Medullary but Not Cortical Thymic Epithelial Cells Present Soluble Antigens to Helper T Cells

By Toshiaki Mizuochi,* Michiyuki Kasai,‡ Takehiro Kokuho,§ Terutaka Kakiuchi,§ and Katsuiku Hirokawa‡

*From the *Department of Blood Products, National Institute of Health, 2-10-35, Kamiosaki, Shinagawa-ku, Tokyo, 141; the ‡Department of Pathology, Tokyo Metropolitan Institute of Gerontology, 35-2, Sakaecho, Itabashi-ku, Tokyo, 173; and the §Department of Allergology, Institute of Medical Science, University of Tokyo, Shirokanedai, Minato-ku, Tokyo, 108, Japan*

Summary

Thymic epithelial cell lines (TECs) were established from newborn C57BL/6 mice. They were classified into two types (medullary and cortical TECs) by using the monoclonal antibody (Th-3) that recognizes the meshwork structure of thymic cortical epithelial cells. Antigen-presenting activity of each TEC was determined by using ovalbumin-specific, I-A^b-restricted helper T cell lines. It was demonstrated that the medullary but not the cortical TECs functioned as antigen-presenting cells. This is the first evidence for the functional difference between the cortical and the medullary TEC.

The thymic environment is the main site of T cell maturation/differentiation (1–5). On arrival in the thymus, bone marrow–derived T cell precursors maturate into thymocytes that undergo intrathymic selection. It is generally believed that cortical thymic epithelial cells (TECs)[1] are the main cell type that mediate positive selection, the process of selecting clones capable of self MHC-restricted antigen recognition, whereas medullary macrophages/dendritic cells or TECs are responsible for negative selection, the process of clonal elimination of autoreactive cells (6–8). There has been, however, no direct information that accounts for the functional difference of cortical and medullary TECs. In this report we demonstrated that cortical TECs are incapable of presenting soluble antigens to mature helper T cell lines while medullary TECs can function as APC, corroborating the proposed distinct roles played by these two types of TECs in selecting T cell repertoires.

Materials and Methods

Mice. Male and female C57BL/6 mice were purchased from Charles River Japan Inc. (Shizuoka, Japan) and were mated and reared in our specific pathogen-free mouse colony.

Establishment of TECs. Thymi obtained from newborn to 2-wk-old mice were digested in PBS containing 0.25% trypsin and 0.02% EDTA, and were suspended in Ca^{2+}-free MEM. The cell suspension (10^4–10^5 per dish) was then plated on 4,000-rad irradiated Swiss 3T3 cells (7×10^5 per dish) in 60-mm dishes (3802; Falcon Labware, Oxnard, CA) at 35°C in a CO_2 (5%) incubator. The culture dishes were washed with PBS containing 0.02% EDTA to remove 3T3 cells and thymic fibroblastic cells, and then were washed with PBS containing 0.25% trypsin and 0.02% EDTA to make a suspension of TEC. Under an inverted microscope, single or clusters of the TECs were picked up and transferred to 24-well culture plates (3847; Falcon Labware) coated with 4,000-rad irradiated Swiss 3T3 cells and cultured in Ca^{2+}-free MEM at 37°C. This cloning procedure was repeated several times to obtain TECs.

Antibodies. Th-3 mouse mAb against mouse thymic cortical epithelial cells was produced in our laboratory (9). M5/114 rat mAb against mouse I-A^b (10) was supplied by Dr. Uchida (NIH, Tokyo, Japan). Rabbit anti-human keratin antibody was purchased from Dakopatts (Glostrup, Denmark).

Immunocytochemistry. The established TECs were cultured on eight-hole heavy Teflon-coated slides (Bokusui Brown, New York, NY). The slides were washed twice with PBS(+) at room temperature (RT) and then fixed with 3.7% paraformaldehyde in PBS(−) for 5 min at RT. They were washed three times with PBS(−) at 4°C, and refixed with acetone for 5 min at 4°C. Then the slides were reacted with primary antibody for 2 h at RT, washed twice with chilled PBS(−) at 4°C, and reacted with FITC-conjugated second antibody for 2 h at RT. The slides were washed with chilled PBS(−), and then mounted in PBS(−)-glycerin (1:9). The immunofluorescence photographs were taken on a Nikon Optiphot equipped with an MRC-500 laser confocal scanning microscope (Japan Bio-Rad Lab. Co. Ltd., Tokyo, Japan).

Assay for APC Activity. OVA-specific, I-A^b-restricted helper T cell lines, MD-23 and KT-17, were used as indicator T cells. TECs were cultured in the presence of IFN-γ (100 U/ml) for 48 h to augment the expression of MHC class II molecules. They were irradiated at 10,000 rad and were used as APC. 10^4 IFN-γ-

[1] *Abbreviation used in this paper:* TEC, thymic epithelial cell.

pretreated TECs were cocultured with 10^4 helper T line cells in the presence of various concentrations of antigen, OVA, for 24 h in a 96-well flat-bottomed microculture plate (0.2 ml/well) (25860; Corning, Cambridge, MA). Culture supernatant (0.1 ml) of each cultured well was assayed for IL-2/IL-4 content by measurement of [^{3}H]thymidine incorporation by an IL-2/IL-4–dependent cell line, CTLL-2. Standard errors were generally <5% of the mean and so have not been included in the Fig. 3 and Table 1.

Results and Discussion

We have recently produced a mAb, Th-3, that specifically reacts with the meshwork structure of epithelial cells in the thymic cortex (9). This mAb is, therefore, expected to classify TECs into cortical and medullary type (see below). We have established 42 TEC lines from the thymus of newborn C57BL/6 mice and morphologically characterized them to be of epithelial nature by their cobblestone appearance (11). Among them, TEC1-4C18 and TEC1C6 were chosen for further experiments, because of their morphological appearances representing cortical (with thick cytoplasm and distinct cell borders, as shown in Fig. 1 *a*) and medullary TEC (with thin/broad cytoplasm and indistinct cell borders, as shown in Fig. 1 *b*), respectively. These two TEC lines were positive with antikeratin antibody showing fine filamentous structures (Fig. 1, *c* and *d*), and had typical desmosomes and tonofibrils in electron micrographs (data not shown). They were then examined for reactivity with Th-3 mAb. As shown in Fig. 1, *e* and *f*, TEC1-4C18 was positive with Th-3 mAb, whereas TEC1C6 was negative. Fig. 1 *g* confirmed that the Th-3 mAb reacts specifically with the meshwork structure in the thymic cortex but does not react with thymic medulla. According to these results and our previous observations (9, 11), it was reasoned that TEC1-4C18 was derived from epithelial cells in the thymic cortex and that TEC1C6 was derived from those in the thymic medulla.

We next examined the antigen-presenting activity of each TEC line by using OVA-specific, I-A^b-restricted helper T cell

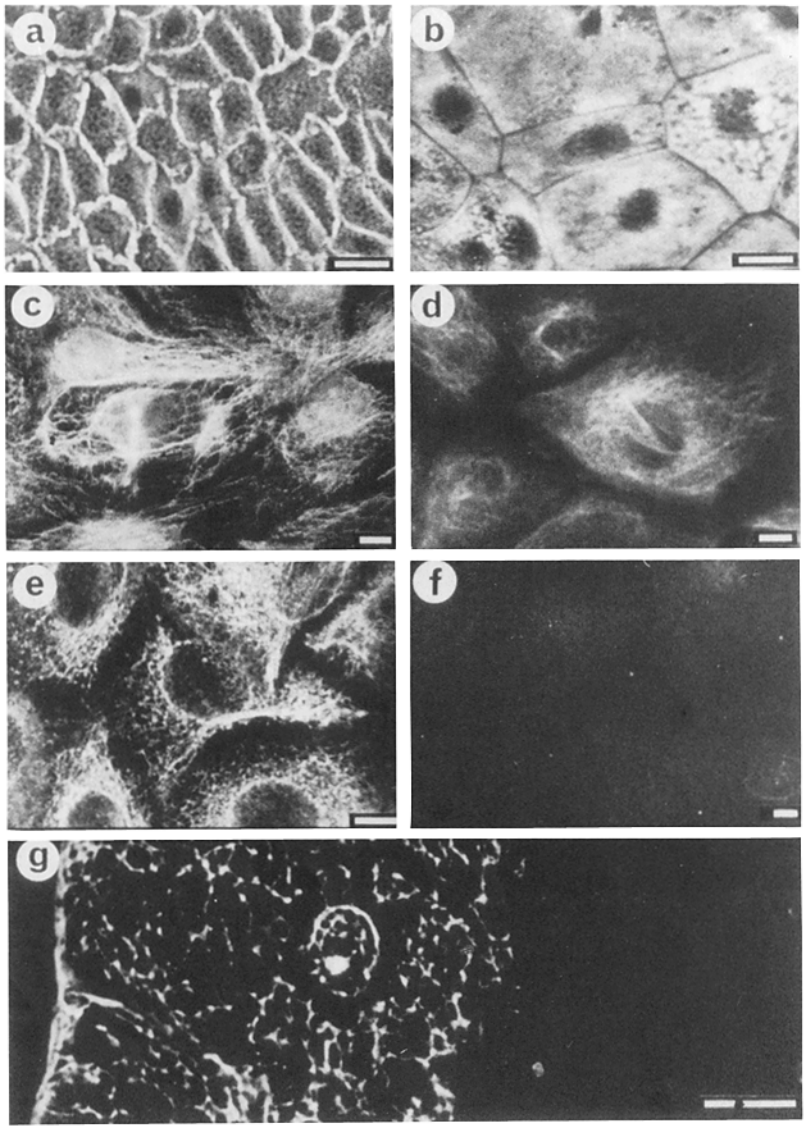

Figure 1. The phase contrast microscopy (*a* and *b*) and the immunofluorescence microscopy (*c–g*) of TEC1-4C18 and TEC1C6. (*a*) TEC1-4C18 showed a cobblestone pattern and distinct cell borders due to thick cytoplasm (scale bar, 20 μm). (*b*) TEC1C6 also showed a cobblestone pattern and relatively indistinct cell borders due to thin cytoplasm (scale bar, 20 μm). (*c*) TEC1-4C18 was positive with antikeratin antibody showing finely filamentous pattern (scale bar, 10 μm). (*d*) TEC1C6 was also positive with antikeratin antibody showing finely filamentous pattern (scale bar, 10 μm). (*e*) TEC1-4C18 was positive with Th-3 mAb (see the text) showing finely filamentous pattern (scale bar, 10 μm). (*f*) TEC1C6 was negative with Th-3 mAb (scale bar, 10 μm). (*g*) The frozen section of C57BL/6 mouse thymus stained with Th-3 mAb. The cortex was positive with the Th-3 mAb showing the meshwork pattern (*left half*), but the medulla was negative (*right half*) (scale bar, 50 μm).

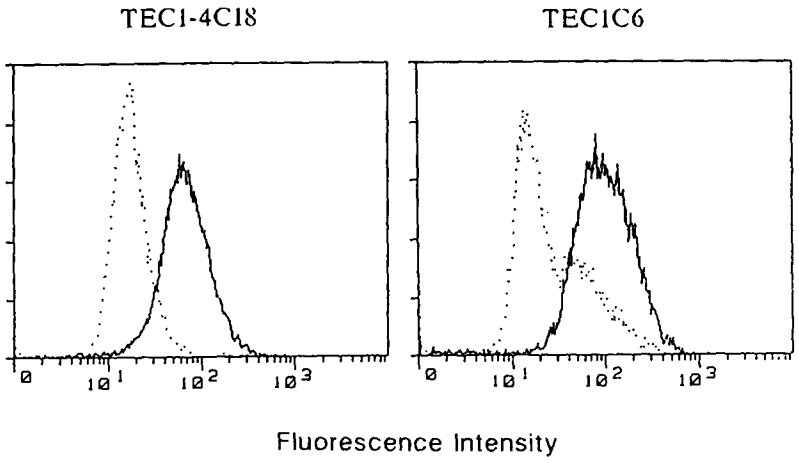

Figure 2. FACS® analysis of cell surface class II MHC antigens expression on TEC lines. The first-step mAb used was M5/114 reacted with the I-A^b molecule. FITC mouse anti-rat Ig κ chain (MAR 18.5) was used as the second-step mAb. TEC cells were cultured in vitro for 48 h without IFN-γ (*dotted line*) or with IFN-γ (*solid line*) before FACS® analysis.

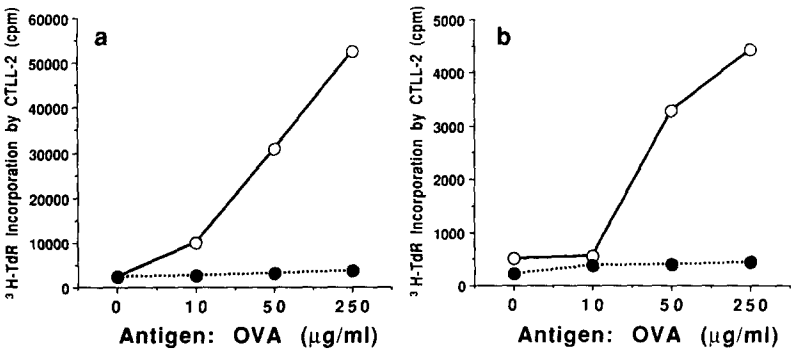

Figure 3. Antigen-presenting activity of TEC lines pretreated with IFN-γ assessed by the IL-2/IL-4 production from helper T cells. Two independently established OVA-specific, I-A^b-restricted helper T cell lines, MD-23 (*a*) and KT-17 (*b*), were used as indicator cells. TEC 1C6 (*open circle, solid line*) and TEC 1-4C18 (*closed circle, dotted line*) were used as APC.

lines. TEC lines were stimulated in vitro with IFN-γ for 48–72 h to induce a high level of class II MHC antigens (12). Fig. 2 illustrates the expression of class II MHC on each TEC line before and after the IFN-γ treatment. Despite comparable expression of class II MHC molecules by the two TEC lines, there is a clear difference between TEC1-4C18 and TEC1C6 in their ability to present the soluble antigen to two independently established OVA-specific, I-A^b-restricted helper T cell lines, MD-23 (Fig. 3 *a*) and KT-17 (Fig. 3 *b*).

Other TEC lines were then examined for their phenotypes and the ability to present OVA by using the MD-23 as indicator cells. Table 1 summarizes the data obtained by analyzing 10 TEC lines, including TEC1-4C18 and TEC1C6. The immunostaining studies using Th-3 indicated that the five lines (TEC1-4C18, TEC1-2, TEC1-2C1, TEC1C8, and TEC1C9) were of cortical nature, and the other five lines (TEC1C6, TEC1-3, TEC2-3, TEC3-10, and TEC1-2C2) were of medullary nature. Without a single exception, only medullary-type TEC lines showed the antigen-presenting activity, whereas cortical-type TEC lines failed to present soluble antigen to helper T cells. Since stimulation of the OVA-specific helper T cell line was determined by the level of IL-2 produced in the culture supernatant, the observed failure in presenting OVA antigen of cortical-type TEC can hardly be explained by lack of costimulatory lymphokine. The failure of cortical-type TEC to function as APC was not based on a single dose of TEC used in the culture, since essentially the same results were obtained by using a wide range of TEC cell numbers

Table 1. *Medullary but Not Cortical Thymic Epithelial Cells Are Able to Present Soluble Antigens to Helper T Cells*

Thymic epithelial cell	Classification	APC activity (IL-2 production by MD-23)	
		No antigen	250 μg/ml antigen
		cpm	
1-4C18	Cortex	96	178
1-2	Cortex	120	95
1-2C1	Cortex	171	427
1C8	Cortex	101	158
1C9	Cortex	113	134
1C6	Medulla	83	47,902
1-3	Medulla	119	20,902
2-3	Medulla	158	19,026
3-10	Medulla	196	22,709
1-2C2	Medulla	147	18,792

APC activity of TEC lines were assayed by the method described in Materials and Methods ([^{3}H]TdR uptake by CTLL-2). The TEC lines stained by Th-3 mAb are considered to be derived from the thymic cortex. The other TECs are considered to be derived from the thymic medulla (see Fig. 1).

(2.5×10^3 to 6×10^5 cells) per culture (data not shown). Finally, the possibility that the release of toxic cytokines or some other harmful products by cortical-type TEC was excluded by the mixing experiment; that is, the addition of cortical-type TEC did not affect the antigen-presenting activity of medullary type TEC at all (data not shown). These results strongly indicate that cortical-type TECs are in general incapable of presenting exogenous soluble protein antigens.

The differential reactivity of the mAb, i.e. Th-3, with cortical and medullary TEC in situ has been firmly established (Fig. 1 *g*, and reference 9), supporting the validity of typing the isolated in vitro lines of TEC with this mAb. Thus, our data strongly indicate that cortical and medullary TECs are two functionally distinct populations. The medullary TECs are more or less like conventional class II MHC-positive APC found in peripheral lymphoid tissues. The cortical TECs, on the contrary, are incapable of presenting exogenous soluble protein antigens despite the expression of class II MHC molecules on the surface.

We envision four possibilities to account for the observed inability of cortical-type TEC in antigen presentation. The first possibility is that this type of class II MHC-positive cells may not be furnished with the machinery for internalization and processing protein antigens, as is the case for keratinocytes induced to express class II MHC molecules by IFN-γ (13). If this is the case, these TECs would be capable of presenting peptide fragments ready for binding to class II MHC molecules. In this regard, a recent report by Ransom et al. (14) demonstrated that a thymic-derived epithelial cell line has defects in its ability to process antigens to produce immunodominant peptides recognized by some T cell hybridoma, although they did not phenotype the TEC. It may be possible that the TEC line they established was of cortical rather than medullary nature. Alternatively, it is also possible that the cortical TECs do process antigen but much slower than medullary TECs. Our preliminary study, however, did not support such a possibility, since even when the cortical TEC was prepulsed with antigen for 1–3 d before the APC assay, it could not present antigens to helper T cells (data not shown). Second, even when the cortical TEC can process protein antigens, these cells might be deficient in giving accessory signals mediated by either lymphokines or by certain accessory molecules. This possibility, nevertheless, is unlikely, since the stimulation of the T cell line was assessed by IL-2/IL-4 production, which is generally known to be fully triggered by solely cross-linking (multi-valent ligand binding) of the TCR-T3 complex (15). The third possibility is the blockade of peptide binding to class II MHC molecules expressed on the surface of cortical-type TEC. This model predicts inability of these cells to present peptide fragments. Such a blockade could be due to occupancy of the groove of surface class II MHC molecules by something such as an invariant chain or by internal self-peptide, in case the cells do not synthesize invariant chains. It is also possible that some unidentified molecules are bound on the surface class II MHC molecules enforcing a tertiary structure not suitable for peptide binding. The fourth and the most simple explanation is the differential expression of the MHC class II molecules in the cortical and in the medullary TECs. Interestingly, two groups have reported the expression of a novel MHC class II molecule in thymic medulla but not cortex (16, 17). This novel MHC class II molecule might be involved in the presentation of soluble antigens to helper T cells, particularly in the thymic environment.

It may sound rather surprising that the cortical-type TEC lines failed to present soluble antigens to helper T cells, because it is generally believed that the thymic cortex is the microenvironment where the positive selection of MHC-restricted nominal antigen recognition structure of T cells is operated. One assumption is that class II MHC antigens expressed on cortical TEC lines are free of invariant chain (Ii) and preoccupied by self-derived peptides, which might determine the "self-MHC restriction," and therefore antigenic peptides derived from exogenous nominal antigen are unable to be presented by cortical TECs. It would be advantageous for the host immune system that this mechanism might prevent an undesirable stimulation by too many exogenous antigens that may permeate the thymic cortex (18).

In conclusion, we classified TEC lines, which we recently established from the thymus of newborn C57BL/6 mice, into cortical and medullary TEC by immunostaining with Th-3 mAb, and then demonstrated that the medullary TEC lines are able to present soluble antigens to helper T cells, whereas the cortical TEC lines are unable to do so. These results strongly suggest that the cortical and the medullary TEC may play functionally distinct roles in the selection of the T cell repertoire.

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Address correspondence to Toshiaki Mizuochi, Department of Blood Products, National Institute of Health, 2-10-35 Kamiosaki, Shinagawa-ku, Tokyo, 141, Japan

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