

T-cell Development and Function Are Modulated by Dual Specificity Phosphatase DUSP5*[§]

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Interleukin-2 (IL-2) is a pleiotropic cytokine that regulates lymphocyte proliferation and peripheral tolerance. IL-2 activates mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase, and signal transducer and activator of transcription (STAT) pathways and modulates expression of target genes. Systematic analysis of IL-2 target genes has revealed regulation of potential feedback inhibitors of IL-2 signaling, including several suppressor of cytokine signaling (SOCS) family members as well as MAPK pathway-regulating dual specificity phosphatases (DUSPs). Here we have evaluated the *in vivo* actions of DUSP5, an extracellular signal-regulated kinase 1/2 (ERK1/2)-specific phosphatase, by generating transgenic mice overexpressing DUSP5 within the lymphoid compartment. We show that transgenic DUSP5 expression results in a block in thymocyte development at the double positive stage. We also demonstrate that DUSP5-expressing mature T cells exhibit decreased IL-2-dependent proliferation and defective IL-2-mediated induction of genes. Finally, DUSP5 transgenic mice develop autoimmune symptoms, suggesting a role for the MAPK pathway in the regulation of tolerance. Thus, proper regulation of DUSP5 activity is critical for normal immune system development, IL-2 actions, and tolerance.

Interleukin-2 (IL-2)⁴ is a cytokine with pleiotropic actions (1). IL-2 was originally characterized as a T-cell growth factor (2), but subsequently was shown to mediate activation-induced cell death (3), to support the development and maintenance of

CD4⁺CD25⁺ regulatory T cells involved in the regulation of peripheral tolerance (4), to expand CD8⁺ T cells during secondary immune responses (5), and to have a range of actions beyond T cells as well (6).

IL-2 mediates its biologic actions by triggering a signaling cascade after binding to intermediate or high-affinity IL-2 receptors (IL-2R) (7–9). This causes the phosphorylation and activation of receptor-associated tyrosine kinases, Jak1 and Jak3. These in turn mediate the phosphorylation of tyrosine residues in the IL-2R β cytoplasmic domain that serve as docking sites for Shc, which couples to the Ras/mitogen-activated protein kinase (MAPK) pathway, and signal transducer and activator of transcription (STAT) proteins. IL-2 also activates phosphatidylinositol 3-kinase. Collectively, these pathways contribute to T-cell survival and proliferation and the generation of regulatory T cells (8).

The different signaling pathways regulate the actions of IL-2 in part by regulating expression of IL-2 target genes, such as IL-2R α , cyclin D2, Pim-1, Bcl-2, and granzyme B (8). Interestingly, IL-2 induces the expression of several feedback inhibitors of signaling, including suppressors of cytokine signaling family members SOCS1 and CIS, which provide general inhibitory signals or more specifically inhibit Stat5 proteins, respectively (10–12), as well as the dual specificity phosphatases DUSP5 and DUSP6 (13, 14), which can inhibit the activity of extracellular signal-regulated kinases 1/2 (ERK1/2), suggesting careful control by IL-2 of MAPK activity.

MAPKs are evolutionarily conserved serine-threonine kinases that convert extracellular stimuli to intracellular signaling events, regulating cellular processes such as proliferation, differentiation, and apoptosis (15, 16). Four major groups of MAPKs have been identified, including ERKs (ERK1, ERK2, and ERK5), c-Jun N-terminal kinases (JNK; JNK1, JNK2, and JNK3), p38 MAPKs (p38 α , p38 β , p38 γ and p38 δ), and ERK5/Big MAP kinase 1 (BMK1). MAPKs are activated by a phosphorylation cascade that involves activation of MAPK kinases (MAPKK; MEK1, MEK2, MKK3–7) by extracellular activators including growth factors, cytokines, or oxidative stress via distinct signaling molecules such as Ras and Raf. Distinct MAPKKs activate different MAPKs. For example, MEK1 and MEK2 activate ERK1 and ERK2, MKK3 and MKK4 activate p38 MAPK, MKK4 and MKK7 activate JNK, and MKK5 activates ERK5. The substrates of MAPKs include transcription factors such as Elk-1, ATF2, and c-Jun, as well as other signaling molecules such as ribosomal S6 kinases (Rsk) (15).

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⁴ The abbreviations used are: IL, interleukin; MAPK, mitogen-activated protein kinase; STAT, signal transducer and activator of transcription; ERK, extracellular signal-regulated kinase; WT, wild type; FBS, fetal bovine serum; PBS, phosphate-buffered saline; JNK, c-Jun N-terminal kinase; DUSP, dual specificity phosphatase.

MAPKs are essential for normal immune function, contributing to T-cell development and function (17, 18). For example, p38 MAPK negatively regulates early T-lymphocyte development in the thymus (19), ERK1/2 have an important role in T-cell positive selection (20, 21), and JNK is needed for T-lymphocyte negative selection (22). JNK-deficient T cells are defective in IFN- γ production and Th1 cell function (23). p38 MAPK also appears to regulate Th1 cytokine production (24, 25).

As noted above, MAPKs can be regulated by DUSPs. Ten distinct DUSPs with MAPK phosphatase activity (DUSP1, 2, 4, 5, 6, 7, 8, 9, 10, and 16) contain an N-terminal MAPK targeting domain and a C-terminal catalytic domain that can dephosphorylate both serine-threonine and tyrosine residues (26–28). Different DUSPs are specific for distinct MAPKs. For example, DUSP5 and DUSP6 are specific for ERK1 and ERK2, whereas DUSP8, DUSP10, and DUSP16 regulate JNK and p38 MAPK.

We identified DUSP5 as an IL-2-regulated gene that can act as a negative feedback regulator of the MAPK pathway in the context of IL-2 signaling (13). DUSP5 is a nuclear phosphatase that acts on ERK1/2 but not on JNK or p38 MAPK (29, 30). In T cells, DUSP5 is also induced by IL-7 and IL-15 but not by IL-4 (13). To clarify the actions of DUSP5, we generated transgenic (Tg) mice that overexpress DUSP5 in T cells. These mice exhibit defective thymic T-cell development, with a block at the double positive (DP) stage and decreased numbers of mature CD4⁺ and CD8⁺ T cells, indicating a role for MAPK in the establishment of these cell populations.

EXPERIMENTAL PROCEDURES

Generation of DUSP5 Tg Mice—A human DUSP5 cDNA with a C-terminal c-Myc epitope tag (kindly provided by Dr. Jack E. Dixon) was cloned between the XbaI and ClaI sites of the pHSE vector containing the H2-K^b promoter, β -globin exons 2 and 3 plus polyadenylation signal, and immunoglobulin heavy chain enhancer (Fig. 1A). The transgenic DNA fragment was directly microinjected into the pronuclei of fertilized eggs collected from superovulated female B6CBAF1/J mice (The Jackson Laboratory, Bar Harbor, ME). The eggs were cultured overnight to the two-cell stage of development and transferred into oviducts of pseudopregnant foster mothers according to standard procedures (31). Mice born to these foster mothers were screened for the presence of transgenes by PCR using specific primers (5'-ACCCACCTACACTACAAGTGGATC-3'; 5'-TGTGGAGGGCAGGATCTCAGATTC-3'). Two independent founder lines were further bred and backcrossed to the C57Bl/6J genetic background. Protein expression was confirmed by Western blotting using polyclonal antibodies to DUSP5 that we generated (BSYN4105). We did not observe any sex-related differences between the WT and DUSP5 Tg mice. Both Tg lines had similar phenotypes. All experiments were performed under protocols approved by the NHLBI Animal Care and Use Committee and followed National Institutes of Health guidelines.

Anti-DUSP5 Polyclonal Antibodies—A C-terminal peptide of human DUSP5 (NH₂-GHLQTLSPDMQGYC-OH), which has 93.3% homology sequence with mouse DUSP5, was used to immunize rabbits (at BioSynthesis Inc., Lewisville, TX). Six weeks later, rabbit sera were titrated against the immobilized

antigenic peptide, and antibody-producing rabbits were sacrificed 17 weeks after immunization. We evaluated pre-immune and specific sera by Western blotting using a GST-hDUSP5 fusion protein and sequentially purified specific antibodies using columns of immobilized antigenic peptide (CarboxyLink Kit, Pierce) and Dynabeads Protein A (DynaL Biotech, Lake Success, NY). Purified rabbit polyclonal antibodies (BSYN4105) were used to detect DUSP5 expression in freshly isolated mouse total thymocytes, purified splenic T cells (pan T cell isolation kit, Miltenyi Biotec, Auburn, CA) (95–97% purity), and purified splenic B cells (B220 MicroBeads, Miltenyi Biotec) (95–97% purity).

Western Blot Analysis—Cells were lysed in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P40, 1 mM Na₃VO₄, 1 mM EDTA, plus Complete Protease Inhibitor Mixture (1 minitab for 10 ml; Roche Applied Science, Mannheim, Germany) or in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.25% sodium deoxycholate, 1 mM NaF, 1 mM EGTA, plus 1% phosphatase inhibitor cocktails 1 and 2 (Sigma). Total protein extracts were combined with 4 \times NuPAGE LDS sample buffer (Invitrogen, Carlsbad, CA), supplemented with 50 mM Tris(2-carboxyethyl) phosphine hydrochloride, and boiled for 5 min. The samples were loaded on NuPAGE Bis-Tris 10% or 4–12% polyacrylamide gels and electrophoresis performed using NuPAGE MOPS running buffer (Invitrogen). Proteins were transferred to Immobilon-P or Immobilon-FL membranes (Millipore, Bedford, MA). Membranes were blocked with 5% nonfat milk in TBS (Bio-Rad) for anti-DUSP5 (BSYN4105) and anti-actin (sc-1615, Santa Cruz Biotechnology, Santa Cruz, CA) blots or 10% bovine serum albumin in TBS for anti-ERK1/2 antibodies (9101, Cell Signaling Technology, Danvers, MA; sc-7383 and sc-94-G, Santa Cruz Biotechnology), anti-Stat5 antibodies (9351, Cell Signaling Technology; S21520, Transduction Laboratories, BD Biosciences, San Jose, CA), and anti-Akt antibodies (4058 and 9272, Cell Signaling Technology). The membranes were incubated with primary antibody, washed, and incubated either with horseradish peroxidase-conjugated secondary antibody (NA934, GE Healthcare, Piscataway, NJ; sc-2304, Santa Cruz Biotechnology), and chemiluminescence was used for detection (SuperSignal West Pico Luminol, Pierce), or with Alexa-Fluor-680-conjugated secondary antibody (A10043, A21058, and A21084, Molecular Probes, Invitrogen), and infrared signals were detected using an Odyssey system (LI-COR, Lincoln, NE).

Flow Cytometry—Single cell suspensions from thymus and spleen were stained and analyzed using a FACSort and a FACSCalibur with CellQuest software (BD Biosciences) or FlowJo software (FlowJo LLC, Ashland, OR). The following antibodies were used (all from BD Biosciences): anti-CD4-FITC, -PE, and -cychrome; anti-CD8 α -PE and -allophycocyanin; anti-CD25-FITC and -PE; anti-CD44-cychrome; anti-IL-2R β -PE; anti-TCR $\gamma\delta$ -FITC; anti-CD3-PE and -allophycocyanin; anti-B220(CD45R)-cychrome; anti-Syndecan-1 (CD138)-PE; and DX5(anti-CD49b)-PE. All flow cytometric data shown are representative of at least three independent experiments from each DUSP5 Tg line.

Cell Treatment and Activation—For ERK1/2 anti-CD3 ϵ activation assays, single cell suspensions (40 \times 10⁶/ml) of freshly

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enriched DP thymocytes (CD4 MicroBeads, Miltenyi Biotec) (95–97% purity) from WT and DUSP5 Tg mice were treated or not with anti-mouse-CD3 ϵ antibody (10 μ g/ml; clone 145-2C11) pre-cross-linked with goat-anti-hamster antibodies (10 μ g/ml; 107-005-142, Jackson ImmunoResearch Laboratories, West Grove, PA), and incubated at 37 °C for 10 min. Cells were lysed, and total protein extracts were assayed for ERK1/2.

For activation of splenic T lymphocytes, single cell suspensions of purified T cells (pan T cell isolation kit) (95–97% purity) or purified CD8 $^+$ cells (CD8 α MicroBeads, Miltenyi Biotec) (95–97% purity) from WT and DUSP5 Tg mice were treated for 3 days with plate-bound anti-CD3 ϵ (2 μ g/ml) and soluble anti-mouse-CD28 (1 μ g/ml; BD Biosciences) mAbs and cultured in complete medium (RPMI 1640 medium containing 10% FBS, 2 mM L-glutamine, and 1% penicillin/streptomycin) supplemented with hIL-2 (100 units/ml, Roche Applied Science) for a total time of 7 days. (Cells were diluted at 5×10^5 cells/ml on day 3 and day 5 in complete medium with IL-2.) Cells were then rested for 18 h at 1×10^6 cells/ml in complete medium before experiments.

For Stat5, Akt, and ERK1/2 IL-2-activation assays, single cell suspensions (12×10^6 /ml) of activated/rested splenic T cells from WT and DUSP5 Tg mice (see above) were treated or not with IL-2 (1,000 units/ml), and incubated at 37 °C for 5, 10, and 60 min. Cells were lysed, and total protein extracts were assayed for Stat5, Akt, and ERK1/2.

Thymocyte Depletion Assay—To analyze the role of DUSP5 in thymic selection events, we examined the *in vivo* depletion of DP thymocytes induced by anti-CD3 ϵ mAb. WT and DUSP5 Tg animals were injected intraperitoneally with PBS or 100 μ g of anti-CD3 ϵ in 200 μ l of PBS, and thymuses were harvested for counting and flow cytometric analysis 2 days after injection.

Proliferation Assays—Single cell suspensions of freshly isolated thymocytes, freshly purified splenic T lymphocytes, and activated splenic T cells (see above) from WT or DUSP5 Tg mice were cultured for 72 h (thymocytes and splenic T cells) or 48 h (activated splenic T cells) in 96-well flat-bottom plates, in 200 μ l/well at 2×10^5 cells/well (thymocytes) or 1×10^5 cells/well (freshly purified and activated splenic T cells). Where indicated, plate-bound anti-CD3 ϵ (10 μ g/ml) or IL-2 (100 units/ml) was used. Cells were pulsed with 1 μ Ci of [3 H]thymidine (6.7 Ci/mmol; PerkinElmer Life Sciences) for the last 24 h of culture.

mRNA Preparation, GeneChip Hybridization, and Microarray Data Analysis—Splenic CD8 $^+$ T cells from four WT and four DUSP5 Tg mice were purified and activated for 7 days, as described above. Cells were rested for 18 h, then restimulated with 100 units/ml IL-2 for 4 h. Total RNA was isolated using RNeasy (Qiagen, Valencia, CA) and processed to cRNA probes for GeneChip analysis following manufacturer's protocols (Affymetrix, Santa Clara, CA).

The probes were hybridized to GeneChip Mouse Genome 430A 2.0 Arrays (Affymetrix), washed, and scanned (Hewlett Packard, GeneArray scanner G2500A). Signal values and calls of "present," "marginal," or "absent" were generated using GeneChip Operating Software 1.4.0 (Affymetrix).

For each array, a log transform was applied to the probe set signals followed by normalization to the array median log signal. The paired Student's *t* test was applied separately to the

data for cells from WT and DUSP5 Tg mice to test for differential expression after IL-2 stimulation. These analyses were performed using the A-SCAN 3.0 computer program. Transcripts were defined as present if at least three of four experiments for a given condition had either a "present" or "marginal" call. An estimated false discovery rate of <20%, a fold change cutoff of 2.0, and a designation of "present" were used to define the sets of differentially expressed genes (32, 33).

Histology and Anti-DNA Antibody Assays—Mice were sacrificed and tissues fixed in neutral buffered formalin, embedded in paraffin, and sectioned at 4–6 microns. Slides were stained with hematoxylin and eosin.

Flat-bottom 96-well plates were coated with sheared salmon sperm DNA (10 μ g/ml in PBS, 50 μ l/well, overnight at 4 °C), washed with 0.05% Tween-20 in PBS, and blocked 1 h at room temperature using 10% FBS in PBS (200 μ l/well). Plate-bound DNA was then washed and incubated for 1 h at room temperature with WT or DUSP5 Tg mouse serum samples diluted in PBS with 10% FBS (200 μ l/well). After washing, wells were incubated 1 h at room temperature with horseradish peroxidase-conjugated anti-IgG1 or anti-IgM antibodies (2 μ g/ml in 10% FBS in PBS, 100 μ l/well; BD Biosciences). Plates were then extensively washed before the enzymatic reaction substrate was added (BD OptEIA, BD Biosciences). After development, the colorimetric reaction was stopped with 10% H $_3$ PO $_4$, and absorbance measured at 450 nm. Optical densities obtained from two dilutions of each mouse serum were used to generate graphic representation of data.

RESULTS

We previously showed that IL-2 regulates DUSP5 mRNA and protein levels in T lymphocytes (13, 14). To learn more about the actions of DUSP5, we generated Tg mice in which DUSP5 was under the control of the H2-K b promoter and immunoglobulin heavy chain enhancer (Fig. 1A), which directs expression in T, B, and NK cells (34, 35). We established two founder lines with DUSP5 expression in both thymus and spleen (representative data from one founder line are shown, Fig. 1B), which did not affect total thymic or splenic cell numbers (Fig. 1C).

Because no gross abnormalities in thymic or splenic development were observed (Fig. 1C), we further investigated a possible effect of the DUSP5 transgene on lymphocyte development by examining subpopulations. The DUSP5 Tg mice in fact had diminished CD4 $^+$ and CD8 $^+$ single positive (SP) thymocytes, with a corresponding relative increase in the frequency of DP lymphocytes (Fig. 2A). The numbers of early double negative (DN) DN1 to DN4 thymocytes were similar to those in WT animals (data not shown), but consistent with the reduced number of SP thymocytes, DUSP5 Tg mice had diminished levels of CD4 $^+$ and CD8 $^+$ splenic T cells (Fig. 2B). Furthermore, a higher fraction of CD8 $^+$ T cells from DUSP5 Tg mice exhibited a CD44 hi IL-2R β^{hi} memory phenotype than was found in WT animals (Fig. 2C). An increased proportion of the CD4 $^+$ T cells from DUSP5 Tg animals also displayed an activated memory phenotype (data not shown). The numbers of TCR $\gamma\delta$ T cells (Fig. 2D) and NK cells (Fig. 2E) were only slightly increased in DUSP5 Tg mice. The percentage of plasma cells was more

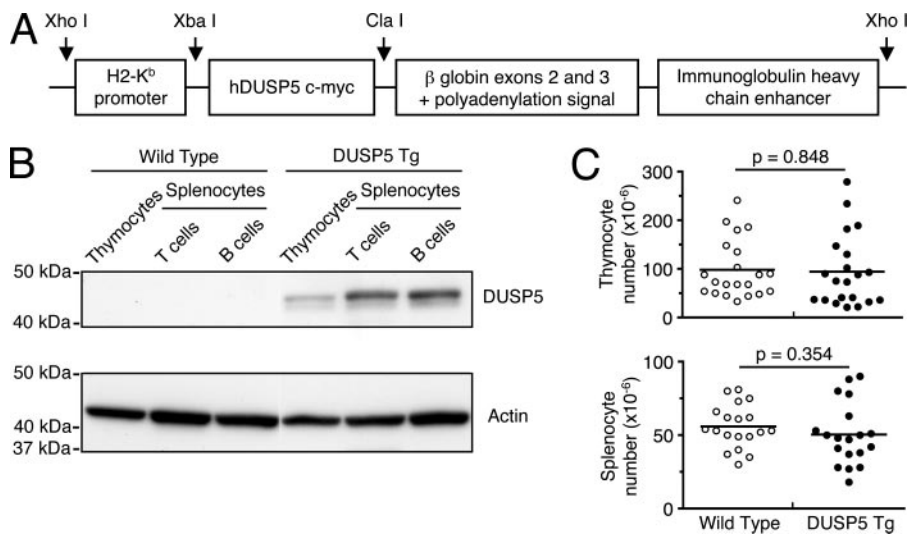


FIGURE 1. Generation of DUSP5 Tg mice. A, schematic of the DUSP5 transgenic construct. Human DUSP5 cDNA with a C-terminal Myc epitope tag was cloned into the pHSE transgenic vector between XbaI and ClaI sites. B, Western blot analysis of total cell lysates showing the transgenic overexpression of DUSP5 in thymocytes and splenic T and B cells (10 μ g of total protein extract per lane). C, similar total thymocyte and splenocyte cell numbers in WT and DUSP5 Tg mice. Cell counts were obtained on age- and sex-matched animals.

Because thymocyte development in DUSP5 Tg mice was blocked at the DP stage, we tested the effect of DUSP5 transgenic expression on ERK1/2 phosphorylation in thymocytes (Fig. 3A). Enriched DP thymocytes from WT and DUSP5 Tg mice were activated with anti-CD3 ϵ , and the phosphorylation of ERK1/2 was determined by Western blotting. Whereas anti-CD3 ϵ treatment induced an increase in ERK1/2 phosphorylation in WT cells, this was considerably reduced in cells from DUSP5 Tg mice, indicating that transgenic DUSP5 indeed blocked ERK1/2 activation. Because ERKs were previously reported to affect positive but not negative selection (20, 21), we next investigated the ability of anti-CD3 ϵ antibodies to mediate the depletion of immature DP thymocytes (22). Both WT and DUSP5 Tg mice exhibited similar reductions in total thymocyte cell numbers after anti-CD3 ϵ intraperitoneal injection (Fig. 3B), consistent with the lack of a role for DUSP5 and ERKs in this depletion.

Because ERK activation is associated with proliferation, we hypothesized that the decreased number of mature CD4⁺ and CD8⁺ T cells in DUSP5 Tg mice (Fig. 2B) might have resulted from diminished T-cell homeostatic proliferation, a process that involves both TCR- and cytokine-dependent signals. It is known that cytokines that share the common cytokine receptor γ chain (γ_c) are important for this process (37–39). As total thymocytes from ERK1-deficient mice showed diminished proliferation in response to plate-bound anti-CD3 ϵ (21), we first compared the effect of TCR stimulation and IL-2 on thymocytes from WT or DUSP5 Tg animals. Freshly isolated thymocytes from DUSP5 Tg mice showed impaired proliferation in response to TCR stimulation with or without IL-2, as compared with cells from

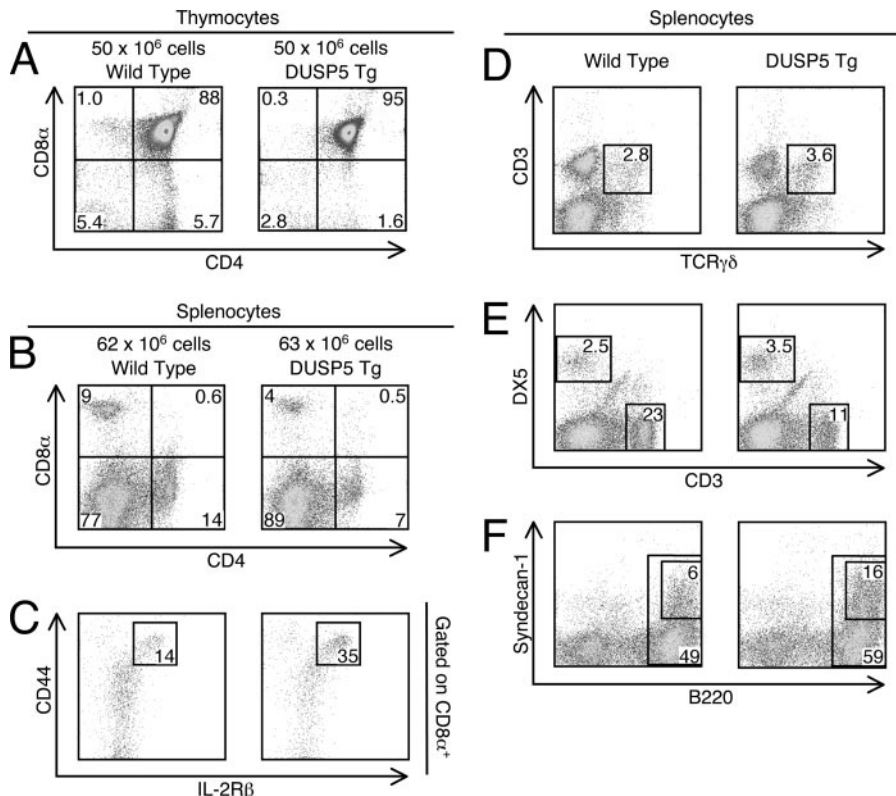


FIGURE 2. Defective T-cell development in DUSP5 Tg mice. Flow cytometric analysis of thymocytes from DUSP5 Tg mice shows a block in the DP to CD4⁺ and CD8⁺ SP T-cell transition (A). DUSP5 Tg mice have decreased numbers of CD4⁺ and CD8⁺ splenocytes (B), with CD8⁺ T cells containing an increased proportion of IL-2R β^{hi} CD44 $^{\text{hi}}$ memory phenotype cells (C). The numbers of TCR $\gamma\delta$ cells (D) and NK cells (E) were similar to modestly increased, and the number of Syndecan-1-expressing B220⁺ B lymphocytes was increased (F).

markedly elevated in the DUSP5 Tg mice (Fig. 2F), supporting a role for ERK1/2 in inhibiting plasma cell differentiation, as was recently suggested (36). We did not observe consistent differences in the frequency of CD4⁺CD25⁺ regulatory T cells (data not shown).

WT mice (Fig. 4A). Because reduced numbers of SP or increased numbers of DP thymocytes from DUSP5 Tg mice (Fig. 2A) could have been responsible for the impaired proliferation, we further investigated the effect of TCR-stimulation and IL-2 on mature T lymphocytes purified from WT or DUSP5 Tg

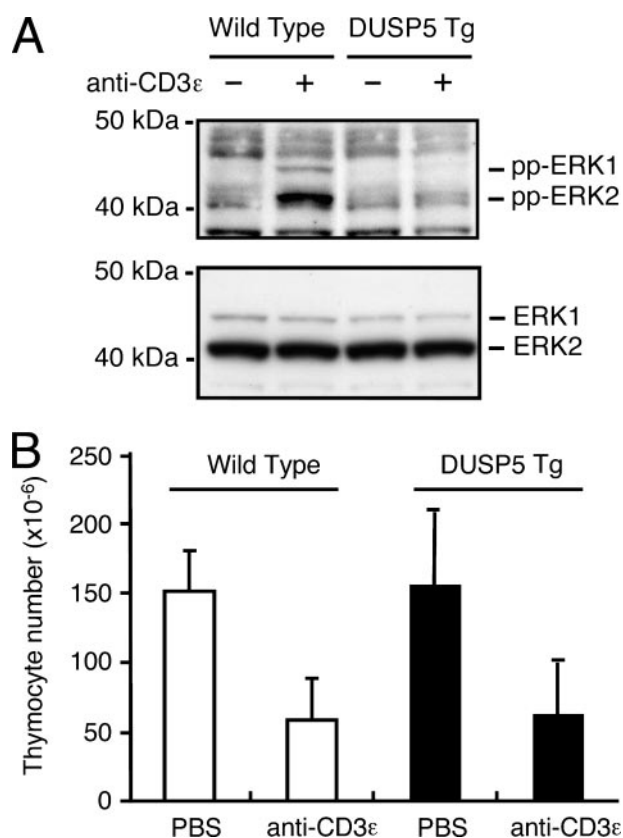


FIGURE 3. Transgenic DUSP5 modulates thymocyte ERK signaling but does not affect anti-CD3 ϵ -induced thymocyte depletion. *A*, effect of DUSP5 on TCR-driven phosphorylation of ERK1/2. Enriched DP cells from WT and DUSP5 Tg animals were stimulated or not (+ or -, respectively) with anti-CD3 ϵ antibodies (10 μ g/ml) for 10 min at 37 °C. Total ERK1/2 and phosphothreonine-phosphotyrosine ERK1/2 were detected by Western blotting (40 μ g of total protein extract per lane). *B*, WT and DUSP5 Tg mice were injected intraperitoneally with PBS or anti-CD3 ϵ , and the thymuses were examined on day 2 (three mice in each group). Both WT and DUSP5 Tg mice injected with anti-CD3 ϵ showed a significant decrease in thymocyte number.

animals. Although freshly isolated splenic T cells from WT or DUSP5 Tg mice showed similar proliferation in response to TCR stimulation plus or minus IL-2 (Fig. 4*B*), after TCR activation and IL-2 stimulation, T cells from DUSP5 Tg mice exhibited less proliferation than WT controls in response to IL-2 (Fig. 4*C*).

IL-2 is known to activate several signaling pathways in addition to Ras/MAPK, including the JAK-STAT and phosphatidylinositol 3-kinase/Akt pathways. To examine the signaling pathways that might be defective in DUSP5 Tg T cells, we first determined by Western blotting the phosphorylation status of Stat5, Akt, and ERK1/2 in activated/rested splenic T cells stimulated with IL-2. Although the IL-2-induced phosphorylation of Stat5 and Akt was relatively similar in T cells from WT or DUSP5 Tg mice, IL-2-induced ERK1/2 phosphorylation was lower in DUSP5 Tg than in WT T cells (Fig. 4*D*). To further study the signaling pathways that might be defective in DUSP5 Tg T cells, we isolated RNA from IL-2-stimulated CD8⁺ T cells derived from WT and DUSP5 Tg mice, and analyzed their gene expression profiles using GeneChip Mouse Genome 430A 2.0 Arrays (Affymetrix; complete data are in supplemental Table S1). We identified 974 genes whose expression was regulated by IL-2 in WT mice (supplemental Table S2), some of which are

known Stat5 target genes (13) and were detected in T cells from both WT and DUSP5 Tg mice (expression data on select genes is shown in Fig. 4*E*, upper panel). Interestingly, ~67% (655 of the 974 genes) were not differentially expressed after IL-2 stimulation in T cells from DUSP5 Tg mice (supplemental Table S3). We hypothesize that at least some of these 655 IL-2 target genes are directly or indirectly regulated by ERK1/2 (expression data on select genes not induced by IL-2 in T cells from DUSP5 Tg mice are shown in Fig. 4*E*, lower panel). Additional work will be required to determine which of these genes potentially contribute to the defects observed in the DUSP5 Tg mice.

Above, we have described transgenic DUSP5 expression as affecting single positive T-cell development and regulating a range of target genes. It was interesting that T cells from DUSP5 Tg mice also tended to exhibit an activated memory phenotype and that there was an increase in the number of plasma cells, based on staining with Syndecan-1 (Fig. 2*F*). Examination of mice in the colony unexpectedly revealed the development of alopecia in a number of animals that was associated with skin ulceration (Fig. 5*A*). Within a population of 238 mice (120 WT mice and 118 DUSP5 Tg mice), 34 Tg animal developed skin disease at an average age of 14 \pm 3 weeks (from 9 to 21-week-old). Other DUSP5 Tg mice did not show skin ulceration at the time they were sacrificed (average age of 15 \pm 8 weeks; from 5 to 37-week-old). None of the 120 WT littermates showed skin ulceration at the time they were sacrificed (average age of 16 \pm 8 weeks; from 4 to 27-week-old). Moreover the DUSP5 Tg mice with skin ulceration had elevated serum levels of anti-DNA specific IgG1 antibodies, although anti-DNA IgM levels were not altered (Fig. 5*B*; 10 WT mice versus 12 DUSP5 Tg mice), consistent with an autoimmune/inflammatory process. Histologic examination of 7 DUSP5 Tg mice versus 6 WT mice (Fig. 5, *C–F*) revealed ulcerative dermatitis with lesions and epidermal damage, inflammatory cell infiltration, and adjacent epithelial hyperplasia (Fig. 5, *D* versus *C*). The transgenic mice with skin ulceration also developed glomerular lesions in their kidneys (Fig. 5, *F* versus *E*) and were not responsive to antibiotics (trimethoprim/sulfamethoxazole, cephalexin) or anti-inflammatory treatments (diphenhydramine, hydrocortisone); death typically occurred within 2 months of development of the skin disease. These data reveal that DUSP5 transgenic expression indeed predisposes to an autoimmune/inflammatory process.

DISCUSSION

We previously observed that IL-2 regulates the mRNA levels of several potential feedback regulators of signaling including DUSP5 (13, 14). Here we have further studied the role of DUSP5 in T-cell development and function by generating DUSP5 Tg mice. These mice show decreased numbers of both thymic SP and splenic CD4⁺ and CD8⁺ lymphocytes. Consistent with the inhibition of ERK1/2 by DUSP5, ERK1/2 has been shown to be important for positive selection.

The negative feedback signals involved in the regulation of MAPK activity in the thymus have not been well-characterized. However, MAPK phosphatases are good candidates for this role. Many MAPK phosphatases belong to the family of dual specificity phosphatases, which exhibit both patterns of tissue specific expression as well as distinct specificities against differ-

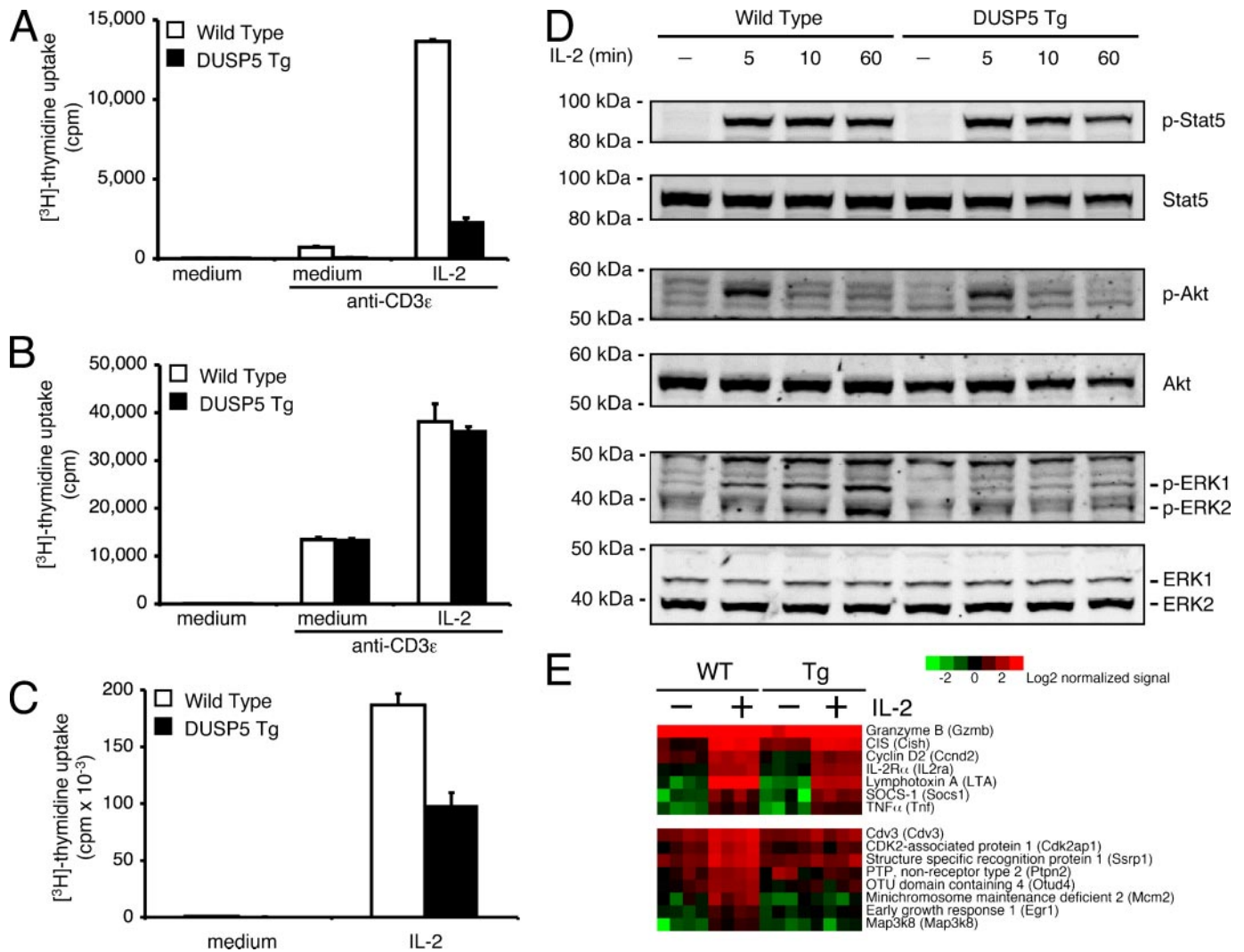


FIGURE 4. Activated DUSP5-expressing T cells show decreased IL-2-dependent proliferation, lower activation of ERK1/2 by IL-2, and defective IL-2 induction of specific genes. *A*, freshly isolated thymocytes from DUSP5 Tg mice show decreased proliferation in response to TCR stimulation and IL-2. Cells were stimulated or not with anti-CD3 ϵ (10 μ g/ml) and IL-2 (100 units/ml) for 72 h, and proliferation was measured by [3 H]thymidine incorporation. The results shown are representative of six WT and DUSP5 Tg mice and three independent experiments. *B*, freshly purified splenic T cells from WT or DUSP5 Tg mice show similar proliferation in response to TCR stimulation and IL-2. Cells were stimulated or not with anti-CD3 ϵ (10 μ g/ml) and IL-2 (100 units/ml) for 72 h, and proliferation was measured by [3 H]thymidine incorporation. The results shown are representative of four WT and DUSP5 Tg mice and two independent experiments. *C*, activated splenic T cells from DUSP5 Tg mice show decreased IL-2-stimulated proliferation. Splenic T cells were purified and activated as described under "Experimental Procedures," rested, and restimulated or not with IL-2 (100 units/ml) for 48 h. Cell proliferation was measured by [3 H]thymidine incorporation. The results shown are representative of four WT and DUSP5 Tg mice and two independent experiments. *D*, activated splenic T lymphocytes show IL-2-induced activation of Stat5 and Akt in cells from WT or DUSP5 Tg mice but decreased phosphorylation of ERK1/2 in cells from DUSP5 Tg mice. Splenic T cells were purified and activated as described under "Experimental Procedures," rested, and restimulated or not with IL-2 (1,000 units/ml). Total and phosphorylated Stat5, Akt, and ERK1/2 were detected by Western blotting (40 μ g of total protein extract per lane). The results shown are representative of two WT and DUSP5 Tg mice. *E*, activated splenic CD8 $^+$ T cells show defective induction of select IL-2-regulated genes. Splenic CD8 $^+$ T cells were purified from four WT and DUSP5 Tg animals and activated as described under "Experimental Procedures." Cells were rested and restimulated or not (+ or -, respectively) with 100 units/ml of IL-2 for 4 h. The expression patterns of resting and IL-2-stimulated T cells from WT and DUSP5 Tg mice were analyzed. Shown are expression data (Affymetrix signal values) for a selection of IL-2 target genes known to be regulated by Stat5 (*upper panel*) and some genes for which expression is more induced in T lymphocytes from WT mice than from DUSP5 Tg animals (*lower panel*). *Green squares* correspond to genes with relatively low-level expression and *red squares* to genes with relatively high-level expression, compared with the average expression of all genes on the microarray. The signal data were log $_2$ -transformed, and the heat map was generated using the Cluster 3.0/TreeView program (58). A log $_2$ value of 0 in the scale corresponds to an Affymetrix signal value of 230.

ent MAPKs (28, 40). The thymic expression of different DUSPs was recently evaluated, and DUSP1, 2, 5, 6, 10, and 11 were observed to be expressed at a relatively high level (41). DUSP5 and DUSP6 showed TCR-dependent inducibility in response to stimulation with anti-CD3 ϵ , with stronger regulation of DUSP5. DUSP5 is specific for ERK1/2, thus making it a potential *in vivo* regulator of positive selection. Indeed, we found that DUSP5 overexpression blocked thymocyte development at the

DP stage, suggesting that the ERK1/2 pathway is not essential to DP to DP transition or that low activation of ERK1/2 is sufficient for pre-TCR signaling. In contrast, TCR-dependent thymocyte depletion proceeded well in the presence of transgenic DUSP5, suggesting that ERK1/2 does not regulate negative selection.

Lymphocyte homeostasis is controlled by both TCR- and cytokine-dependent signals (37, 42). IL-7 and TSLP regulate the

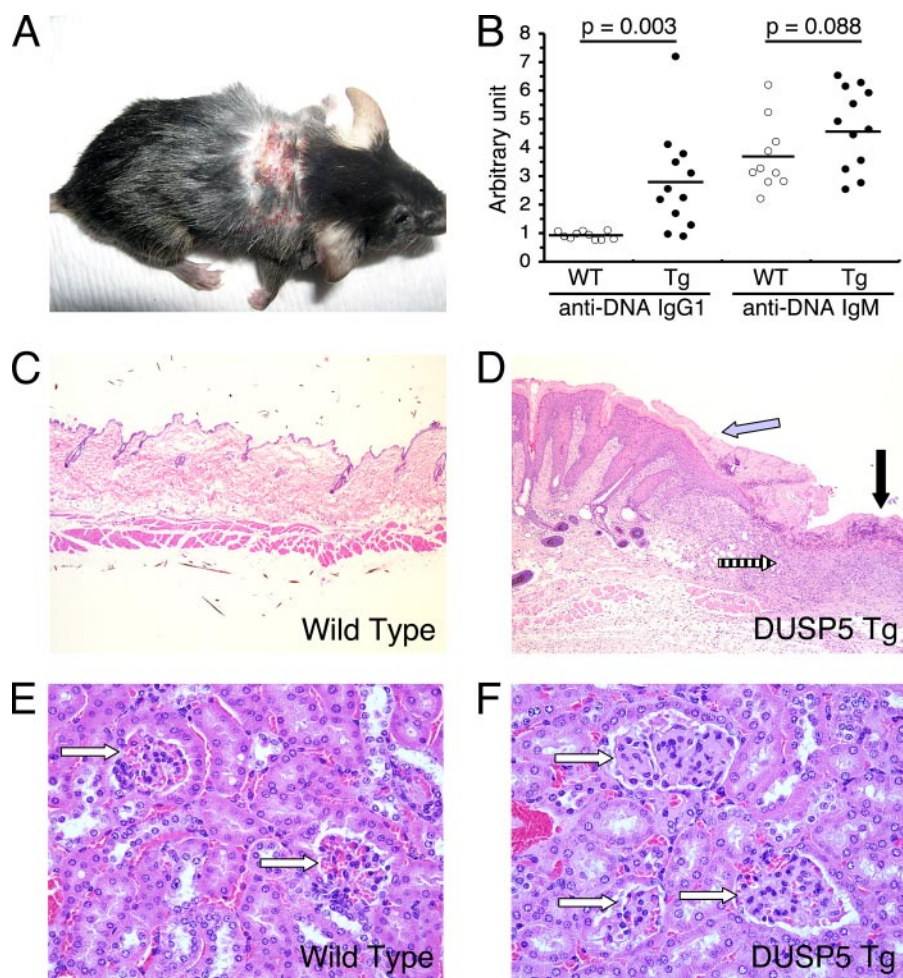


FIGURE 5. Autoimmune symptoms of DUSP5 Tg mice. DUSP5 Tg mice develop skin lesions within areas of alopecia (A) and show increased plasma IgG1 anti-DNA antibody levels (B), as well as morphologic changes of skin (C and D) and kidney glomeruli (E and F) (indicated by white arrows). C, WT mouse skin (original magnification: $\times 40$). D, DUSP5 Tg mouse skin shows ulcerative dermatitis with lesion (black arrow), inflammatory cell infiltration (hatched arrow), and adjacent epithelium hyperplasia (gray arrow). E, WT mouse kidney glomeruli appear very vascular, and glomerular capillaries contain many erythrocytes (red-stained cells without purple-stained nuclei) (original magnification: $\times 400$). F, DUSP5 Tg mice with skin abnormalities also exhibit advanced kidney glomerular lesions. Glomerular capillaries contain lower numbers of erythrocytes and display abundant infiltrates (pink-stained material).

homeostatic growth of CD4⁺ T cells (43–45), whereas IL-7 and IL-15, and to some extent IL-2 have been suggested to regulate the homeostasis of naïve and memory CD8⁺ T cells (46–48). DUSP5 Tg mice showed decreased numbers of splenic CD4⁺ and CD8⁺ T cells, which may reflect, at least in part, decreased thymic output. However, we hypothesized that DUSP5 might also interfere with homeostatic growth. In support of this idea, activated T cells from DUSP5 Tg mice exhibited decreased proliferation in response to IL-2, associated with a lower activation of ERK1/2 following IL-2 stimulation. To evaluate if the growth defect was associated with defective induction of genes, we investigated the IL-2 regulation of gene expression in the CD8⁺ T cells from DUSP5 Tg animals. We found that DUSP5 transgenic expression altered the expression of most genes regulated by IL-2 in activated T cells from WT mice; however, as anticipated, these genes did not include known targets of Stat5 (Fig. 4E, upper panel), which is a well characterized mediator of IL-2-dependent transcription. Several genes whose expression was affected have been shown to regulate cell proliferation, includ-

ing *Egr1* and *Map3k8* (Fig. 4E, lower panel), which are known transcriptional targets of MAPK in other cellular systems (49, 50).

Splenic T cells from DUSP5 Tg mice showed an activated memory phenotype, with enhanced expression of IL-2R β and CD44 on both CD4⁺ and CD8⁺ T cells. As the mice aged, they started to develop autoimmune symptoms. Analysis of histological sections revealed infiltration in both skin and kidneys, suggesting an autoimmune response. We also found elevated levels of anti-DNA antibodies (IgG1) in the serum of DUSP5 Tg mice. Similar findings have been described in mice defective in Ras-Grp1 function (51, 52), suggesting that ERK1/2 activity in T cells is essential to prevent autoimmunity. The molecular mechanism underlying autoimmune manifestations in DUSP5 Tg mice is not clear but could reflect defective selection events in the thymus. ERK1/2 activity may also be required for sustaining proper peripheral tolerance, as IL-2 signaling is required for maintaining the CD4⁺CD25⁺ regulatory T-cell pool. The number of CD4⁺CD25⁺ regulatory T cells was not decreased in DUSP5 Tg mice, arguing against a major defect in the regulatory T-cell function. It is possible that ERK1/2 activation is involved in tuning the sensitivity of potentially autoreactive T cells

to apoptosis. Consistent with this hypothesis, T cells from DUSP5 Tg mice exhibited defective regulation of several apoptosis-related genes, including for example, the gene encoding the anti-apoptotic protein, apoptosis inhibitor 5 (supplemental Table S3).

Roles for certain MAPK phosphatases in immune system function have been recently demonstrated. For example, mice defective in DUSP1 show a defect in the expression of a subset of lipopolysaccharide-responsive genes and altered response to endotoxin shock (53–55). DUSP2 deficiency results in increased JNK activity and decreased p38 MAPK activity with reduced inflammatory responses, whereas DUSP10-deficient mouse cells exhibit selectively increased JNK activity with enhanced innate and adaptive immune responses (56, 57).

In this study, we have used mice expressing transgenic DUSP5, an ERK1/2-specific phosphatase that is expressed both in thymocytes and mature T cells and is induced by TCR signals as well as by IL-2, IL-7, and IL-15. Our findings indicate that DUSP5 via its action on ERK1/2 can influence positive but not

negative selection, and that proper regulation of ERK1/2 activity is needed for lymphocyte homeostatic growth signals as well as for preventing autoimmunity.

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