Supplemental Materials and Methods

Reaggregate Thymus Organ Culture (RTOC) System

To analyse the development of thymocyte in the thymus, we used an in vitro model called the RTOC system [1]. The RTOC system enables *in vitro* study monitoring the effects of exogenously expressed molecules on the development of thymocytes by using thymocytes co-cultured with thymus epithelial cells. An overview of this system is provided in Supplemental Fig. 1B.

Cell culture

The cells used in the RTOC system were grown in CML culture medium (RPMI 1640 supplemented with 10% heat-inactivated FCS (Invitrogen, Carlsbad, CA), 50 μM 2-mercaptoethanol (2-ME), 10 mM HEPES, 2 mM L-glutamine, 1 x nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin).

Preparation of Viral Supernatants for Infection

The cDNA which encodes full-length wild-type of human DAP3 (WT-DAP3) or the dominant negative form of human DAP3 (DN-DAP3) [2], was inserted into the EcoRI-NotI site of pMX-IRES-EGFP (Supplemental Fig. 1A) [3]. For packaging retroviral vectors, the packaging cell lines PLAT-E and Phonix were used for the derivatives of pMRX-IRES-EGFP and pBabe-puro, respectively. Packaging and infection of the retrovirus vectors were performed as previously described [1].

Preparation of thymic stromal cells

Thymus lobes from C57BL/6 fetal mice at day 15 of gestation (Japan SLC, Hamamatsu, Japan) were cultured in the presence of 1.35 mM 2-deoxyguanosine (dGuo; Sigma, St Louis, MO) for 7 days to eliminate lymphoid cells. Thymic epithelial cells were obtained by trypsinisation of dGuo-treated lobes.

Retroviral Gene Transfer into Thymocytes

Total thymocytes were prepared from neonatal C57BL/6 mice (day 21; Japan SLC, Hamamatsu, Japan). Thymocytes (2×10^6 cells) were suspended in 1 ml of packaged retroviral vector stock with addition of polybrene (final concentration 30 μg/ml) into each well of a 24-well plate. For the spin-fection, the plate was sealed with Parafilm (Structure Probe, West Chester, PA) and spun at 1,000 x g for 1 hour at room temperature in a centrifuge. Next the cells were transferred into a microtube, and retroviral vector-containing supernatant was removed by centrifugation. The cells were re-suspended in fresh CML culture medium and grown at 37 ºC with 5% CO_2. After 24 hours, the retroviral vector integrated thymocytes were separated by monitoring the fluorescence of enhanced green fluorescent protein (EGFP) which is carried with the vector, using a FACSVantage cell sorter (Beckton Dickinson, Lincoln Park, NJ), and then used for the RTOC.

Analysis of subpopulation of thymocyte

The thymic epithelial cells and the retroviral vector-infected neonatal thymocytes were mixed at a 1:1 cell ratio. The mixtures of the cells were pelleted by centrifugation using 1.5 ml micro-centrifuge tubes, and were seeded onto the surface of a polycarbonate filter membrane (Nucleopore, 0.8 μm pore size; Whatman, Clifton, NJ) on collagen sponges (Collagen sponge INTEGRAN Sheet type; Nippon Zoki Pharmaceuticals, Osaka, Japan) in CML culture medium, and cultured at 37 ºC with 5% CO_2. After 3 days, the subpopulations of the reconstituted cultures were analyzed by a flow cytometric analyzer (FACScan; Beckton Dickinson, Lincoln Park, NJ). The retroviral vector integrated cells were detected by the fluorescence of EGFP.

Supplemental reference

[1] Ueno T, Liu C, Nitta T, Takahama Y (2005) Development of T-lymphocytes in mouse fetal thymus organ culture. *Methods Mol Biol* **290:** 117-133

[2] Miyazaki T, Reed JC (2001) A GTP-binding adapter protein couples TRAIL receptors to apoptosis-inducing proteins. *Nat Immunol* **2**(6)**:** 493-500

[3] Saitoh T, Nakano H, Yamamoto N, Yamaoka S (2002) Lymphotoxin-beta receptor mediates NEMO-independent NF-kappaB activation. *FEBS Lett* **532**(1-2)**:** 45-51

Supplemental Figure Legend

Supplemental Fig. 1. Retrovirus gene transfer into developing thymocytes in RTOC.

(A) The schematic diagram of the gene structure of retroviral vectors, pMRX-IRES-EGFP-WT-DAP3 and pMRX-IRES-EGFP-DN-DAP3. The structure of each retroviral vector for expression of DAP3 used in the experiments using the RTOC system is indicated. The cDNA which encodes wild-type human DAP3 (WT-DAP3) or dominant-negative form of human DAP3 (DN-DAP3) was inserted in the retroviral vector, pMRX-IRES-EGFP [3]. Predicted domain structures of DAP3 are indicated by black (NR-like Box, nuclear receptor-interacting domain) and hatched (DED-like Domain, death effecter domain-binding domain) box. The regions for amplification of the vector in Escherichia coli are omitted in the diagram. These retroviral vectors were packaged by using the packaging cell line, PLAT-E. CMV: Cytomegalovirus promoter; LTR: Long terminal repeat; Ψ: Packaging signal; IRES: Internal ribosome entry site; EGFP: Enhanced green fluorescent protein.

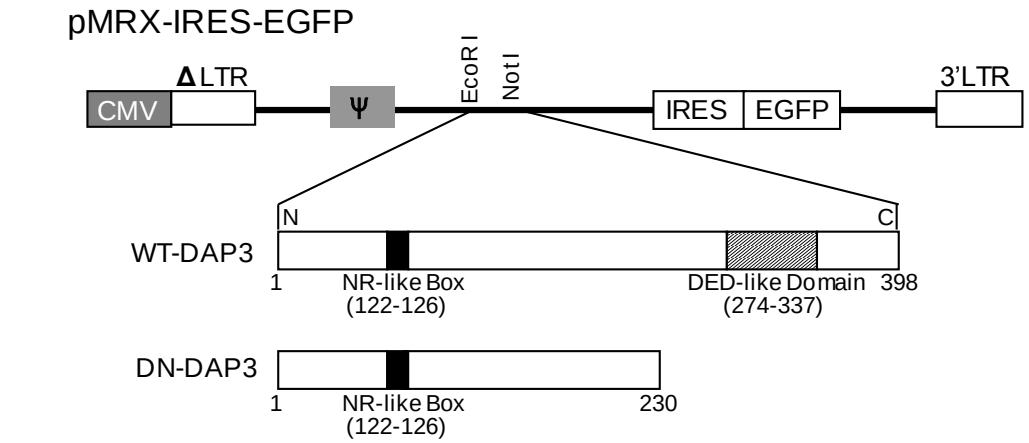
(B) Schematic illustration of the experimental procedure for the analysis of developing thymocytes in RTOC. Total thymocytes which were prepared from the neonatal mice (C57BL/6; day21) were infected with the DAP3-expressed retroviral vector by the spin-fection method (top left). In parallel, thymic epithelial cells were prepared from the fetal mice (C57BL/6; day 15 of gestation). The fetal thymus lobes isolated from the fetal mice were treated with 2-deoxyguanosine for elimination of lymphoid cells (top right). Then the retroviral vector-infected thymocytes were cultured together with the thymic epithelial cells at a 1:1 cell ratio. After 3 days, subpopulations of the thymocytes in the cultured cells were analysed using a flow cytometer (center bottom).

Supplemental Fig. 2. Analysis of the effect of the DN-DAP3 expression on development of thymocytes using RTOC system.

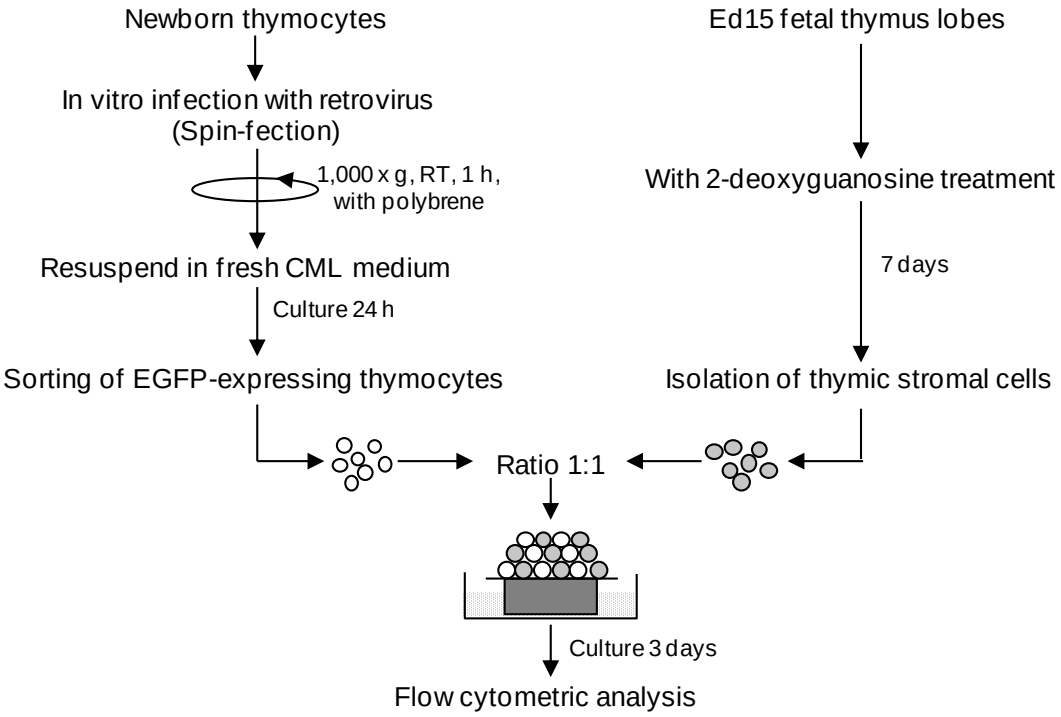
The thymocytes infected with the retroviral vector, pMRX-IRES-EGFP (Empty), pMRX-IRES-EGFP-WT-DAP3 (WT-DAP3) or pMRX-IRES-EGFP-DN-DAP3 (DN-DAP3) were cultured in RTOC. After 3 days, the subpopulation of thymocytes was analyzed by three-color flow cytometry. The data represent the ratio of each population estimate from the total cell number. DN: $CD4^-CD8^-$ double negative cells, DP: $CD4^+CD8^+$ double positive cells, CD4: $CD4^+$ single positive cells, and CD8: $CD8^+$ single positive cells.

Supplemental Fig. 1

A



B



Supplemental Fig. 2