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Critical function of death associated protein 3 in T cell receptor-mediated apoptosis induction

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Abstract

Death associated protein 3 (DAP3) is crucial for promoting apoptosis induced by various stimulations. This report demonstrates that DAP3 is also important for T cell receptor (TCR)-mediated apoptosis induction in immature thymocytes. Enforced expression of DAP3 accelerated the negative selection in developing thymocytes, using the reaggregate thymus organ culture system. In addition, expression of DAP3 accelerated TCR-mediated apoptosis induction in DO11.10 cells. We also demonstrated that DAP3 translocates into the nucleus during TCR-mediated apoptosis in a Nur77 dependent manner. It is concluded that DAP3 is critical for TCR-mediated induction of apoptosis at the downstream of Nur77.

Keywords: apoptosis / death associated protein 3 / negative selection / Nur77 / T cell receptor /

Abbreviations: IFN, interferon; NR box, nuclear receptor-interacting domain; WT, wild-type; DN, dominant-negative form; RTOC, reaggregate thymus organ culture; FADD, FAS-associated death domain protein

Introduction

Negative selection is a quite important event for acquisition of self-tolerance by elimination of self-reactive thymocytes [1, 2]. During T cell development, immature CD4⁺CD8⁺ thymocytes undergo negative selection events based on the specificities of the $\alpha\beta$ T cell receptor (TCR) complexes.

Death-associated protein 3 (DAP3) is ubiquitously expressed in various tissue including the immune system, such as in the thymus of human and mice [3, 4]. DAP3 is a GTP-binding protein that has been identified as a positive mediator in interferon (IFN)- γ -induced cell death [5]. The gene of DAP3 encodes for a 46 kDa protein with a potential P-loop motif, a potential nuclear receptor-interacting domain (NR box), and a putative cleavage site for the N-terminal mitochondrial targeting sequence [5-7]. It has been reported that mitochondrial DAP3 regulates cellular senescence through an oxidative stress response [8]. Moreover, DAP3 is reportedly phosphorylated in an Akt-dependent manner, correlating with the suppression of DAP3-facilitated apoptosis in anoikis [9], and IFN- β promoter stimulator 1 (IPS-1) binds DAP3, resulting in induced anoikis by caspase activation [10]. Although the accumulating evidence shows that DAP3 plays roles in apoptosis induction, the role of DAP3 in thymocyte development is still unknown.

An orphan nuclear receptor, Nur77, belonging to the steroid/thyroid hormone receptor

superfamily, is a transcription factor responsible for inducing apoptosis [11]. Nur77 is activated by various kinds of stimulation for apoptosis induction, and TCR stimulation is known to be a potent activator of Nur77 transcription [12, 13]. A previous report indicated that thymocytes from transgenic mice that express a dominant-negative form of Nur77 (DN-Nur77) are protected from negative selection, and conversely transgenic mice that express wild-type Nur77 exhibit promoted negative selection [14]. Therefore, Nur77 is assumed to be an important factor in the development of thymocytes.

This study demonstrates the physiological importance of DAP3 on negative selection of immature thymocytes. The data indicate that DAP3 is a critical factor for induction of apoptosis induced by TCR stimulation at the downstream of Nur77.

Material and Methods

Antibodies

The specific antibodies used in this study, anti-DAP3 (Clone 10; BD Biosciences, San Jose, CA), anti-Crk (clone 22; BD Biosciences), anti-Hsp60 (Clone LK-1; StressGen, Victoria BC, Canada), anti-nucleoporin p62 (clone 53; BD Biosciences), anti-Nur77 (clone 12.14; BD Biosciences), anti-mouse CD3 antibody (clone 145-2C11; BD Biosciences) and anti-mouse CD28 antibody (BD Biosciences) were purchased from commercially available products.

Cell Culture

The T cell hybridoma, DO11.10 cells were kindly provided by Dr. Makoto Iwata (Tokushima Bunri University), and were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS (Sigma, St Louis, MO), 50 μ M 2-mercaptoethanol (2-ME).

Preparation of Viral Supernatants for Infection

The pBabe-DN-Nur77 was kindly provided by Dr. Nobutaka Suzuki (Takeda Pharmaceutical, Osaka, Japan) [15]. The retroviral vectors derived from pMRX-IRES-EGFP to express wild type DAP3 (WT-DAP3) or its dominant negative form (DN-DAP3) in TRAIL signal were constructed as shown in Supplemental Fig. 1. To obtain the information of the construction and packaging of other retroviral vectors used in this study, please refer to the Supplemental Materials and Methods.

Reaggregate Thymus Organ Culture (RTOC) System

Analysis of the subpopulations of retrovirus-infected thymocytes using the RTOC system were carried out as described elsewhere [16]. Please refer to Supplemental Materials and Methods for the detailed procedures for the use of this system.

Isolation of each population of thymocytes

CD4⁺CD8⁺ double positive cells were purified from freshly isolated thymocytes of

C57BL/6 mice (5 weeks of age; Japan SLC, Hamamatsu, Japan) by the panning method [17], and the other populations of thymocytes were prepared by using a magnetic cell sorting system (MidiMACS Separator; Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocols. All protocols for experiments on animals were approved by Committee on Animal Experimentation, Graduate School of Medicine, Hokkaido University.

Flow Cytometry

For the flow cytometric analysis, cells were stained with phycoerythrin (PE)-anti-CD4 (RM4-4; BD Biosciences) and Allo-phycocyanin (APC)-anti-CD8 (53-6.7; BD Biosciences). For detection of apoptosis, DO11.10 cells were stained with FITC-annexin V (Roche, Mannheim, Germany) or PE-annexin V (BD Biosciences), and propidium iodide (PI; Sigma). Flow cytometric analysis was carried out by using FACSscan (Beckton Dickinson, Lincoln Park, NJ) and analyzed with CELLQuest software (Becton Dickinson).

Semi-quantitative RT-PCRs

Total RNA was extracted from the thymocytes using TRIzol (Invitrogen, Carlsbad, CA). The reactions of the reverse-transcription were performed by Superscript II RT (Invitrogen) using random oligonucleotide hexamers. Each procedure was carried out

according to the manufacturer's protocols. The following primer set for the mouse DAP3 gene was used in this study: 5'- GCAAGACATGACTGGCTGAT -3' and 5'- TGTGGACAAGGGAGAGTTCC -3'.

Cell fractionation

Nuclear fractionation experiments were performed using the Nuclear/Cytosol fractionation Kit (BioVision, Mountain View, CA). Fractionation experiments of the cytosol and mitochondria fractions were carried out using an ApoAlert Cell Fractionation kit (Clontech, Palo Alto, CA). Each procedure was performed in accordance with the manufacturer's instructions.

Results and discussion

DAP3 is critical for negative selection.

To understand DAP3 functioning on T cell development in the thymus, the DAP3 expression in each population of thymocytes was initially analyzed by RT-PCR analysis (Fig. 1A). Here, DAP3 was shown to be expressed in CD4⁺CD8⁺ double positive cells, CD4⁺ single positive cells, and in CD8⁺ single positive cells, but not in CD4⁻CD8⁻ double negative cells; CD4⁺CD8⁺ double positive cells are known to be screened against autoreactivity during development in the thymus. The results suggest that DAP3

is involved in the development of thymocytes in the negative selection in the thymus. To investigate whether DAP3 expression is changed by TCR stimulation during negative selection, an in vitro stimulation assay which mimics negative selection was performed. Thymocytes were stimulated with anti-CD3 and anti-CD28 antibodies, subsequently expression of DAP3 was analyzed by immunoblotting. As shown in Fig. 1B, the DAP3 expression in thymocytes was not significantly changed by anti-CD3 and anti-CD28 antibody stimulation. These results suggest that DAP3 is expressed in thymocytes throughout the negative selection.

Next, the functional role of DAP3 expression in $CD4^+CD8^+$ double positive cells was investigated. Thymocytes expressing DAP3 by the infection of a retrovirus vector were analyzed using an RTOC system. The RTOC system provides a model in which the cellular interactions required for T cell development can be studied under controlled in vitro conditions [18]. The DAP3-expressed retroviral vectors were infected to total thymocytes which were isolated from mice, and then the thymocytes were cultured in RTOC. Under this condition, retroviral infection occurs predominantly in the $CD4^+CD8^+$ double positive subpopulation [16]. After culturing in RTOC, the thymocytes were analysed by flow cytometry. The retrovirus infected cells were detected by the fluorescence of the enhanced green fluorescent protein (EGFP) which is carried with the retrovirus vector as a marker protein. The results show that the population of $CD4^+CD8^+$ double positive cells expressing DAP3 by the infection of

pMRX-IRES-EGFP -WT-DAP3 decreased significantly ($P<0.005$) when compared with that of the empty vector, pMRX-IRES-EGFP (Fig. 1C, D). In addition, the number of $CD4^+CD8^+$ double positive cells expressing DAP3 were dramatically lower when compared with that of empty vector integrated cells, whereas the number of $CD4^+$ single positive cells and $CD8^+$ single positive cells was not significantly changed (Fig. 1E). These findings suggest that the expression of DAP3 enhances TCR-mediated induction of apoptosis during negative selection.

DAP3 expression promotes TCR-mediated apoptosis.

To investigate whether the decrement in double positive cells determined by the RTOC system were caused by apoptosis induced by TCR stimulation during the negative selection, the effects of the DAP3 expression on the TCR-mediated apoptosis induction in DO11.10 cells hybridomas were analyzed. The retroviral vectors which express wild type DAP3 (WT-DAP3) or the dominant negative form of DAP3 (DN-DAP3) were infected to DO11.10 cells, and then the cells were stimulated by anti-CD3 antibody. After a 24 hour post-stimulation incubation period, the cells were stained by PI or Annexin-V, and apoptotic cells were defined as Annexin-V⁺ cells (Fig. 2A). The results show that the number of apoptotic cells induced by the expression of WT-DAP3 was significantly ($P<0.005$) higher than that of the control cells after anti-CD3 antibody stimulation (Fig. 2B). In addition, the number of apoptotic cells induced by the

expression of DN-DAP3, was significantly ($P < 0.005$) fewer than that of the WT-DAP3. Different from this, the results of unstimulated cells indicate that only very few apoptotic cells were detected in either WT-DAP3 or DN-DAP3 expressed cells, suggesting that spontaneous induction of apoptosis was not affected by the enforced expression of DAP3. These results indicate that DAP3 expression accelerates TCR-mediated apoptosis induction.

DAP3 is moved into the nucleus by TCR stimulation.

The subcellular localization of DAP3 is mainly observed in mitochondria; however, a previous report indicated that DAP3 is also functional in cytoplasm. Further, our own preliminary data using the hepatocellular carcinoma cell line, Hep3B, suggested that DAP3 is also localized in the nucleus during the progress of apoptosis induced by the stimulation of TRAIL (data not shown). To fully elucidate this, the subcellular localization of DAP3 in DO11.10 cells after anti-CD3 antibody stimulation was analyzed. Anti-Crk, anti-Hsp60, and anti-nucleoporin p62 antibodies were used as marker proteins for the cytosol fraction, the mitochondria fraction, and nucleus fraction, respectively [19-21]. *The results show DAP3 is detected in the cytosol and mitochondria fraction, but only a little in the nucleus fraction in the cells before anti-CD3 antibody stimulation (Fig. 3).* The DAP3 expression was increased in the nucleus fraction 4 hours after the anti-CD3 antibody stimulation. These data suggest that DAP3 translocated to

the nucleus in DO11.10 cells after the anti-CD3 antibody stimulation.

It has been reported that Nur77, an orphan nuclear receptor, is required for TCR-mediated apoptosis in immature thymocytes undergoing negative selection steps [12, 13], and the subcellular localization of Nur77 in DO11.10 cells after anti-CD3 antibody stimulation was analyzed. *As shown in Fig. 3, a little hyper-phosphorylated Nur77 (80 kDa) and hypo-phosphorylated Nur77 (70 kDa) were slightly detected in the cytosol and mitochondria fraction at 1 hour, and in the peak level at 2 hours after anti-CD3 antibody stimulation.* Hypo-phosphorylated Nur77 in the nucleus fraction was detected at 2 hours as weak signals and peaked at 4 hours after stimulation. These results indicate that the subcellular localization changes of Nur77 after anti-CD3 antibody stimulation closely resemble DAP3, suggesting that DAP3 is functionally associated with Nur77 on TCR mediated apoptosis.

Nur77 is necessary for nuclear transport of DAP3.

The DN-Nur77 expressed retroviral vector was infected to DO11.10 cells, and the retrovirus vector-integrated cells were selected in medium containing 2 µg/ml puromycin. As shown in Fig. 4A, DN-Nur77 protein was well expressed in DO11.10 cells infected with the retrovirus vector. The retroviral vector infected DO11.10 cells were then stimulated with anti-CD3 antibody. After 24 hours, the apoptotic cells were evaluated by PI and Annexin V staining. In agreement with the findings of the previous

study, the results show that the population of apoptotic cells was significantly decreased in DN-Nur77 expressed DO11.10 cells, by a comparison with empty vector infected cells (Fig. 4B). Next, we investigated whether the translocation of DAP3 to the nucleus is affected by the expression of DN-Nur77. As shown in Fig. 4C, the data showed that the amount of nuclear localized DAP3 protein is significantly decreased by the expression of DN-Nur77. These data suggested that translocation of DAP3 to the nucleus depends on the activation of Nur77 during the course of TCR-mediated apoptosis.

Recruitment of FAS-associated death domain (FADD) followed by activation of caspase-8 is thought to be a major molecular mechanism on the DAP3-mediated induction of apoptosis. However, previous studies have shown that FADD [22] and caspase-8 [23] are not always necessary for negative selection of thymocytes. Thus, these molecules can be ruled out in the molecular mechanism of the DAP3-mediated signaling pathway for the induction of apoptosis against immature thymocytes induced by TCR stimulation. In agreement with the previous observations, our data for DN-DAP3 which lacks FADD binding domain also suggests that FADD independent pathway is involved in DAP3 mediated acceleration of negative selection. As shown in Fig. 2, although significantly lower induction of apoptosis was observed in the DN-DAP3 expressed DO11.10 cells after the anti-CD3 antibody stimulation compared with the WT-DAP3 expressed cells, DN-DAP3 did not inhibit the apoptosis by

comparison with the empty vector infected cells. In addition, same results were obtained using the DN-DAP3 expressed thymocytes in RTOC system (supplemental Fig. 2). In conclusion, the data presented here suggest that there is an unknown pathway for apoptosis induction mediated by DAP3 independent of the FADD and caspase-8 dependent pathway.

This report shows that Nur77 is crucial for nuclear translocation of DAP3 in the apoptosis induced by TCR stimulation. It is known that DAP3 has a NR box-like structure which is located close to the N-terminal side of the P-loop motif [7]. Therefore, it is likely that Nur77 directly binds to DAP3 and transports DAP3 into the nucleus. Although the data presented here suggest that nuclear translocation of DAP3 is important for TCR induced apoptosis, molecular function of DAP3 in the nucleus for apoptosis induction is still not clearly established. Further work is required for understanding the physiological importance of the nuclear translocation of DAP3, and the functional relationship between DAP3 and Nur77 in the induction of TCR-mediated apoptosis.

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Figure Legends

Fig. 1. DAP3 expression enhances cell death during thymocyte development in RTOC.

(A) Total RNA extracted from each population of thymocytes isolated from C57BL/6 mice was subjected to RT-PCR analysis. DN: CD4⁻CD8⁻ double negative cells, DP: CD4⁺CD8⁺ double positive cells, CD4: CD4⁺ single positive cells, and CD8: CD8⁺ single positive cells. (B) Freshly isolated thymocytes from C57BL/6 mice were treated with the indicated concentrations of immobilized anti-CD3 together with anti-CD28 antibody (50 µg/ml). After 20 hours, the cells were harvested and the whole cell extracts were subjected to immunoblotting analysis. (C) The thymocytes infected with the

retroviral vector, pMRX-IRES-EGFP (Empty or EM) or pMRX-IRES-EGFP-WT-DAP3 (WT-DAP3 or WT) were cultured in RTOC. After 3 days, the subpopulation of thymocytes was analyzed by three-color flow cytometry. The data represent CD4/CD8 staining profiles of cells within electronically gated gene-transferred (EGFP⁺) cells. (D, E) The ratio of each population (D) and number of each population estimate from the total cell number (E). Data shown in (A), (B), (C), and (E) are representative results from three individual experiments. Data in (D) represent mean \pm S.E. *: $P < 0.01$, **: $P < 0.005$ and N.S.: not significant; the two-way ANOVA compares with the empty vector infected cells.

Fig. 2. Enhanced sensitivity to TCR-mediated induction of apoptosis by expression of DAP3 in DO11.10 cells.

The DO11.10 cells were infected with retroviral vector, pMRX-IRES-EGFP (Empty or EM), pMRX-IRES-EGFP-WT-DAP3 (WT-DAP3 or WT), or pMRX-IRES-EGFP-DN-DAP3 (DN-DAP3 or DN). After a 72 hour post-infection incubation period, the cells were stimulated with 0.3 μ g/ml of immobilized anti-CD3 antibody. After an additional 24 hours of incubation, the cells were stained with PE-Annexin-V and PI, and analyzed by three-color flow cytometry. Annexin-V/PI staining profiles of cells within electronically gated gene-transferred (EGFP⁺) cells are displayed. Data shown in (A) are representative of three individual experiments. Data of

(B) represent mean \pm S.E. *: $P < 0.01$, **: $P < 0.005$, and N.S.: not significant; the two-way ANOVA compared with WT-DAP3 expressing cells.

Fig. 3. Subcellular localization of DAP3 and Nur77 in DO11.10 cells treated with anti-CD3 antibody.

The DO11.10 cells were stimulated with 3 $\mu\text{g/ml}$ of immobilized anti-CD3 antibody. At the end of each incubation period indicated in the figure, the cells were harvested, lysed and then separated into cytosol, mitochondria, and nucleus fractions. Each subcellular fraction was analyzed by immunoblotting using specific antibodies against DAP3, Nur77, Crk, Hsp60, or nucleoporin-p62 (P62). The experiment is representative of three independent experiments.

Fig. 4. Effects of DN-Nur77 expression in translocation of DAP3 to the nucleus in DO11.10 cells treated with anti-CD3 antibody.

The DO11.10 cells were infected with a retroviral vector, the pBabe-puro (Empty) or pBabe-DN-Nur77 (DN-Nur77). The retroviral vector integrated cells were selected by the medium containing puromycin (2 $\mu\text{g/ml}$). (A) Immunoblotting analysis of retroviral vector expressed DN-Nur77. Xpress tagged DN-Nur77 protein was detected by immunoblotting after immunoprecipitation using anti-Xpress, and endogenously expressed DAP3 protein in the whole cell extract was monitored using a DAP3 specific

antibody. (B) The retroviral vector integrated cells were stimulated with 3 $\mu\text{g/ml}$ of immobilized anti-CD3 antibody. After 24 hours, the cells were stained with FITC-Annexin-V and PI, and analyzed by two-color flow cytometry. (C) The cells were stimulated with 3 $\mu\text{g/ml}$ of immobilized anti-CD3 antibody. At the end of each incubation period indicated in the figure, the cells were harvested and separated into cytosol, mitochondria, and nucleus fractions. Each fraction was analyzed by immunoblotting using DAP3 specific antibody. The experiment is representative of two independent experiments.

Fig. 1

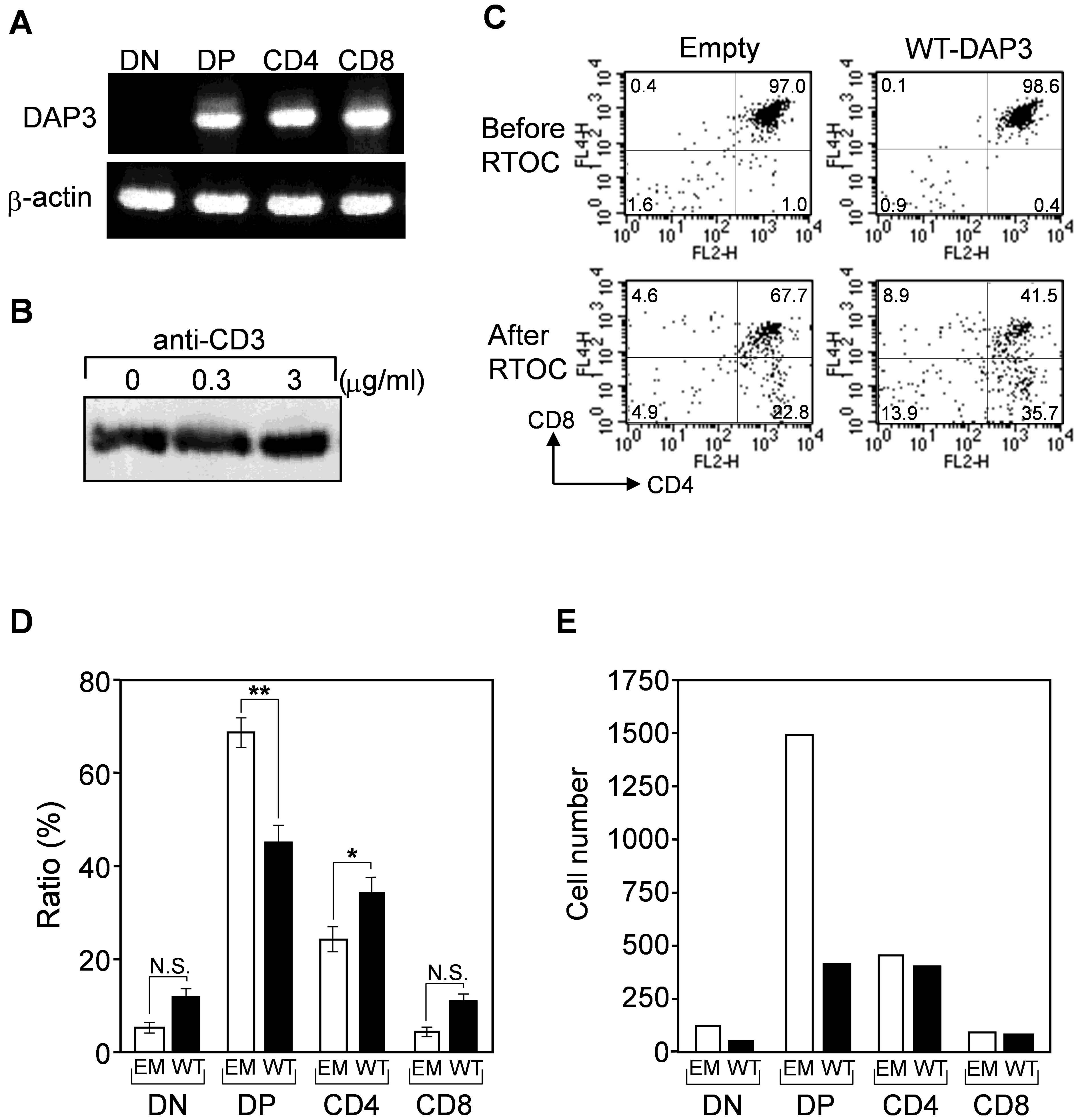
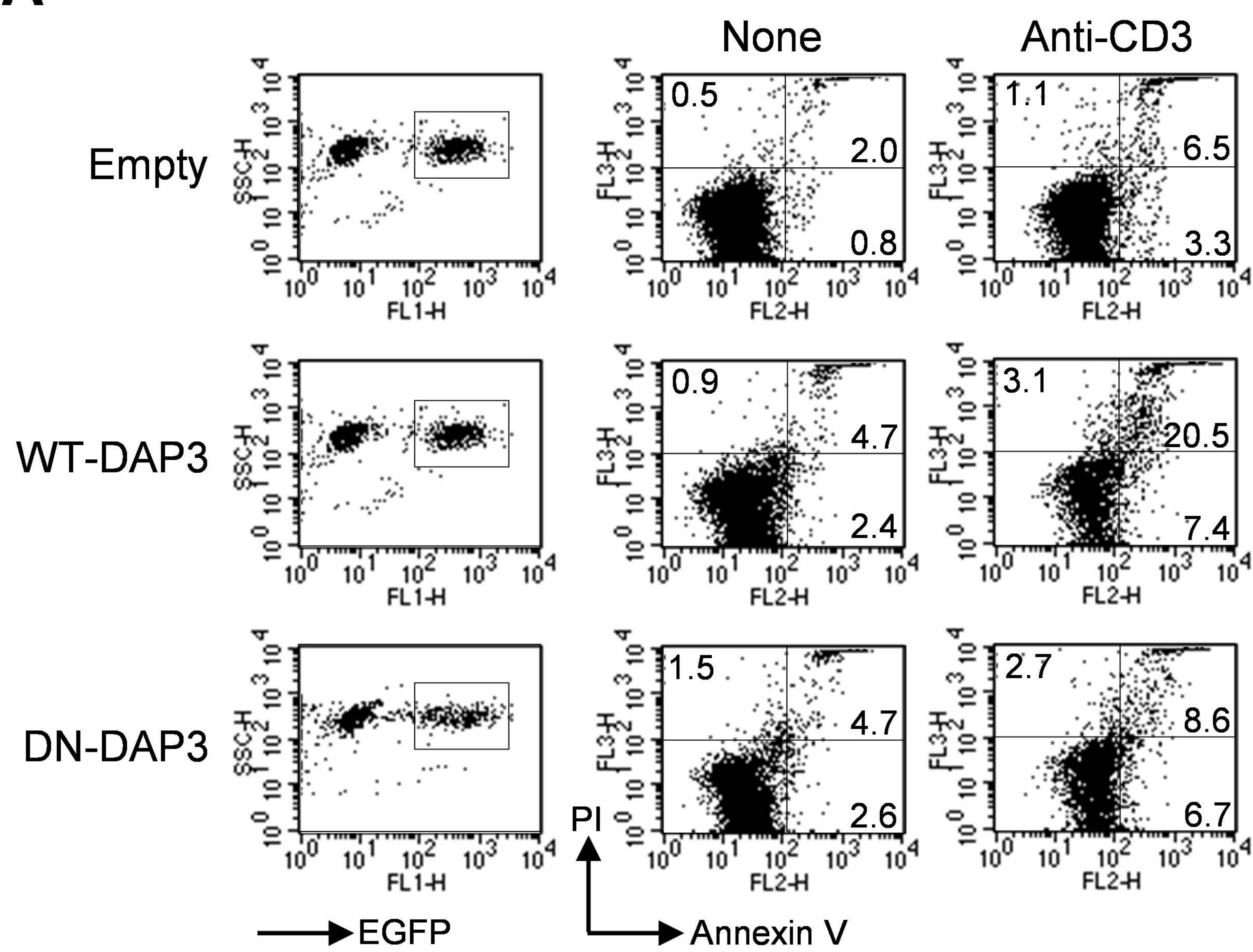


Fig. 2

A



B

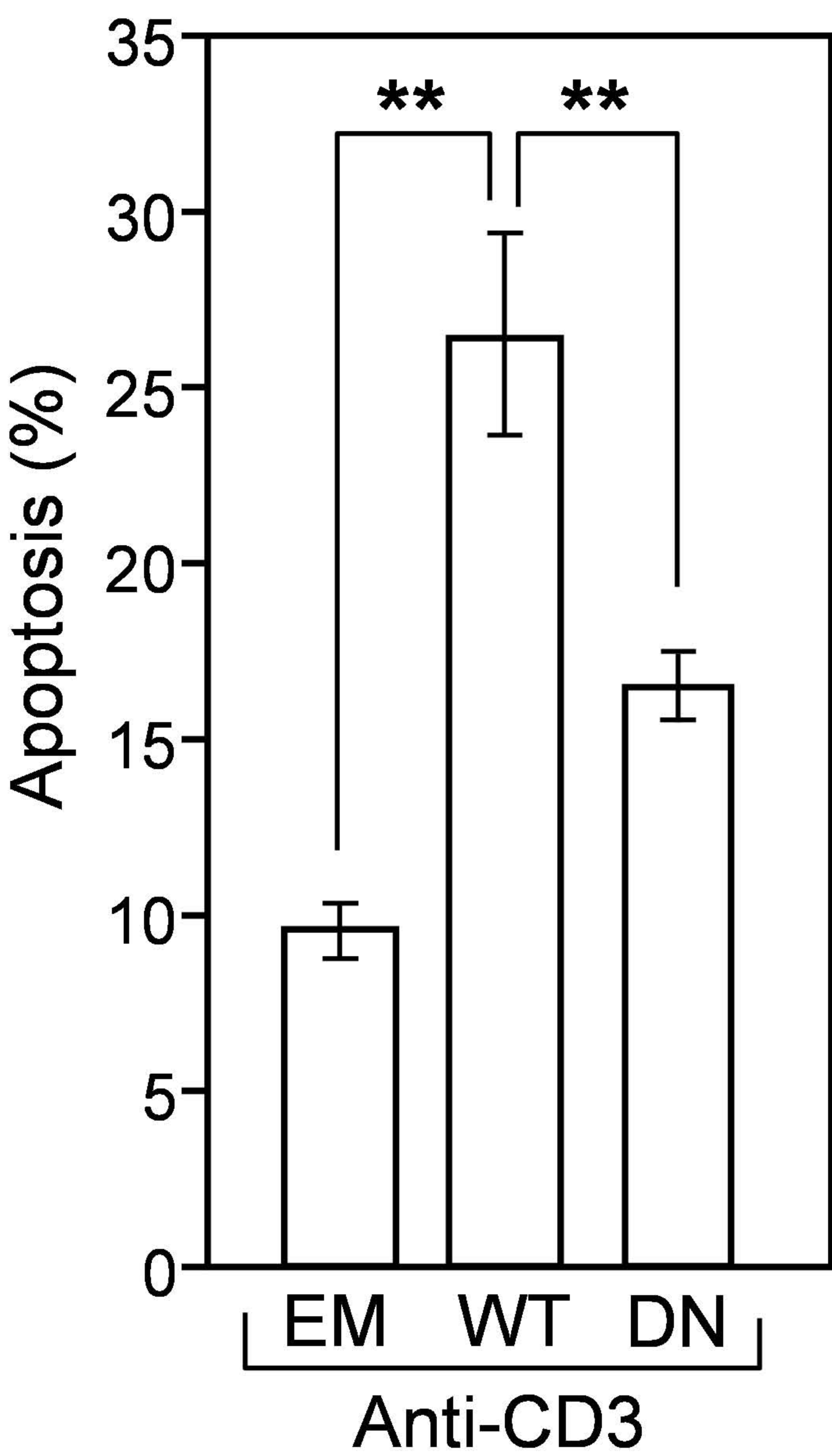


Fig. 3

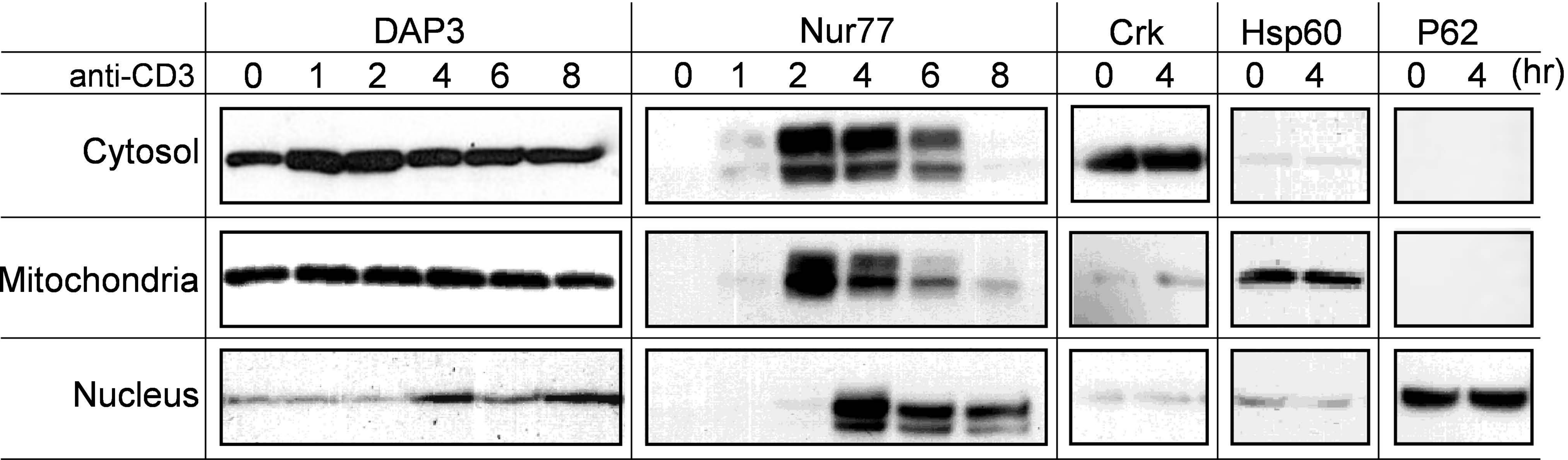


Fig. 4

