

Activation of the p38 Mitogen-activated Protein Kinase Pathway Arrests Cell Cycle Progression and Differentiation of Immature Thymocytes In Vivo

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Abstract

The development of T cells in the thymus is coordinated by cell-specific gene expression programs that involve multiple transcription factors and signaling pathways. Here, we show that the p38 mitogen-activated protein (MAP) kinase signaling pathway is strictly regulated during the differentiation of CD4⁻CD8⁻ thymocytes. Persistent activation of p38 MAP kinase blocks fetal thymocyte development at the CD25⁺CD44⁻ stage in vivo, and results in the lack of T cells in the peripheral immune system of adult mice. Inactivation of p38 MAP kinase is required for further differentiation of these cells into CD4⁺CD8⁺ thymocytes. The arrest of cell cycle in mitosis is partially responsible for the blockade of differentiation. Therefore, the p38 MAP kinase pathway is a critical regulatory element of differentiation and proliferation during the early stages of in vivo thymocyte development.

Key words: transgenic mice • thymocyte development • mitosis • apoptosis • T cells

Introduction

The generation of T cells in the thymus is mediated by a complex biological mechanism that combines differentiation, proliferation, death, selection, and lineage commitment. These processes require TCR signals together with signals delivered by cell–cell interactions and soluble factors provided by the thymic environment. Stem cells seed the thymus and differentiate into immature CD4⁻CD8⁻ double negative (DN)¹ thymocytes, which represent a minor population (3–5%) in the adult thymus. DN thymocytes express CD8 on the cell surface first, progressively acquire the expression of CD4 (CD8⁺CD4^{low}), and become CD4⁺

CD8⁺ thymocytes. The immature CD8⁺CD4^{low} population is clearly detected during fetal thymocyte development (embryonic days E16–18), but is almost undetectable in the adult thymus. Based on the expression of the cell surface markers CD25 (IL-2 receptor α chain), CD44, and c-Kit, the differentiation of DN thymocytes follows the sequence c-Kit⁺CD25⁻CD44⁺ (most immature), c-Kit⁺CD25⁺CD44⁺, c-Kit⁻CD25⁺CD44⁻, and c-Kit⁻CD25⁻CD44⁻ (for a review, see reference 1). Downmodulation of CD25 from the cell surface is associated with the expression of the pre-TCR, which is formed by the association of the TCR β chain with gp33/pre-T α . Signals mediated by the pre-TCR complex induce the differentiation of DN thymocytes into CD4⁺CD8⁺ double positive (DP) cells. DP cells undergo both positive and negative selection, which involve signals delivered by the interaction of the TCR with the corresponding MHC, and additional signals provided by stromal cells. As a result of these processes, and the downmodulation of either CD4 or CD8 coreceptors, DP thymocytes differentiate into mature single CD4⁺ or CD8⁺ thymocytes.

Impairments of the appropriate signals occurring during the generation of T cells in the thymus can lead to immune disorders. Defective deletion of autoreactive T cells in the

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¹Abbreviations used in this paper: BrdU, bromodeoxyuridine; DN, double negative; DP, double positive; ERK, extracellular signal regulatory kinase; FTOC, fetal thymocyte development organ culture; GST, glutathione S-transferase; JNK, c-Jun NH₂-terminal kinase; HSA, heat stable antigen; MAP, mitogen-activated kinase; MKK, MAP kinase kinase; RAG, recombination activating gene; TUNEL, terminal deoxynucleotidyl transferase-mediated FITC-dUTP nick end labeling.

thymus during negative selection results in the presence of these cells in the peripheral immune system and can lead to autoimmune diseases. The failure to successfully undergo positive selection may be reflected by the absence of antigen-specific T cells in the peripheral immune system. More importantly, impairment of early DN thymocyte development can result in a severe T cell immunodeficiency.

Thymocyte development is coordinated by cell-specific gene expression programs. Several transcription factors and signaling pathways provide critical checkpoints (for reviews, see references 1–4). The mitogen-activated protein (MAP) kinase signaling cascades have been implicated in cell growth, differentiation, and death (5). This family of kinases also plays an important role in thymocyte selection. The extracellular signal regulatory kinase (ERK) MAP kinase signaling pathway is required for positive selection and lineage commitment, but is not necessary for negative selection (6). c-Jun NH₂-terminal kinase (JNK) and p38 MAP kinase pathways have been implicated in negative selection of DP thymocytes (7, 8). ERK activation is also required for differentiation of DN into DP thymocytes (9). The roles of JNK and p38 MAP kinases in early thymocyte differentiation have not yet been determined.

p38 MAP kinase is activated by phosphorylation on Thr and Tyr residues by the MAP kinase kinases MKK3, MKK4, and MKK6 (10–13). Intrathymic signals appear to activate the p38 MAP kinase pathway in thymocytes from recombination-activating gene (RAG)-2-deficient mice (14), suggesting that this pathway could also play a role in early thymocyte development. Previous studies have established that the p38 MAP kinase pathway can be regulated by several cytokines, including TNF- α , IL-1, CSF-1, GM-CSF, IL-3, IL-2, and IL-7 (15–20). Some of these cytokines, notably TNF- α , IL-1, and IL-7, are involved in differentiation and cell expansion of DN thymocytes. Here, we show that the p38 MAP kinase pathway is strictly regulated during differentiation of DN thymocytes. The persistent activation of p38 MAP kinase in vivo arrests cell cycle progression and differentiation of immature fetal thymocytes at the CD8⁺CD4^{low}CD25⁺CD44⁻ stage, leading to a lack of T cells in the peripheral immune system. Therefore, the p38 MAP kinase plays an essential regulatory role in early thymocyte development.

Materials and Methods

Generation of Transgenic Mice. The constitutively activated MKK6 cDNA was generated by replacing Ser²⁰⁷ and Thr²¹¹ by Glu (MKK6 [Glu]) (13). The dn p38 MAP kinase mutant was generated by replacing Thr¹⁸⁰ and Tyr¹⁸² by Ala and Phe, respectively (16). The Flag epitope tag was inserted between codons 1 and 2 of the MKK6 and p38 cDNAs, which were subcloned downstream of the proximal *lck* MKK6(Glu) promoter (6, 21, 22). The DNA fragments containing the proximal *lck* promoter, the MKK6(Glu) gene, and the β -globin intron and polyadenylation signals, or the proximal *lck* promoter, dn p38 gene, and the human growth hormone intron and polyadenylation sequence were injected into fertilized (C57BL/6 \times C₃H)F₂ eggs. Transgenic mice were gen-

erated as described previously (23). Transgene expression was analyzed by slot blot using a 500-bp fragment of the proximal *lck* promoter (MKK6[Glu]) or a 500-bp fragment from the human growth hormone sequence (dn p38). The founders were backcrossed into B10.BR mice (The Jackson Laboratory) to establish stable transgenic lines.

Flow Cytometry Analysis. The distribution of populations in the thymus, spleen, and lymph nodes was examined by cell surface staining and flow cytometry (EPICS; Coulter). The following antibodies and conjugates were used: PE-conjugated anti-CD4 mAb, a red⁶¹³-conjugated anti-CD8 mAb, red⁶¹³-streptavidin (GIBCO BRL); FITC-conjugated anti-CD25, biotinylated anti-TCR (H57), biotinylated anti-CD69, biotinylated anti-heat stable antigen (HSA) or anti-CD25, red⁶⁷⁰-streptavidin (PharMingen); PE-anti-CD44 (Caltag); and Quantum red-anti-CD4 and Quantum red-anti-CD8 (Sigma Chemical Co.). Isolation of CD8⁺CD4^{low}CD25⁺CD44⁻ and CD8⁺CD4^{low}CD25⁻CD44⁻ populations was performed by staining with the corresponding mAbs and cell sorting (EPICS; Coulter). Staining was performed in the presence of Fc Block (PharMingen) in all conditions.

Intracellular staining for cyclin A and p27 was performed as described (24). The cells were stained for cell surface expression of CD4 and CD8, fixed with 1% paraformaldehyde, and permeabilized with cold 0.1% (wt/vol) saponin, 1% bovine serum albumin (BSA) fraction V (Sigma Chemical Co.) in PBS. The cells were incubated sequentially with either a rabbit anti-mouse cyclin A or goat anti-mouse p27 (Santa Cruz Biotechnology), followed by either FITC-conjugated anti-rabbit IgG or FITC-conjugated anti-goat IgG, respectively. To examine intracellular staining for TCR β chain, the thymocytes were stained for surface CD4, CD8, CD25, and CD44 and fixed with 1% (vol/vol) methanol-free formaldehyde in PBS for 15 min at 4°C. Fixed thymocytes were then permeabilized with cold 0.03% (wt/vol) saponin in PBS/1% BSA and stained using an FITC-conjugated anti-TCR- β mAb (H57; PharMingen) or an FITC-hamster Ig as an isotype control. Both cell surface and intracellular staining were performed in the presence of Fc Block (PharMingen).

Histological Analysis. Tissues were fixed in 1% paraformaldehyde, embedded in TissueTek, sectioned, and stained with hematoxylin and eosin. Freshly isolated or treated thymocytes were cytopun, fixed in methanol for 7 min, and stained with Giemsa.

Cell Cycle Analysis. Total thymocytes (10⁶ cells) were resuspended in low salt staining solution (3 g/ml polyethylene glycol PEG 8000, 50 μ g/ml propidium iodide, 180 U/ml RNase A, 0.1% Triton X-100, 4 mM sodium citrate) and incubated at 4°C for 30 min. An equal volume of high salt solution (3 g/ml polyethylene glycol PEG 8000, 50 μ g/ml propidium iodide, 180 U/ml RNase A, 0.1% Triton X-100, 400 mM sodium chloride) was added. Propidium iodide incorporation was analyzed by flow cytometry.

Bromodeoxyuridine Staining. Bromodeoxyuridine (BrdU) incorporation was examined as described previously (25). Mice were administered three intraperitoneal injections of 1 mg of BrdU in PBS at 4-h intervals on day 1. On day 2, an additional intraperitoneal injection was administered 1 h before killing the mouse. Thymocytes were fixed in 70% ethanol, washed with PBS, fixed again in 1% paraformaldehyde, washed, and incubated for 30 min at 37°C in 0.15 M NaCl, 4.2 mM MgCl₂, and 100 U/ml DNase. Cells were then stained with FITC-conjugated anti-BrdU (Becton Dickinson) at room temperature, washed, and analyzed by flow cytometry.

TUNEL Assay. To determine incidence of apoptosis, total thymocytes were fixed in 1% paraformaldehyde, permeabilized in

70% ethanol, and assayed for apoptosis via terminal deoxynucleotidyl transferase-mediated FITC-dUTP nick end labeling (TUNEL), as recommended by the manufacturer (PharMingen).

p38 and JNK MAP Kinase Assays. Cells were lysed with buffer A (20 mM Tris, pH 7.5, 10% glycerol, 1% Triton X-100, 0.137 M NaCl, 25 mM β -glycerophosphate, 2 mM EDTA, 0.5 mM dithiothreitol, 1 mM sodium orthovanadate, 2 mM sodium pyrophosphate, 10 μ g/ml leupeptin, 1 mM PMSF) as described (26, 27). Endogenous p38 MAP kinase and JNK were immunoprecipitated using anti-p38 polyclonal antibody (16) or anti-JNK polyclonal antibody (Santa Cruz Biotechnology), respectively. These antibodies were prebound to protein A-sepharose. The immunoprecipitates were washed twice with buffer A and twice with kinase buffer (25 mM HEPES, pH 7.4, 25 mM β -glycerophosphate, 25 mM magnesium chloride, 0.5 mM dithiothreitol, 0.1 mM sodium orthovanadate). The protein kinase reactions were initiated by addition of 1 μ g of recombinant substrate protein (glutathione *S*-transferase [GST]-ATF2 for p38 and GST-c-Jun for JNK) and 50 μ M [γ -³²P]ATP (10 Ci/mmol). The reactions were terminated after 30 min at 30°C by addition of Laemmli sample buffer. Phosphorylation of the substrate protein was examined after SDS-PAGE by autoradiography and PhosphorImager analysis (Molecular Dynamics).

p38 MAP kinase activation was also determined by intracellular staining. Total thymocytes were stained with anti-CD4, anti-CD8, anti-CD25, and anti-CD44 mAbs, in the presence of Fc Block (PharMingen). Stained cells were fixed for 20 min in 4% paraformaldehyde, and incubated in permeabilization buffer (1% FCS, 0.1% saponin/PBS) in the presence of an FITC-antiphospho-p38 MAP kinase mAb (Santa Cruz Biotechnology). The cells were examined by flow cytometry.

Western Blot Analysis. Proteins were fractionated by SDS-PAGE, electrophoretically transferred to an Immobilon-P membrane (Millipore), and probed with an anti-p38 polyclonal antibody (Santa Cruz Biotechnology) to detect endogenous p38 MAP kinase, or anti-Flag mAb (Sigma Chemical Co.) to detect MKK6 (Glu). Immunocomplexes were detected using chemiluminescence (LumiGLO™; Kirkegaard & Perry).

Results

Increased p38 MAP Kinase Activity in Immature Thymocytes. To investigate the role of the p38 MAP kinase pathway in thymocyte development, we first examined p38 MAP kinase activity in freshly isolated DN, DP, and mature CD4⁺ thymocytes from wild-type mice. Consistent with a previous report (14), we found that p38 MAP kinase was highly activated in DN thymocytes (Fig. 1 A). The level of p38 MAP kinase activity in DP thymocytes was lower than the activity found in DN thymocytes. Only low levels of p38 MAP kinase activity were detected in mature CD4⁺ thymocytes (Fig. 1 A). These results suggested that inactivation of p38 MAP kinase may be important for thymocyte maturation. To address this possibility, we have developed a mouse model in which p38 MAP kinase is persistently activated during early thymocyte development.

Abnormal Thymocyte Cellularity in the MKK6(Glu) Transgenic Mice. MKK6 is an upstream activator of p38 MAP kinase (11–13) and does not activate other MAP kinase family members such as JNK or ERK. MKK6 is activated by dual phosphorylation within subdomain VIII. The re-

placement of Ser²⁰⁷ and Thr²¹¹ with Glu generates an activated MKK6 (MKK6[Glu]) that is able to phosphorylate p38 MAP kinase in the absence of prior stimulation (13). Here, we generated transgenic mice that express MKK6(Glu) in thymocytes using the proximal *lck* promoter, which drives a high level of expression in all thymocyte populations. Thus, thymocytes in these mice maintain p38 MAP kinase constitutively activated.

We examined the expression of MKK6(Glu) protein in three transgenic mouse lines by immunoblot analysis using an antibody that recognizes the Flag epitope incorporated into the transgene. The MKK6(Glu) was expressed in thymocytes from all three transgenic mouse lines. The level of expression obtained in line 6 was dramatically higher (10–15-fold) than the other two lines (Fig. 1 B). A low level of transgene expression was also observed in the spleen (Fig. 1 B). The expression of constitutively activated MKK6 resulted in increased p38 MAP kinase activity in thymocytes from the MKK6(Glu) transgenic mice (Fig. 1 C), but did not affect JNK activity (Fig. 1 C), demonstrating the specific effect of MKK6 on the p38 MAP kinase pathway *in vivo*. The thymus from 2–3-mo-old mice exhibited reduced cellularity in each of three transgenic lines compared with negative littermate control mice (Fig. 1 D). Consistent with the level of MKK6(Glu) expression and p38 MAP kinase activity (Fig. 1, B and C), the most dramatic effect was observed in transgenic mice from line 6 (Fig. 1 D).

Interestingly, despite the reduced cell number, the size of thymi from 2–3-mo-old MKK6(Glu) transgenic mice (line 6) was slightly larger than thymi of negative littermate control mice (Fig. 1 E). The weights of thymi from 2–3-mo-old MKK6(Glu) transgenic mice were approximately two-fold greater than those of age-matched negative littermate controls (Fig. 1 F), although the number of thymocytes was reduced (Fig. 1 G). The difference in thymus size between transgenic and control mice increased with age. At 5–5.5 mo, the thymi of the MKK6(Glu) transgenic mice were dramatically enlarged (Fig. 1 E). The weight of the thymi (Fig. 1 F) and the number of thymocytes (Fig. 1 G) were, respectively, 8–10-fold and 3-fold greater than the weight and the number of thymocytes in age-matched negative littermate control mice. The MKK6(Glu) transgenic mice (line 6) died between 5.5 and 6 mo of age. 1 or 2 wk before death, the mice exhibited signs of respiratory distress characterized by a reduced respiratory rate and gasping. Death ensued, apparently due to either compression of the trachea or extinction of lung expansion (Fig. 1 E, right). Death did not appear to be secondary to extrinsic compression of the heart, as there was no evidence of hepatic congestion, ascites, or peripheral edema characteristic of heart failure.

Absence of T Cells in the Peripheral Lymphoid Organs of the MKK6(Glu) Transgenic Mice. In contrast to the dramatically enlarged thymus, the spleen and lymph nodes of the MKK6(Glu) transgenic mice were significantly smaller (Fig. 2 A). Very few cells could be obtained from lymph nodes from the MKK6(Glu) transgenic mice (100-fold reduction; Fig. 2 B). The number of cells in the spleen from these mice was also significantly reduced (Fig. 2 B). Histo-

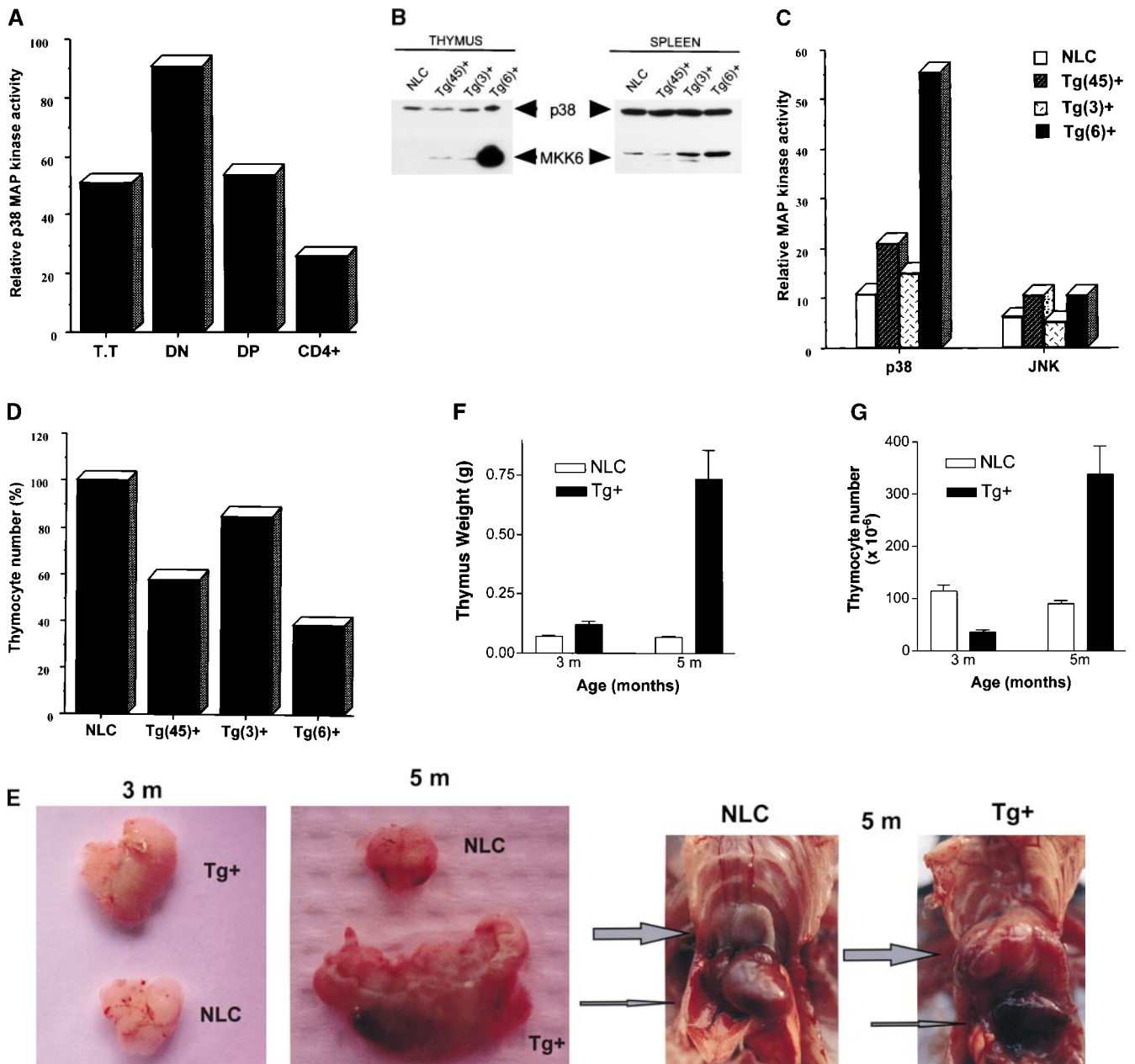


Figure 1. Progressive enlargement of the thymus in the MKK6(Glu) transgenic mice. (A) p38 MAP kinase activity in thymocyte populations from wild-type mice. Whole cell extracts (4×10^5) of freshly isolated total thymocytes (T.T), DN, DP, and mature single CD4⁺ thymocytes from wild-type mice were assayed for p38 MAP kinase activity using the substrate GST-ATF2. (B) Expression of MKK6(Glu) in the MKK6(Glu) transgenic mice. The expression of endogenous p38 MAP kinase and the MKK6(Glu) transgene (MKK6) was analyzed in thymocytes and spleen cells from negative littermate control mice (NLC) and mice from three different MKK6(Glu) transgenic (Tg+) lines (lines 45, 3, and 6) by Western blot analysis. Blots were probed with either an anti-Flag antibody (MKK6) or an anti-p38 MAP kinase polyclonal antibody (p38). (C) Activation of p38 MAP kinase by the expression of MKK6(Glu) in thymocytes. Total thymocytes (5×10^5 cells) from MKK6(Glu) transgenic mice and negative littermate control mice were lysed, and whole extracts were assayed for p38 activity using the substrate GST-ATF2 or for JNK activity using the substrate GST-c-Jun. (D) Diminished thymocyte number in the MKK6(Glu) transgenic mice. Data represent the percentage of the total number of cells in the thymus from the MKK6(Glu) transgenic mice from different lines compared with the total number of thymocytes in negative littermate control mice. (E) Enlarged thymus in the MKK6(Glu) transgenic mice. Thymi from 3- and 5.5-mo-old (3 m and 5 m, respectively) negative littermate control and MKK6(Glu) transgenic (line 6) mice (left two panels). Thymus (thick arrow) and lungs (thin arrow) from 5.5-mo-old (5 m) negative littermate control and MKK6(Glu) transgenic mice (right two panels). (F) Weight of thymi from 2.5–3.5- and 4.5–5.5-mo-old (3 m and 5 m, respectively) negative littermate control and MKK6(Glu) transgenic mice. Values represent average weight ($n = 4$). (G) Progressive accumulation of thymocytes in the MKK6(Glu) transgenic mice. Values represent the average ($n = 4$) of the number of total thymocytes in 3- and 5-mo-old negative littermate control and MKK6(Glu) transgenic mice.

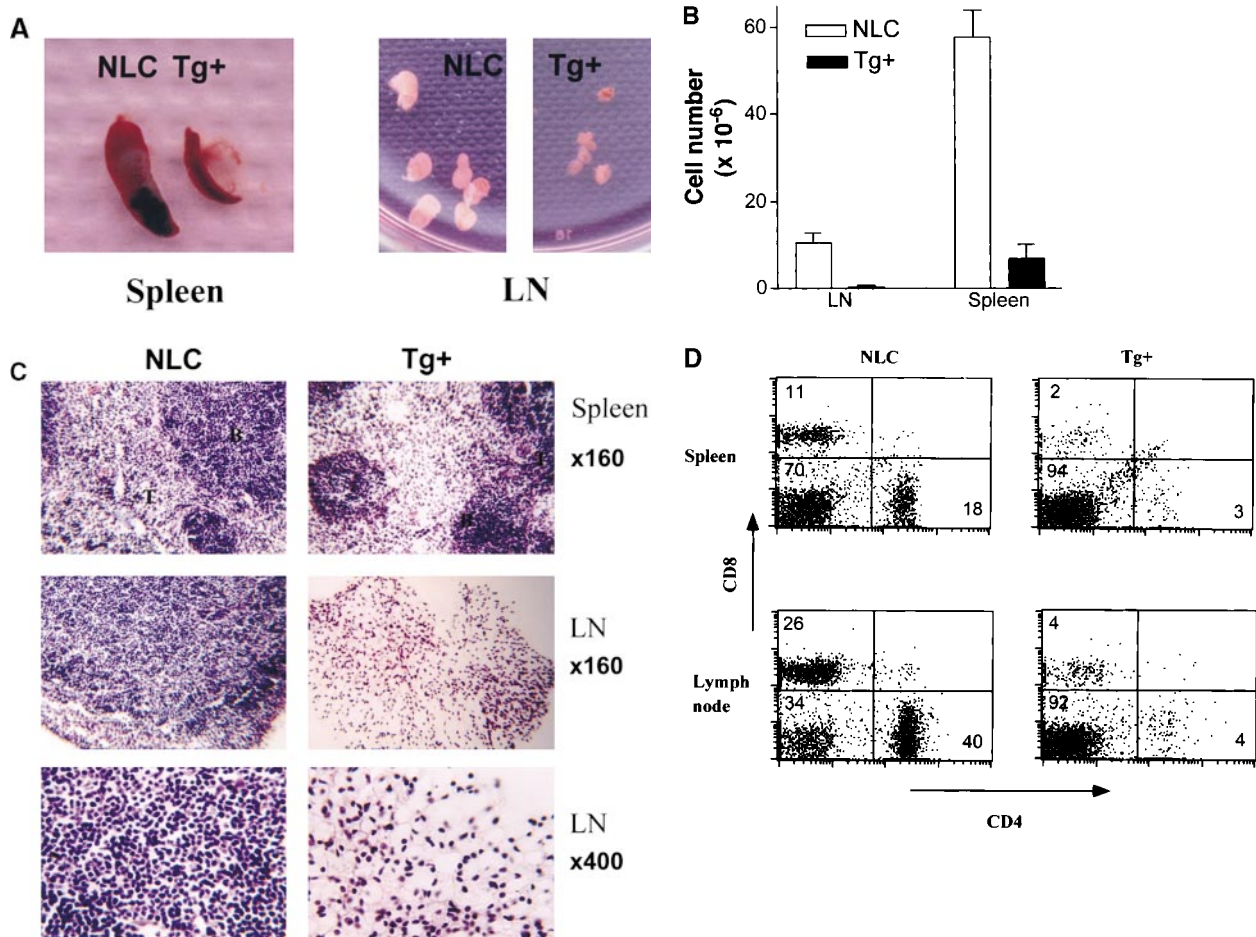


Figure 2. Expression of activated MKK6 in thymocytes results in the lack of T cells in peripheral lymphoid organs. (A) Small lymph nodes and spleen in the MKK6(Glu) transgenic mice. Lymph nodes (LN) and spleen from 5.5-mo-old negative littermate control (NLC) and MKK6(Glu) transgenic (Tg+) mice. (B) Decreased numbers of cells in lymph nodes and spleen. Values represent the average ($n = 4$) of the number of total lymph node and spleen cells in negative littermate control and MKK6(Glu) transgenic (line 6) mice (4–5.5 mo of age). (C) Lack of T cell area in peripheral lymphoid organs. Hematoxylin and eosin-stained tissue sections from spleen and lymph nodes of negative littermate control and MKK6(Glu) transgenic mice. B cell (B) and T cell (T) areas are labeled. (D) Lack of CD4⁺ and CD8⁺ T cells. Cells from lymph nodes and spleen from negative littermate control or MKK6(Glu) transgenic mice were isolated, stained for CD4 and CD8, and analyzed by flow cytometry. Numbers represent the percentage of cells in each quadrant.

logical analysis confirmed that the spleen from the MKK6(Glu) transgenic mice contained only a few cells in the T cell zone, but B cell zones were not affected (Fig. 2 C). Lymph nodes from the MKK6(Glu) mice contained stromal cells, but were virtually devoid of lymphocytes (Fig. 2 C). Cell surface staining and analysis by flow cytometry demonstrated that CD4⁺ and CD8⁺ T cells were almost absent in spleen and lymph nodes from the MKK6(Glu) transgenic mice (Fig. 2 D). B cells were the major population present in these organs from the MKK6(Glu) transgenic mice (data not shown). These results indicated that the expression of constitutively activated MKK6 in thymocytes caused a T lymphocyte immunodeficiency.

Immature Thymocyte Development Arrested by the Persistent Activation of p38 MAP Kinase. The absence of T cells in the peripheral immune organs (Fig. 2) and the progressive enlargement of the thymus (Fig. 1 E) suggested an impairment of thymocyte development. Histological analysis sup-

ported this hypothesis (Fig. 3 A). Thymi from the MKK6(Glu) transgenic mice contained uniform cortical areas (immature thymocytes) and lacked defined medulla (mature thymocytes) (Fig. 3 A), indicating that the expression of MKK6(Glu) resulted in an accumulation of immature thymocytes. To determine the specific stage of differentiation of the thymocytes, we examined the expression of CD4 and CD8 cell surface markers. The DP population defined by a high cell surface expression of both CD4 and CD8 (CD8⁺CD4⁺) was not detected in the MKK6(Glu) transgenic mice (Fig. 3 B). Surprisingly, most thymocytes from the MKK6(Glu) transgenic mice were CD8⁺CD4^{low} (Fig. 3 B). The immature CD8⁺CD4^{low} population is a transient stage clearly identified during fetal thymic differentiation of DN thymocytes into DP thymocytes, although very few CD8⁺CD4^{low} cells can be detected in adult thymus in wild-type mice. These results suggest that the expression of MKK6(Glu) may arrest immature thymocyte differentiation.

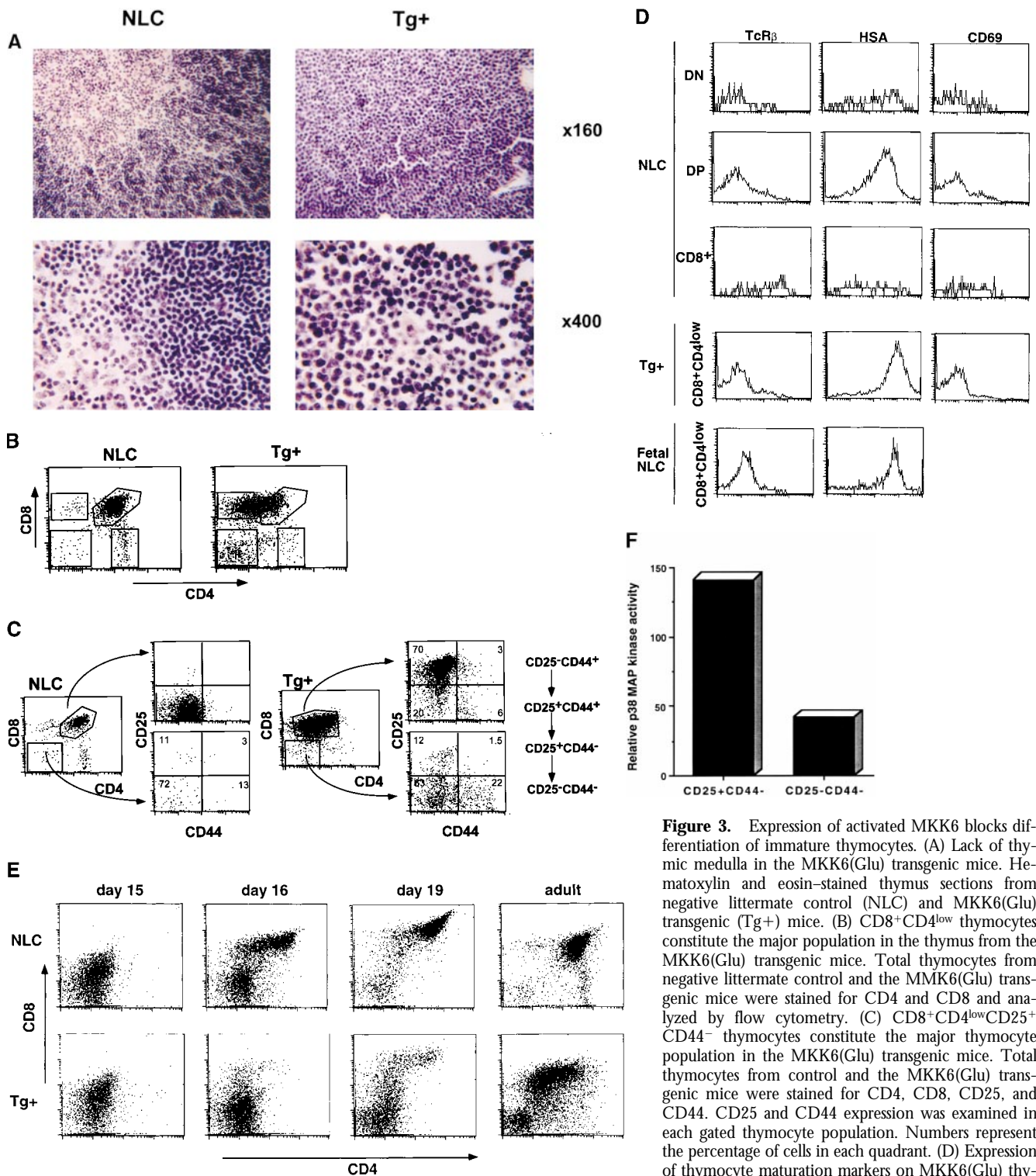


Figure 3. Expression of activated MKK6 blocks differentiation of immature thymocytes. (A) Lack of thymic medulla in the MKK6(Glu) transgenic mice. Hematoxylin and eosin-stained thymus sections from negative littermate control (NLC) and MKK6(Glu) transgenic (Tg+) mice. (B) CD8⁺CD4^{low} thymocytes constitute the major population in the thymus from the MKK6(Glu) transgenic mice. Total thymocytes from negative littermate control and the MKK6(Glu) transgenic mice were stained for CD4 and CD8 and analyzed by flow cytometry. (C) CD8⁺CD4^{low}CD25⁺CD44⁻ thymocytes constitute the major thymocyte population in the MKK6(Glu) transgenic mice. Total thymocytes from control and the MKK6(Glu) transgenic mice were stained for CD4, CD8, CD25, and CD44. CD25 and CD44 expression was examined in each gated thymocyte population. Numbers represent the percentage of cells in each quadrant. (D) Expression of thymocyte maturation markers on MKK6(Glu) thymocytes. Histograms represent the cell surface expression of TCR- β , CD69, and HSA on CD4⁻CD8⁻ DN, CD4⁺CD8⁺ DP, and CD8⁺ single-positive thymocytes from negative littermate control mice; CD8⁺CD4^{low} thymocytes from the MKK6(Glu) transgenic mice; and CD8⁺CD4^{low} fetal thymocytes from day 16 negative littermate control embryos (Fetal NLC) analyzed by flow cytometry. (E) Expression of activated MKK6 blocks fetal thymocyte development. Fetal thymi were isolated from negative littermate control and the MKK6(Glu) transgenic embryos at E15, E16, or E19. Total fetal thymocytes were stained for CD4 and CD8 and analyzed by flow cytometry. (F) Elevated p38 MAP kinase activity in CD25⁺CD44⁻ thymocytes. Fetal thymocytes from wild-type E18 embryos were isolated, pooled, and stained for CD4, CD8, CD25, and CD44. CD8⁺CD4^{low}CD25⁺CD44⁻ and CD25⁻CD44⁻ populations were purified by cell sorting, lysed, and assayed for p38 MAP kinase activity using GST-ATF2 as a substrate as described in the legend to Fig. 1 B.

The expression of CD25 and CD44 characterizes the specific differentiation stages within the DN population (Fig. 3 C). The expression of CD25 during thymocyte development is restricted to this immature stage. Interestingly, CD8⁺CD4^{low} thymocytes from the MKK6(Glu) transgenic mice expressed high levels of CD25, but did not express CD44 (Fig. 3 C). Thus, the expression of activated MKK6 blocked thymocyte differentiation and led to an accumulation of immature CD8⁺CD4^{low}CD25⁺CD44⁻ thymocytes. The high level of HSA characteristic of immature cells and the lack of expression of CD69 (mature thymocytes) (Fig. 3 D) confirm the immature phenotype of the thymocytes in the MKK6(Glu) transgenic mice. The high level of HSA present on thymocytes from the MKK6(Glu) transgenic mice was comparable to the level expressed in E16 fetal thymocytes from control mice (Fig. 3 D), supporting the immature phenotype of the thymocytes from the MKK6(Glu) transgenic mice. Low but significant levels of TCR- β expression were detected in the CD8⁺CD4^{low} population from the MKK6(Glu) transgenic mice (Fig. 3 D), suggesting that the pre-TCR could already be present on these immature thymocytes.

To further demonstrate that activated MKK6 arrested thymocyte development at an early stage, we examined *in vivo* fetal thymic development during embryogenesis. CD8⁺CD4^{low} thymocytes constituted the predominant population at E16–17, whereas CD8⁺CD4^{low} thymocytes had already differentiated into DP thymocytes in E19 wild-type embryos (Fig. 3 E). In contrast, at E19 only CD8⁺CD4^{low} thymocytes were found in the MKK6(Glu) embryos (Fig. 3 E). In control mice, the differentiation of CD8⁺CD4^{low} fetal thymocytes into DP fetal thymocytes is associated with a remarkable increase in cell number (~10-fold per day). Consistent with the arrest at the CD8⁺CD4^{low} stage, the number of fetal thymocytes at E19 was dramatically lower (three- to fourfold) in the MKK6(Glu) embryos compared with the number found in control fetal thymi (data not shown). The number of total thymocytes in the MKK6(Glu) transgenic mice was consistently lower than the number of thymocytes in control mice during the first 2–3 mo of age. These results indicated that the early arrest of thymocyte differentiation at the CD8⁺CD4^{low}CD25⁺CD44⁻ stage in the MKK6(Glu) transgenic mice was associated with an inhibition of the expansion of these immature cells. The increased number of thymocytes observed in MKK6(Glu) transgenic mice by 5–6 mo of age may be the result of the continuous entry of new cohorts of bone marrow-derived precursor thymocytes and their limited expansion before the CD25⁺CD44⁻ stage.

Our data demonstrated that a persistent activation of p38 MAP kinase arrested the differentiation and expansion of immature CD8⁺CD4^{low}CD25⁺CD44⁻ thymocytes and suggested that p38 MAP kinase must be inactivated during the transition from the CD25⁺CD44⁻ to the CD25⁻CD44⁻ stage. To test this hypothesis, we examined p38 MAP kinase activity in CD8⁺CD4^{low}CD25⁺CD44⁻ and CD8⁺CD4^{low}CD25⁻CD44⁻ thymocytes from wild-type mice. p38 MAP kinase activity was substantially higher in the

CD25⁺CD44⁻ subpopulation compared with the activity found in the CD25⁻CD44⁻ cells (Fig. 3 F). Therefore, p38 MAP kinase was inactivated during the differentiation of CD25⁺CD44⁻ into CD25⁻CD44⁻ thymocytes, and the persistent activation of this pathway in the MKK6(Glu) transgenic mice prevented this differentiation step.

Inhibition of Mitotic Progression of CD25⁺CD44⁻ Thymocytes by Activation of p38 MAP Kinase. Two subpopulations, defined by cell size, have been described within the CD25⁺CD44⁻ immature thymocyte subset (28). The CD25⁺CD44⁻ “E” (expected size) stage, characterized by a small cell size, precedes the CD25⁺CD44⁻ “L” (large size) stage, characterized by large cells (Fig. 4 A). The CD25⁺CD44⁻ L and E stages also define cells before (E) or after (L) a successful β chain rearrangement and expression of the pre-TCR. Progression from E to L stage is inhibited in mice deficient in specific components of the pre-TCR (e.g., RAG, pre-T α , TCR- β [28]). Histological analysis showed that the cells present in the thymus from the MKK6(Glu) transgenic mice were larger than thymocytes from negative littermate control mice (Fig. 3 A). This size difference was confirmed by flow cytometry analysis of forward and side scatter (Fig. 4 B), suggesting that thymocytes from the MKK6(Glu) transgenic mice could be arrested at the L stage. The low level of cell surface expression of TCR- β detected on CD8⁺CD4^{low}CD25⁺CD44⁻ thymocytes from the MKK6(Glu) transgenic mice (Fig. 3 D) suggested that successful β chain rearrangement could occur in these cells. To further demonstrate the presence of functional TCR- β , we examined the intracellular expression of TCR- β by flow cytometry. In correlation with previous studies, two populations of CD25⁺CD44⁻ thymocytes could be clearly distinguished based on the intracellular expression of the β chain in negative littermate control mice (Fig. 4 C). Most CD8⁺CD4^{low}CD25⁺CD44⁻ thymocytes from the MKK6(Glu) transgenic mice expressed the TCR- β chain, indicating that β rearrangement had successfully occurred in these cells (Fig. 4 C). These results indicated that the activation of p38 MAP kinase resulted in an accumulation of immature CD25⁺CD44⁻ L thymocytes.

A substantial percentage of the CD25⁺CD44⁻ L thymocytes from wild-type mice has been described to be cycling cells (28). Therefore, we examined the cell cycle status of thymocytes from the MKK6(Glu) transgenic mice by measuring DNA content using propidium iodide. Only a small number of thymocytes was in S and G₂/M phases in negative littermate control mice (Fig. 4 D). In contrast, the thymi from MKK6(Glu) transgenic mice contained an elevated proportion of cells in the S and G₂/M phases and a low percentage of cells in G₀/G₁ phase (Fig. 4 D). The increased percentage of thymocytes with >2N DNA content was observed in both younger (2–3 mo) and older (5–6 mo) MKK6(Glu) transgenic mice.

Despite the elevated number of cells with >2N DNA content, the total number of thymocytes during fetal development through the first 3–4 mo after birth of the MKK6(Glu) transgenic mice was significantly lower than the number of thymocytes found in negative littermate control mice.

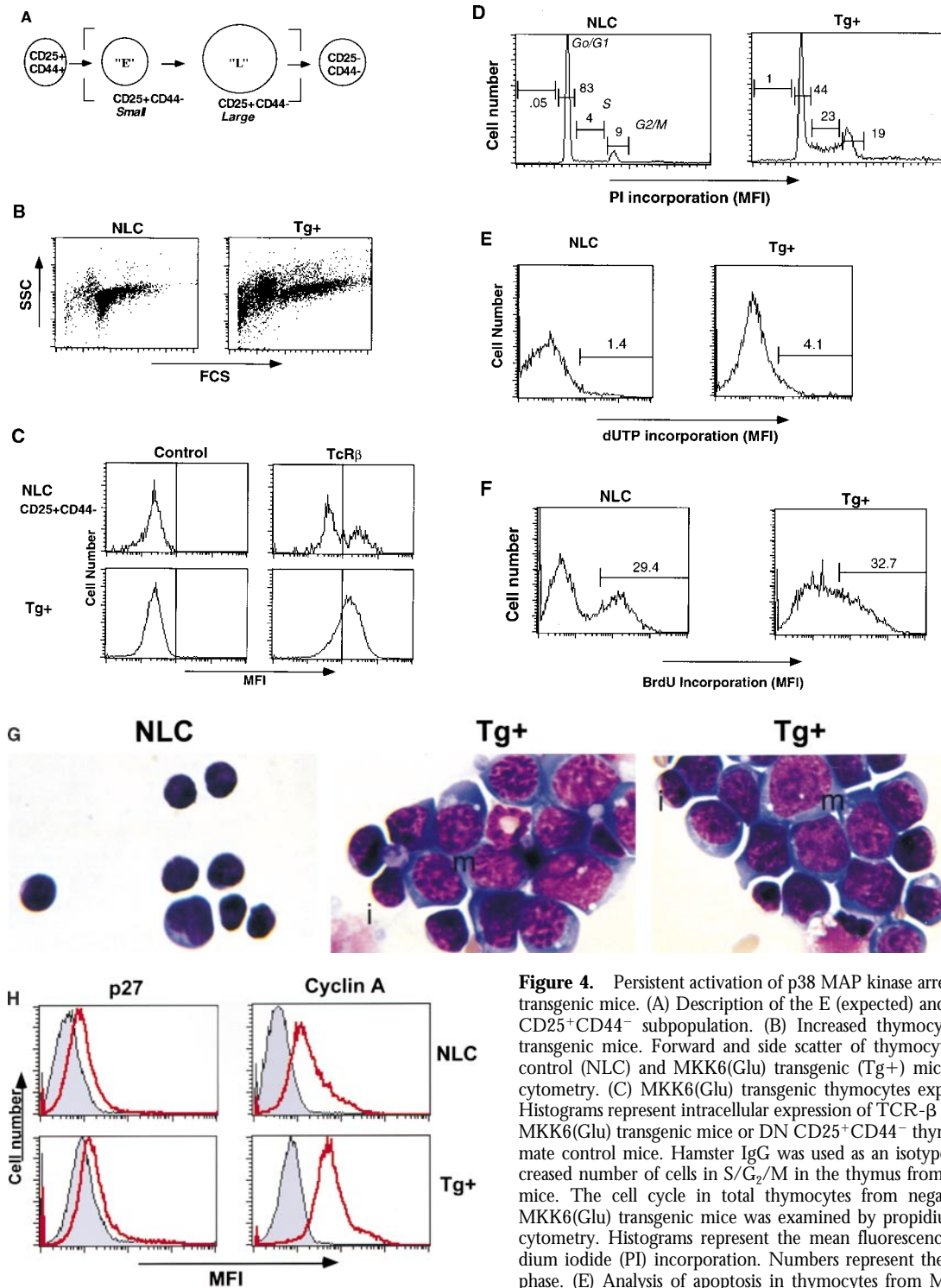


Figure 4. Persistent activation of p38 MAP kinase arrests cell cycle in MKK6(Glu) transgenic mice. (A) Description of the E (expected) and L (large) subsets within the CD25⁺CD44⁻ subpopulation. (B) Increased thymocyte size in the MKK6(Glu) transgenic mice. Forward and side scatter of thymocytes from negative littermate control (NLC) and MKK6(Glu) transgenic (Tg+) mice were determined by flow cytometry. (C) MKK6(Glu) transgenic thymocytes express TCR β chain protein. Histograms represent intracellular expression of TCR-β in total thymocytes from the MKK6(Glu) transgenic mice or DN CD25⁺CD44⁻ thymocytes from negative littermate control mice. Hamster IgG was used as an isotype-matching control. (D) Increased number of cells in S/G₂/M in the thymus from the MKK6(Glu) transgenic mice. The cell cycle in total thymocytes from negative littermate control and MKK6(Glu) transgenic mice was examined by propidium iodide staining and flow cytometry. Histograms represent the mean fluorescence intensity (MFI) of propidium iodide (PI) incorporation. Numbers represent the percentage of cells in each phase. (E) Analysis of apoptosis in thymocytes from MKK6(Glu) transgenic mice. Apoptosis of freshly isolated total thymocytes from negative littermate control and

MKK6(Glu) transgenic mice was examined by TUNEL assay. Histograms represent the mean fluorescence intensity of the incorporation of FITC-dUTP. Numbers represent the percentage of dUTP⁺ cells. (F) Normal proliferation rate of MKK6(Glu) thymocytes. In vivo BrdU incorporation in thymocytes from negative littermate control and MKK6(Glu) transgenic mice was determined by intracellular staining using an anti-BrdU mAb. Numbers represent the percentage of cells that have incorporated BrdU. (G) Accumulation of mitotic thymocytes in the MKK6(Glu) transgenic mice. Total thymocytes from negative littermate control and MKK6(Glu) transgenic mice were cytopun and stained with Giemsa. Two fields of the same preparation are shown for the transgenic mice. Cells in mitosis (m) or interphase (i) are labeled. (H) Increased expression of cyclin A in the MKK6(Glu) transgenic thymocytes. p27 and cyclin A expression in thymocytes from negative littermate control and MKK6(Glu) transgenic mice were determined by intracellular staining using unconjugated anti-p27 and anti-cyclin A antisera followed by staining with the corresponding conjugated secondary antibody (red line, open histograms). Thymocytes stained with the secondary antibody alone are included as a control (gray line, filled histograms). The experiment shown represent the results from four independent experiments.

These results could be explained by (a) an accelerated entry of G_0/G_1 cells into S phase (increased rate of proliferation) accompanied by an increased cell death, or (b) a partial inhibition of cell cycle progression from G_2/M into G_0/G_1 phase and the progressive accumulation of new bone marrow-derived precursor thymocytes.

Using TUNEL assay, we examined apoptosis of freshly isolated thymocytes from negative littermate control and MKK6(Glu) transgenic mice. No significant numbers of apoptotic thymocytes were observed in either negative control or MKK6(Glu) transgenic mice (Fig. 4 E), suggesting that the constitutive activation of p38 MAP kinase did not cause increased cell death of thymocytes in vivo. To determine whether thymocytes from the MKK6(Glu) transgenic mice had a higher rate of proliferation, we examined the fraction of cells synthesizing DNA by BrdU incorporation in vivo. As described previously, a large percentage of thymocytes from control mice incorporated BrdU, indicating that they have undergone S phase transition during the 24-h labeling period (Fig. 4 F). The percentage of BrdU⁺ thymocytes in the MKK6(Glu) transgenic mice was comparable to the percentage found in control mice (Fig. 4 F), indicating that the number of cells that had progressed through S phase was similar. Together, these results indicate that the accumulation of cells with >2N DNA content and the corresponding reduction of the number of cells in G_0/G_1 phase in the MKK6(Glu) transgenic mice were not caused by an accelerated entry of G_0/G_1 cells into S phase, suggesting that cell cycle progression could be partially inhibited at G_2/M phase.

Therefore, the large $CD8^+CD4^{low}CD25^+CD44^-$ thymocytes with >2N DNA content that accumulate in the MKK6(Glu) transgenic mice could be mitotic cells, suggesting that activation of p38 MAP kinase inhibited mitotic progression of immature $CD25^+CD44^-$ thymocytes. In correlation with this hypothesis, it has been shown that activation of p38 MAP kinase, but not ERK or JNK, can arrest the cell cycle in meiosis (M phase) in *Xenopus* embryos (29). We examined whether activated MKK6 could inhibit the progression of mitosis in immature thymocytes by Giemsa staining, since propidium iodide staining does not discriminate G_2 from M phases. Most thymocytes (90–95%) from negative littermate control mice were in interphase (G_0/G_1 , S, or G_2), as indicated by the compact nuclei present in these cells (Fig. 4 G). In contrast, a significant proportion of the thymocytes from the MKK6(Glu) transgenic mice were in early/late prophase of mitosis, indicated by the visualization of chromosomes and increased cell size (Fig. 4 G). In addition, intracellular staining and flow cytometry analysis showed that the level of cyclin A normally expressed during mitosis was increased in thymocytes from the MKK6(Glu) transgenic mice compared with control thymocytes (Fig. 4 H). The level of cyclin-dependent kinase inhibitor p27 levels was not affected (Fig. 4 H). These results supported the hypothesis that the activation of p38 MAP kinase inhibited progression of mitosis in $CD8^+CD4^{low}CD25^+CD44^-$ thymocytes that progressively accumulate in the thymus.

Inhibition of p38 MAP Kinase Restores Cell Cycle Progression and Differentiation of $CD25^+CD44^-$ Thymocytes. Differentiation and proliferation are closely regulated during the development of T cells in the thymus. Differentiation of $CD25^-CD44^+$ thymocytes into $CD25^+CD44^+$ and $CD25^+CD44^-$ thymocytes is associated with some proliferation, but the principal expansion of cells occurs during the differentiation of $CD25^+CD44^-$ thymocytes into $CD25^-CD44^-$ thymocytes (for a review, see reference 1). However, it is unclear which of these processes, cell cycle progression or differentiation, initiates the transition between two differentiation stages. Our results indicated that activation of p38 MAP kinase prevented cell cycle progression and differentiation of $CD25^+CD44^-$ thymocytes. To address which of these two biological functions is the cause of the arrest of thymocyte development, we examined the effect of p38 MAP kinase inhibition on thymocytes from the MKK6(Glu) transgenic mice. Thymocytes from adult MKK6(Glu) transgenic mice were incubated in vitro with medium alone or in the presence of different concentrations of SB203580, a pyridinyl imidazol drug that selectively inhibits p38 MAP kinase (18). After 6 h, the nuclear morphology of MKK6(Glu) thymocytes was examined by Giemsa staining. As shown above, in the absence of the inhibitor a large proportion of these cells remained in prophase (Fig. 5 A). However, after treatment with 1 μ M SB203580, a significant number of thymocytes in late anaphase/early telophase was observed (Fig. 5 A). Cells in late telophase and small cells in interphase were also detected upon incubation with 5 μ M SB203580 (Fig. 5 A). No SB203580 effect was observed in control thymocytes (data not shown). Down-regulation of cyclin A expression was also observed in the MKK6(Glu) thymocytes treated with SB203580 (Fig. 5 B). These results indicated that inhibition of p38 MAP kinase is required for progression of mitosis in $CD25^+CD44^-$ thymocytes.

We examined whether the presence of SB203580 allowed further differentiation of $CD25^+CD44^-$ thymocytes. No significant differences in the expression of differentiation cell markers were observed between 12 and 24 h of treatment with SB203580 (data not shown). However, after 2 d of treatment, immature $CD25^+CD44^-$ thymocytes differentiated into DP thymocytes in a dose-dependent manner (Fig. 5 C). The presence of SB203580 did not affect the distribution of negative littermate control thymocytes (Fig. 5 C). The differentiation of $CD8^+CD4^{low}$ thymocytes from the MKK6(Glu) transgenic mice into DP thymocytes in the presence of SB203580 was associated with cell expansion (Fig. 5 D). Therefore, inactivation of p38 MAP kinase must occur for $CD25^+CD44^-$ thymocytes to progress through mitosis, expand, and ultimately differentiate into $CD25^-CD44^-$ and DP thymocytes.

Activation of the p38 MAP Kinase Pathway Is Important for the Earliest Stages of Thymocyte Development. The presence of high levels of p38 MAP kinase activity in immature $CD25^+CD44^-$ thymocytes compared with the activity in $CD25^-CD44^-$ thymocytes (Fig. 3 F) suggested that activation of p38 MAP kinase may be important for develop-

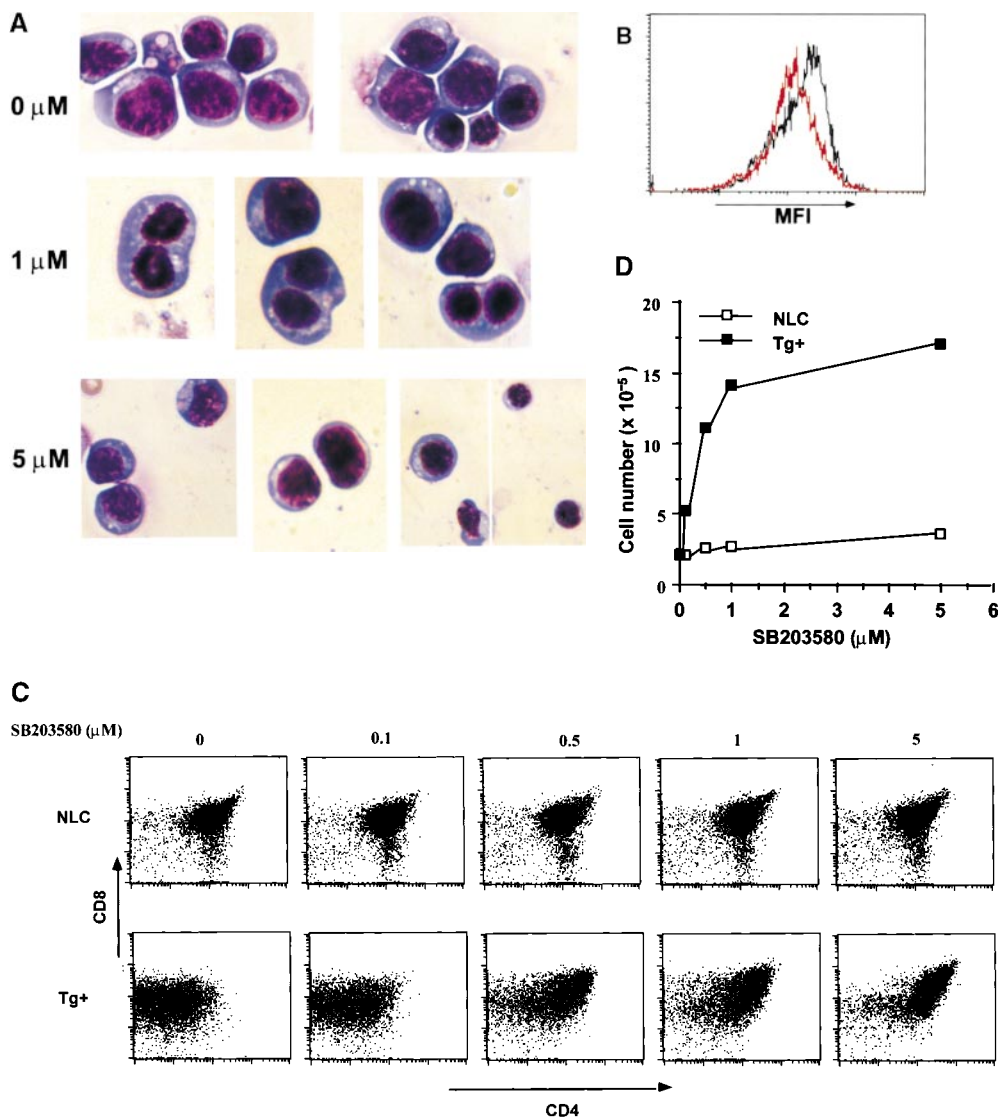


Figure 5. Inactivation of p38 MAP kinase is required for CD25⁺CD44⁻ cell cycle progression and differentiation. (A) Inactivation of p38 MAP kinase allows progression of mitosis in CD25⁺CD44⁻ MKK6(Glu) thymocytes. Thymocytes from the MKK6(Glu) transgenic mice were incubated in the presence of the p38 MAP kinase inhibitor, SB203580, at the indicated dose. After 6 h, the cells were cytospun and stained with Giemsa. Two or three fields from the same slide are presented. (B) Decreased cyclin A expression in MKK6(Glu) thymocytes upon inactivation of p38 MAP kinase. Total thymocytes from the MKK6(Glu) transgenic mice were treated in the presence (red line) or absence (black line) of SB203580 (1 μM) for 12 h. Cyclin A expression was examined by intracellular staining. (C) Inactivation of p38 MAP kinase restores differentiation of CD25⁺CD44⁻ MKK6(Glu) thymocytes. Total thymocytes from adult negative littermate control (NLC) or MKK6(Glu) transgenic (Tg⁺) mice were incubated for 2 d with the indicated concentrations of the p38 MAP kinase inhibitor, SB203580. The recovered cells were stained for CD4 and CD8 and analyzed by flow cytometry. (D) Inhibition of p38 MAP kinase in MKK6(Glu) thymocytes restores cell proliferation. Total thymocytes (10⁶ cells) were treated as described in C. After 2 d, the number of live cells was determined by trypan blue staining.

ment of the CD25⁺CD44⁻ population. To address this hypothesis, we generated transgenic mice that overexpress a dominant-negative p38 MAP kinase (dn p38) in thymocytes. The activating phosphorylation sites Thr¹⁸⁰ and Tyr¹⁸² were replaced by Phe and Ala, respectively, in the dn p38 mutant (16). We have previously generated transgenic mice that overexpress the dn p38 MAP kinase using the distal *lck* promoter (30). No effect on thymic cellularity or the distribution of thymic population was observed in these mice, since the distal *lck* promoter does not drive transgene expression in DN thymocytes. Thus, we have generated new transgenic mice in which dn p38 MAP kinase expression was driven by the proximal *lck* promoter.

p38 MAP kinase activity was examined in thymocytes from two different transgenic mouse lines. Decreased p38 MAP kinase activity was observed in thymocytes from mice expressing the dn p38 MAP kinase (Fig. 6 A). Analysis of the total number of thymocytes showed decreased thymic cellularity in mice from two dn p38 transgenic lines (Fig. 6 B). Although there were no significant differences in the per-

centages of DP and mature CD4⁺ and CD8⁺ populations, the absolute number of each of these thymocyte populations was reduced in the dn p38 transgenic mice compared with control mice (Fig. 6 C). These results suggested an impairment in the very earliest stages of thymocyte development that did not completely prevent further differentiation but slowed the process. We also examined the presence of the different DN thymocyte subsets defined by CD25 and CD44 expression in the dn p38 transgenic mice. No significant reduction of the number of CD25⁻CD44⁻ was observed in these mice. In contrast, the total number of CD25⁻CD44⁺, CD25⁺CD44⁺, and CD25⁺CD44⁻ thymocytes was reduced, supporting the hypothesis that activation of p38 MAP kinase is probably needed very early during thymocyte development (Fig. 6 D).

To determine the regulation of p38 MAP kinase in the different DN subsets, we examined the presence of activated p38 MAP kinase by intracellular staining and flow cytometry using an antibody that specifically recognizes the phosphorylated form of p38 MAP kinase. Competition ex-

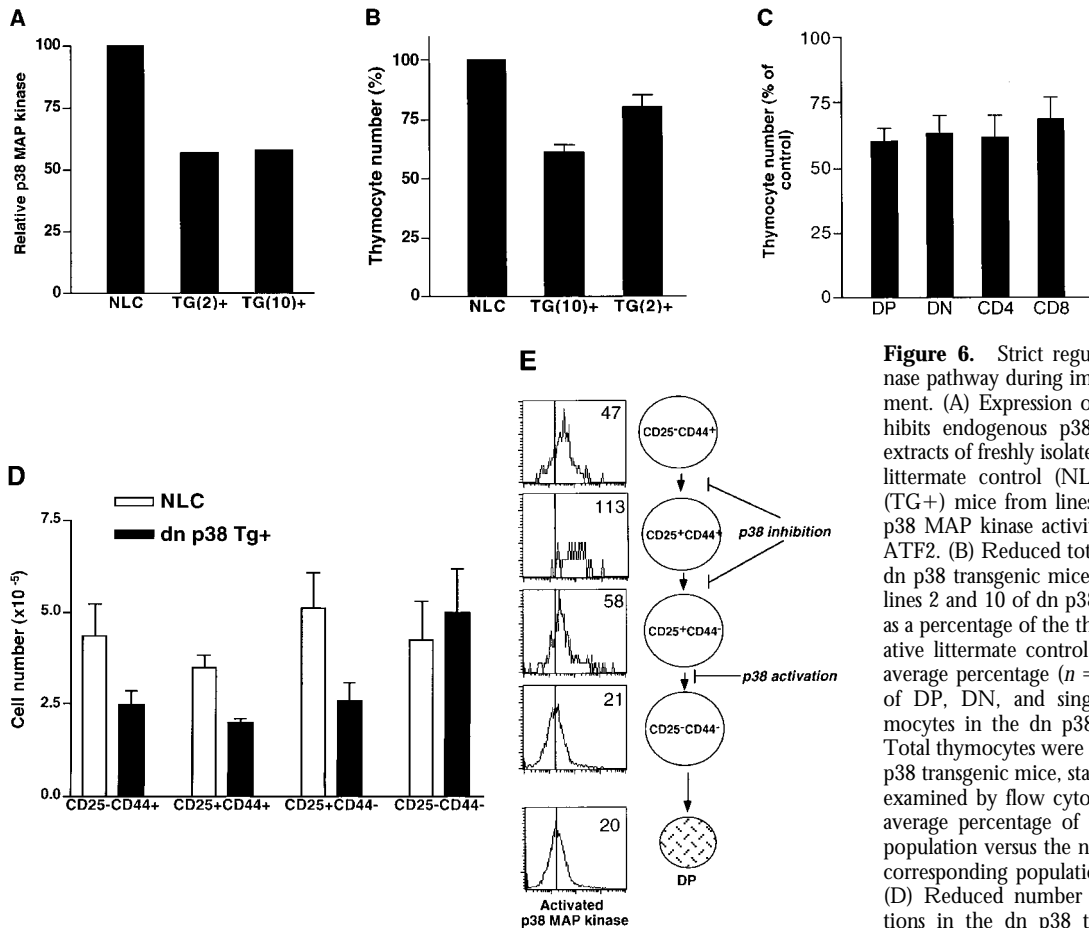


Figure 6. Strict regulation of the p38 MAP kinase pathway during immature thymocyte development. (A) Expression of dn p38 in thymocytes inhibits endogenous p38 MAP kinase. Whole cell extracts of freshly isolated thymocytes from negative littermate control (NLC) and dn p38 transgenic (TG+) mice from lines 2 and 10 were assayed for p38 MAP kinase activity using the substrate GST-ATF2. (B) Reduced total thymocyte number in the dn p38 transgenic mice. Thymocyte numbers from lines 2 and 10 of dn p38 transgenic mice are shown as a percentage of the thymocyte number from negative littermate control mice. Values represent the average percentage ($n = 4$). (C) Reduced numbers of DP, DN, and single CD4⁺ and CD8⁺ thymocytes in the dn p38 transgenic mice (line 10). Total thymocytes were isolated from control and dn p38 transgenic mice, stained for CD4 and CD8, and examined by flow cytometry. Values represent the average percentage of thymocyte number in each population versus the number of thymocytes of the corresponding population in control mice ($n = 3$). (D) Reduced number of DN thymocyte populations in the dn p38 transgenic mice. Total thymocytes were isolated from negative littermate control and dn p38 transgenic mice, stained for CD4, CD8, CD25, and CD44, and examined by flow cytometry. Values represent the average of the absolute number of thymocytes in each DN subpopulation, based on the total number of thymocytes ($n = 3$). (E) Activation and inactivation of p38 MAP kinase during earliest stage of thymocyte development. Total thymocytes from wild-type mice were stained for CD4, CD8, CD44, and CD25. Cell surface staining was followed by intracellular staining for the active form of p38 MAP kinase using an FITC-antiphospho-p38 MAP kinase mAb. Histograms (left) represent the presence of activated p38 MAP kinase in the different DN subpopulations (right) and DP cells. Vertical line represents the negative control. Numbers express the mean fluorescence intensity of phospho-p38 MAP kinase positive cells. The results are representative of five independent experiments.

control and dn p38 transgenic mice, stained for CD4, CD8, CD25, and CD44, and examined by flow cytometry. Values represent the average of the absolute number of thymocytes in each DN subpopulation, based on the total number of thymocytes ($n = 3$). (E) Activation and inactivation of p38 MAP kinase during earliest stage of thymocyte development. Total thymocytes from wild-type mice were stained for CD4, CD8, CD44, and CD25. Cell surface staining was followed by intracellular staining for the active form of p38 MAP kinase using an FITC-antiphospho-p38 MAP kinase mAb. Histograms (left) represent the presence of activated p38 MAP kinase in the different DN subpopulations (right) and DP cells. Vertical line represents the negative control. Numbers express the mean fluorescence intensity of phospho-p38 MAP kinase positive cells. The results are representative of five independent experiments.

periments using a phospho-p38 peptide demonstrated the specificity of this antibody (data not shown). In correlation with the increase of p38 MAP kinase activity (threefold) determined by the conventional immunocomplex kinase assay (Fig. 3 F), intracellular staining and flow cytometry analysis revealed increased levels (threefold) of activated (phosphorylated) p38 MAP kinase in DN CD25⁺CD44⁻ thymocytes compared with DN CD25⁻CD44⁻ thymocytes (Fig. 6 E). Interestingly, p38 MAP kinase activity was dramatically augmented in the DN CD25⁺CD44⁺ population compared with DN CD25⁻CD44⁺ thymocytes (Fig. 6 E), indicating that p38 MAP kinase is strictly regulated during the differentiation of DN thymocytes.

Together, our results indicate that activation of the p38 MAP kinase pathway is important for differentiation and/or survival of immature CD25⁻CD44⁺ thymocytes into CD25⁺CD44⁺ and CD25⁺CD44⁻ thymocytes, but that inactivation of p38 MAP kinase is required to proceed from the CD25⁺CD44⁻ to the CD25⁻CD44⁻ stage (Fig. 6 E).

Discussion

Reprogramming of gene expression patterns has been implicated as a mechanism for the morphologic and functional commitment of cells to specific lineages during the development of T cells in the thymus. Transcription factors and signaling pathways play critical roles in the control of the differentiation, proliferation, and death of thymocytes. Here, we have shown that the p38 MAP kinase pathway is very strictly regulated during the early stages of thymocyte development. The level of p38 MAP kinase activity is critical for both the differentiation and expansion of immature thymocytes.

Using the MKK6(Glu) transgenic mice, we have shown that persistent activation of p38 MAP kinase in thymocytes *in vivo* results in a progressive increase of thymus size. The enlarged thymus might theoretically be the result of a thymoma induced by the expression of activated MKK6. Several lines of evidence presented in this study argue against this possibility. First, splenomegaly and lymphadenopathy

are frequently associated with the development of thymoma. However, the spleen and lymph nodes from these mice are dramatically reduced in size and cell number and do not contain T cells. No metastases are found in other organs of the MKK6(Glu) transgenic animals. Second, thymocytes from the MKK6(Glu) transgenic mice do not exhibit increased proliferation either in vivo or in vitro. Proliferation of MKK6(Glu) thymocytes in vitro is observed only when p38 MAP kinase is inactivated by the presence of a specific inhibitor, SB203580. Third, despite the increased size of the thymus, the total number of thymocytes is reduced in embryos and young MKK6(Glu) transgenic mice. Increased thymocyte cell number was observed only after these mice reached 4–5 mo of age. The discrepancy between the size of the thymus and the total number of thymocytes can be explained by the difference in thymocyte size. Flow cytometry and histological analysis (Figs. 3 A and 4 B) demonstrate that thymocytes from the MKK6(Glu) transgenic mice are larger than thymocytes from control mice and would therefore occupy more space. Finally, analysis of the TCR repertoire by examining different TCR V β (V β 5, V β 6, V β 8, and V β 11) expression demonstrates that the thymocytes that accumulate in the MKK6(Glu) transgenic mice do not represent a monoclonal population (data not shown). Together, these observations make it unlikely that the accumulation of thymocytes and progressive enlargement of the thymus in the MKK6(Glu) transgenic mice are the result of development of a thymoma.

Instead, our studies demonstrate that the persistent activation of the p38 MAP kinase pathway arrests the differentiation of immature thymocytes early in development. Several lines of evidence confirm the immature phenotype of the CD8⁺CD4^{low} population present in the thymus of the MKK6(Glu) transgenic mice. First, the high level of HSA and low level of CD69 and TCR- β expression detected on thymocytes from the MKK6(Glu) transgenic mice are characteristic of immature thymocytes (Fig. 3 D). Second, MKK6(Glu) CD8⁺CD4^{low} thymocytes display the CD25⁺CD44⁻ phenotype characteristic of a transient stage of immature thymocytes before differentiation into DP thymocytes (Fig. 3 C). Most importantly, we have demonstrated that the arrest of thymocyte differentiation in the MKK6(Glu) transgenic mice occurs during fetal development at approximately E16–17 (Fig. 3 E). Finally, inhibition of p38 MAP kinase in vitro restores differentiation of the MKK6(Glu) CD8⁺CD4^{low}CD25⁺CD44⁻ thymocytes into DP thymocytes (Fig. 5 C).

Recently, Sugawara et al. (8) have shown that the p38 MAP kinase inhibitor SB203580 inhibits deletion of DP thymocytes by anti-CD3 mAb in fetal thymocyte development organ culture (FTOC) in vitro. However, they also show that the presence of SB203580 results in a decreased percentage of DP thymocytes and increased percentage of CD8⁺, CD4⁺, and DN thymocytes in FTOC (8). Thus, the interpretation of these results is unclear. A differential effect of SB203580 in stromal cells and thymocytes may explain these apparently contradictory results. In the same study, it was suggested that the expression of MKK6 induces nega-

tive selection of DP thymocytes. However, the presence of the CD8⁺CD4^{low} population, increased percentage of DN thymocytes, and absence of CD4⁺ thymocytes in wild-type MKK6 retrovirus-infected FTOC (8) do not support this model. In contrast, these results suggest an inhibition of the differentiation of immature fetal thymocytes into DP thymocytes by the expression of wild-type MKK6. Thus, the expression of wild-type MKK6 in FTOC in vitro may mimic the phenotype obtained in mice expressing constitutively activated MKK6 in vivo.

The E and L subsets have been defined within the DN CD25⁺CD44⁻ population in the adult thymus based on their size, TCR- β expression, and cell cycle status (28). Differentiation of thymocyte development is partially or completely inhibited at the CD25⁺CD44⁻ E stage (normal size thymocytes) in mice deficient for components of the pre-TCR complex (e.g., pre-T α , TCR- β , CD3 ϵ , RAG, T cell factor [TCF]/lymphoid enhancer-binding factor [LEF]-deficient mice) or the pre-TCR signaling pathways (e.g., *lck*, ZAP-70, SH2 domain-containing leukocyte phosphoprotein of 76 kD [SLP-76]-deficient mice) (1, 31–36). Thus, pre-TCR-mediated signals appear to be critical for the progression from E to L stage. In our study, we show that activation of the p38 MAP kinase pathway does not prevent the expression of the TCR- β and progression into the L stage. However, a persistent activation of p38 MAP kinase completely arrests the differentiation of CD25⁺CD44⁻ L thymocytes into CD25⁻CD44⁻ and DP thymocytes. Therefore, endogenous p38 MAP kinase must be inactivated before differentiation to the CD25⁻CD44⁻ stage. The analysis of p38 MAP kinase activity in these populations confirms this model (Figs. 3 F and 6 E). The p38 MAP kinase pathway therefore is the first signaling pathway that has been described to play a critical role at the CD25⁺CD44⁻ L checkpoint during thymocyte development.

The specific nuclear morphology (prophase), large size, and low rate of proliferation in CD25⁺CD44⁻ thymocytes from the MKK6(Glu) transgenic mice in vivo indicate that MKK6(Glu) CD25⁺CD44⁻ thymocytes are arrested in mitosis. Moreover, progression of MKK6(Glu) thymocytes towards telophase, cytokinesis, and G₁ phases is restored upon inactivation of p38 MAP kinase in the presence of SB203580. Therefore, activation of p38 MAP kinase in vivo prevents progression through the late phases of mitosis in CD25⁺CD44⁻ thymocytes. The study by Takenaka et al. (29) showing that microinjection of activated p38 MAP kinase into blastomeres of *Xenopus* embryos causes mitotic arrest supports our results in immature thymocytes. Recently, it has been proposed that activation of the p38 MAP kinase pathway can inhibit progression of both G₁ and mitosis in NIH-3T3 fibroblasts (37).

The inhibition of cell cycle progression of CD25⁺CD44⁻ thymocytes from the MKK6(Glu) transgenic mice appears contradictory to the increased thymocyte number observed in 5–6-mo-old MKK6(Glu) transgenic mice. Although only a few cells (50–100/d) from the bone marrow repopulate the thymus in control mice (38), these immature thymocytes (predominantly CD25⁻CD44⁺ thymocytes) can proliferate during their differentiation into CD25⁺CD44⁺ and CD25⁺CD44⁻ thymocytes. The major expansion of cell

numbers appears to occur during the differentiation of CD25⁺CD44⁻ into CD25⁻CD44⁻ thymocytes. The continuous entry of a new cohort of precursor cells into the thymus and the differentiation and expansion of CD25⁻CD44⁺ and CD25⁺CD44⁺ thymocytes can explain the slow but progressive accumulation of thymocytes with time in the MKK6(Glu) transgenic mice. Although the number of fetal thymocytes in E15 MKK6(Glu) embryos was similar to the number in age-matched control embryos, four- to fivefold fewer thymocytes were found in E19 MKK6(Glu) embryos compared with control embryos. Only 1.5–2-fold fewer cells were found in 2–3-mo-old MKK6(Glu) transgenic mice, and 2–3-fold more thymocytes were found in 5–6-mo MKK6(Glu) mice compared with age-matched control mice.

Proinflammatory cytokines (e.g., TNF- α and IL-1), hematopoietic factors (e.g., GM-CSF and IL-3), and environmental stress can induce p38 MAP kinase activity in various cell types (15–20). Interestingly, in correlation with the elevated p38 MAP kinase activity in the CD25⁺CD44⁺ thymocytes, it has been shown that TNF- α and IL-1 produced by stromal cells can stimulate the expression of surface CD25 on immature CD25⁻CD44⁺ thymocytes. Furthermore, these two cytokines are required for the differentiation of immature fetal thymocytes into DP thymocytes (39). It is possible that p38 MAP kinase activity in immature thymocytes may be regulated by stromal cell-derived cytokines.

Previous studies have demonstrated the involvement of the p38 MAP kinase pathway in proliferation and cell death. Our studies show the importance of the p38 MAP kinase pathway in the control of differentiation and cell cycle progression of CD25⁺CD44⁻ thymocytes in vivo. Proliferation is closely associated with differentiation during thymocyte development, and it remains unclear which of these processes is the initial event in each developmental step. We have demonstrated that the inhibition of p38 MAP kinase in MKK6(Glu) thymocytes in vitro rapidly (several hours) restores cell cycle progression, whereas a longer period of time (24–48 h) is required to observe significant changes on the cell surface phenotype. Therefore, we propose that inhibition of cell cycle progression by the p38 MAP kinase prevents further differentiation of immature CD25⁺CD44⁻ thymocytes.

Our study demonstrates that p38 MAP kinase is strictly regulated during the early stages of thymocyte development. Although an initial activation of p38 MAP kinase appears to be important for thymic cellularity, persistent activation of this pathway completely blocks the generation of T cells and results in T cell immunodeficiency. The regulation of the p38 MAP kinase pathway is critical in thymocyte development and generation of mature T cells.

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References

- Rodewald, H.-R., and H.J. Fehling. 1998. Molecular and cellular events in early thymocyte development. *Adv. Immunol.* 69:1–113.
- Glimcher, L.H., and H. Singh. 1999. Transcription factors in lymphocyte development—T and B cells get together. *Cell.* 96:13–23.
- Clevers, H., and P. Ferrier. 1998. Transcriptional control during T-cell development. *Curr. Opin. Immunol.* 10:166–171.
- Killeen, N., B.A. Irving, S. Pippig, and K. Ziegler. 1998. Signaling checkpoints during the development of T lymphocytes. *Curr. Opin. Immunol.* 10:360–367.
- Ip, Y.T., and R.J. Davis. 1998. Signal transduction by the c-Jun N-terminal kinase (JNK)—from inflammation to development. *Curr. Opin. Cell Biol.* 10:205–219.
- Alberola-Ila, J., K.A. Forbush, R. Seger, E.G. Krebs, and R.M. Perlmutter. 1995. Selective requirement for MAP kinase activation in thymocyte differentiation. *Nature.* 373:620–623.
- Rincón, M., A. Whitmarsh, D.D. Yang, L. Weiss, B. Dérjard, P. Jayaraj, R.J. Davis, and R.A. Flavell. 1998. The JNK pathway regulates the in vivo deletion of immature CD4⁺CD8⁺ thymocytes. *J. Exp. Med.* 188:1817–1830.
- Sugawara, T., T. Moriguchi, E. Nishida, and Y. Takahama. 1998. Differential roles of ERK and p38 MAP kinase pathways in positive and negative selection of T lymphocytes. *Immunity.* 9:565–574.
- Crompton, T., K.C. Gilmour, and M.J. Owen. 1996. The MAP kinase pathway controls differentiation from double-negative to double-positive thymocyte. *Cell.* 86:243–251.
- Dérjard, B., J. Rainjeaud, T. Barret, I.-H. Wu, J. Han, R.J. Ulevitch, and R.J. Davis. 1995. Independent human MAP kinase signal transduction pathways defined by MEK and MKK isoforms. *Science.* 267:683–685.
- Han, J., J.-D. Lee, Y. Jiang, Z. Li, L. Feng, and R.J. Ulevitch. 1996. Characterization of the structure and function of a novel MAP kinases kinase (MKK6). *J. Biol. Chem.* 271:2886–2891.
- Moriguchi, T., F. Toyoshima, Y. Gotoh, A. Iwamatsu, K. Irie, E. Mori, N. Kuroyanagi, M. Hagiwara, K. Matsumoto, and E. Nishida. 1996. Purification and identification of a major activator for p38 from osmotically shocked cells. Activation of mitogen-activated protein kinase 6 by osmotic shock, tumor necrosis factor- α and H₂O₂. *J. Biol. Chem.* 271:26981–26988.
- Rainjeaud, J., A.J. Whitmarsh, T. Barret, B. Dérjard, and R.J. Davis. 1996. MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. *Mol. Cell. Biol.* 16:1247–1255.
- Sen, J., R. Kapeller, R. Fragoso, R. Sen, L.I. Zon, and S.J. Burakoff. 1996. Intrathymic signals in thymocytes are mediated by p38 mitogen-activated protein kinase. *J. Immunol.* 156:4535–4538.
- Freshney, N.W., L. Rawlinson, F. Guesdon, E. Jones, S. Cowley, J. Hsuan, and J. Saklatvala. 1994. Interleukin-1 activates a novel protein kinase cascade that results in the phos-

- phorylation of the Hsp27. *Cell*. 78:1039–1049.
16. Raingeaud, J., S. Gupta, J. Roger, M. Dickens, J. Han, R.J. Ulevitch, and R.J. Davis. 1995. Pro-inflammatory cytokines and environmental stress cause p38 MAP kinase activation by dual phosphorylation on tyrosine and threonine. *J. Biol. Chem.* 270:7420–7426.
 17. Han, J., J.-D. Lee, L. Bibbs, and R.J. Ulevitch. 1994. A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science*. 265:808–811.
 18. Lee, J.C., T. Laydon, P.C. McDonnell, T.F. Gallagher, S. Kumar, D. Gree, D. McNulty, M.J. Blumenthal, J.R. Heys, S.W. Landvatter, et al. 1994. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature*. 372:739–746.
 19. Rouse, J., P. Cohen, S. Trigon, M. Morange, A. Alonso-Llamazares, D. Zamanillo, T. Hunt, and A.R. Nebreda. 1994. A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. *Cell*. 78:1027–1037.
 20. Foltz, I.N., J.C. Lee, P.R. Young, and J.W. Schrader. 1997. Hemopoietic growth factors with the exception of interleukin-4 activate the p38 mitogen-activated protein kinase pathway. *J. Biol. Chem.* 272:3296–3301.
 21. Wildin, R.S., A.M. Garvin, S. Pawar, D.B. Lewis, K.M. Abraham, K.A. Forbush, S.F. Ziegler, J.M. Allen, and R.M. Perlmutter. 1991. Developmental regulation of *lck* gene expression in T lymphocytes. *J. Exp. Med.* 173:383–393.
 22. Swan, K.A., J. Alberola-Ila, J.A. Gross, M.W. Appleby, K.A. Forbush, J.F. Thomas, and R.M. Perlmutter. 1995. Involvement of p21ras distinguishes positive and negative selection in thymocytes. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:276–285.
 23. Hogan, B., F. Constantini, and E. Lacy. 1986. *Manipulating the Mouse Embryo*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
 24. Cibotti, R., J.A. Punk, K.S. Dash, S.O. Sharrow, and A. Singer. 1997. Surface molecules that drive T cell development *in vitro* in the absence of thymic epithelium and in the absence of lineage-specific signals. *Immunity*. 6:245–255.
 25. Lucas, B., F. Vasseur, and C. Penit. 1995. Stochastic coreceptor shut-off is restricted to the CD4 lineage maturation pathway. *J. Exp. Med.* 181:1623–1633.
 26. Rincón, M., B. Dérjard, C.-W. Chow, R.J. Davis, and R.A. Flavell. 1997. Reprogramming the signaling requirement for AP-1 (activator protein-1) activation during differentiation of precursor CD4⁺ T cells to effector Th1 and Th2 cells. *Genes Funct.* 1:51–68.
 27. Dérjard, B., M. Hibi, I.-H. Wu, T. Barret, B. Su, T. Deng, M. Karin, and R.J. Davis. 1994. JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell*. 76:1025–1037.
 28. Hoffman, E.S., L. Passoni, T. Crompton, T.M.J. Leu, D.G. Schatz, A. Koff, M. Owen, and A.C. Hayday. 1996. Productive T-cell receptor β -chain gene rearrangement: coincident regulation of cell cycle and clonality during development *in vivo*. *Genes Dev.* 10:948–962.
 29. Takenaka, K., T. Moriguchi, and E. Nishida. 1998. Activation of the protein kinase p38 in the spindle assembly checkpoint and mitotic arrest. *Science*. 280:599–602.
 30. Rincón, M., H. Enslen, J. Raingeaud, M. Recht, T. Zapton, M.S.-S. Su, L.A. Penix, R.J. Davis, and R.A. Flavell. 1998. Interferon- γ expression by Th1 effector T cells mediated by the p38 MAP kinase signaling pathway. *EMBO (Eur. Mol. Biol. Organ.) J.* 17:2817–2829.
 31. Brickner, A.G., D.L. Gossage, M.R. Dusing, and D.A. Wiginton. 1995. Identification of a murine homolog of the human adenosine deaminase thymic enhancer. *Gene*. 167:261–266.
 32. Oosterwegel, M.A., M. van de Wetering, D. Dooijes, L. Klomp, A. Winoto, K. Georgopoulos, F. Meijlink, and H. Clevers. 1991. Cloning of murine TCF-1, a T cell-specific transcription factor interacting with functional motifs in the CD3 ϵ and T cell receptor α enhancers. *J. Exp. Med.* 173:1133–1142.
 33. Sawada, S., and D.R. Littman. 1991. Identification and characterization of a T-cell specific enhancer adjacent to the murine CD4 gene. *Mol. Cell. Biol.* 11:5027–5035.
 34. Travis, A., A. Amsterdam, C. Belanger, and R. Grosschedl. 1991. LEF-1, a gene encoding a lymphoid-specific protein with an HMG domain, regulates T-cell receptor α enhancer function. *Genes Dev.* 5:880–894.
 35. van de Wetering, M., J. Gastrop, V. Korinek, and H. Clevers. 1996. Extensive alternative splicing and dual promoter usage generate Tcf-1 protein isoforms with differential transcription control properties. *Mol. Cell. Biol.* 16:745–752.
 36. Waterman, M.L., W.H. Fischer, and K.A. Jones. 1991. A thymus specific member of the HMG protein family regulates the human T-cell receptor α enhancer. *Genes Dev.* 5:656–669.
 37. Ellinger-Ziegelbauer, H. 1999. Cell cycle arrest and reversion of Ras-induced transformation by a conditionally activated form of mitogen-activated protein kinase kinase kinase 3. *Mol. Cell. Biol.* 19:3857–3868.
 38. Scollay, R., J. Smith, and V. Stauffer. 1986. Dynamics of early T cells: prothymocyte migration and proliferation in the adult mouse thymus. *Immunol. Rev.* 91:129–157.
 39. Zúñiga-Pflucker, J.C., J. Di, and M.J. Lenardo. 1998. Requirement for TNF α and IL-1 α in fetal thymocyte commitment and differentiation. *Science*. 268:1906–1909.