Characterization of the expression of cytokeratins 5, 8, and 14 in mouse thymic epithelial cells during thymus regeneration following acute thymic involution

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Abstract: The thymus is a central lymphoid organ for T cell development. Thymic epithelial cells (TECs) constitute a major component of the thymic stroma, which provides a specialized microenvironment for survival, proliferation, and differentiation of immature T cells. In this study, subsets of TECs were examined immunohistochemically to investigate their cytokeratin (CK) expression patterns during thymus regeneration following thymic involution induced by cyclophosphamide treatment. The results demonstrated that both normal and regenerating mouse thymuses showed a similar CK expression pattern. The major medullary TECs (mTEC) subset, which is stellate in appearance, exhibited CK5 and CK14 staining, and the minor mTEC subset, which is globular in appearance, exhibited CK8 staining, whereas the vast majority of cortical TECs (cTECs) expressed CK8 during thymus regeneration. Remarkably, the levels of CK5 and CK14 expression were enhanced in mTECs, and CK8 expression was upregulated in cTECs during mouse thymus regeneration after cyclophosphamide-induced acute thymic involution. Of special interest, a relatively high number of $CK5^+CK8^+$ TEC progenitors occurred in the thymic cortex during thymus regeneration. Taken together, these findings shed more light on the role of CK5, CK8, and CK14 in the physiology of TECs during mouse thymus regeneration, and on the characterization of TEC progenitors for restoration of the epithelial network and for concomitant regeneration of the adult thymus.

Key words: Cytokeratin, Thymic epithelial cell, Thymic epithelial progenitor, Thymus regeneration

Introduction

The thymus is a central lymphoid organ for development of T cells from bone marrow-derived immature T cells. Thymic epithelial cells (TECs) constitute a major component of the thymic stroma, which provides a specialized micro-

environment for T cell development [1]. TECs in the thymic cortex are responsible for attraction of T cell precursors, commitment to the T cell lineage, expansion of immature double negative thymocytes, and positive selection of double positive thymocytes [2-4]. The thymic medulla is composed of a heterogeneous population of epithelial cells that function in negative selection of single positive thymocytes [3-5].

The morphological heterogeneity of TECs has been well documented at the light and electron microscopic level [6-8]. It is important to understand the identification of the different types of TECs, as each type has distinct properties and functions in governing T cell development. TECs are

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traditionally divided into two major categories; cortical TECs (cTECs) and medullary TECs (mTECs), according to their location in the thymic lobule. A subset of mTECs, particularly in humans, is often concentrically arranged and forms Hassall's corpuscles. Furthermore, human TECs can be classified into several types based on their ultrastructural characteristics, including thymic subcapsular (subcapsular/paraseptal/perivascular), cortical or pale, intermediate, dark, undifferentiated, large medullary or cystic, and spindle-shaped medullary epithelial cells, and Hassall's corpuscles [9]. Generally, TECs are broadly classified into four types: subcapsular/paraseptal/perivascular, cortical and medullary epithelial cells, and Hassall's corpuscles, based on their immunohistochemical features [6, 7, 10, 11].

A unique characteristic of TECs among the various cell populations of the thymus is the presence of cytokeratin (CK) intermediate filaments, which form tonofilaments in the cytoplasm. Thus, antibodies against various types of CKs have been very useful for identifying TECs in the thymus. In humans, CKs comprise a family of at least 30 subtypes [12, 13]. Each CK subset expressed by an epithelial cell depends mainly on the type of epithelium, the stage of development and differentiation, and the functional states and pathological conditions [14, 15]. Immunohistochemical determination of CK polypeptides with highly specific antibodies is very useful for classifying and phenotypically characterizing subsets of TEC populations. Variations in CK composition in different parts of the human thymus have been demonstrated [16, 17]. Simple epithelium CKs such as CK8, CK18, and CK19 are present in the cortex, and stellate TECs in the medulla are positive for CK19 [18]. Most mTECs express complex epithelium CKs such as CK13, CK14, and CK17, in contrast to the cortex, where only a few cells are positive for these CKs [18]. In mice, antibodies specific to CK5, CK8, and CK14 have been most commonly used in studies to identify the main TEC subtypes [19-22].

Thus, in the present study, we characterized the CK5, CK8, and CK 14 expression patterns in TECs during thymus regeneration following acute involution induced by cyclophosphamide in mice.

Materials and Methods

Experimental acute thymic involution and regeneration model

Adult male, specific pathogen-free, C57BL/6 mice were purchased from Dae Han Bio Link (Seoul, Korea). They were housed three to four per cage and maintained under a 12 hour light/dark cycle at 24°C, in a specific pathogen-free, humidity-controlled facility, and were provided with standard sterile food and water *ad libitum*. The mice were allowed to acclimate to their environment for 1 week, and were used at 8-10-weeks-of-age. Because cyclophosphamide, a DNA alkylating agent commonly used in chemotherapy, is a long-established method for investigating thymic regeneration [23-28], the animals were given a single intraperitoneal dose of cyclophosphamide (400 mg/kg body weight, Sigma, St. Louis, MO, USA) in normal saline and were killed in groups of four or more at 3, 7, and 14 days after injection. Mice given the same volume of normal saline were used as controls. Animal care and all experimental procedures were conducted in accordance with the "Guide for Animal Experiments" published by the Korean Academy of Medical Sciences.

Tissue preparation for immunohistochemistry

Mice were anesthetized with a single intraperitoneal injection of sodium pentobarbital (5 mg/kg body weight). For cryosections, the thymus was removed and rapidly frozen in isopentane cooled with liquid nitrogen. Frozen sections (5-μm thick) were cut on a Reichert cryostat and placed on 3-aminopropyltriethoxysilane-coated slides. After being dried, the cryosections were fixed in cold acetone for 10 minutes at −20°C.

Immunohistochemistry

Immunostaining was performed using the streptavidin-biotin complex (ABC) method, as described previously [25-27]. Briefly, the sections were incubated for 20 minutes in a solution of phosphate-buffered saline (PBS), containing 0.3% H_2O_2, to eliminate endogenous peroxidases. After washing in PBS, the sections were incubated with 2% bovine serum albumin (Sigma). Excess solution was shaken off, and the sections were incubated for 16-18 hours at 4°C with a rabbit anti-CK5 monoclonal antibody (mAb) raised against a synthetic peptide mapping to the carboxy terminus of human CK8 (EP1601Y, Thermo Scientific, Rockford, IL, USA), a rabbit anti-CK8 mAb raised against a synthetic peptide

mapping to the carboxy terminus of human CK8 (EP1628Y, Thermo Scientific), and a rabbit polyclonal anti-CK14 antibody raised against a synthetic peptide mapping to the carboxy terminus of human CK14 (Thermo Scientific).

Following incubation with the primary antibody, the sections were washed three times with PBS for 5 minutes and incubated for 2 hours at room temperature with an affinity-purified F(ab´)$_2$ fragment donkey anti-rabbit biotinylated antibody (1 : 200, Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The sections were then rinsed in PBS and incubated for 60 minutes at room temperature with an ABC reagent (Vectastain Elite Kit, Vector Laboratories, Burlingame, CA, USA), according to the manufacturer's instructions. The sections were developed in 0.025% 3,3'-diaminobenzidine and 0.003% H$_2$O$_2$ medium under microscopic control at room temperature to visualize peroxidase activity. The sections were rinsed in distilled water, counterstained with or without Mayer's hematoxylin, and mounted in a xylene-based mounting medium (Entellan, Darmstadt, Germany). Controls for the staining procedure included the following: (1) omission of the primary antibody from the reaction sequence and its replacement with non-immune rabbit serum or (2) omission of the secondary antibody or the ABC solution from the reaction sequence. Light microscopic slides were observed and photographed using an Olympus BX50 microscope (Olympus, Tokyo, Japan). All photomicrographs were taken with an Olympus C-3030 digital camera.

Immunofluorescent staining

Two-color double immunofluorescent staining was performed to identify CK5$^+$CK8$^+$ TECs. After the sections were rinsed in PBS and incubated with 2% bovine serum albumin (Sigma) for 60 minutes, they were incubated with the first primary antibody (anti-CK5) for 16-18 hours at 4°C. Following the primary antibody incubation, the sections were incubated with an affinity-purified F(ab´)$_2$ fragment donkey anti-rabbit rhodamine-conjugated antibody (1 : 100, Jackson ImmunoResearch Laboratories). After the sections were rinsed in PBS, they were further incubated with the second primary antibody, which was a rat anti-CK8 mAb raised against mouse cytokeratin Endo-A (Troma-1, Developmental Studies Hybridoma Bank from The University of Iowa, Ames, IA, USA). Following the second primary antibody incubation, the sections were incubated with an affinity-purified F(ab´)$_2$ fragment donkey anti-rabbit FITC-conjugated antibody (1 :

80, Jackson ImmunoResearch Laboratories). Thereafter, the labeled cells were examined with a Zeiss Axio Imager light microscope (Carl Zeiss, Oberkochen, Germany) fitted with a filter combination to visualize green and red fluorescing cells. Photomicrographs were captured digitally at a 1,300×1,030 pixel resolution with a Photometrix CoolSnap Fx CCD-camera (Roper Scientific, Trenton, NJ, USA) on a Zeiss Axio Imager microscope equipped with fluorescent epi-illumination. The digital images were processed using an image analysis program software (MetaMorph, Universal Imaging, Downingtown, PA, USA).

Results

The entire thymic epithelial network was stained with anti-CK5, -CK8, and -CK14 antibodies using an immuno-peroxidase technique. Identical immunofluorescent staining of both the cortical and medullary regions was obtained with these three antibodies (data not shown). Cells immunolabeled for CK5, CK8, and CK14 were observed in the normal and regenerating rat thymus and were identifiable as TECs by their morphological features, which showed a reticular network of cells in the cortex and medulla, and by their CK expression (Figs. 1-3). In contrast to the delicate and dendritic morphology of cTECs, most mTECs were stellate in appearance and closely packed, displaying voluminous cytoplasm and short processes (Figs. 1-3).

CK5 and CK14 were distributed in similar patterns in normal and regenerating thymuses with a stronger CK14 immunostaining intensity than that for CK5 (Figs. 1, 2). In normal mouse thymus, CK5 and CK14 immunoreactivity was observed in virtually all of the stellate mTECs, whereas the cTECs barely expressed CK5 and CK14 in the thymic cortex of normal mice (Figs. 1, 2, 4). We previously showed that the thymus undergoes time-dependent changes after cyclophosphamide treatment, which involve both lymphoid and stromal cells [24]. Essentially the same localization pattern of CK5 and CK14 immunoreactivity was observed in the thymuses of normal and cyclophosphamide-treated mice at all times (Figs. 1, 2). Characteristically, however, CK5 and CK14 protein expression was strongly upregulated in mTECs during thymus regeneration from acute thymic involution induced by cyclophosphamide treatment (Figs. 1, 2). Furthermore, and of particular interest, CK5- and CK14-positive TECs increased in number and in intensity in the
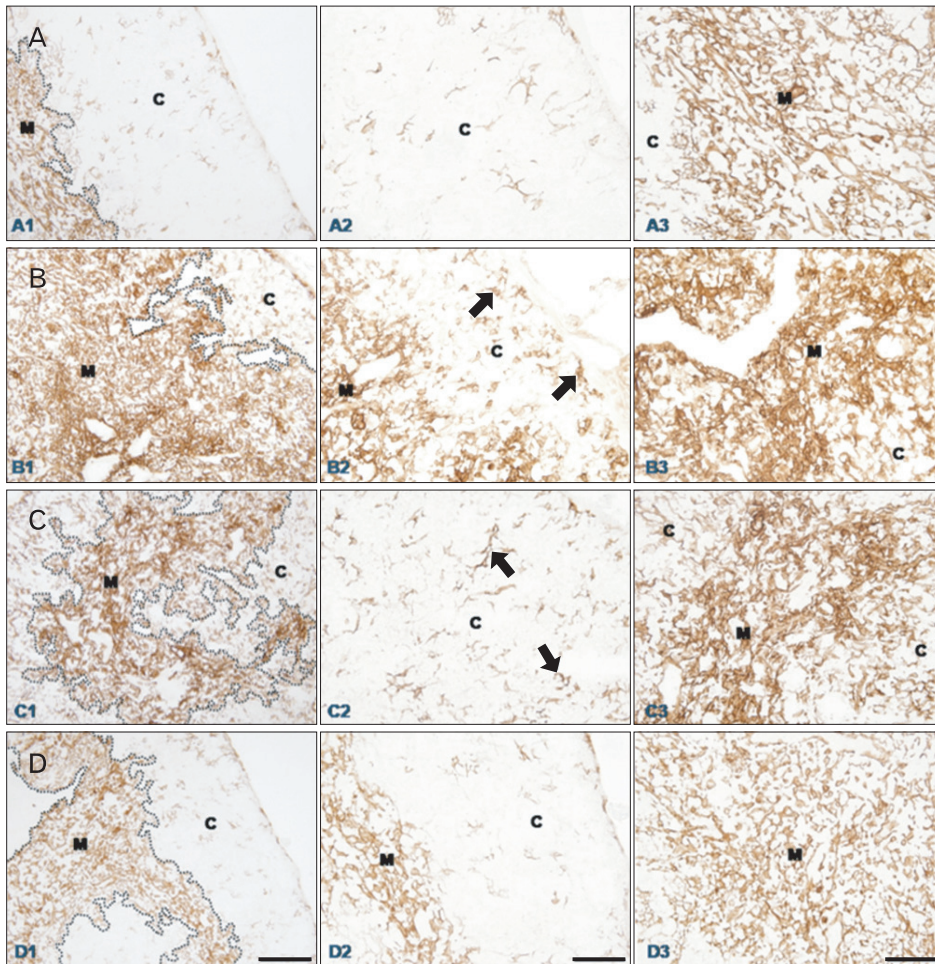
Fig. 1. (A-D) Immunohistochemical localization of cytokeratin (CK)5 in frozen sections of mouse thymus from control animals (A1-3, n=4), and at 3 (B1-3, n=7), 7 (C1-3, n=6), and 14 (D1-3, n=4) days after cyclophosphamide treatment. C, cortex; M, medulla; arrows, CK5$^+$ thymic epithelial cells. Scale bars=100 μm (A1, B1, C1, D1), 50 μm (A2, A3, B2, B3, C2, C3, D2, D3). (E) Data on the relative intensity of CK5 expression in the thymic medulla are also plotted in a bar graph and expressed as mean±standard deviation. CY 3d, CY 1w, and CY 2w represent 3, 7, and 14 days after cyclophosphamide treatment, respectively. *$P<0.001$ compared with the control, as determined by the t-test.
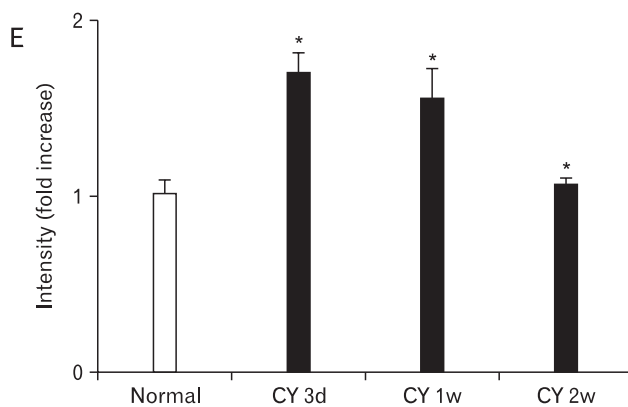
cortex during regeneration from acute thymic involution induced by cyclophosphamide treatment (Figs. 1, 2). The CK5 and CK14 staining intensity and pattern and the number of CK5 and CK14 immunoreactive cTECs returned to levels similar to those of the normal mouse thymus 2 weeks after cyclophosphamide treatment, which coincided with the completion of thymic regeneration (Figs. 1, 2). No

immunoreactivity was seen in control sections after omitting the primary antibody, secondary antibody, or the ABC reagent from the reaction sequence, or after replacing the primary antibody with a normal (nonimmune) rabbit IgG (data not shown).

CK8 immunoreactivity was observed in both cTECs and mTECs in normal mouse thymus (Fig. 3). The subcapsular/
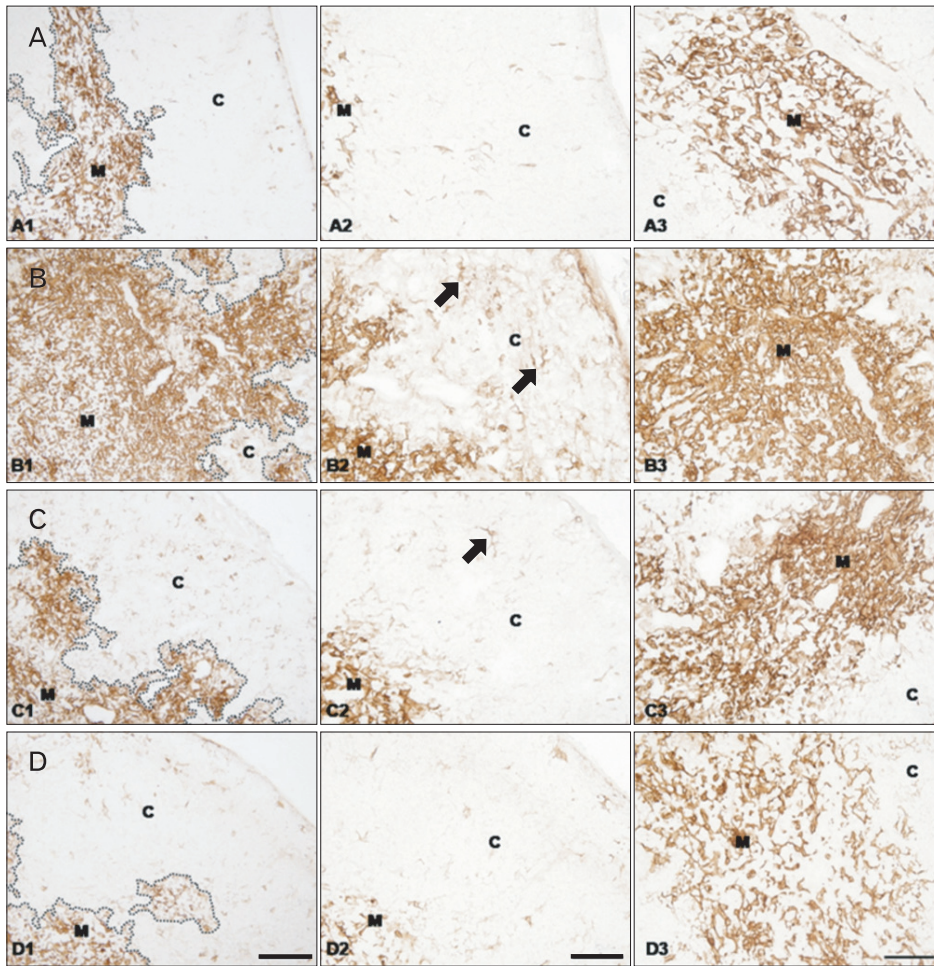
Fig. 2. (A-D) Immunohistochemical localization of cytokeratin (CK)14 in frozen sections of mouse thymus from control animals (A1-3, n=4), and at 3 (B1-3, n=7), 7 (C1-3, n=6), and 14 (D1-3, n=4) days after cyclophosphamide treatment. C, cortex; M, medulla; arrows, CK14+ thymic cortical epithelial cells. Scale bars=100 μm (A1, B1, C1, D1), 50 μm (A2, A3, B2, B3, C2, C3, D2, D3). (E) Data on the relative intensity of CK14 expression in the thymic medulla are also plotted in a bar graph and expressed as mean±standard deviation. CY 3d, CY 1w, and CY 2w represent 3, 7, and 14 days after cyclophosphamide treatment, respectively. *$P<0.001$ compared with the control, as determined by the t-test.
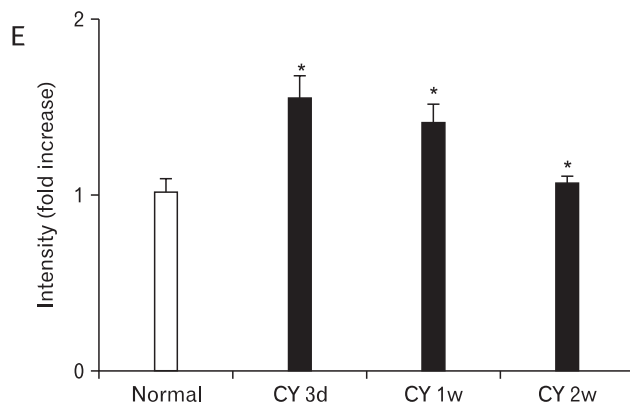
parasegtal subset of cTECs was flat and their cytoplasmic processes were very thin in control thymus (Fig. 3). These cTECs lined the capsule and the interlobular septum as a single, continuous layer, which had a smooth, even surface contour (Fig. 3). Perivascular TECs, which belong to the same subcapsular/paraseptal TEC subset, and resembled subcapsular/paraseptal TECs morphologically, surrounded blood vessels completely as a single, continuous layer (Fig. 3). Most cTECs in the thymic cortex, other than the subcapsular/paraseptal/perivascular cTEC subset, were thin and sheet like, displaying slender cytoplasmic processes and providing a large surface area for cell-cell interactions between TECs and developing thymocytes (Fig. 3). Additionally, CK8 was also expressed by the minor subset of mTECs in the thymic
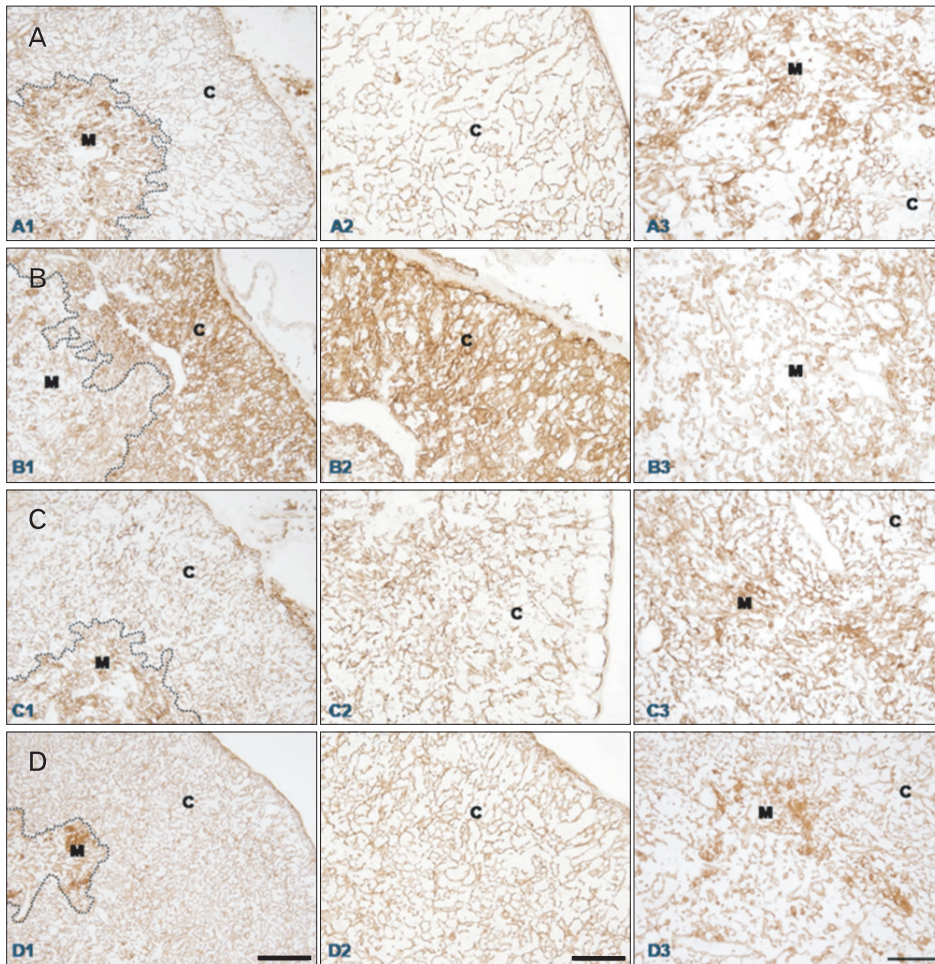
Fig. 3. (A-D) Immunohistochemical localization of cytokeratin (CK)8 in frozen sections of mouse thymus from control animals (A1-3, n=4), and at 3 (B1-3, n=7), 7 (C1-3, n=6), and 14 (D1-3, n=4) days after cyclophosphamide treatment. C, cortex; M, medulla. Scale bars=100 μm (A1, B1, C1, D1), 50 μm (A2, A3, B2, B3, C2, C3, D2, D3). (E) Data on the relative intensity of CK8 expression in the thymic cortex are also plotted in a bar graph and expressed as mean±standard deviation. CY 3d, CY 1w, and CY 2w represent 3, 7, and 14 days after cyclophosphamide treatment, respectively. *$P<0.001$ compared with the control, as determined by the t-test.

medulla of control mice, most of which were globular in appearance (Fig. 4).

Substantially the same localization pattern of CK8 immunoreactivity was observed in the thymuses of normal and cyclophosphamide-treated mice at all times (Fig. 3). Characteristically, however, strongly enhanced CK8 protein expression was observed in cTECs during thymus

regeneration from acute thymic involution induced by cyclophosphamide treatment, particularly at 3 days after cyclophosphamide treatment (Fig. 3). However, CK8 expression by the minor globular mTEC subset decreased considerably during thymic regeneration, particularly at 3 days after cyclophosphamide treatment (Fig. 3). Morphological alterations in subcapsular/paraseptal/
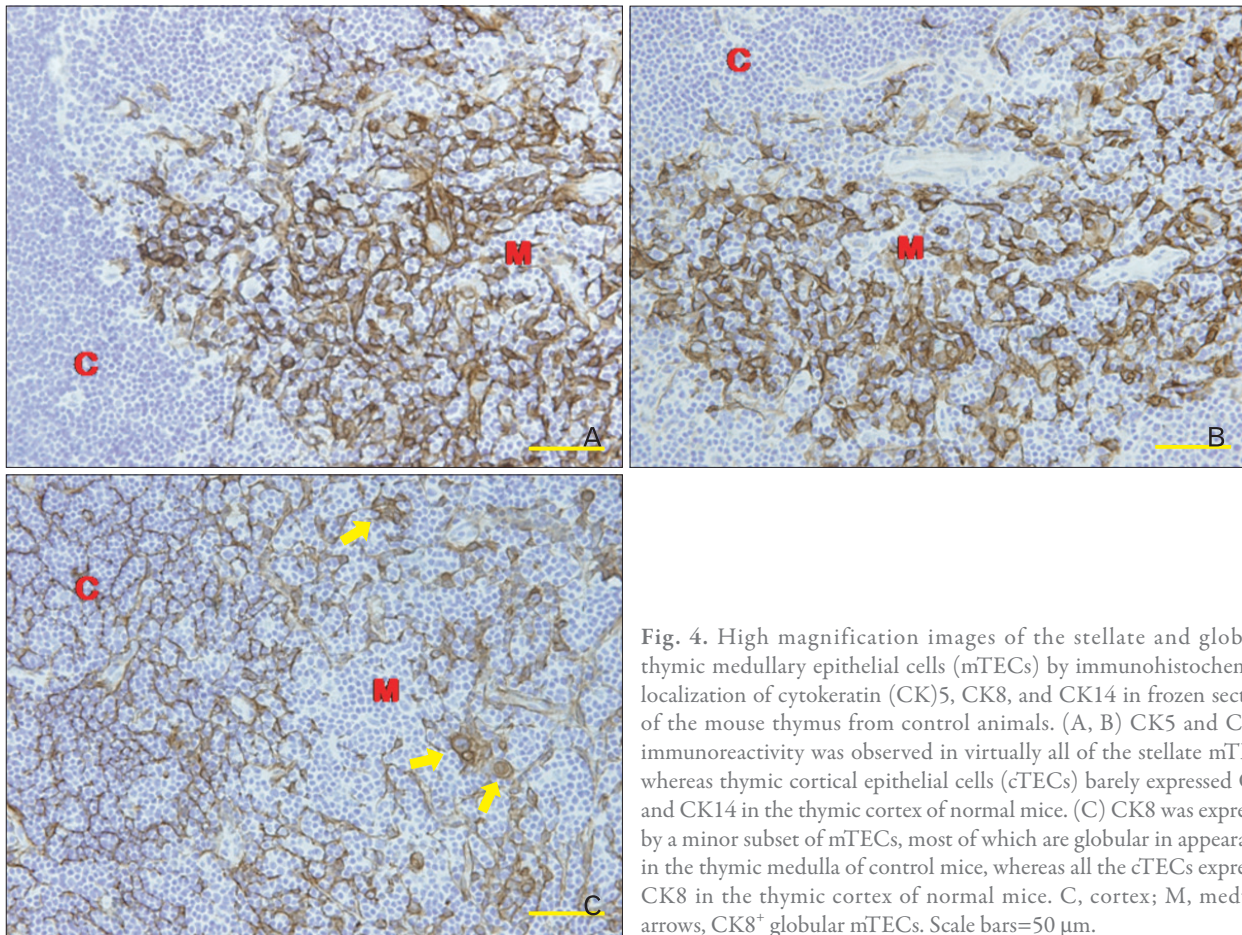
Fig. 4. High magnification images of the stellate and globular thymic medullary epithelial cells (mTECs) by immunohistochemical localization of cytokeratin (CK)5, CK8, and CK14 in frozen sections of the mouse thymus from control animals. (A, B) CK5 and CK14 immunoreactivity was observed in virtually all of the stellate mTECs, whereas thymic cortical epithelial cells (cTECs) barely expressed CK5 and CK14 in the thymic cortex of normal mice. (C) CK8 was expressed by a minor subset of mTECs, most of which are globular in appearance, in the thymic medulla of control mice, whereas all the cTECs expressed CK8 in the thymic cortex of normal mice. C, cortex; M, medulla; arrows, CK8$^+$ globular mTECs. Scale bars=50 μm.

perivascular TECs during thymic regeneration were the same as those described in a previous study [25]. The staining intensity and expression pattern in CK8 immunoreactive TECs were similar to those of the control mouse thymus 1 week after cyclophosphamide treatment (Fig. 3).

Double immunofluorescence staining of CK5 and CK8 clearly defined CK5$^+$CK8$^+$ TECs, which increased in number in the thymic cortex during thymus regeneration (Fig. 5).

Discussion

This study was designed to examine the properties of mouse TECs from the viewpoint of CK5, CK8, and CK14 expression using immunohistochemical and immunofluorescent methods to clarify whether TEC phenotypes and expression levels change during mouse thymus regeneration following thymic involution induced by cyclophosphamide treatment. Our findings confirmed

that both normal and regenerating mouse thymuses showed a substantially similar pattern of CK expression. The major mTEC subset, which was stellate in appearance, exhibited CK5 and CK14 staining, and the minor mTEC subset, which was globular in appearance, exhibited CK8 staining, whereas the vast majority of cTECs expressed CK8 during thymus regeneration. However, CK5$^+$ and CK14$^+$ cTECs, which were barely seen in the normal mouse thymic cortex, occurred in a relatively high number in the cortex during mouse thymus regeneration. Remarkably, the level of CK5 and CK14 expression was enhanced in mTECs, and CK8 expression was upregulated in cTECs, whereas the CK8 expression level was fairly diminished in globular mTECs during mouse thymus regeneration after cyclophosphamide-induced acute thymic involution. Generally, these changes in CK5, CK8, and CK14 expression level became similar to those of normal thymus 2 weeks after cyclophosphamide treatment at approximately the same time when thymus regeneration was almost completed. The levels of CK5, CK8, and CK14 increased in TECs during
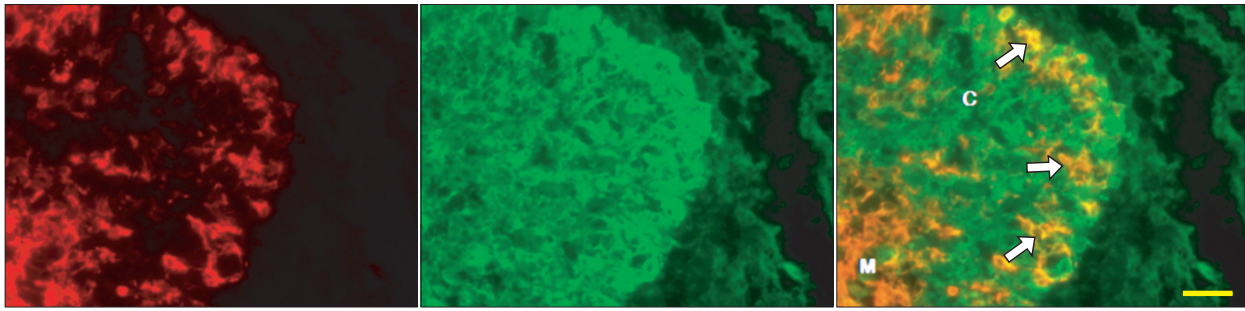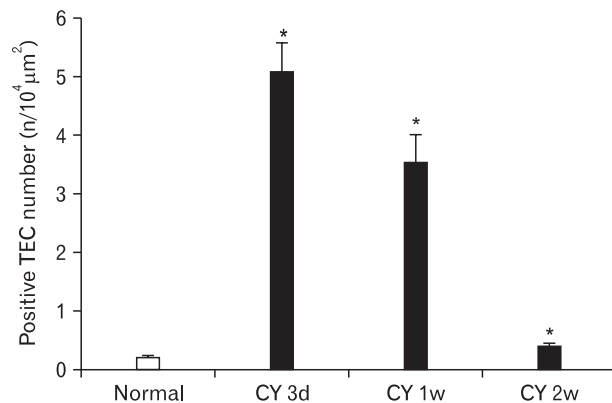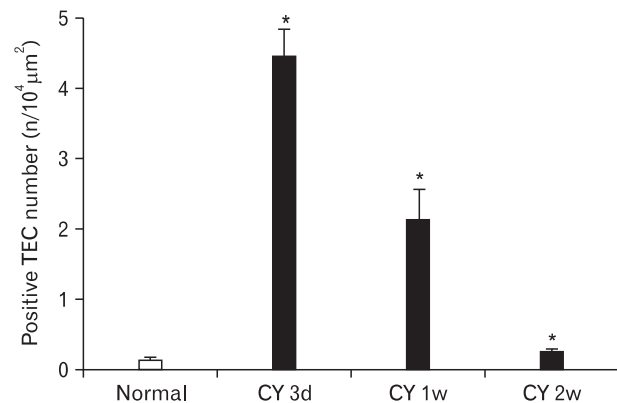
Fig. 5. (A) Two-color double-label immunofluorescent localization of cytokeratin (CK)8 (green) and CK5 (red) in frozen sections of mouse thymus 3 days after cyclophosphamide treatment. C, cortex; M, medulla; arrows, CK5$^+$CK8$^+$ thymic epithelial cells. Scale bar=50 μm. Data on the relative number of CK5$^+$CK8$^+$ (B) and CK14$^+$CK8$^+$ (C) Cotical thymic epithelial cells (cTECs) are also plotted in a bar graph and expressed as mean±standard deviation. *P<0.001 compared with the control, as determined by the t-test.

thymus regeneration. It has been demonstrated that CK5, CK8, and CK14 are overexpressed in various cancer cells including human breast carcinoma, lung squamous cell carcinoma, and gastric adenocarcinoma [29-34], suggesting that elevation of these CKs could promote cell survival and proliferation. Moreover, CK8 and CK14 suppress apoptosis induced by a variety of agents in various epithelial cells [35, 36]. Thus, it is speculated that the enhanced expression of CK5, CK8, and CK14 may be involved in various cellular activities, including survival of TECs during thymus regeneration.

Our findings strongly indicate that the anti-CK5 and anti-CK14 antibodies can be utilized as a tool for immunohistological analysis of mTECs, and that the anti-CK8 mAb used in this study can be applied for immunohistological analysis of entire cTEC populations and minor globular mTECs in both normal and regenerating mouse thymuses. Additionally and most importantly, the anti-CK5 and anti-CK14 antibodies used in this study can

also be employed to identify CK5- or CK14-positive cTECs by immunohistochemical or immunofluorescence methods in both normal and regenerating mouse thymuses throughout the entire period of mouse thymus regeneration.

Our observations are in agreement with those of many other studies in which various antibodies were directed against CK5, CK8, and CK14. For example, CK5 and CK14 are expressed by the predominant mTECs using a rabbit polyclonal antibody (AF 138) specific to mouse CK5 and a rabbit polyclonal antibody (AF 64) specific to mouse CK14 obtained from Covance Research (Princeton, NJ, USA) [20-22]. These anti-CK5 and anti-CK14 antibodies were originally developed by Roop et al. [37] using synthetic peptides corresponding to the carboxyl-terminal amino acid sequences of mouse CK5 and CK14, and their immunoreactive properties against mouse thymus are well characterized [19]. Our data using a rabbit anti-CK5 mAb (clone EP1601Y, Thermo Scientific) directed against a synthetic peptide corresponding to residues on the carboxyl-terminal of

human CK5, and a rabbit polyclonal antibody directed against a synthetic peptide derived from the carboxyl-terminal of human CK14 (Thermo Scientific) coincide with previous results showing that in normal mouse thymus, CK5 expression is found together with CK14 expression in stellate medullary TECs.

In agreement with previous results using a rat mAb specific for mouse CK8 (Troma-1, the Developmental Studies Hybridoma Bank), our data using a rabbit anti-CK8 mAb (EP1628Y, Thermo Scientific) directed against a synthetic peptide corresponding to residues on the carboxyl-terminal of human CK8 confirmed that in normal mouse thymus, CK8 is expressed by the vast majority of cTECs, including subcapsular TECs, which have a distinctive reticular appearance, and by the minority of mTECs, which have a distinctive globular appearance [19, 20]. This anti-CK8 mAb (Troma-1) was originally developed by Kemler et al. [38]. Thus, the medulla of the thymic lobule in mice consisted of two main populations of TECs: $CK5^+$ or $CK14^+$ stellate mTECs, the major mTEC subset, which did not express CK8, and $CK8^+$ globular mTECs, the minor mTEC subset, which did not express CK5 or CK14. These two mTEC subsets were also defined by differential reactivity with the lectin UEA-1 and mAbs that recognize classical versus nonpolymorphic major histocompatibility complex molecules. The major $CK5^+$ or $CK14^+$ stellate mTEC subset does not express UEA-1 or Ia, but the minor $CK8^+$ globular mTECs express UEA-1 and Ia [19, 39, 40].

One of the most salient features observed in our experimental thymus regeneration model was the occurrence of a relatively high number of $CK5^+CK8^+$ TECs in the thymic cortex during thymus regeneration. An emerging body of data supports the existence of thymic epithelial progenitor/stem cells during both the embryonic and postnatal period, which have the $CK5^+CK8^+$ phenotype [41]. Similar to our results, García-Ceca et al. [42] demonstrated that high numbers of $CK5^+CK8^+$ TEC progenitors occur in both wild-type and EphB-deficient thymic parenchyma after fetal thymus lobes were grafted under the kidney capsule of mice, and these $CK5^+CK8^+$ TEC progenitors reached higher numbers in the grafted EphB-deficient thymus lobes than in the wild-type ones. High numbers of $CK5^+CK8^+$ TEC progenitors have also been reported in other mice with defective maturation of thymic epithelium, such as $Foxn1^{\Delta/\Delta}$ mice [43], conditioned Stat3-deficient mice [44], and $Krm1^{-/-}$ mice [45]. Popa et al. [46] confirmed that $CK5^+CK8^+$ TEC progenitors in the thymic cortex expand significantly during thymus atrophy induced by steroid treatment or by irradiation and prior to thymic regeneration. They also showed that in involuted thymuses, cMyc and Trp63 transcription factors, known to be expressed in early epithelial cell progenitors, are expressed in a subset of cortical $CK5^+CK8^+$ TEC progenitors [46]. Thus, $CK5^+CK8^+$ cTECs, which emerged in the thymic cortex during mouse thymus regeneration, are obviously regarded as thymic epithelial progenitor cells. Although the precise role of $CK5^+CK8^+$ TEC progenitors during thymus regeneration after cyclophosphamide-induced acute thymic involution remains to be elucidated, our results suggest that these TEC progenitors may be involved in replenishing TECs during thymus regeneration.

Taken together, our data shed more light on the role of CK5, CK8, and CK14 in TECs and strongly indicate that the antibodies specific to these CKs used in this study can be utilized as a tool for immunohistological analysis of TEC subsets in the thymus during mouse thymus regeneration. In addition, our results demonstrated that critical steps in the recovery of the adult thymus include expansion of $CK5^+CK8^+$ TEC progenitors and elevated expression of CK5 and CK14 in mTECs and CK8 in cTECs during thymus regeneration, suggesting that $CK5^+CK8^+$ TEC progenitors are activated during thymus regeneration and may be involved in restoration of the epithelial network and consequent regeneration of the adult thymus.

Acknowledgements

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