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Thymic nurse cells exhibit epithelial progenitor phenotype and create unique extra-cytoplasmic membrane space for thymocyte selection

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ABSTRACT

Thymic nurse cells (TNCs) are epithelial cells in the thymic cortex that contain as many as 50 thymocytes within specialized cytoplasmic vacuoles. The function of this cell-in-cell interaction has created controversy since their discovery in 1980. Further, some skepticism exists about the idea that apoptotic thymocytes within the TNC complex result from negative selection, a process believed to occur exclusively within the medulla. In this report, we have microscopic evidence that defines a unique membranous environment wherein lipid raft aggregates around the αβTCR expressed on captured thymocytes and class II MHC molecules expressed on TNCs. Further, immunohistological examination of thymic sections show TNCs located within the cortico-medullary junction to express cytokeratins five and eight (K5 and K8), and the transcription factor Trp-63, the phenotype defined elsewhere as the thymic epithelial progenitor subset. Our results suggest that the microenvironment provided by TNCs plays an important role in thymocyte selection as well as the potential for TNCs to be involved in the maintenance of thymic epithelia.

1. Introduction

Although the internalization of a viable cell by another cell has been described for over a century, not until very recently has this phenomenon been accepted as scientifically plausible [1]. Although phagocytes, cells with the ability to take up dead or dying cells, have for a long time been a part of scientific dogma, it has been difficult to advance the idea that a viable cell can internalize another viable cell, and in some cases release the trapped cell from its intra-cytoplasmic space [1]. Thymic nurse cells (TNCs) were discovered in mice by Wekerle and Ketelson in 1980 [2,3]. Their initial report described TNCs as keratin expressing cells containing several thymocytes completely enclosed within specialized cytoplasmic vacuoles. The number of thymocytes enclosed was reported to vary from about 7–50. TNCs were also shown to express both class I and class II MHC antigens on their cell surfaces as well as on the surfaces of the vacuoles surrounding internalized thymocytes. The expression of membrane class II MHC antigens is atypical for epithelial cells. The expression of class II MHC antigens is generally thought to be restricted to cells of the immune system. Typically, epithelial cells do not function within the immune system. Following their initial discovery in mice, TNCs were isolated from the thymus of fish, frogs, chicken, sheep, pigs, rats and humans [2–8]. Since then, the major focus of their study has been to determine the immunological function of the TNC/thymocyte interaction within the thymic cortex. Initial studies of TNCs were performed using freshly isolated cells [2–5]. However, more than twenty years passed before new information was obtained about TNC function because, upon isolation, cytoplasmic thymocytes are released, which does not allow for the identification of the internalized subset. Further, once freshly isolated TNCs release their internalized thymocytes they do not retain the capacity to further internalize thymocytes, making functional studies of the interaction impossible.

Much information has been reported recently in support of TNCs ability to engulf another cell, as well as to define a role for this interaction in shaping the T cell repertoire [4,9–11]. The most convincing evidence has been obtained from the generation of TNC lines that produce cells with the ability to internalize thymocytes *in vitro* [11,12]. Upon addition of freshly isolated thymocytes to cells of the TNC lines, only $\alpha\beta TCR^{low}CD4^{+}CD8^{+}$ cells were found to be bound and internalized [12]. TNCs were shown to selectively rescue a subset of triple positive thymocytes from apoptosis, and antibodies against MHC I and MHC II molecules prevented this rescue activity, suggesting that the rescue was a function of MHC

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driven selection [11]. The rescued population matured to the $\alpha\beta TCR^{hi}CD69^{+}$ stage of development before being released from the TNC complex. In studies using the TNC-specific monoclonal antibody (mAb) pH91, which blocks the TNC/thymocyte interaction, it was demonstrated that this interaction is required for thymocyte viability during the triple positive stage of development [13]. In fetal thymic organ culture, the presence of pH91 reduced the viability of developing thymocytes by 80%, with the largest reduction found in the $\alpha\beta TCR^{hi}CD69^{+}$ thymocyte subset [14].

Collectively, these data have been difficult to accept because they define a subset of epithelial cells in the thymic cortex that facilitate the MHC restriction process using a cell-in-cell activity, a not well-accepted biological phenomenon [1]. Further, these data suggest that both positive and negative selection can occur within TNC complexes, which reside in the thymic cortex. Current dogma insists that positive selection but not negative selection occurs in the cortex of the mouse thymus [15]. MHC restriction is defined as the positive selection of triple positive thymocytes (rescue from apoptosis), or negative selection (induction of apoptosis) that results from an interaction between the αβTCR on developing thymocytes and MHC molecules on antigen presenting cells (APC) [16,17]. While it has been accepted that TNC cytoplasmic thymocytes undergo apoptosis, most reports suggest that cells of the thymic cortex are not functionally equipped to perform negative selection [15]. More specifically, it is believed that negative selection requires the expression of the AIRE protein, which has been reported to control the expression of tissue-restricted antigens (TRA) [18–20]. Both of these functions have been reported to be restricted to cells located within the medulla. However, very recently both AIRE and TRA expression was detected within the TNC complex [21]. These current findings along with the data presented here showing an αβTCR/MHC interaction within the TNC complex adds support to data suggesting that TNCs have the capacity to facilitate MHC restriction, both positive and negative selection. Further, we define the structures involved in thymocyte uptake and show that the initial internalization event results in the delivery of trapped thymocytes into specialized membrane spaces created as a result of extensive cytoplasmic membrane folding. These membrane spaces are external to the TNC cytoplasm but create the two dimensional illusion that trapped thymocytes are cytoplasmic. We propose that these unique membrane structures provide a microenvironment for the αβTCR/MHC interaction and easy release of positively selected thymocytes, while allowing the cytoplasmic uptake of negatively selected thymocytes. If this is correct, the only thymocyte subset that truly becomes cytoplasmic is apoptotic and destined for destruction through lysosomal fusion [22]. Finally, and most unexpectedly, *in vitro* and *in vivo* staining results show a subset of TNCs to express the thymic epithelial cell progenitor phenotype which has been shown to have the capacity to generate an entire functional thymus when transplanted under the kidney capsule [23].

2. Materials and methods

2.1. Isolation of TNCs and thymocytes

C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were dissected aseptically and the thymi were removed. Thymi were slightly disrupted with fine needles and subjected to enzymatic digestion in a solution of 0.015% collagenase D (Sigma Aldrich, St. Louis, MO), 0.01% DNAse I (Sigma Aldrich), and 25 ml of trypsin (GIBCO, Carlsbad, CA) along with gentle agitation. The solution was changed every 10 min until the thymi were completely digested. The resulting cells were subjected to 1×g gradient separation in fetal bovine serum (Atlas Biological, Fort Collins, CO) at 4 °C to enrich TNC numbers. Thymocytes were obtained by the mechanical disruption of thymi obtained from 4 to 6 week old C57BL/6 mice. Macrophage depletion was accomplished by negative sorting using CD11b Microbeads (Miltenyi Biotech, Auburn, CA).

2.2. Scanning electron microscopy

One million thymocytes were allowed to incubate with 1×10^5 TNCs from our temperature sensitive cell line, tsTNC-1 [24], at 37 °C in Terisaki culture plates for 0–12 h and transferred to microscope slides. Freshly isolated TNCs were allowed to incubate on microscope slides for 2 h at 37 °C. The samples were fixed with 3.2% gluteraldehyde in 0.1 M sodium cacodylate buffer pH 7.2 (Electron Microscopy Sciences, Hatsfield, PA), and stored at 4 °C for 12–24 h. The cells were then rinsed in distilled water, dehydrated in a graded series of ethanol (Electron Microscopy Sciences), rinsed twice in amyl acetate (Electron Microscopy Sciences) and critical point dried. The cells were then sputter coated with 6–10 nm of gold and observed in a Zeiss DSM 940 Scanning Electron Microscope. Secondary electron images were captured using the Spirit image acquisition system (version 1.07) at a 1024×1024 pixel resolution.

2.3. Transmission electron microscopy

For studies requiring co-incubation, 5×10^6 TNCs were incubated with 5×10^7 thymocytes for 0–20 h in glass petri dishes at 37 °C. Co-incubated cells and isolated TNCs were fixed in 0.1 M cacodylate, 2% gluteraldehyde, and 1% osmium tetroxide (Electron Microscopy Sciences), pH 7.4 at 4 °C for 30 min. Cells were then dehydrated in ascending concentrations of acetone (Electron Microscopy Sciences). After dehydration, cells were embedded in Embed 812 (Electron Microscopy Sciences). Ultra thin sections were made on a LKB Ultrotome III and stained with uranyl acetate followed by lead citrate (Electron Microscopy Sciences). Cells were viewed on a Zeiss EM 902 Electron Microscope using a SIS Mega-View III digital camera at a resolution of 1376×1032 pixels.

2.4. Video microscopy

Phase contrast videography of 1×10^4 TNCs co-incubated with 2×10^6 thymocytes was viewed using a Nikon Diaphat Microscope with a Hoffman Modulation Contrast System. The microscope was attached to a Nikon CCD-72 camera. The samples were visualized on a Sony 19 inch color monitor coupled to a JVC VCR. Videography using a light microscope was observed using an IX70 Olympus microscope attached to a DP11 Olympus camera. Video images were captured in real time and immediately digitized. All video microscopy was performed at 37 °C.

2.5. Thymic sections

Thymi were dissected aseptically from C57BL/6 mice. Individual lobes were embedded in OTC medium (Richard Allan Scientific, Kalamazoo, MI). Thymic sections 7 μm in thickness were made using a Leica CM1950 Cryostat. Sections were mounted on Bond-Rite microscope slides (Richard Allan Scientific) for immunostaining.

2.6. Immunostaining of TNCs and thymic sections

Isolated TNCs were deposited onto glass slides using a Thermo Scientific Shandon Cytospin 4. Thymic sections or isolated TNCs were fixed in 2% paraformaldehyde (Baker, Phillipsburg, PA) for 30 min followed by three washes with phosphate buffered saline