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Research paper

Enhanced isolation of adult thymic epithelial cell subsets for multiparameter flow cytometry and gene expression analysis

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ABSTRACT

The epithelial cells (TECs) are microenvironmental niche cells which support T lymphocyte development in the thymus. Most studies of TEC biology have focused on TEC at the fetal stage of development, whereas the biology of adult-stage TECs is not as well-understood. Delineating the molecular mechanisms that control adult TEC differentiation has implications for the success of T-lymphocyte based therapies for autoimmune diseases and induction of immunological tolerance to stem cell-derived tissues. Detailed analysis of adult TECs is technically challenging due to their rarity, their diminishing numbers with age, and the limited number of markers to distinguish between unique TEC subpopulations. Here, we have devised an improved isolation protocol for adult mouse TECs and combined it with six-color multiparameter flow cytometry. Using these techniques, we have identified four distinct subsets of CD45− EpCAM+ TECs in adult mice: a) UEA1^{low} CDR1^{low} (UC^{low}); b) UEA1^{high} CDR1^{high}(UC^{high}); c) UEA1^{low} CDR1^{high} MHC^{high} (cTEC); and d) UEA1^{high}CDR1^{low} MHC$^{int/high}$ (mTEC). PCR analysis verified that these TEC subsets differentially expressed known TEC genes. TEC subsets were further analyzed using high-throughput quantitative PCR arrays to reveal novel genes that could be important for TEC subset maintenance. Intracellular staining for keratin-5 and keratin-8 can also be added, but our results suggest that keratin expression alone cannot be used to distinguish adult TEC subsets. Our enhanced isolation allows for detailed analysis of rare TEC subpopulations in the adult mouse at the cellular and molecular levels.

1. Introduction

There are several clinical justifications for research into regeneration of the thymus. The thymus involutes with age, and this atrophy has been associated with a decrease in thymocyte output and increased susceptibility to infections and cancer in aged individuals. There is particular interest and focus on the thymic microenvironmental niche and the thymic epithelial cells (TEC) that are important for structural support as well as molecular signaling to the developing T cell repertoire. Regeneration of TECs can promote T cell expansion in lymphopenic patients and may enhance the induction of immunological tolerance towards stem cell derived therapies in the adult (Chidgey et al., 2008).

TECs have been traditionally divided into two categories: the cortical TEC (cTEC) and medullary TEC (mTEC) populations which are found in distinct regions in the thymus (reviewed by Zhang et al., 2007 and Alves et al., 2009a). Mature cTECs are thought to provide positive selection signals to developing thymocytes and shape the T cell receptor repertoire, whereas mTECs have been implicated in negative selection, which results in the deletion of self-reactive thymocytes. cTECs and mTECs can be distinguished using extracellular surface markers as well as intracellular markers. All TECs express epithelial cell adhesion molecule (EpCAM). cTECs stain positively for CDR1 (Ly51), MHC class II

Abbreviations: cTEC, cortical thymic epithelial cell; EpCAM, epithelial cell adhesion molecule; FACS, fluorescence activated cell sorting; HBSS, Hank's Balanced Salt Solution; K5, keratin-5; K8, keratin-8; mTEC, medullary thymic epithelial cell; PBS, phosphate-buffered saline; TEC, thymic epithelial cell; UC^{low}, UEA^{low},CDR1^{low}; UC^{high}, UEA^{high},CDR1^{high}; UEA-1, Ulex europaeus agglutinin-I.

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(MHC II), and keratin-8 (K8), but do not stain positively for keratin-5 (K5) or with UEA1. In contrast, mature mTECs are predominantly CDR1^{neg}, UEA1$^{int/high}$, MHCII$^{int/high}$K5^{+} K8^{-}. The cell marker profiles of TEC populations are largely based on studies of fetal thymic epithelial cells, not adult or aged thymi, and often only utilize 2 or 3 markers simultaneously in flow cytometry or immunohistochemistry. Clearly, detailed analysis and further subfractionation of TEC subsets are needed to identify the molecular mechanisms that regulate TEC development in the adult.

In this study, we developed a TEC isolation protocol which combines enzymatic plus gentle mechanical disruption, and combined it with six-color flow cytometric sorting to purify four distinct TEC subsets from adult mice. Amongst these four TEC subsets, we have purified a UEA1^{hi} CDR1^{hi} population that has been noted previously by other groups but has not been well-characterized. The isolation method provides enough material to perform high throughput qPCR array analysis of gene expression in the rare TEC subsets, and has revealed the expression of genes whose function in TECs has not yet been investigated.

2. Materials and methods

2.1. Mice

C57BL/6 (B6) mice were purchased from Taconic or Jackson Laboratories (Bar Harbor, ME) or bred in house. Mice were housed in sterile microisolator cages with sterile feed and autoclaved water. Mice were euthanized by CO_2 asphyxiation followed by cervical dislocation and all procedures were approved by the UC Merced Institutional Animal Care and Use Committee.

2.2. Antibodies

Hybridomas which produce anti-EpCAM (clone G8.8, Developmental Studies Hybridoma Bank, University of Iowa), anti-K8 (clone Troma-1, Developmental Studies Hybridoma Bank, University of Iowa), and anti-Ly51 (clone CDR1, purchased from ATCC) were cultured in DMEM media containing 10% FCS until confluent. Hybridoma supernatants were collected and antibodies purified using goat anti-rat CNBr Sepharose Beads (Jackson Immunohistochemical, Amersham Biosciences). EpCAM (rat IgG2a) antibody was conjugated to phycoerythrin using the Lightning Link PE kit (Novus Biological). CDR1 (rat IgG2a) antibody was conjugated to Cy5 using Cy5 monoreactive dye (Amersham). Anti-K8 Ab (rat IgG2a, kappa) was fluorescently labeled with an Alexa Fluor 680 succinimidyl ester (Invitrogen). Anti-MHC II-PeCy5 (clone MM5/114.15.2, rat IgG2b, kappa) was purchased from Biolegend, and Ulex agglutitin-1 (UEA1)-FITC was purchased from Vector Laboratories. Anti-K5 is a rabbit polyclonal antibody (Covance) and was detected with goat anti-rabbit PE-Alexa 610 (Invitrogen).

2.3. TEC isolation

Thymi from mice between 6 and 8 weeks of age were harvested in Medium 199 (Invitrogen) containing 2% FCS (Atlanta Biologicals), which we term "M199+". For each experiment, 2 to 10 thymi were pooled. Thymic lobes were nicked and placed in a glass beaker containing cold M199+ and mixed under constant slow magnetic stirring to release thymocytes from the lobes (Gray et al., 2002, 2008). Stromal tissue was then dissociated into a single cell suspension using two methods.

In the first method (which we term "enzymatic digestion"), is similar to that described previously (Gray et al., 2002) Briefly, stromal tissue was exposed to 10 ml collagenase D (0.125% w/v)/DNAse I (0.1% v/v) (Roche) for 15 min at 37 C in a 15 mL conical tube, agitated with a glass Pasteur pipette, and the thymic fragments were then allowed to settle. The supernatant (containing isolated cells) was separated from the fragments and placed in a new 15 ml conical tube on ice, and then fresh collagenase D/DNAse I added to the remaining fragments. This enzymatic digestion was repeated a total of 4 times. In the final step, thymic fragments were allowed to settle, and a mixture of collagenase D/dispase (0.125% w/v) with DNAse I was added for 15 min at 37 °C. Then, all fractions were combined, cells pelleted at 450×*g* at 4 °C for 5 min in a refrigerated centrifuge, and resuspended in 1×PBS/5 mM EDTA/1% FCS/0.02% sodium azide for 10 min at 4 °C and then filtered through 70 μm nylon mesh.

In the alternative method (which we term "enzymatic digestion plus mechanical disruption"), stromal tissue was exposed to 10 ml collagenase D (0.125% w/v)/DNAse I (0.1% v/v) in a GentleMACS C-Tube (Miltenyi Biotec) and initially disrupted using "Program B" (intermediate strength mixing in 30 second pulses) 2–3 times. The C-tube was then incubated at 37 °C for 15 min in a circulating water bath, with periodic Program B pulses every 5 min. Cells were pelleted in the C-tube at 805×*g* for 5 min at room temperature. The supernatant was poured off from the cell pellet and then, 10 mL of fresh collagenase/DNAse I solution was added to the pellet and the sequence repeated (two Program B pulses, incubation at 37 °C for 15 min, with periodic Program B pulses every 5 min, and cell centrifugation). The supernatant was poured off from the cell pellet and then 5 mL collagenase/dispase/DNAse I enzyme mix was added to the cell pellet and incubated in the 37 °C water bath for an additional 15 min with periodic Program B pulses every 5 min. The final single cell suspension was pelleted at 805×*g* at 4 °C for 3 min in a refrigerated centrifuge (Eppendorf), resuspended in 1×PBS/5 mM EDTA/1%FCS/0.02% sodium azide, and incubated at 4 °C for 10 min.

After preparation of the thymic stromal cells by both methods, cells were treated with ACK lysis buffer to eliminate erythrocytes, and then washed and resuspended in M199+. Hemocytometer cell counting with Trypan Blue staining was performed to determine the number of live cells. Cells were then stained with biotinylated anti-CD45 mAb (clone 104, Biolegend) and streptavidin microbeads (20 μL per 10^7 cells) (Miltenyi Biotec) and separated in one round of depletion using the AutoMACS (Miltenyi Biotec) with the "Deplete_S" protocol to separate the CD45+ cells from the CD45− TEC-enriched cell fraction. The negative fraction was collected and then stained for flow cytometric sorting.

2.4. Flow cytometric sorting and analysis

Enriched TECs were resuspended in FACS staining buffer containing 1× HBSS, 4.2 mM sodium bicarbonate, 0.1% w/v