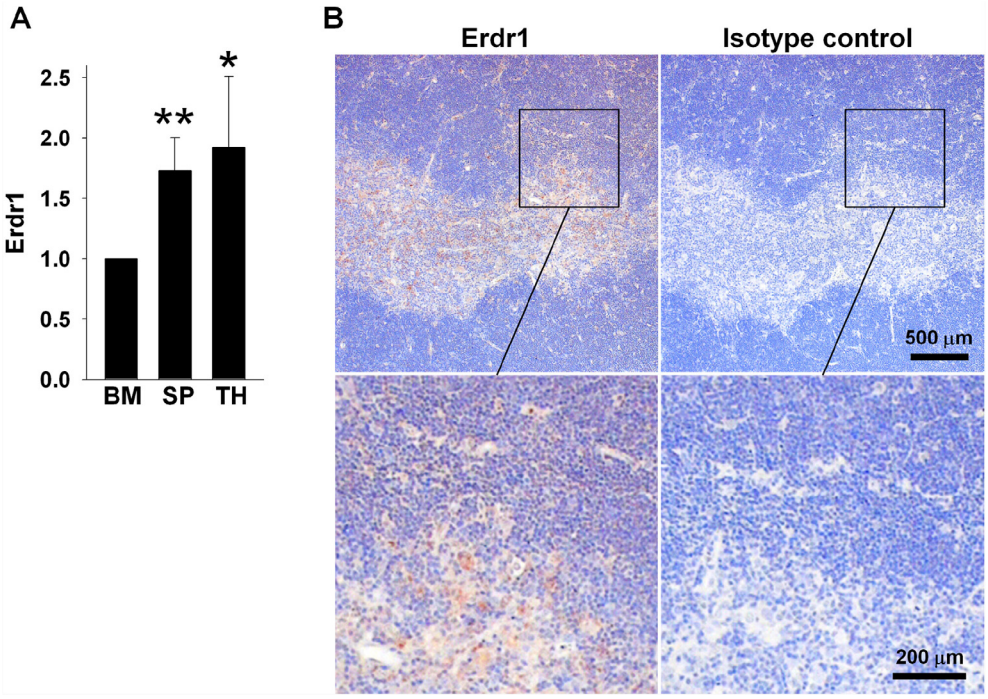

Fig. 1. The expression of Erdr1 in thymus. (A) Total RNA of bone marrow (BM), spleen (SP), and thymus (TH) was isolated from 8-week old mice and Erdr1 expression was analyzed by real-time PCR. β actin was used as a house keeping gene, and the results are presented as mean ± SD. The results are representative of data from 4 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$. (B) The expression of Erdr1 in thymus from 8-week old mice was visualized by immunohistochemistry. Thymic sections were incubated with Rabbit anti-mouse Erdr1 (left) or isotype control antibody (right) for overnight at 4 °C. HRP-conjuaged goat anti-rabbit IgG was treated for 30 min at RT. 3,3′-diaminobenzidine was used as a substrate. Nuclei were stained by hematoxylin.

thymocytes (Fig. 2B). Elevated levels of CD69 were also detected on SP and DN thymocytes stimulated with Erdr1, although the percentage increases were low (Fig. 2B). Thus, these data indicate that Erdr1 enhances the expression of CD69 mainly on DP thymocytes. In addition, Erdr1 was thought to amplify TCR signaling, as the induction of CD69 expression was observed only in the presence of TCR stimulation (Fig. 2A and B). Because both positive and negative selection occur in the DP stage, depending on the strength of TCR signaling [1], our results indicate that Erdr1 can affect the T cell developoment by modulation of TCR-mediated signaling in thymocytes.

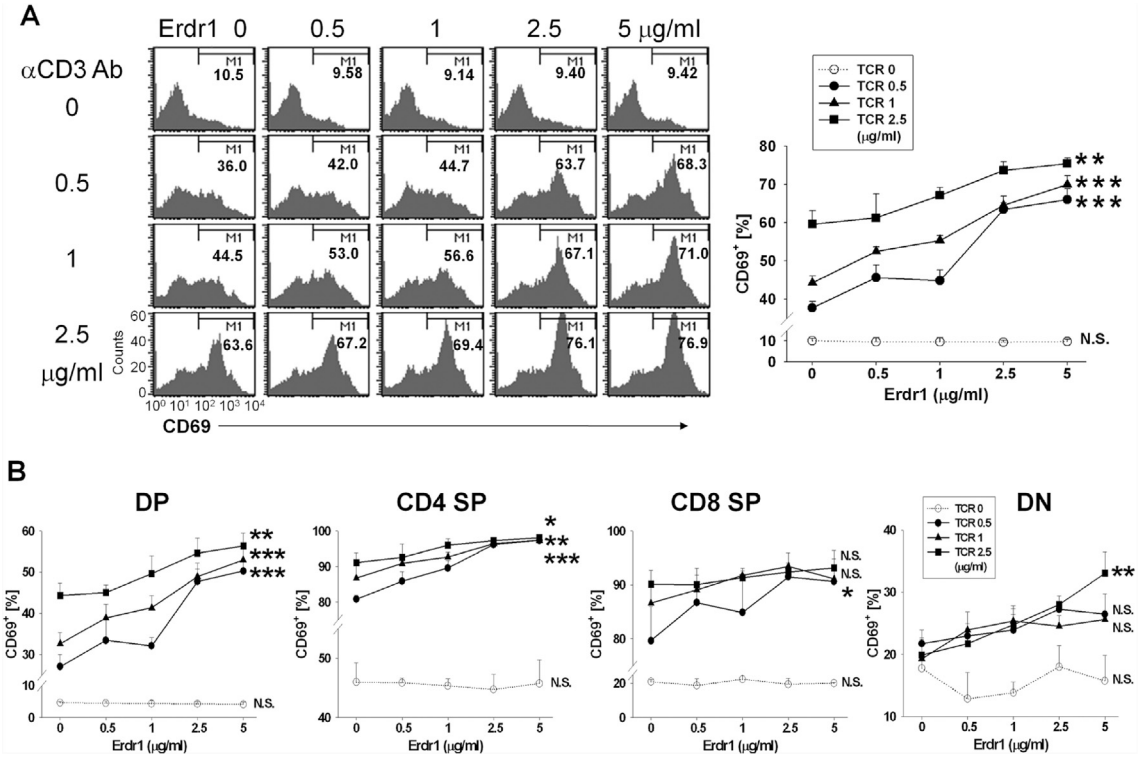

Fig. 2. CD69 expression was induced significantly in DP thymocytes by Erdr1. Thymocytes from 8-week old mice were cultivated with various concentrations of Erdr1 (0–5 μg/ml) in the presence or absence of anti-CD3 ε antibody (coated on culture plates, 0–2.5 μg/ml) for 18 h. (A) The expression of CD69 on total thymocytes was analyzed by flow cytometry, and the results are presented as mean ± SD. (B) The levels of CD69 on DP, CD4 SP, CD8 SP, and DN thymocytes were summarized as mean ± SD. The numbers in the histogram plots are the percentages of the marker. The flow cytometric results are representative of data from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (vs Erdr1 0 μg/ml).

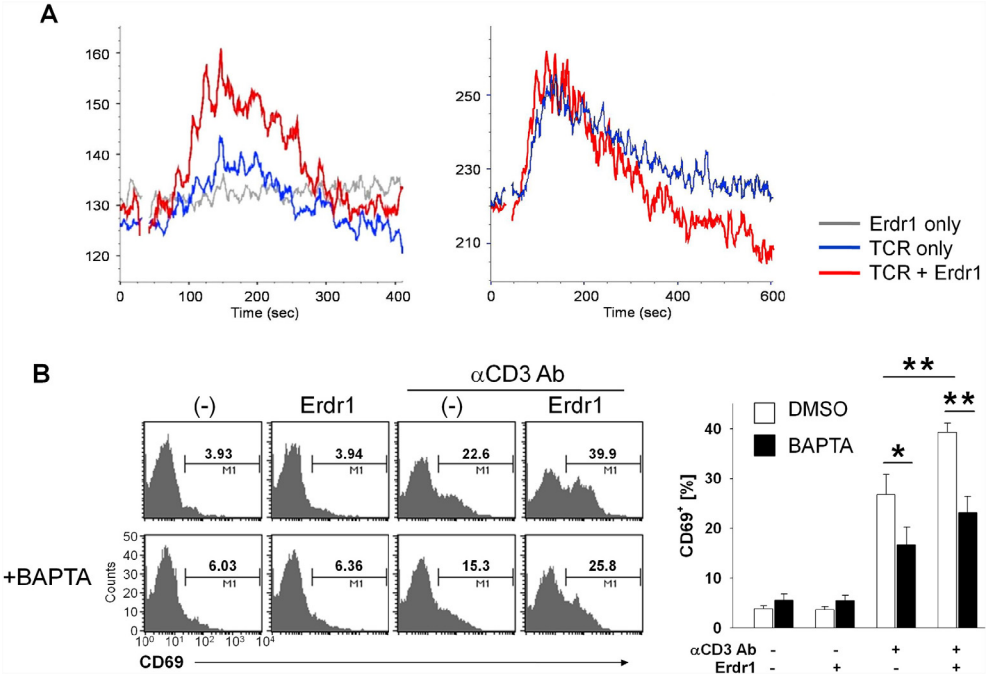

Fig. 3. Erdr1 enhanced TCR strength of DP thymocytes via increase of Ca^{2+} influx. Thymocytes were isolated from 8-week old mice. (A) Flou-4 (3 μM) loaded thymocytes were incubated with 1 μg/ml (left panel) or 2 μg/ml (right panel) of anti-CD3 ε antibody (hamster IgG). The Ca^{2+} influx of DP thymocytes was evaluated immediately after the addition of anti-hamster IgG1 antibody (5 μg/ml) with or without Erdr1 (2.5 μg/ml) by flow cytometry. (B) Thymocytes were pre-treated with DMSO or BAPTA-AM (10 μM) for 20 min, and then cultured in the anti-CD3 ε antibody-coated plate (0.5 μg/ml) with or without Erdr1 (1 μg/ml) for 18 h. The expression of CD69 on thymocytes was determined by flow cytometric analysis, and the results were summarized as mean ± SD. The numbers in the histogram plots are the percentages of the marker. The flow cytometric results are representative of data from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

3.3. *Erdr1 strengthens TCR signaling in DP thymocytes via induction of Ca^{2+} influx*

TCR signaling is accompanied by Ca^{2+} influx, and the Ca^{2+} signaling pathway plays important roles in both positive and negative selection [3–5]. We therefore investigated the effects of Erdr1 on Ca^{2+} influx in DP thymocytes. Results described that Erdr1 in combination with a relatively low dose of TCR stimulus dramatically increased the Ca^{2+} influx in DP thymocytes, where as Erdr1 alone had no effect (Fig. 3A). In addition, when administered with a higher concentration of TCR antibody, Erdr1 induced an earlier and more robust spike in Ca^{2+} signaling, with a faster Ca^{2+} influx and a more rapid drop (Fig. 3A). These data suggest that Erdr1 amplified Ca^{2+} influx in response to TCR stimulation. To determine whether the Erdr1-mediated induction in CD69 expression is dependent on this increase in Ca^{2+} influx, the intracellular Ca^{2+} chelator, BAPTA-AM, was utilized [16,17]. When thymocytes were treated with BAPTA-AM, the enhanced expression of CD69 on DP thymocytes in response to Erdr1 was significantly inhibited (Fig. 3B). Thus, ERDR1 promotes increased Ca^{2+} influx and thereby enhances CD69 expression on thymocytes.

3.4. *Erdr1 enhances expression of positive selection markers in thymocytes in vivo*

Based on the observation that Erdr1can modulate TCR signaling in DP thymocytes *in vitro*, the role of Erdr1 in T cell development *in vivo* was examined by administrating this protein to normal mice. Because thymus size is closely correlated with selection and cellularity of thymocytes [18,19], both thymus size and the subpopulations of thymocytes in ERDR1-treated mice were compared with to vehicle-treated controls. Interestingly, the thymic size was increased in Erdr1-treated 8-week old mice (Fig. 4A), and further, thymocyte numbers were also higher in the ERDR1-treated group (Fig. 4B). More importantly, both the percentages and the total numbers of $CD3TCR^{hi}CD69^{+}$ cells in the $CD4^{hi}CD8^{hi}$ (DP^{hi}) late stage of DP thymocyte development [20], were significantly higher in Erdr1-treated mice than in control mice (Fig. 4C). In addition, the total numbers of $CD3TCR^{hi}CD69^{+}$ DP thymocytes were also increased by Erdr1 injection (Fig. 4C). To compare age differences, we also performed the same *in vivo* experiments using 3-month old mice. Results revealed that the thymic size was also tended to increase by Erdr1 administration (Fig. 4D). Moreover, the percentages and the numbers of $CD3TCR^{hi}CD69^{+}$ thymocytes in DP^{hi} and in total DP cells were significantly higher in Erdr1-injected mice (Fig. 4E). Because $CD3TCR^{hi}CD69^{+}$ thymocytes are generated in response to positive selection [11,17,21], these results demonstrate that Erdr1 enhances the positive selection of thymocytes.

4. Discussion

In the present study, we demonstrate that Erdr1 enhances the strength of TCR signaling in thymocytes by modulating Ca^{2+} influx. Although the expression of Erdr1 has been reported in thymus [15], to our knowledge, this is the first time that Erdr1 has been shown to affect thymocyte development. Specifically, our results indicate that Erdr1 significantly induces the expression of activation markers by enhancing Ca^{2+} influx in the presence of TCR stimuli *in vitro*. Further, the *in vivo* administration of Erdr1 in mice also promotes the cellularity and the expression of thymocyte activation markers.

To fully understand the precise role and functioni of a particular protein *in vivo*, knockout mice are generally used. Because Erdr1 is expressed in both the cortex and medulla of thymus (Fig. 1B), *Erdr1* knockout mice might show specific phenotypes associated with thymocte function. However, to date, there is no knockout model for *Erdr1*. In addition, a recent study revealed that a whole-body knockout or a conditional knockout mouse for *Erdr1* could not be produced, likely due to fetal death and the repetitiveness of locus, respectively [22]. For these reasons, we treated wild-type thymocytes and normal mice with Erdr1 and determined the effect on TCR signaling and thymocyte selection. Notably, we found that administration of Erdr1 enahnced TCR-mediated Ca^{2+} influx in DP thymocytes and promoted the positive selection of thymocytes *in vivo*.

The regulation of TCR signaling strength, which is closely correlated with the level of Ca^{2+} influx, is a major mechanism utilized for generating a proper T cell repertoire [3–5,10,11]. Interestingly, administration of Erdr1, in combination with a TCR stimulus, enhanced Ca^{2+} influx in DP thymocytes, whereas Erdr1 alone had no effect (Fig. 3). Therefore, Erdr1 seems to amplify the Ca^{2+} influx in response to TCR stimuli, suggesting that Erdr1 might be able to rescue a population of thymocytes with very weak TCR interactions from death by neglect. In addition, because the effect of Erdr1 on Ca^{2+} influx in SP thymocytes,

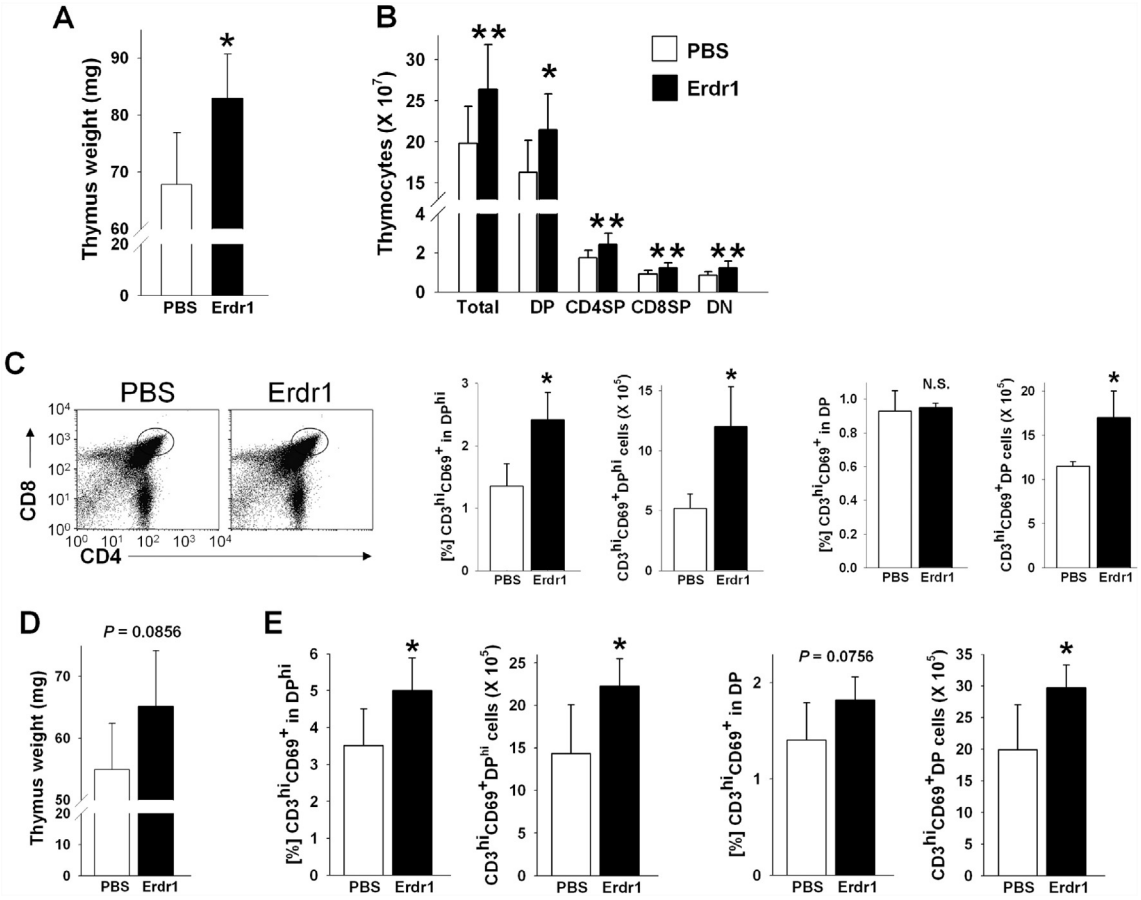

Fig. 4. Erdr1 promoted positive selection of thymocytes *in vivo*. PBS or Erdr1 (500 μg/kg) was i.p. injected into 8-week old mice total 2 times with 24 h interval. After 24 h from the last injection, thymi were collected (A-C). (A) The weights of thymi were compared, and the results are presented as mean ± SD. (B) The numbers of total thymocytes were counted and the numbers of DP, CD4 SP, CD8 SP, and DN thymocytes were analyzed based on the percentages from flow cytometry. The data are summarized as mean ± SD. (C) The expression of selection markers, CD69 and CD3-TCR complex, was examined in total DP or mature DP (DP^{hi}, $CD4^{hi}CD8^{hi}$) thymocytes by flow cytometric anlysis. The gate on DP^{hi} was shown in the left panel. The numbers and percentages of $CD3TCR^{hi}CD69^{+}$ cells in total DP and DP^{hi} thymocytes were presented as mean ± SD, respectively. The results are representative of data from two independent experiments with 5 mice per a group. $^{*}P < 0.05$, $^{**}P < 0.01$. The same experiments were performed using 3-month old mice, n = 5 (D and E). (D) The weights of thymi were compared, and the results are presented as mean ± SD. (E) The percentages and numbers of $CD3TCR^{hi}CD69^{+}$ cells in DP^{hi} or in total DP thymocytes were presented as mean ± SD. $^{*}P < 0.05$.

which have higher basal concentration of Ca^{2+} in cytosol [4], are mild (data not shown), the basal level of Ca^{2+} is also thought to be involved in the effectiveness of Erdr1 on thymocytes.

Erdr1 was previously reported to be secreted in response to various stressors. For example, release of Erdr1 from erythroleukemia cells and fibroblasts was detected in response to trypsin, hydrogen peroxide, or heat shock stress [12]. Erdr1 was also found to be secreted from keratinocytes after exposure to ultraviolet B irradiation [23]. Because the thymus is a sensitive organ to stress [24,25], the observation that Erdr1 improves thymocyt cellularity (Fig. 4) suggests that Erdr1 may protect thymocytes from stress-mediated cell death. Since various stressful conditions, including inflammation, starvation, and sepsis, can cause thymic atrophy [26,27], Erdr1 may be considered as a novel candidate for the treatment of stress-induced thymic involution.

5. Conclusions

In this study, we found that Erdr1 enhances the strength of TCR signaling in thymocytes by modulating calcium influx. Erdr1 significantly induces CD69 expression and enhances Ca^{2+} influx in the presence of TCR stimuli *in vitro*. Further, administration of Erdr1 to mice *in vivo* increases thymic size and elevates numbers of $CD3TCR^{hi}CD69^{+}$ DP thymocytes, suggesting that Erdr1 can improve the cellularity and induce positive selection of thymocytes. To our knowledge, this study provides the first evidence demonstrating that Erdr1 can affect TCR signaling and thymocyte development and thus indicates a critical role for Erdr1 in T cell development.

Conflict of interest disclosure

There is no conflict of interest to disclose.

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Author contributions

M.S.K. – study concept, experimental design, acquisition, analysis, and interpretation of data, and drafting of the manuscript; S.L. –

experimental design, acquisition, analysis; S.J. – experimental design, acquisition, analysis; S.P. – study concept, experimental design; K.E.K. – study concept, experimental design; T.S.K. – study concept, experimental design; H.J.P. – study concept, experimental design, revision of the manuscript, and study supervision; D.C. – study concept, experimental design, revision of the manuscript, and study supervision

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