이학석사학위 청구논문

TSCOT 유전자를 발현하는 흉선상피세포에 대한 연구

Analysis of TSCOT-Expressing Thymic Epithelial Cells

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이 논문을 석사학위 논문으로 제출함

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Abstract

Intrathymic T-cell development requires an intact stromal micro-environment, of which thymic epithelial cell (TEC). Using mouse model, called TDLacZ, established by knock-in the LacZ gene into TSCOT gene, I explored the developmental change and compartmentalization of TSCOT-expressing cells. In addition some markers were utilized to define the characteristics of TSCOT+ cells. These results demonstrate that the compartment of TSCOT gene expression is developmentally regulated, and the number of TSCOT-expressing cell change in stages. Furthermore, TSCOT-expressing cells appear to have properties of thymic epithelial precursor cell (TEPC) and to possess a capacity of antigen-presenting. To perform in-depth study, I attempt to make cell line including TSCOT gene expression.
Introduction

Thymus has a central role in the immune development, as it is crucially required for T cell differentiation and repertoire selection. These processes are mediated by the thymic stroma, which has a complex cellular composition [1]. However, the unique functions of the thymus reside mainly in the thymic epithelium, which forms the major sub-compartment of the stroma. The stroma itself is commonly divided into two main regions, the cortex and the medulla, based on the histological analysis and each of these regions contains several ultrastructurally and phenotypically distinct types of thymic epithelial cells (TECs).

T-cell development is characterized by the progression through several phenotypically distinct stages, defined as double negative (DN), double positive (DP) and single positive (SP) based on the expression of the co-receptors CD4 and CD8; the DN subset is further subdivided into four stages (DN1–4) by differential expression of CD44 and CD25 [2]. Thymocytes at different stages of developmental stages occupy distinct spatially restricted domains in the adult thymus, indicating that differentiation occurs concomitantly with a highly ordered migration. T cell precursors enter the thymus at the cortico-medullary junction, then migrate progressively to the subcapsular zone of the outer cortex, back through the cortex and into the medulla, from where they egress to the periphery. Thymocytes and TECs are in close contact throughout this differentiation programme [2]. It is believed that this organization provides the different microenvironment that affects T cell development as these cells transit through the thymus. Although thymocytes development has been extensively studied, not much is known regarding how stromal cells affect this process.

Previously, in an effort to separate TEC components, a new marker (Ly110) was isolated and designated as thymic stromal co-transporter (TSCOT), which is expressed in a specific TEC subpopulation. TSCOT is a putative 12-transmembrane protein, located mainly in the thymic cortex [3]. TSCOT is not detected in any other tissues by northern blotting [1] and quantitative reverse transcription PCR (RT-PCR) [4]. It is also not expressed in thymocytes
TSCOT’ thymic stromal cells are all MHC II’ and CDR1’/6C3’, well-defined cortical epithelial markers [5]. A targeted new mouse model system was established and called as TSCOT delta LacZ (TDLacZ) that expresses a β-galactosidase (β-gal) in the TEC sub-population. This model system provides a new tool for the study of TEC development and function [6].

Although it is well established that morphologically and antigenically unique subsets of TECs occupy distinct microenvironmental niches, the developmental regulation imposed by early DN thymocytes on cortical TEC maturation and the lineage relationships involved in this process are not well defined. Keratins are intermediate filament proteins that are expressed in all epithelial cells as heterodimers of acidic type I and basic type II polypeptides [7]. However, the expression of different keratin species is regulated in a tissue-specific manner and also depends on epithelial cell differentiation stage and proliferative activity. Thus, keratin expression patterns have been used as the markers to identify epithelial cell subsets and determine their lineage relationships in a variety of tissues [7-9].

T cells are selected for recognizing self MHC molecules and against responding to self antigen during their development in the thymus. Analysis of T-cell development in the thymus has led to the identification of a series of checkpoints during early thymocyte development [10]. During later stages of T-cell development, the positive and negative selection of CD4’CD8’ thymocytes, which leads to the production of a self-tolerant T-cell repertoire, is of critical importance in avoiding unwanted T-cell responses to self antigens that can manifest as autoimmunity. The mechanism that regulate the development and selection of αβTCR-expressing T cells are governed by signals from thymic stromal cells [11].

Mouse strains expressing the site-specific recombinase Cre was used for conditional ablation of gene function when one or several exons of the gene of interest are flanked by loxP sites [12]. Cre expression achieved by classic transgenesis or targeting to an appropriate locus might be tissue specific, temporally restricted or inducible [13, 14]. In such experimental outlines, it is necessary to monitor Cre activity at desired time points as well as to verify that Cre was not active previously during development. Other investigators have
generated transgenic or knock-in lines in which $lacZ$ expression is conditional on the removal of an intervening segment [12]. However, such lines are most useful if $lacZ$ can be expressed in all cell types and hence is driven off a constitutively active promoter in the mouse [12].

In this study, I try to examine about TSCOT expressing cells using TDLacZ mouse model. The results indicate that the TSCOT expression was developmentally changed in developing thymus of TDLacZ$^{Δ/Δ}$ mouse. And the number of γδ TCR-bearing thymocyte were increased in the thymus of TDLacZ$^{Δ/Δ}$ mouse compared with TDLacZ$^{+/Δ}$ mouse. This result offers the possibility that it can be used tool for study of γδ T cells. Moreover, the TSCOT expression detected in K5*K8+ cell and CD80, which is one of the costimulatory molecule, expression was strong in CDR1’TSCOT+ cTECs compared with CDR1− mTECs. To perform an analysis of TSCOT+ cells, according to these results, I attempt to establish a cell line containing TSCOT expression. It is possible an in-depth study about cell expressing TSCOT gene if there is a TSCOT+ cTEC cell line.
Materials and Methods

Mice

TDLacZ mouse model were described [6]. For PCR genotyping, tail samples were employed using the red Extract-N-Amp Tissue PCR kit (Sigma) and three primers, for the TSCOT locus, Neo primer: ACCGCTATCAGGACATAGCGTTGG, 1C12 F1: TTACTCAAAAGTGATGCTGGACTGG, 1C12 B2: CCGAGGGTTCCTTGTTGACATT.

3.1T-EGFP transgenic mouse line expresses enhanced green fluorescent protein (EGFP) in the thymic epithelial cells by 3.1kb TSCOT promoter activity [4]. Primers used for EGFP genotyping, EGFP forward: GCCACAAGTTCAGCGTGTCC, EGFP reverse: GCTTCTCGTTGCGGTTCTT.

4.4T-CRE express cre recombinase specifically in TEC by 4.4kb TSCOT promoter activity (unpublished result). Primers for cre transgene genotyping are Cre-a: GCCGCTCTGGGAGCATTACATC, Cre-b: GTGAAACAGCATGCCTGACTT.

β-gal Activity Assay

Either whole embryos or isolated thymuses were washed in PBS and fixed in 1% para-formaldehyde, 0.2% glutaraldehyde, 0.02% NP-40 and 1mM MgCl₂ in PBS for 10 min or 2 h. Staining was conducted using 1μl/ml X-gal solution with 100mM D-galactose in 2mM MgCl₂, 5mM potassium ferricyanide and 5mM potassium ferrocyanide in PBS for overnight at 37°C. The embryos or thymuses were washed with PBS for 10 min and then post-fixed with 4% para-formaldehyde in PBS for 10 min. The thymuses were embedded in Tissue Freezing Medium (Triangle Biomedical Sciences), then 4μm sections were prepared using Cryostat (Leica).
**Thymic Stromal Cell Preparation**

Thymus were finely chopped and minced with straight scissor in cold PBS and thymocytes were removed by allowing the suspension to stand for 1 min until the supernatant becomes clear. The remaining fragments were subjected to digestion with 450μl of 0.25% trypsin and 100μl of 20 mg/ml deoxyribonuclease 1 (DNase 1) for 15 min on 37°C at 950 r.p.m.. After adding 1% Fetal Bovine Serum (FBS) in PBS, the resulting suspension were filtered with nylon mesh and then centrifuged at 6500 r.p.m. for 30 s. The pellet was resuspended with 10% FBS in PBS and DNase 1, then centrifuged at 6500 r.p.m. for 30s. The pellet was incubated with 5μl of anti-Fc receptor (100x 2.4G2) and 10μl of anti-mouse CD45-microbeads per 5x10⁷ cells for 20 min at 4°C, and CD45-positive cells were collected using Magnetic Bead Cell Sorter (MACS). The passed stromal cells were centrifuged at 2000 r.p.m. for 8 min. The pellet was resuspended with 200μl of FACS buffer and analyzed by flow cytometry after antibody staining.

**Flow Cytometry**

Thymocytes were washed in cold FACS buffer (PBS +1%BSA) and centrifuged at 1200 rpm for 2 min. The cells were stained on ice with 2.4G2 and primary antibodies for 20 min, and washed with FACS buffer on 96-well-plate by centrifuge at 1200 rpm for 2min. Subsequently supernatant was removed and the pellet was suspended with FACS buffer and moved to FACS tube for analysis on FACS Aria machine (BD). Analysis was done using FlwJo 6.0 (http://flowjo.com). Antibodies used for flow cytometric analysis were as follows: for thymocytes, FITC-conjugated anti-CD8 and anti-γδ TCR, PE-conjugated anti-CD44 and CD8, mouse CD4 and CD8 PE-Cy5 conjugate, and mouse CD25 and CD4 PE-Cy7 conjugate.

**Immunofluorescence and Confocal microscope**

Isolated TECs were cultured on chamber-slide in culture medium (IMDM 10% FBS) for 1 week. The cells were fixed with 4% para-formaldehyde and
incubated with ImaGene Green C12 FDG LacZ Gene Expression kit (Molecular Probes). After 1 hr, the cells were stained with rat anti-keratin8 (TROMA-1) and TexasRed-conjugated goat anti-rat IgG (H+L) (Vector laboratories), and rabbit anti-keratin5 and Cy5-conjugated goat anti-rabbit (AF 138, Covance). Images were collected on LSM 510 META (Zeiss), and processed with LSM Image Examiner (Zeiss) and Photoshop.

Isolated TECs (about 10^5 cells) were washed in cold FACS buffer, subsequently stained on ice with 2.4G2 and APC-conjugated anti-CDRI, biotinylated anti-CD80 (B7-1, Armenian hamster IgG2κ) followed by streptavidin-AlexaFluor568 (Molecular Probes). For the detection of LacZ-expressing cells, ImaGene Green C12 FDG lacZ Gene Expression kit (Molecular Probes) was used. Stained samples were placed on the slides by cyto spin at 1200 rpm for 2 min. Images were collected on LSM 510 META (Zeiss), and analyzed with LSM Image Examiner (Zeiss) and Photoshop.

**Retrovirus Infection**

Retroviral vector containing SV40 T antigen sequence between two LTRs was transfected into the packaging cell line (293gpg cell line from Cheong HY in Hanyang University) with lipofectamin source. After thymocyte were removed as much as possible, stromal cell and remained thymocyte were re-aggregated on transwell-plate with culture medium (IMDM 10%FBS), and infected with supernatant in culture medium. The cells were observed under the fluorescence phase contrast microscope (Olympus CKX41 and Olympus IX71) and documented by DP controller (Olympus) and Photoshop.
Result

Where is TSCOT expressed in fetal and developing thymus?

In order to address the question, above TDLacZ mouse line was used (Figure 1). In this system, LacZ is expressed in the cells that express TSCOT since two genes are in the same mRNA (Figure 1A and B). The genotype profiles are shown in Figure 1C. To examine when the TSCOT gene expression initiated and where embryos, at different stages, were stained with X-gal. The LacZ expression in TDLacZ mouse was detected in E11.5, when the thymic rudiment start to pattern from third pharyngeal pouch [2], as well as in E12.5, when the thymic rudiment outgrowth and separate from third pharyngeal pouch, and start the differentiation [2] (Figure 2A and B). During developmental stage, furthermore, LacZ expression patterns were slightly changed. In the 4-week-old and 6-week-old thymus of TDLacZ\(\Delta/\Delta\), LacZ was generally expressed (Figure 2C and D) but principally visible in the cortical area of the thymus (Figure 2F). In the 8-week-old thymus that is fully developed stage, LacZ expression was restricted in the outer cortex region (Figure 2E). It is consistent with previous data that TSCOT expression was shown only in the thymic cortex region by in situ hybridization [3]. Surprisingly, strong LacZ expression was detected in the uterus of female TDLacZ\(\Delta/\Delta\) (Figure 2G).

Is there any effect on thymocyte in TDLacZ\(\Delta/\Delta\) mouse?

To investigate whether thymocyte development was affected by deletion in TM5–TM12 portion of the TSCOT protein, thymocyte of TDLacZ at fetal and new-born stage were analyzed by flow cytometry. Whereas previous date showed that the total cell number of 6–week–old TDLacZ\(\Delta/\Delta\) were reduced about 30 percentage than TDLacZ\(+/\Delta\) and TDLacZ\(+/+) [6], total cell number of TDLacZ\(\Delta/\Delta\) in E14.5 and new-born mouse was similar with those of TDLacZ\(+/\Delta\) (Figure 3A). T–cell development is analysed using the markers of the co–receptors CD4 and CD8, and CD44 and CD25. Subsequently, the thymocyte of TDLacZ\(\Delta/\Delta\) and TDLacZ\(+/\Delta\) in E14.5 and new–born stage were analyzed using markers for
developmental thymocyte subsets (Figure 3B–C). In E14.5, all of thymocyte is double-negative for CD4 and CD8 in TDLacZ+Δ and TDLacZΔ/Δ mouse whereas majority of thymocyte in new-born stage was double-positive (Figure 3B and C, upper). The profile of DN stage of TDLacZ+Δ and TDLacZΔ/Δ was also similar in E14.5 and new-born stage (Figure 3B and C, middle). In E14.5 stage of TDLacZ+Δ and TDLacZΔ/Δ, about 55% and 61% of total thymocyte were γδ TCR. In new-born mouse, however, only about 1% of total thymocyte were γδ TCR (Figure 3B and C, low). It is consistent that the γδ T-cell is differentiated in early fetal stage, but general cell number is decreased as progression of development stages. The γδTCR thymocyte cell number of TDLacZΔ/Δ in new-born stage were increased about 60% than those of TDLacZ+Δ mouse, whereas there was no differency in E14.5 stage (Figure 3D).

**Keratin Expression in LacZ’ Cells of TDLacZ**

In previous study, TSCOT gene expression detected in the thymic rudiment of nude mouse which is mutated in the FoxN1 gene involved in thymic epithelial cell differentiation and maturation (unpublished data). I expected TSCOT is active in the precursor stage. To examine whether TSCOT’ cells have a characteristics of thymic epithelial precursor cell (TEPC), I used keratin marker. It was reported that double-positive cell for keratin5 (K5) and keratin8 (K8) has a characteristics of TEPC. Isolated thymic stromal cells from TDLacZΔ/Δ mouse were analysed by confocal microscope (Figure 4). Among the K5 K8’ cells, one cell appears to be express LacZ. The K5 K8’LacZ’ cell strongly exhibited the LacZ expression whereas LacZ-no treated cells were weakly expressed, and has large size comparing with the other cells. Also, among K5’K8’ cells, just one cell appears to be express LacZ.

**TSCOT’ Cell Express Co-stimulatory Molecules**

We further assessed the presence or absence of selected costimulatory and adhesion molecules in the TSCOT-expressing cells. Although there has been reports that cortical epithelium does not express costimulatory molecule by histological analysis, we had reasons to believe that this conclusion may be
false based on our observation of disparity between histology and flow cytometry [17]. Flow cytometry revealed that TSCOT\(^+\) cells are all positive for MHC\(\text{II}\), CD40, and CD54 expression [6]. An important costimulatory molecule, CD80, was expressed in some TSCOT\(^+\) cells. In order to compare the relative levels of CD80 between mTEC and TSCOT\(^+\) cells, the multi-parameter analyses in confocal microscopy were applied including LacZ staining with the stromal cells prepared from TDLacZ thymus (Figure 5). The mTECs defined by CDR1\(^-\)LacZ\(^+\) expressed CD80 on their cell surface as well known whereas TSCOT\(^-\)cTECs defined by CDR1\(^-\)LacZ\(^+\) expressed CD80 strongly.

**Establishing Thymic Epithelial Cell Line With TSCOT Expression**

We tried to make cell line including TSCOT gene for further molecular experiments using mouse model that EGFP expression is restricted within TSCOT-expressing TECs. To establish this cell line, we choose retrovirus transduction system. SV40 T antigen sequence put into BamH1 site between 5’ and 3’ LTR in pBABE retroviral vector (Figure 6A). Stromal cell, but partly thymocyte-contained, were prepared by trypsinization the thymus from new-born mouse, and re-aggregated on the transwell-plate for about 1 month or 2 month. During re-aggregate culture, the cells were infected by generated retrovirus including SV40 T antigen (Figure 6B).

To perform this strategy, I utilized mouse which is crossed between 4.4T-CRE and ROSA reporter line. The offspring mouse from these two mouse line express EGFP only in TECs by 4.4kb TSCOT promoter activity, and were observed under the phase contrast and fluorescence microscope (Figure 7). When re-aggregated cells were observed under the phase contrast microscope, the cells were spread-out to outside of clump in day 2 (Figure 7A, upper line). The cells formed thymus-like shape within aggregates in day 2 (Figure 7A, middle line), and, in day 14, the cells were generally spread and leaved the trace of thymus-like shape as edge (Figure 7A, low line). Same experiment was executed with another mouse model, 3.1T-EFGP transgenic mouse (Figure 8). The result of this experiment show similar pattern with those of 4.4T-CRE
mouse except strong expression level of 3.1T-EGFP mouse (Figure 8A, upper and middle line). In spite of virus infection, as well as 4.4T-CRE mouse, the EGFP+ TECs disappeared after 2 month (Figure 8A, low line). During re-aggregate culture, it was observed that aggregated TECs hold a number of thymocytes (Figure 8B). And dead or apoptotic cells having autofluorescence were gathered together in a center of clump (Figure 8C).
Discussion

In this thesis, I have examined the nature of TSCOT-TECs. The TSCOT gene expression was identified at E11.5, the initial stage of thymus development, by detecting LacZ expression in TDLacZ mouse. The location and number of LacZ+ cells were developmentally changed. Whereas LacZ expression was showed in outer cortical region at 8 weeks, it was detected in medullary region and cortico-medullary junction as well as cortical region at 4 weeks (Figure 2C and E). The number of LacZ+ cell appears to be decreased according to development. TSCOT-expressing cell defined by LacZ expression appears to express K8, or K5 and K8 coincidently (Figure 4). It was reported that double-positive for K5 and K8 has a characteristics of TEPC. These results offer a possibility that TSCOT can be used marker to identify the TEPC. Using confocal analysis, moreover, I try to define the function of TSCOT-TECs. In this experiment, it was confirmed that TSCOT+ cTEC has antigen-presenting function and express important molecule, CD80, for raising tolerance to self-antigen [6].

Because there are some restricts in a study of TSCOT cells obtained directly from thymus of mouse, I attempt to establish cell line containing TSCOT expression for profound study. Isolated stromal cells from thymus, however, dead or disappeared when the cells were cultured in vitro a long period of time about 2 month. It is well known that some factors from thymocyte and fibroblast are required for thymic stromal cell maintenance. Consistent with this information, aggregated TECs contained a lot of thymocytes, and the thymocytes appear to be dead rapidly and concentrated on center region of aggregate (Figure 8B and C).

TDLacZ mouse has incomplete TSCOT gene expression because LacZ gene put into TSCOT locus. Because It is well known that there is crosstalk between thymocytes and TECs during thymic organogenesis, I expected that some effects may exist in development fo TDLacZ mouse. As shown in Figure 3, however, a head part of TSCOT gene seems not to be necessary for development of normal thymus. Further studies are needed to define the effect by whole gene
knock-out to thymus development.
Figure 1. Targeting LacZ into the TSCOT locus for expression in thymic epithelium (Adapted from Ahn et al. [6]).

(A) Schematic presentation of wild-type (top), the targeting construct (middle), and the targeted allele (bottom). The restriction sites BCII (BCII) and BamHI (B) and location of coding regions are shown. PCR genotyping primer positions are shown as small arrows.

(B) Northern blot for the TSCOT-LacZ fusion message. The probe (TSCOT, LacZ and GAPCH control) is at the top left.

(C) PCR genotyping for TDLacZΔ/Δ, TDLacZ+/Δ and TDLacZ+/A mouse. KO and WT band size are 240bp and 417bp, respectively.
Figure 2. LacZ and TSCOT expression in thymus

(A and B) LacZ activity in the developing thymus (E11.5 and E12.5, respectively) of fetuses from TDLacZΔΔ and TDLacZ+/+. Only the targeted thymus was stained (within the red circles).

(C, D and E) LacZ activity in the thymus of 4-week, 6-week, and 8-week-old mouse from TDLacZΔΔ, respectively. Pictures show boundary region of thymus.

(F) Horizontal section of thymus of 4-week-old TDLacZΔΔ mouse. LacZ generally express in thymus, but mainly express cortex region of thymus.

(G) LacZ activity in the uterus of adult TDLacZΔΔ and TDLacZ+/+ mouse. TDLacZΔΔ uterus has strong LacZ expression.
Figure 3. Analysis of thymocyte of TDLacZ$^{\Delta\Delta}$ and TDLacZ$^{\gamma/\Delta}$

(A) Total thymocyte cell number of TDLacZ$^{\Delta\Delta}$ and TDLacZ$^{\gamma/\Delta}$ in E14.5 and new-born mouse, respectively. Average cell number are similar between TDLacZ$^{\Delta/\Delta}$ and TDLacZ$^{\gamma/\Delta}$ mouse.
(B and C) Profiles of thymocyte of TDLacZ$^{\Delta\Delta}$ and TDLacZ$^{\gamma/\Delta}$ by developing markers, CD4 and CD8, CD44 and CD25, and γδTCR.
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Figure 4. Confocal images of TEC costained with Keratin5, Keratin8 and LacZ in TD LacZΔΔ.
Figure 5. Confocal images of CD80, Co-stimulatory molecule, and CDR1, cTEC marker, of TSCOT-expressing cell.
Figure 6. Schematic presentation of retrovirus infection
(A) Retroviral vector containing SV40 T-antigen
(B) Procedure of retrovirus infection
A.

SV40 T antigen

5' LTR gag SV40 early promoter puro 3' LTR

B.

Thymus from New Born mouse

Trypsinize

Thymic Stromal Cell Preparation

Re-aggregate Culture on the Transwell-plate

After 1 day

Retrovirus sup. + Polybrene (8μg/ml) In Culture Media
Figure 7. Pictures from TEC of 4.4T-Cre crossed with EGFP reporter mouse during reaggregate culture
(A) Pictures obtained from phase contrast microscope. Each lines present middle, boundary and outer region of reaggregate spot.
(B) Pictures obtained from fluorescence microscope.
Figure 8. Pictures from TEC of 3.1T-EGFP during reaggregate culture
(A) Pictures obtained from phase contrast fluorescent microscope, 2 days, 28 days and 56 days after reaggregate culture.
(B) Reggregated TECs include a number of thymocytes.
(C) Dying or dead cells are gathered together. These cells have yellowish fluorescence.
References


