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Transgenic overexpression of human *LY6K* in mice suppresses mature T cell development in the thymus

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Abstract. Lymphocyte antigen 6 family member K (LY6K) is upregulated in a number of types of cancer and promotes tumor cell proliferation and metastasis. In addition, LY6K is involved in tamoxifen resistance in breast cancer. However, the *in vivo* molecular mechanism of LY6K has not yet been investigated. In the present study, transgenic mice overexpressing human LY6K (hLY6K) were generated using the pMAMneo vector, and the effect of LY6K upregulation *in vivo* was investigated. A total of 4 transgenic mice were generated, and the gene copy number was examined using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT-qPCR demonstrated that mRNA of hLY6K was overexpressed in the thymus and spleen of the transgenic mice compared with wild-type mice. Flow cytometric analysis demonstrated that the proportions of B and T cells in the spleen were similar in wild-type and transgenic mice; however, the proportion of thymic mature T cells decreased in the transgenic mice, while there was an increase in the proportion of naïve T cells. These findings suggest that the overexpression of LY6K suppresses T cell development, and that LY6K is a potential therapeutic target for cancer.

Introduction

Ly-6/urokinase-type plasminogen activator receptors (uPARs) are a membrane protein superfamily characterized by the three-finger fold structural motif and a putative glycosylphosphatidylinositol (GPI)-anchoring site (1,2). uPARs regulate the proteolytic degradation processes in the extracellular matrix (ECM) by binding the extracellular protease urokinase-type plasminogen activator, which subsequently activates plasminogen (3). ECM molecules constitute the cellular microenvironment and provide mechanical support for cells. Furthermore, they also regulate intracellular signaling pathways by interacting with transmembrane proteins (4). For example, it was reported that ECM-integrin interaction may promote β cell proliferation by activating the phosphoinositide 3-kinase cascade (5). uPAR-mediated modulation of the ECM is known to alter many cellular processes, including cell adhesion, proliferation, differentiation and migration (3,6); therefore, uPARs have been implicated in a number of human diseases, including cancer and other inflammatory diseases. Previous studies have demonstrated that uPARs are generally upregulated in cancer cells and during other inflammatory conditions or infections, and this makes them attractive therapeutic targets for the impairment of ECM-cell interactions and the signaling pathways of abnormal cells (1,7-9)

Lymphocyte antigen 6 family member K (Ly6K) is a recently discovered member of the uPAR family and was first identified as a molecular marker for head-and-neck squamous cell carcinoma (10). Previous studies have demonstrated that Ly6K is upregulated in numerous types of cancer, including esophageal squamous cell carcinoma, bladder cancer and breast cancer, and contributes to cell growth, migration, invasion, and immune escape (11-14). In breast cancer, it is reported that Ly6K expression is regulated by the transcription factor activator protein-1, and that Ly6K promotes cell proliferation and metastasis by activating the Raf-1/MEK/ERK signaling pathway in cancer (15). Therefore, Ly6K has been suggested as a cancer biomarker and therapeutic target. Nevertheless, the *in vivo* molecular mechanism of Ly6K remains ill-defined, and an appropriate *in vivo* mouse model to study the role of Ly6K has not yet been generated.

Lymphocytes are the blood cells responsible for immune responses. They comprise three main types: T cells, B cells, and natural killer cells (NK cells), which all develop from hematopoietic stem cells (HSC) in bone marrow (16). Lymphocyte development is achieved through several specific stages, involving coordinated regulation of lineage-associated gene expression, which is dependent on the type of cell (17). B cells,

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which mediate the humoral immune response by producing antibodies, undergo full development in the bone marrow via immunoglobulin gene rearrangement, whereas T cell development occurs distal to the bone marrow (18). HSCs migrate from the bone marrow to the thymus and continue to proliferate to generate a large group of immature thymocytes, which lack T-cell receptor (TCR) expression. These cells do not express CD4 or CD8, and are therefore termed double-negative (DN) cells (19). Following several developmental stages, cells that achieve pre-TCR expression develop into double-positive $CD4^+CD8^+$ cells. These cells then become single-positive thymocytes ($CD4^+CD8^-$ or $CD4^-CD8^+$), depending on the interaction between the TCR and the major histocompatibility complex (MHC) ligand on the epithelial cells (20,21).

Despite the strong association between Ly6K and cancer development, *in vivo* models to examine the effect of Ly6K expression on tumors have not yet been developed. In the present study, a transgenic mouse model overexpressing the human *LY6K* (*hLY6K*) gene was generated to investigate the changes resulting from *LY6K* overexpression *in vivo*. Mouse mammary tumor virus (MMTV)/*hLY6K* transgenic mice expressed *LY6K* mRNA strongly in the thymus and spleen. Flow cytometric analysis demonstrated that the levels and distribution of cytotoxic and helper T cells were decreased in the transgenic mouse, which indicated that T cell development was defective when *LY6K* was overexpressed.

Materials and methods

Generation of LY6K transgenic mice. The construct to generate the transgenic mice was produced by inserting human *LY6K* cDNA, which was previously described (22), into the pMAMneo vector (Clontech Laboratories, Inc., Mountainview, CA, USA). The full-length human *LY6K* cDNA fragment was amplified by polymerase chain reaction (PCR) under the following thermocycling conditions: Initial denaturation at 98˚C for 5 min, 35 cycles at 98˚C for 10 sec, 50˚C for 50 sec and 72˚C for 20 sec; and final extension at 72˚C for 5 min. For PCR, Taq Polymerase, dNTPs and reaction buffers (Real Biotech Corporation, Taipei, Taiwan) were used according to the manufacturer's protocol. Specifically-designed primers containing *Nhe*I or *Xho*I restriction enzyme sites were used and the sequences were as follows: Forward, 5'-CTAGCTAGC ATGGCGCTGCT-3'; and reverse, 5'-GGCCTCGAGTCAAGA CAGGC-3'. Fragments digested with *Nhe*I and *Xho*I restriction enzymes (New England Biolabs, Ipswich, MA, USA) were purified by the HiYield Gel/PCR extraction kit (Real Biotech Corporation) according to the manufacturer's protocol, and then inserted into the corresponding restriction sites of the vector. The recombinant construct carrying the transgene was microinjected into the pronuclei of fertilized eggs of B6 mice (Animal Facility, Sookmyung Women's University, Seoul, Korea), and the eggs were then transferred into the oviducts of pseudo-pregnant female mice. Transgenic founder mice were bred with B6 mice to establish transgenic lines; a total of 7 founder mice (8-9 weeks-old; average weight, ~20 g) were generated. All mice were housed in a specific pathogen-free barrier facility with a 12 h light/dark cycle and maintained at a humidity of 40-60% at 23˚C. Mice had *ad libitum* access to food and water. After 6 weeks, mice were euthanized by cervical dislocation for further analysis. All animal experiments were approved by the Institutional Animal Care and Use Committee at the Sookmyung Women's University (Seoul, South Korea).

Genomic DNA extraction and PCR. Genomic DNA was extracted from the tail biopsies of each mouse using a Quick-DNA™ Universal Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol. To confirm the presence of the transgene, PCR was performed to amplify specific fragments of the *hLY6K* gene. The sequences of the primers used were as follows: Forward, 5'-TGTGGTTTA GGTTGGAGTGTAGTG-3'; and reverse, 5'-CTCATCAAA AAAATCTCCCCAAC-3'. EmeraldAmp® GT PCR Master Mix (Clontech Laboratories, Inc.) was used to amplify DNAs according to the manufacturer's protocol. The thermocycling conditions were as follows: Initial denaturation step for 2 min at 95˚C, 30 cycles for 30 sec at 95˚C, 30 sec at 58˚C and 30 sec at 72˚C, and the final extension step for 5 min at 72˚C. The amplified DNA samples were resolved on a 1.0% agarose gel and separated by electrophoresis at 200 V for 40 min, which produced bands of 360 bp. To compare the amount of the transgene, standard samples containing 1, 10 and 100 copies of the transgene were prepared and mixed with the genomic DNA of wild-type B6 mice, then amplified and separated on the same agarose gel.

Quantitative PCR (qPCR) evaluation of transgene copy number. To examine the copy number of the transgene, a standard curve was generated using 1, 10 and 100 copies of the transgene diluted in the genomic DNA of wild-type mice. qPCR was performed using SYBR-Green qPCR Master Mix (PCR Biosystems, Ltd., London, UK), and each reaction sample contained the following components: 100 ng genomic DNA, 10 μM of each primer and 5 μl SYBR Green qPCR Master Mix. The sequence of the primers used were as follows: *hLY6K* forward, 5'-AGCCCATGCCCTTCTTTT ACCTCA-3'; and *hLY6K* reverse, 5'-CCAGCCACAGCCCAC CACAG-3'; mouse *Gapdh* (*mGapdh)* forward, 5'-GCTGAG TATGTCGTGGAGTC-3'; and *mGapdh* reverse, 5'-ATGGAC TGTGGTCATGAGC-3'. The *mGapdh* gene was amplified as an endogenous control. qPCR cycling conditions were as follows: A pre-incubation step of 10 min at 95˚C; followed by 45 amplification cycles of 10 sec at 95˚C, 10 sec at 60˚C, and 10 sec at 72˚C; and a final elongation step of 1 min at 65˚C. The standard curve was determined by plotting ΔCq ($\Delta Cq=Cq_{hLY6K}-Cq_{Gapdh}$) against the log of *hLY6K* gene copies of the corresponding standard samples, and the copy numbers of the transgene in transgenic mice were determined by the $2^{-\Delta\Delta Cq}$ method (23). The formula was as follows: $2^{-\Delta\Delta Cq}=2^{-(\Delta Cq \text{ of transgenic samples}-\Delta Cq \text{ of wild-type samples})}$ (24).

RNA isolation and reverse transcription (RT). RNAs were extracted from each tissue of 3 mice per group (wild-type and transgenic mice) using a NucleoSpin® DNA/RNA/Protein kit (Machery-Nagel GmbH, Düren, Germany), according to the manufacturer's protocol. RNAs (2 μg) diluted in 12.5 μl of sterile RNase free water were reverse transcribed to cDNAs using an M-MLV Reverse Transcription kit (Promega Corporation, Madison, WI, USA) at 42˚C for 1 h and then inactivated at 70˚C for 10 min.

RT-PCR. Following RT, as described above, cDNA samples (100 ng) were used as templates to amplify specific fragments with RBC Taq Polymerase, and each reaction mixture contained the following: 3 μl 10X RBC Reaction Buffer, 3 μl dNTPs (2.5 mM), 10 mM of each primer, 100 ng of cDNA, 1 μl RBC Taq Polymerase and 21.7 μl double-distilled water. The sequences of the primers used to detect *hLY6K or mGapdh* were as follows: *hLY6K* forward, 5'-TGCTCGCCT TGCTGCTGGTC-3'; *hLY6K* reverse, 5'-TCGCTGCACAAC CAGCGGAG-3'; *mGapdh* forward, 5'-CGGTGCTGAGTA TGTCGTGGAG-3'; and *mGapdh* reverse, 5'-TGTCATCAT ACTTGGCAGGTTTC-3'. Amplification condition were as follows: Initial denaturation step for 2 min at 95˚C, 30 cycles for 30 sec at 95˚C, 30 sec at 58˚C and 30 sec at 72˚C; and the final extension step for 5 min at 72˚C. Following amplification, the PCR products were separated on 1.0% agarose gels by electrophoresis at 200 V for 40 min.

RT-qPCR. To further compare the RNA expression level of *hLY6K* in wild-type and transgenic mice, RT-qPCR was conducted using SYBR Green qPCR Master Mix (PCR Biosystems, Ltd.). cDNA samples (100 ng) were used as templates, and the sequences of the primers to detect *hLY6K* and mouse *Gapdh* were same as those used to examine the transgenic copy number. qPCR cycling conditions were as follows: A pre-incubation step of 10 min at 95˚C; 45 amplification cycles of 10 sec at 95˚C, 10 sec at 60˚C and 10 sec at 72˚C; and a final elongation step of 1 min at 65˚C. The relative expression levels of *hLY6K* were calculated by the $2^{-\Delta\Delta Cq}$ method, normalized against the level of *mGapdh*.

Western blot analysis. To obtain proteins from the mouse tissues, tissues was disrupted and homogenized with lysis buffer RP1 (Macherey-Nagel GmbH & Co., Düren, Germany) containing 1% β-mercaptoethanol (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and stainless steel beads using TissueLyser (Qiagen GmbH, Hilden, Germany) at 1500 oscillations/min for 1 min at room temperature. Protein extracts were prepared using a NucleoSpin® DNA/RNA/Protein kit and buffers included in kit (Macherey-Nagel GmbH & Co.), according to the manufacturer's protocol. Protein extracts were dissolved with Protein Solving Buffer containing the reducing agent TCEP (Macherey-Nagel GmbH & Co.) and boiled at 98˚C for 3 min. The protein concentration was measured using a bicinchoninic acid solution and copper (II) sulfate solution (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). Samples were loaded (30 μg/lane) and separated by SDS-PAGE (10-12% gel) and transferred onto polyvinylidene fluoride membranes. Membranes were blocked with 5% skim milk in PBS containing 0.2% Tween-20 for 1 h at room temperature. Immunoblotting was conducted using an anti-Ly6K antibody (cat no. sc-87282; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for the detection of human and mouse Ly6K, and an anti-β-actin antibody (cat no. A300-491A; Bethyl Laboratories, Montgomery, TX, USA), which were diluted at 1:1,000 in 1% skim milk in PBS containing the detergent 0.2% Tween-20 overnight at 4˚C. Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (cat no. ADI-SAB-300-J; Enzo Life Science, Inc., Farmingdale, NY, USA) was used and diluted at 1:4,000 in 2% skim milk in PBS containing the detergent 0.2% Tween-20 for 1 h at room temperature. Immunoreactive proteins were detected using the enhanced chemiluminescence reagent EzWestLimiplus (cat no. WSE-7120; ATTO Corporation, Tokyo, Japan).

Flow cytometric analysis. Cell populations were isolated from the thymus or spleen of the transgenic mouse and investigated by flow cytometry. Cells were washed with 1X PBS and then fixed with 70% ethanol overnight at 4˚C. To examine the expression of surface markers of thymocytes, cells were stained with specific antibodies targeting surface markers of lymphocytes: Conjugated PerCP-Cy5.5-anti-CD44 (cat no. 45-0441-82; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), conjugated allophycocyanin (APC)-anti-CD25 and conjugated APC-anti-B220 (cat nos. 17-0251-82 and 17-0452-82; all from Invitrogen; Thermo Fisher Scientific, Inc.), conjugated PE-anti-CD4 and conjugated fluorescein isothiocyanate-anti-CD8 (cat nos. ab134354 and ab28010; all from Abcam). All antibodies were diluted 1:200 in FACS buffer (1X PBS containing 1% BSA) and incubated with each sample for 30 min on ice. Analysis was conducted using a FACSCanto II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and data were analyzed using FlowJo 7.6.5 software (Tree Star, Inc., Ashland OR, USA).

Statistical analysis. All experiments were repeated three times independently. Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). The data are presented as the mean ± standard deviation. The RT-qPCR data were analyzed by Student's t-test, and the other data were examined by a two-way analysis of variance followed by a Bonfferoni post-hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

hLY6K was successfully overexpressed in transgenic mice. To investigate the function of human *LY6K* on mammary development *in vivo*, transgenic mice overexpressing the human *LY6K* gene were established. We generated transgene construct for the full-length human *LY6K* gene downstream of the MMTV-long terminal repeat (MMTV-LTR) promoter (Fig. 1A). The transgene was injected into fertilized mouse eggs, which were then transferred into the oviducts of female mice. The potential founder mice were crossed with C57BL/6 (B6) mice, and 7 transgenic mouse lines were generated. Genomic DNAs extracted from the transgenic mice were amplified by PCR with human *LY6K*-specific primers. As shown in Fig. 1B, 4 of the 7 transgenic mice carried the *hLY6K* transgene. To further examine the copy number of the *hLY6K* transgene, qPCR was performed, revealing that transgenic mouse B had the highest expression of the transgene (Fig. 1C), and this was used for further study.

hLY6K expression in the tissues of transgenic mice. To determine the tissue specificity of the transgene expression, the RNA levels of *hLY6K* were examined in wild-type and transgenic mice by RT-PCR (Fig. 2A). Tissues were harvested from the thymus, spleen, mammary gland and other organs of 6-week-old virgin mice, and RNAs were extracted from each

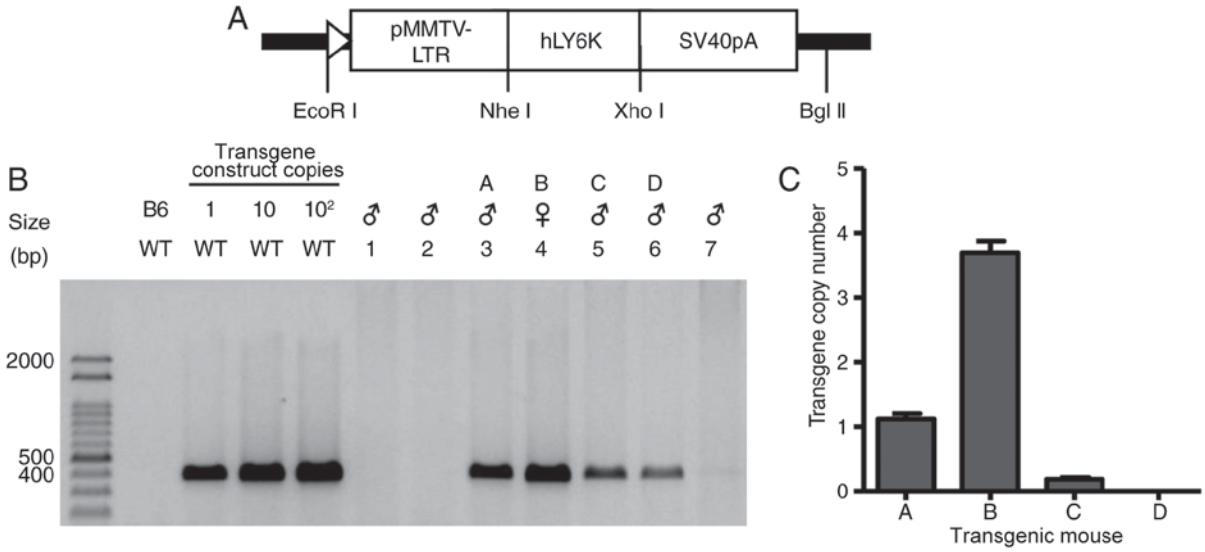

Figure 1. Generation and identification of transgenic mice. (A) Schematic diagram of a transgene construct for the *hLY6K* gene. The full-length *hLY6K* cDNA was placed under the control of the MMTV promoter. (B) PCR analysis using *hLY6K* gene-specific primers. Genomic DNAs extracted from seven transgenic mice (lanes 1-7) were amplified and size-separated on an agarose gel. The presence of the transgene was detected in genomic DNA from 4 of the mice, designated A, B, C, and D. PCR bands of the transgene construct mixed with WT genomic DNA are shown as the standard. (C) The copy number of the *hLY6K* transgene in founder mice. Genomic DNA was subjected to qPCR with primers specific to the *hLY6K* gene, and the signals of the transgene were normalized to those of mouse Gapdh, an endogenous control. Data are presented as mean ± standard deviation. hLY6K, human lymphocyte antigen 6 family member K; WT, wild-type; bp, base pairs; pMMTV-LTR, mouse mammary tumor virus long terminal repeat promoter; qPCR, quantitative polymerase chain reaction.

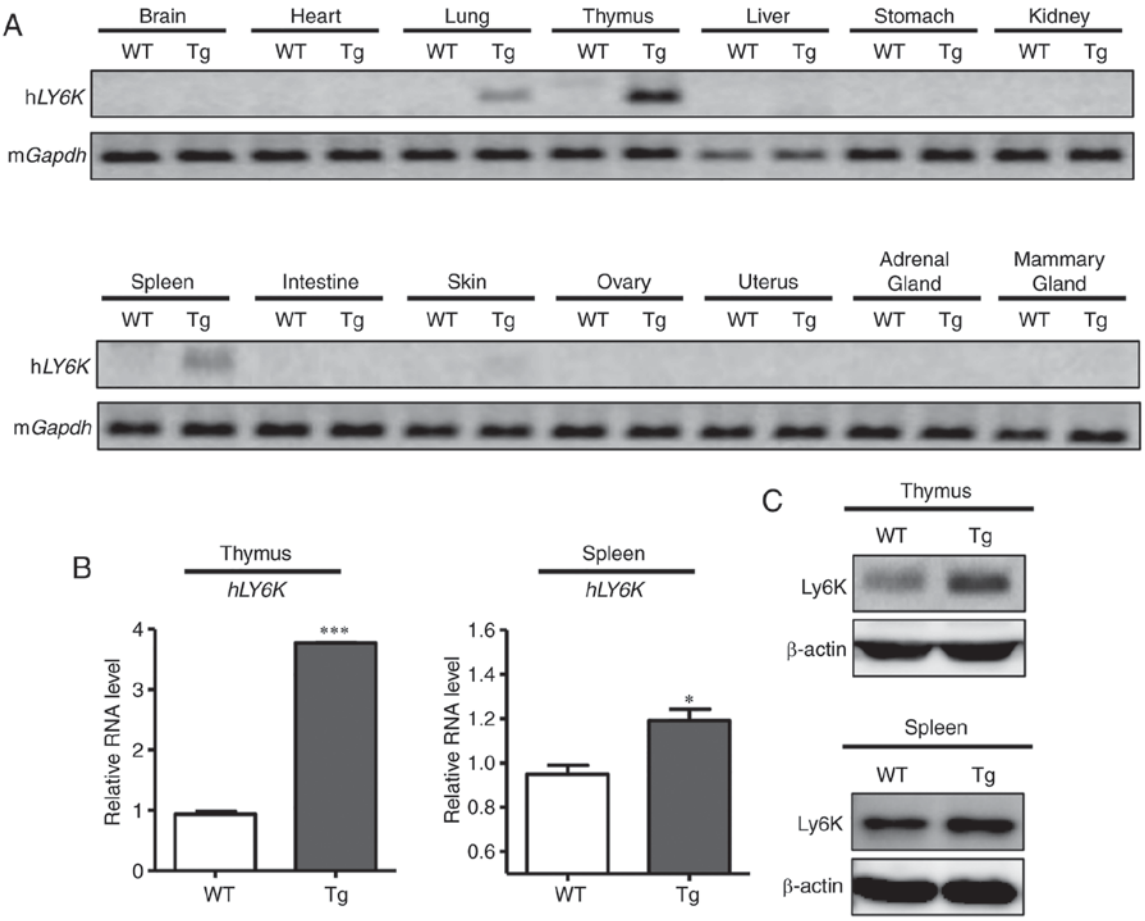

Figure 2. *hLY6K* mRNA levels in tissues of WT mice and Tg mouse B. (A) *hLY6K* mRNAs were extracted from each tissue and subjected to RT-PCR. The amplified fragments were size-separated using an agarose gel. *mGapdh* was used as the loading control. (B) The mRNA expression levels of *hLY6K* in the thymus and spleen were analyzed by RT-qPCR. The relative mRNA levels of hLY6K were determined by normalizing against the level of *mGapdh*. (C) The protein levels of LY6K in the thymus and spleen were analyzed by western blotting. β-actin was used as the loading control. Data are presented as the mean ± standard deviation; $^{***}P<0.001$, and $^{*}P<0.05$ (t-test). hLY6K, human lymphocyte antigen 6 family member K; WT, wild-type; Tg, transgenic; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; mGapdh, mouse glyceraldehyde 3-phosphate dehydrogenase.

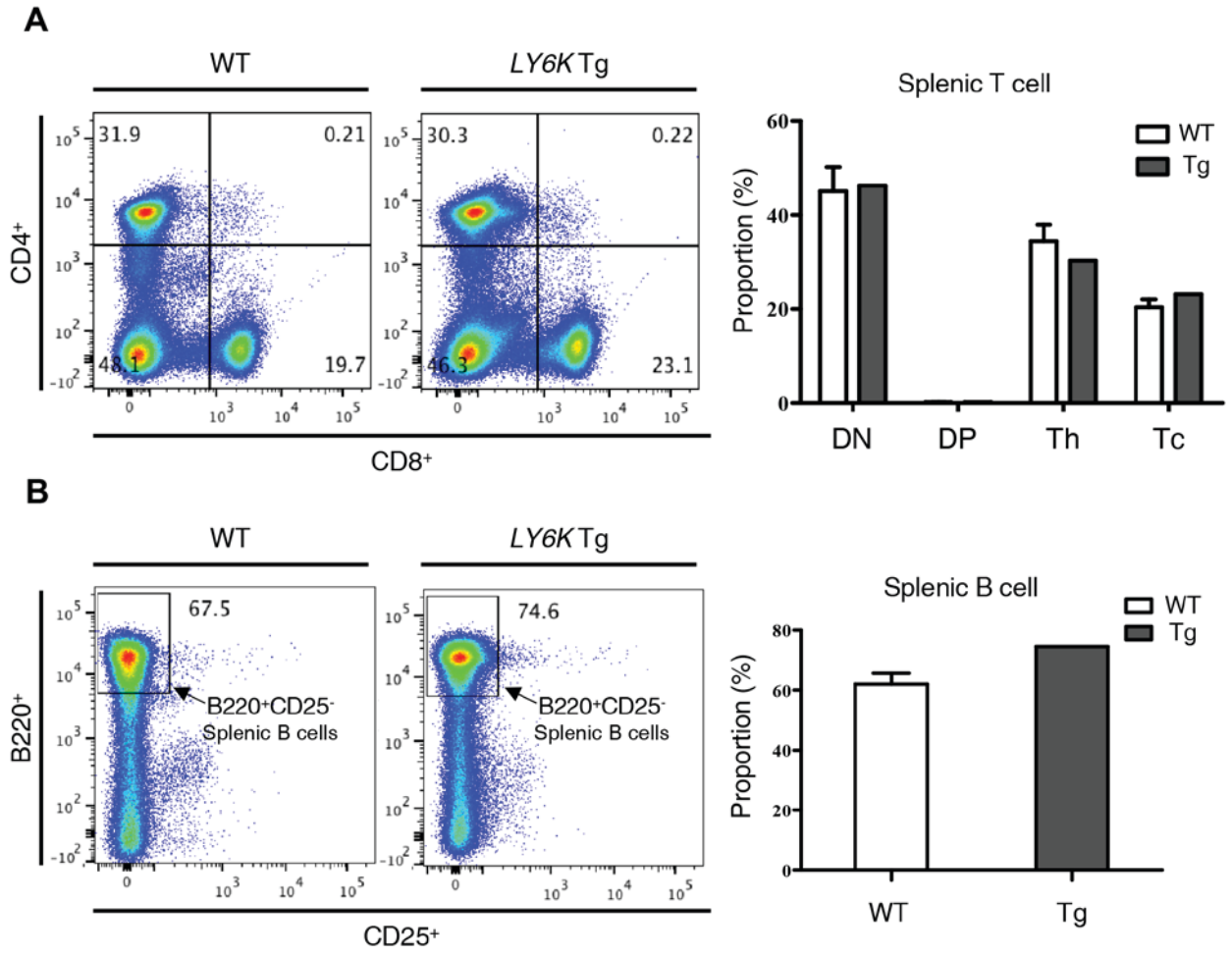

Figure 3. Distribution of T cells and B cells in the spleen of Tg mouse B and WT mice. Flow cytometric analysis was conducted to examine the distribution of T and B cells. The proportion of each cell type is shown in the graph, and the numbers represent the percentages of quadrant analysis. (A) Distribution of splenic T cells in the WT and Tg mice. Antibodies specific for CD4 or CD8 were used to identify the different types of T cells. (B) The distribution of splenic B cells ($B220^+CD25^-$) in the WT and Tg mice. Antibodies against B220 or CD25 were used to identify mature B cells. WT, wild-type; Tg, transgenic; LY6K, lymphocyte antigen 6 family member K; DN, double-negative ($CD4^-CD8^-$); DP, double-positive ($CD4^+CD8^+$); Th, helper T cells ($CD4^+CD8^-$); Tc, cytotoxic T cells ($CD4^-CD8^+$).

tissue sample, which were subsequently analyzed by RT-PCR. To avoid the detection of mouse *LY6K* instead of human *LY6K*, their sequences were compared using NCBI nucleotide BLAST, and there was no significant similarity except for the Ly-6/uPAR domain [(25), https://blast.ncbi.nlm.nih.gov/Blast.cgi; human LY6K gene accession number, NM_017527; mouse LY6K gene accession number, NM_029627]. Thus, PCR primers were designed to recognize only human *LY6K* mRNA but not the endogenous mouse *LY6K*, and it was confirmed that *hLY6K*-specific primers only amplified *LY6K* mRNAs extracted from human cell lines but not those from mouse cells (data not shown). Amplified products were separated on the agarose gel. As Fig. 2A illustrates, the mRNA expression of *hLY6K* was detected strongly in the thymus and weakly in the spleen and lung, while other tissues, including the mammary gland, displayed no expression of the transgene. For further examination, RT-qPCR was performed to quantitatively compare the RNA level of *hLY6K* in the wild-type and transgenic mice. RNAs extracted from the thymus and spleen were examined and, as indicated in Fig. 2B, the relative RNA expression of *hLY6K* was markedly increased in the thymus and spleen of the transgenic mouse compared with those of the wild-type mice. Furthermore, western blot analysis revealed that the transgenic mouse had markedly higher protein levels of Ly6K in the thymus and spleen compared with those of wild-type mice (Fig. 2C). These results indicated that the *hLY6K* transgene with the MMTV promoter was expressed specifically in the thymus and spleen, rather than in other tissues, including the mammary gland.

Effect of hLY6K overexpression on lymphocyte development in the spleen. As previously stated, the *hLY6K* transgene was significantly expressed in the spleen of the transgenic mouse examined. The spleen filters and generates blood cells; however, it is also involved in lymphocyte proliferation and activation as one of the major sites where blood-circulating antigens are presented to lymphocytes in order to generate an immune response (26). Therefore, the present study investigated whether the overexpression of *hLY6K* caused any changes to lymphocytes in the spleen. First, the distribution of T cells was verified according to the expression of CD4 and CD8, the surface markers of mature T cells. As shown in Fig. 3A, including helper T cells ($CD8^-CD4^+$) and cytotoxic T cells ($CD8^+CD4^-$), the proportion of each cell type did not exhibit any significant

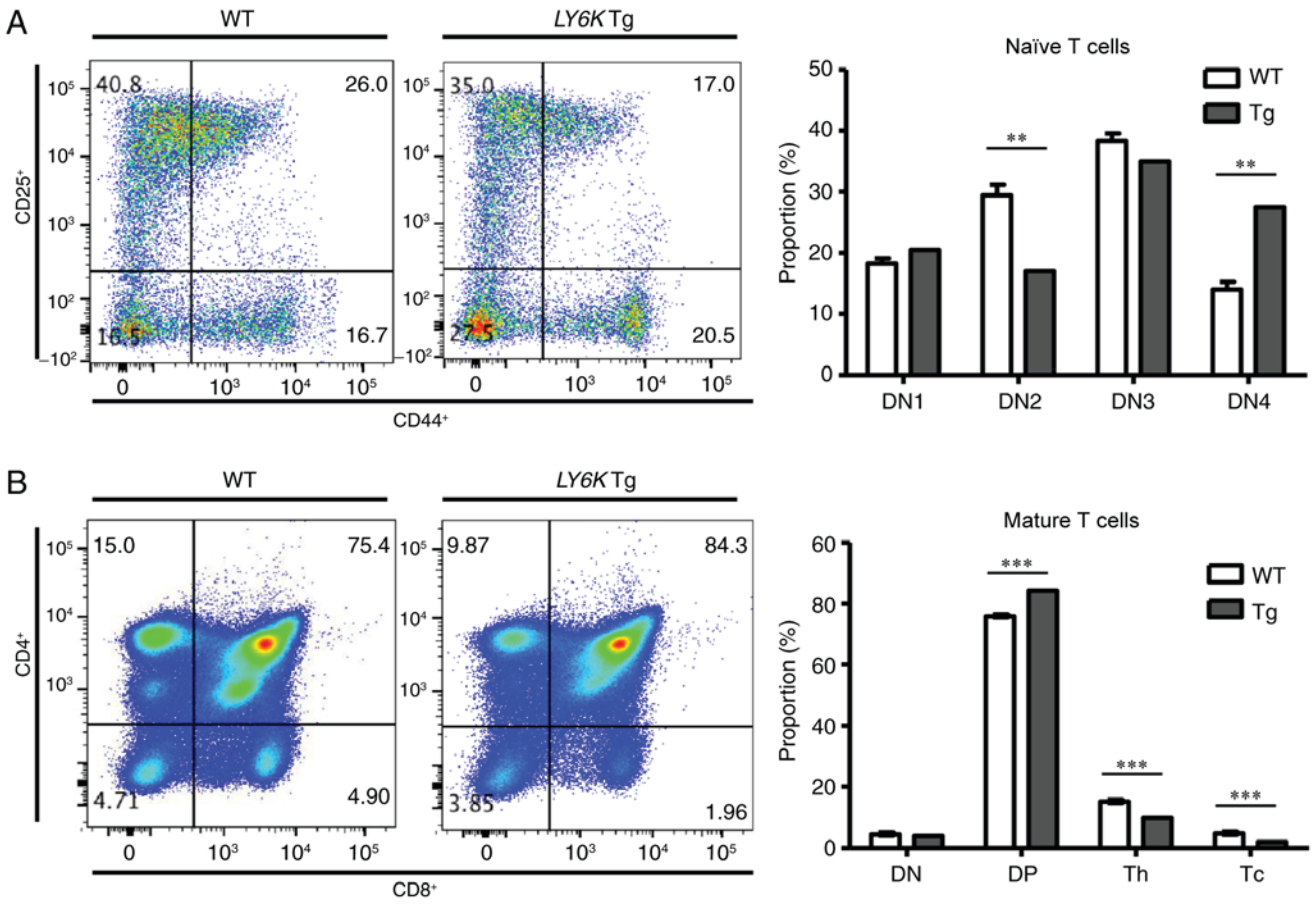

Figure 4. Distribution of T cells in the thymus of the WT and Tg mice. The distribution of T cells was confirmed by flow cytometric analysis. (A) The distribution of naïve T cells in WT and Tg mice was examined using antibodies specific for CD44 or CD25. (B) The proportion of each type of mature T cell was analyzed using antibodies specific for CD4 or CD8. Data are presented as the mean ± SD; ***P<0.001, and **P<0.01 (t-test). WT, wild-type; Tg, transgenic; LY6K, lymphocyte antigen 6 family member K; DN1, $CD44^+CD25^-$; DN2, $CD44^+CD25^+$; DN3, $CD44^-CD25^+$; DN4, $CD44^-CD25^-$; DN, double-negative ($CD4^-CD8^-$); DP, double-positive ($CD4^+CD8^+$); Th, helper T cells ($CD4^+CD8^-$); Tc, cytotoxic T cells ($CD4^-CD8^+$).

change in the spleen compared with the levels in wild-type mice. B cell populations in the spleen were then investigated by analyzing the expression of B220, a murine B cell marker, and CD25, the α chain of the IL-2 receptor (27) (Fig. 3B). Normally, CD25 expression is characteristic of $CD4^+FoxP3^+$ regulatory T cells in mice and only 2% of B cells express CD25 in the spleen (28). As shown in Fig. 3B, there was no significant change in the proportion of $B220^+$ and $CD25^-$ B cells between the wild-type and transgenic mice. These findings implied that the overexpression of *hLY6K* did not have a significant effect on lymphocyte development in the spleen.

Effect of LY6K overexpression on T cell development in the thymus. The expression of *hLY6K* was detected in the spleen and in the thymus (Fig. 2). The thymus is a specialized lymphoid organ where T cell development occurs. Therefore, the present study investigated whether *hLY6K* overexpression influenced T cell development in the transgenic mouse. DN naïve T cells, which are negative for CD4 and CD8 expression, can be subdivided into four subgroups (DN1-DN4) depending on their expression of CD44 and CD25 (29). Flow cytometric analysis was conducted to examine the expression of CD44 and CD25 in thymocytes isolated from wild-type and transgenic mice, and cells were categorized depending on the expression of those markers. Fig. 4A demonstrates the distribution of early naïve T cells in the thymus of wild-type and transgenic mice. The present study identified that the proportion of the DN2 ($CD25^+CD44^+$) subgroup was decreased markedly in the transgenic mouse compared with wild-type mice, whereas the DN1 ($CD25^-CD44^+$) and DN3 ($CD25^+CD44^-$) populations exhibited no significant differences. Notably, the DN4 ($CD25^-CD44^-$) population was markedly increased in the transgenic mouse, suggesting that more naïve T cells had completed TCR rearrangement and had begun to express CD4 and CD8 molecules for the next stage of development. Therefore, the levels of CD4 and CD8 (surface markers of mature T cells) were examined in the thymic T cells of each type of mouse to compare further developmental steps (Fig. 4B). The results revealed that the double positive ($CD4^+CD8^+$) subgroup was increased in the transgenic mouse, as hypothesized. However, the proportions of cytotoxic T cells ($CD8^+CD4^-$) and helper T cells ($CD8^-CD4^+$) were decreased in the transgenic mouse. This implied that fewer T cells were fully differentiated in the transgenic mouse, when *hLY6K* was overexpressed, compared with the wild-type mice, despite an increased number of lymphocytes undergoing TCR rearrangement.

Discussion

Ly6K is known to be upregulated in numerous types of cancer and its increased expression is associated with a poor

outcome in patients with cancer (30). In our previous study, Ly6K expression was identified to be inversely associated with estrogen receptor α (ERα) levels, suggesting that high expression of Ly6K may result in tamoxifen resistance of cancer cells (31). Furthermore, it was recently reported that Ly6K/E signaling involving transforming growth factor β promotes cancer progression and drug resistance in breast cancer (14). Therefore, Ly6K is considered to be both a prognostic marker and a therapeutic target in cancer, particularly in breast cancer (14), and a number of studies have been conducted on the molecular mechanism of LY6K *in vitro*. However, the *in vivo* mechanism of LY6K has received little attention. Therefore, in the present study, a transgenic mouse model overexpressing the *hLY6K* gene was constructed to investigate the effects of *hLY6K* overexpression *in vivo*.

hLY6K cDNA was inserted downstream of the MMTV-LTR promoter, which was subsequently injected into the mouse embryo to generate *hLY6K* transgenic C57BL/6 mice. The C57BL/6 mouse is one of the most commonly used animal models for human diseases (32). Since they have a relatively long life-span, are easy to breed, and are permissive for maximal expression of the majority of mutations, they are widely used to generate transgenic mice (33). MMTV is a milk-transmitted infectious agent involved in mammary epithelial cell tumors in mice (34). It was originally identified as an endogenous murine retrovirus, and has been used to generate animal models for the study of human cancer due to its LTR sequence flanking the MMTV genome and its ability to direct the expression of downstream genes (35,36). In particular, it can induce gene expression in mammary epithelial cells; therefore, MMTV-LTR has been widely used to generate transgenic mice overexpressing an exogenous gene in the mammary gland for the study of human breast cancer (37). Furthermore, as MMTV is expressed in other organs, including the salivary glands, lungs, spleen, and thymus, a transgene under the control of the MMTV-LTR promoter can be expressed in organs other than mammary glands (38,39).

In the present study, the human LY6K gene was inserted downstream of MMTV-LTR to generate transgenic mice, which resulted in the overexpression of *hLY6K* in the thymus and the spleen, but not in the mammary glands. Notably, it has been reported that MMTV-LTR activity is regulated by hormones of pregnancy, such as glucocorticoids and prolactin, and increases during pregnancy and lactation (40). It was also demonstrated that the MMTV promoter did not facilitate transgene expression in the virgin mammary glands, and the gene only began to be expressed during pregnancy and lactation in the examined mice (40,41). Considering that the transgenic mouse used in the present study was a 6-week-old virgin mouse, the lack of transgene expression in the mammary glands was attributed to the lack of pregnancy hormones responsible for the activity of the MMTV promoters. However, high expression of *hLY6K* gene was validated by qPCR analysis in the thymus and spleen of the transgenic mouse using *hLY6K*-specific primers.

The thymus and the spleen are the principal sites for lymphocyte development; therefore, the present study investigated whether the overexpression of *hLY6K* induced any defects in lymphocyte generation. Although *hLY6K* overexpression did not cause any significant changes in the distribution of lymphocytes in the spleen, the proportions of various types of functional mature T cells were decreased markedly in the thymus of the transgenic mouse. The increase in the DN4 ($CD25^-CD44^-$) population in the transgenic mouse, which implied that more premature T cells had undergone TCR rearrangement, suggested that T cell maturation was defective when *hLY6K* was overexpressed.

CD8+ cytotoxic T cells and CD4+ helper T cells are known to perform antitumor functions in numerous types of cancer (42,43). Cytotoxic T cells can specifically eliminate infected cells that present antigens bound to MHC class I molecules, which are recognized by cytotoxic T cells (44). Notably, the majority of cancer cells express MHC class I molecules; therefore, tumor antigen-specific cytotoxic T cells can infiltrate tumor sites and attack tumor cells specifically by recognizing the antigens linked with MHC molecules (45,46). In addition, $CD4^+$ helper T cells, which aid the adaptive immune response of other immune cells by releasing cytokines, are reported to be required for effective antitumor immunity (47,48). In addition to their ability to maintain immune responses to tumor cells by helping cytotoxic T cells and other immune cells, helper T cells are also able to kill cancer cells directly, as mutated or fusion proteins in cancer cells generate MHC class II molecules that are recognized by helper T cells (49,50). Therefore, the immune responses mediated by the different types of T cells execute protective mechanisms against cancer.

The experimental results demonstrating the decrease of the functional T cell population under Ly6K overexpression in the thymus indicated that Ly6K may negatively regulate normal T cell differentiation, which in turn may lead to the suppression of the antitumor effect of immune cells.

As previously stated, a number of studies have demonstrated that Ly6K is upregulated in numerous types of cancer and positively regulates tumor progression. However, the expression patterns and function of Ly6K in the thymus of patients with cancer have not yet been investigated. Considering the potential negative effect of Ly6K on antitumor immunity identified in the present study, further studies are required to investigate whether Ly6K expression is upregulated in the thymus of patients with cancer and whether Ly6K overexpression suppresses the generation of functional lymphocytes.

Notably, it has been reported that uPAR expression is increased to a greater extent in regional lymph node metastasis than in the primary intraprostatic tumor mass (51). Given that thymic metastasis occurs in several types of cancer, including breast cancer (52-54), the expression of Ly6K in the thymus may be altered by the metastasis from the primary tumor mass in these cancer patients. Together with its own oncogenic effect, the potential role of Ly6K in T cell development and anticancer immunity makes it a promising therapeutic target in human cancer.

In conclusion, the present *in vivo* study demonstrated that Ly6K has the potential to suppress the immune response against tumor cells by inhibiting T cell development. However, the present study did not reveal the molecular mechanisms of Ly6K in T cell development. Therefore, further investigation on Ly6K at the molecular level is required in order to demonstrate the involvement of the Ly6K pathway in lymphocyte development. Additionally, the effect of Ly6K on the antitumor immune response in patients with cancer should be studied to fully

understand the role of Ly6K in human cancer. Eventually, Ly6K might provide an effective therapeutic target to treat cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DS served a major role in the experiments and prepared the manuscript draft; HKK was involved in these experiments and prepared the manuscript draft; YK was involved in these experiments; MJS was involved in producing the mouse model; HPL was involved in producing the mouse model; HWL served a major role in producing the mouse model; and JHP designed the experiments and prepared the manuscript draft.

Ethics approval and consent to participate

All animal experiments were approved by the Institutional Animal Care and Use Committee at the Sookmyung Women's University (Seoul, South Korea).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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