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THE ROLE OF THYMIC NURSE CELLS IN T CELL DEVELOPMENT

by

Yang Li

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1995

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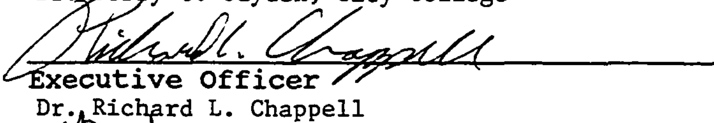

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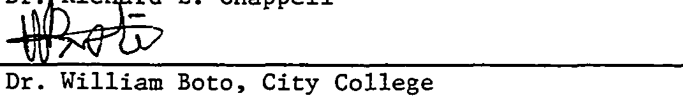

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ABSTRACT

The Role of Thymic Nurse Cells in T Cell Development

by

Yang Li

Adviser: Professor Jerry Guyden

SV40 immortalized Thymic nurse cells (TNCs) were shown to maintain the ability to selectively internalize a subpopulation of $\alpha\beta$ $TCR^+CD4^+CD8^+$ thymocytes *in vitro*. We have now developed temperature sensitive lines of TNCs using the SV40 viral mutant tsA58. One temperature sensitive line of TNCs, tsTNC-1, was shown to be able to rescue a subset of $CD4^+CD8^+$ thymocytes from programmed cell death at 32°C, the temperature at which binding and internalization were detected. Rescue was not detected at 38°C, the temperature at which thymocyte binding was undetectable. The rescued population of thymocytes showed a reduced level of apoptosis as measured by the DNA fragmentation assay. TNC rescue resulted in a shift of $CD4^+CD8^+$ thymocytes from immature TCR^{low} cells to the more mature TCR^{int} phenotype. The rescue activity of tsTNC-1 cells at 32°C was blocked with the addition of antibodies to either class I or class II MHC antigens. These results suggest that TNC rescue is facilitated through an interaction between the TCR and the MHC, and that the resulting survivors mature to a phenotype within the double positive stage of development that is indicative of positive selection.

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To my beloved girlfriend, June Yin,
from whom I have had inspiration and encouragement.

And to my grandfather, Wong Kwok-Chin,
whose constancy, generosity and kindness have been a shining example
throughout my life.

And to my grandmother, mother, father, my sister
whose love and sustained support made it possible to complete this work.

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BACKGROUND

T CELL DEVELOPMENT PROCESS

The thymus is a bilobed organ situated in the anterior mediastinum in mice. Each lobe is divided into multiple lobules by fibrous septa, and each lobule consists of an outer cortex and an inner medulla. The cortex contains a dense collection of T lymphocytes, and the medulla is more sparsely populated with lymphocytes. The lymphocytes in the thymus, also called thymocytes, are developing T cells at various stages of maturation. The thymus is the major site of selection and maturation of both helper T cells and cytolytic T lymphocytes (CTLs) (1,2). This was first suspected because of immunologic deficiencies associated with the removal of the thymus (3,4,5). The thymus provides the microenvironments required both for inducing precursor cells to differentiate into mature T lymphocytes, and for selecting among the developing T cells population those with proper antigen and major histocompatibility complex MHC recognition specificity (6,7,8). The developmental processes within the thymus are very complex. There are many phenotypically distinct subpopulations of thymic lymphocytes which can be isolated at different stages of the T cell developmental pathway (9). Despite the confusion, there has been some agreement on the likely starting point for this complex developmental process. A subpopulation of thymocytes has been identified as having the ability to reconstitute both thymic and peripheral T cell populations (11). These immature lymphocytes enter the cortex of the thymus on day 11 of gestation via blood vessels in mice, and they do not yet express either T cell antigen receptors (TCR), CD3, CD4, or CD8. Thus these cells can not recognize or respond to antigen. They also can not perform effector functions (12). CD4 is a cell surface marker that defines T helper cells and CD8 is the equivalent marker for cytolytic T lymphocytes (CTL). During the T cell

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maturation process, some of the developing cells proliferate, but most of these immature T cells are killed before they migrate from the cortex to the medulla, where only 1% of total thymocytes survive and become mature TCR expressing T helper cells ($CD4^+$) or cytolytic T lymphocytes ($CD8^+$) (9).

The total number of T lymphocyte specific for different antigens in an individual is called the T cell repertoire. Any individual's repertoire of mature helper and cytolytic T lymphocytes (CTLs) has two properties. First, antigen recognition by T cell is self MHC-restricted. Second, the mature T cells are self-tolerant. The process that T cells undergo to learn to distinguish between self and foreign antigens is called thymic educationor MHC restriction, which is believed to involve an interaction between the TCR on the cell surface of thymocytes and the MHC antigen complex on the surface of thymic stromal cells in the thymus (13). These stromal cells can be epithelial cells, macrophages or dendritic cells.

MIGRATION AND PROLIFERATION OF MATURING THYMOCYTES WITHIN THE THYMUS

T cell development in the thymus consist of three closely related processes. They are migration and proliferation, differentiation and selection(14). $CD4^-CD8^-\alpha\beta TCR^-$ (triple negative) immature T cells originate from precursors in the bone marrow and enter the thymus on the 11th day of gestation in mice. These cells have no capacity to recognize antigens or perform effector functions because they do not have the $\alpha\beta$TCR nor the associated signal transduction complex CD3 on their cell surfaces. Within this stage, immature cortical thymocytes can divide rapidly and are killed by high doses of corticosteroids(14). As an aside, this is one reason why chronic steroid

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therapy for a variety of diseases can cause a state of immunodeficiency. There is a high rate of mitosis in the cortex, with each bone marrow-derived precursor giving rise to multiple progenies. Nonetheless, more than 95 percent of the cortical thymocytes die before reaching the medulla(15). During the intrathymic migration, the maturing thymocytes come into close physical contact with a variety of non-lymphoid cells in the thymus. These cells include thymic epithelial cells along with bone marrow-derived macrophages and dendritic cells(16). Deeper within the cortex, epithelial cells form a meshwork of long cytoplasmic processes, around which thymocytes must pass during their migration to the medulla. Other epithelial cells types are present in the medulla. Bone marrow-derived dendritic cells are present at the corticomedullary junction and within the medulla, whereas macrophages are present primarily within the medulla. The migration through this anatomic structure allows sequential interactions between thymocytes and thymic stromal cells, and such interactions are necessary for the maturation of T lymphocytes. It is believed that an interaction between the αβTCR on T cells and the MHC on thymic stromal cells plays an important role in T cell development(17). In mouse thymocytes, both TCR gene types are in the germline configuration until day 13 of fetal life . The rearrangements of first the γδ TCR gene can be detected at day 13 or 14 of normal gestation (18). Transcripts of the γ and δ genes can detetected by day 14 and surface expression of a CD3associated γδ heterodimeric receptor occurs between day 14 and 15 (19, 20). Although nonfunctional DJ rearrangements at the β chain locus begin at the same time as functional β chain VDJ rearrangements, full-length 1.3 kb transcripts can be not detected until day 15 21,22). The α chain genes do not rearrange and generate functional mRNA until day 16 of fetal life. Surface expression of a CD3-associated αβ can be detectable almost two days after γδ

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TCR expression. The surface expression of CD3 associated αβ receptors rapidly overtakes expression of γδ receptors, so that by birth most TCR expressing thymocytes present αβ type receptors.

There is strong evidence that γδ and αβ-expressing thymocytes are separate lineages within common precursors (23). Southern blot analysis of mature αβ -expressing T cells often show out of frame rearrangements of γ genes that are incapable of being transcribed (24). As a result, these cells could never have expressed a γδ type receptor. The δ chain gene segments are located between the V and J gene segments of the α gene, and they are deleted in circles of DNA when the α chain gene rearranges. Analysis of these deleted DNA circles from murine thymocytes shows that the γ chain gene is in the germline configuration in cells which have rearranged their α chain genes. These findings also indicate that αβ -expressing T cells have never expressed γδ receptors, and that the two types of antigen receptors are produced by two distinct lineages of T cells. Most of αβ receptor expressing $CD4^-$ $CD8^-$ T cells will never become $CD4^+$ $CD8^+$ (double positive) thymocytes (14). On the 16th to 17th day of a normal gestation in mice, a subset of T cells expressing CD4, CD8 and αβ TCR can be detected in the cortex of the fetal thymus.

Some immature $CD4^-$ $CD8^-$ T cells may transiently express only CD8 at day 15 or 16 and then become $CD4^+CD8^+$ (25). Small numbers of double negative cells remain $CD4^-$ $CD8^-$ and this population includes cells with rearranged γδ receptors. At the $CD4^+CD8^+TCR^+$ stage of developement, thyomocytes are believed to be restricted or selected in the cortex of the thymus (26). Some cells proliferate after entering the thymic cortex, but most are killed (25). The $CD4^+CD8^+$ cells give rise to mature functional $CD4^+$ T helper cells

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(27,28) or $CD8^+$ T killer cells (29,30). This was first suggested by blocking experiments using anti-CD4 antibody. Exposure of developing thymocytes to anti-CD4 antibody causes the numbers of both mature $CD4^+$ and $CD8^+$ cells to be reduced. This suggests that the antibody induces complement-dependent lysis at the $CD4^+CD8^+$ stage to inhibit the maturation of all single positive cells. On the 18th day of gestation in mice, $CD4^+CD8^-$ TCR $\alpha\beta$ -expressing thymocytes are first detected. These cells display functional helper activity *in vitro* and migrate extra-thymically to peripheral lymphoid tissues to constitute the mature, class II MHC-restricted helper T cell subset. $CD4^-$ $CD8^+$ TCR $\alpha\beta$ -expressing cells appear by day 19 of gestation. These cells have cytolytic activity in *in vitro* assays and enter the blood stream. They are class I MHC-restricted CTLs. The mature single positive thymocytes express higher levels of $\alpha\beta$TCR heterodimers. Only 1% of these cells survive from the migration from the thymic cortex through the medulla and into the periphery (9). Selective cell death ensures that only MHC-restricted, self-tolerant, mature T cells exit the thymus.

THYMIC SELECTIVE PROCESSES

Functional T cells that are foreign antigen specific, self MHC restricted and self tolerant are selected in the thymus. As T cells arise from bone marrow precursors, they have the potential of expressing TCRs that can recognize any antigen in association with any MHC molecule (both self or foreign antigen) (31,32). After different TCRs are expressed on the surface of different clones of developing T cell repertoire, these cells are believed to be selected by two related selective processes. Positive selection kills all the immature T cells that can not bind self MHCs (33) and the negative selection process eliminates potentially

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autoreactive clones, ensuring that mature T cells are self-tolerant, but able to see self MHCs in the context of foreign antigen (34).

Positive selection is believed to be a result of the activation of thymocytes after the binding of their TCRs with self MHC molecules. This process rescues the thymocytes which bind to self MHCs moderately, while the thymocytes whose TCRs have no affinity for self MHC molecules or have high affinity for self MHC molecules are killed. The development of the ability of T-cells to recognize antigenic peptides in association with self-MHC in the thymus gland was first suggested by using radiation-induced bone marrow chimeras (35,36). The host animals were prepared by removing thymuses using irradiation and different combinations of bone marrow and thymic epithelial cells from mice with different MHC haplotypes were transplanted into the irradiated host. The MHC restriction of these mature T cells developed in these mice was examined after stimulating these T cells with antigen plus APCs (thymic epithelial cells). Antigen recognition by these T cells was always restricted by MHC gene products expressed on the transplanted APCs and not necessarily by MHC gene products expressed on extrathymic cells in the host mice (37). Further evidence for the existence of a positive selection mechanism came from using transgenic mice (38,39,40). Transgenic mice expressing a αβ-TCR specific for a D^b -associated H-Y (male specific) antigen which is a foreign antigen for a female mouse, derived from single T cells in these transgenic mice expressing this transgenic TCR. Endogenous TCR gene rearrangements are blocked by allelic exclusion. When the transgenic α and β TCR genes encode a TCR that recognizes a male only peptide (H-Y antigen) in association with a particular allelic MHC D^b molecule , the transgenic T cells matured and populated peripheral lymphoid tissue only in female mice

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expressing MHC D^b allele. If the female mice have MHC D^k haplotype, and do not express the MHC D^b molecule that the transgenic TCR recognizes, no mature transgenic TCR-expressing T cells were detected. This result shows that developing T cells must express TCRs that can bind self MHC molecules in order to survive. The studies using chimeras demonstrate the role of thymic epithelial cells in positive selection of T cell maturation, and shows that it occurs after the maturing T cells express $\alpha\beta$ TCR at the double positive stage of T cell maturation. Experimental evidence shows that positive selection results in the splitting of the T cell population into two major functional classes: T helper (CD4) and T killer (CD8) as a result of recognition of MHC class II and MHC class I, respectively.

Animals are tolerant to self antigens because they lack T lymphocytes specific for self antigens. Self reacting T lymphocytes are deleted or inactivated during maturation in the thymus. This process is called negative selection and it has been established by a variety of experiments (41,42). If developing T cells of $H\text{-}2^k$ haplotype are cultured with fetal thymus of $H\text{-}2^d$ origin, the maturing T cells become tolerant to $H\text{-}2^d$, which can be shown by their inability to give a mixed lymphocyte proliferative response when cultured with stimulators of $H\text{-}2^d$ phenotype. But tolerance in maturing T cells to $H\text{-}2^d$ phenotype can not be induced if cells of the $H\text{-}2^k$ haplotype are incubated with deoxyguanosine treated thymuses of $H\text{-}2^d$ phenotype, because macrophages or dendritic cells in the thymus are sensitive to deoxyguanosine. Negative selection was demonstrated using experiments showing that mice expressing a particular MHC class II molecules (I-E) do not express TCRs with a particular variable segment of the β chain ($V\beta17\alpha$) in the thymic medulla or the peripheral lymphoid organs, but they are $V\beta17\alpha$-expressing T cells in the cortex. While

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strains of mice that do not express I-E molecules can have Vβ17α-expressing T cells in the medulla and periphery (43).

The clonal deletion of self-reactive T cells presumably occurs when the TCR on a $CD4^+CD8^+$ thymocyte binds to a self antigen presented by another thymic cell type (44,45). There is strong evidence that bone marrow-derived cells within the thymus, such as dendritic cells and macrophages, can present self antigen to developing T cells and cause the deletion of self antigen-reactive T cells. The CD4 and CD8 molecules also play a role in negative selection because it has shown that anti-CD4 antibody to I-E expressing mice blocks the elimination of Vβ17α-expressing $CD4^+$ thymocytes. Maturing thymocytes expressing TCR which recognize complexes of self antigens and self MHC in the thymus with high affinity are believed to be deleted or inactivated in the medulla of the thymus (43).

THE ROLE OF THE ACCESSORY MOLECULES IN T CELL ACTIVATION

The interaction between T cells and appropriate antigen presenting cells is mediated through TCR recognition of a specific antigen and MHC complex, but this interaction can be greatly enhanced by the presence of complementary pairs of molecules on the two cells. In addition to CD4 and CD8, several other T cell integral membrane proteins can influence T cell activation and the functional interaction of T cells with other cells. The precise roles of these accessory molecules in antigen-driven activation of T cells are not completely understood, but blocking these proteins by specific monoclonal antibodies have profound effects on T cell development. It has been shown that accessory molecules on T cells specifically bind other molecules present on the surface of other cells, such as antigen presenting cells (APC). These molecules are

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nonpolymorphic and invariant and they can increase the strength of adhesion between a T cell and an APC, which helps the interaction between the TCR and peptide-MHC complexes become stable enough to result in activation of the T cells. Many T cell accessory molecules are members of the Ig gene superfamily or the integrin gene family and they can be used as cell surface marker to identify subset of T cells. Many T cell accessory molecules may transduce biochemical signals to the interior of the T cell that are important in regulating functional responses. Signal transduction presumably occurs as a consequence of ligand binding and may act in concert with other signals generated by the TCR:CD3 complex. CD4 and CD8 are T cell surface glycoproteins that are expressed on mutually exclusive subsets of mature T cells with distinct patterns of MHC restriction.

CD4 serves as a cell-cell adhesion molecule by specifically binding to a nonpolymorphic part of the class II MHC molecule. It is believed that binding of CD4 to class II MHC molecules stabilizes the interaction of a class II MHC-restricted T cell with an APC-bearing class II MHC-associated antigen. This was first suggested by experiment showing that the ability of T cells to functionally respond to class II MHC-expressing APCs only with the presence of CD4 on the T cell surface, there was no functional response when CD4 is blocked by anti-CD4 monoclonal antibody. Monoclonal antibodies against CD4 have stimulatory or inhibitory effects on MHC-independent T cell activation induced by binding of anti-TCR or anti CD3 antibodies. Such activation is independent of recognition of MHC molecules. Also a lymphocyte-specific protein tyrosine kinase, called $p55^{lck}$, is physically associated with the CD4 molecule (46,47). The possible role of such protein kinases is believed to be the transcriptional regulation of genes that are essential to T cell activation.

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CD8 serves as a cell-cell adhesion molecule, by binding to nonpolymorphic immunolgobulin-like domains of class I MHC molecules, stabilizing the interaction of a class I MHC-restricted T killer cell with a target cell bearing class I-MHC associated antigen. This was demonstrated by using $CD8^-$ mutant T cells and experiments with anti-CD8 monoclonal antibody similar to the ones described before for CD4 (46).

CD2 is a 45 to 50 kD glycoprotein that is present on most of mature T cells and on 60% of immature thymocytes. CD2 is a member of the Ig gene superfamily based on sequence homologies (48). The molecule contains two extracellular globular domains that distantly related to Ig homology units, followed by a hydrophobic transmembrane region and a long cytoplasmic tail. It functions as an intercellular adhesion molecule. The ligand for CD2 is the structurally similar 55kD- 70kD glycoprotein called leukocyte function associated antigen-3 (LFA-3) which can be found on the surface of many cells. The binding of CD2 to LFA-3 promotes cell-cell adhesion. This helps the functional binding of helper T cells to APCs, CTLs to their target cells, and maturing thymocytes to thymic epithelial cells. Anti-CD2 antibody can block binding between T cells and LFA-3 expressing cells, CTL activity and antigen-stimulated helper T cell responses. CD2 is also expressed on developing T cells in the thymus earlier than the TCR:CD3 complex and it is a signal-transducing molecule. It has been suggested that CD2 is important for mitotic stimulation of immature T cells. It is believed that CD2 transduces negative or inhibitory signals as well as activating signals, since some anti-CD2 antibodies can block T cell activation induced by anti-TCR or anti-CD3 antibodies. Thus, the consequence of CD2 binding to LFA-3 may depend on the nature of other concurrent stimuli to the T cell.

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LFA-1 is a member of the integrin family of heterodimeric leukocytes surface protein, which function primarily as adhesion molecules (49). LFA-1 is expressed on virtually all bone marrow-derived cells, including more that 90% of thymocytes and mature T cells. One specific ligand for LFA-1 is ICAM-1 (intercellular adhesion molecule-1) which is expressed on many nonhematopoietic cells, including epithelial cells and endothelial cells. LFA-1 dependent cell adhesion phenomena can be blocked by anti-ICAM-1 antibody or by soluble ICAM-1 (50,51).

Thy-1 is a glycoprotein abundantly expressed by murine T cells. Like many other members of the Ig-like superfamily, Thy-1 is believed to participate in cell-cell adhesion (52). The binding of Thy-1 positive T cells to thymic epithelial cells can be inhibited to 40% by soluble Thy-1 molecules purified from phosphatidylinositol-specific phospholipase C-treated mouse thymocytes as well as by Fab' fragments of a Thy-1 specific mAb. The binding of the Thy-1$^-$ mutant T cells to mouse thymic epithelial cells was found to be reduced by 50% as compared with that of the wild type T lymphoma. These data suggest that Thy-1 might play a role in the early T cell maturation by promoting thymocyte adhesion to thymic stroma.

CD44 is an integral membrane glycoprotein, it is expressed on a wide variety of cell types, including T cells. CD44 has been shown to be associated with the cytoskeleton in some cells and can bind extracellular matrix components. These properties suggest that CD44 may functionally link the cytoskeleton with cellular adhesion to extracellular matrix. CD44 on T and B cells also serves as one of several homing receptors that bind to molecules on high endothelial venules (53). It has been shown that CD44 is involved in the activation of T cells mediated by many other surface molecules. In mice, CD44

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is present on bone marrow derived precursors of T cells, before they enter the thymus and its expression declines during murine T cell maturation.

CD45 consists of a group of integral membrane glycoproteins that are expressed on lymphocytes including T cells. The expression of different forms of CD45 is developmentally regulated during the process of maturation of T cells (54). Distinct subsets of CD4 positive T cells express different forms of CD45 at different stages of maturation. The large cytoplasmic domain of CD45 contains an intrinsic tyrosine phosphatas activity, which may be important in the regulation of various activation pathways that involve tyrosine kinase activity.

THE ROLE OF THE THYMUS IN T CELL DEVELOPMENT

The development of T cells from progenitors of extrathymic origin is believed to be crucially dependent upon the thymic microenvironment. Many experiments have shown the importance of the nonlymphoid elements of the thymus in T-cell development (13). The demonstration that mature T cells can develop from a single stem cell after microinjecting into the cortex of the thymus is evidence for the existence of a thymic microenvironments that controls the pathway of this differentiation pathway. Scattered throughout the thymus are non-lymphoid epithelial cells, which have abundant cytoplasm, as well as bone marrow-derived dendritic cells and macrophages. Identification of subclasses of these cells in mice and in humans can aid in understanding of the specific role of each type in thymocyte maturation. One way to determine the function of these stromal cell types is to isolate and culture these cells *in vitro*. Farr *et al.* (55) observed a subpopulation of thymocytes within the mouse thymic cortex expressing low levels of surface T cell antigen receptors. When

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these thymocytes were found in contact with epithelial cells in the cortex, the TCR molecules were localized in the region of thymocyte-epithelial contact. Also macrophages and dendritic cells were found to interact with thymocytes in the thymus. Experiments on bone marrow-reconstituted radiation chimeras show a sequential appearance of thymocyte interactions with macrophages followed by interaction with thymic epithelial cells and dendritic cells. It is believed that the cortical thymic epithelial cells are responsible for positive selection of developing thymocytes which have a strong affinity for self MHC molecules (16). The $CD4^{+}CD8^{+}$ thymocytes are thought to migrate to the cortical and medullary junction where they interact with bone marrow derived macrophage and dendritic cells. Thymocytes that have a very high affinity for self MHC or self MHC in complex with processed self antigens are deleted from the population. This is what we called negative selection. Although some success has been obtained in identifying the cells responsible for important events in the late phase of T cell development (15,17), which include positive and negative selection, little has been clarified about the mechanisms underlying the early phase of T cell development such as commitment to the T cell lineage or induction of differentiation. An important step to reveal the mechanism is to identify the stromal cells involved. Primary cultures of thymic epithelial cells as well as macrophages and dendritic cell lines derived from these cultures have been investigated for their role in T cell development, and some of the lines were shown to support, at least partially, the development of T cells. For example, Palacios *et al* (56) established two thymic epithelial cell lines. One of them supported the differentiation of their pro-T cell lines into $CD4^{+}CD8^{-}$ cells, while another cell line supported generation of both $CD4^{+}CD8^{-}$ and $CD4^{-}CD8^{+}$ cells when transplanted into athymic nude mice. Work of Nishimura *et al* indicated that a nurse cell-like

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line in combination with recombinant interleukin2 and anti-CD3 antibody was able to induce the maturation of immature T cells into $CD4^-CD8^+$ cells (57) . The heterogeneity in the function of these cell lines might indicate that thymic microenvironment comprises various types of stromal cells playing different roles. An alternative possibility is that only one or two types of stromal cells are capable of exerting various functions, while each cell line carries a different part of these functions.

Thymic epithelial cells have also been shown to produce cytokines which are believed to play a role in T cell maturation. Thymic hormones have been shown to promote the appearance of some lineage specific surface molecules on bone marrow cells or immature T cells in culture. They were also shown to enhance T cell functional responses, such as proliferative responses to lectins. Thymic stromal cells secret interleukin-6 (IL-6) which is a cytokine that stimulates the proliferation and maturation of developing T cells in the thymus (58). Our laboratory has focused on the function of thymic nurse cells in T cell developement. The remainder of this section will discuss background literature involving thymic nurse cells (TNCs).

THE FUNCTION OF THYMIC NURSE CELLS IN T CELL DEVELOPMENT

The T cell maturation process requires direct cell-to-cell interactions between stromal cells and the developing T cell (58). TNCs are distinctive stromal epithelial cells located in the cortex of the thymus (59-63). They were found to contain up to 200 dividing T cells in specialized vacuoles within their cytoplasmic membrane. TNCs can be identified from other cells of the thymus by their unique multicellular structure. They have been found in a variety of organisms including human (61), mice (63), rats, sheep and chickens (60).

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The epithelial nature and cortical origin of the TNC have been confirmed using monoclonal antibodies. All freshly isolated TNCs stain positive for ER-TR 4 which exclusively reacts with cortical epithelial cells. TNCs are ER TR-5 negative, which specifically stains medullary epithelial cells. It has also been shown that TNCs are not positive for either of the macrophage markers Mac-1 nor Mac-2, nor the ER-TR-7 antigen of reticular fibroblasts (64).

Although there is no monoclonal antibody specific for TNCs, Vincent Geenen *et al* reported that a monoclonal antibody against neuroendocrine markers called A2B5 stained TNCs specifically in thymus (65). TNCs have been shown to express both class I and class II MHC antigens on their cell surfaces, as well as on the membranes of the specialized vacuoles surrounding internalized thymocytes (59). It has been shown that TNCs can present self-antigens to T cells (66), which suggests that they may have a function in thymic education. The specialized vacuoles may provide an optimal microenvironment for the process of T cell selection. By microinjecting single TNC in the avian chorionallantoic membrane, Penninger *et al* s(67) showed that a significant portion of intra-TNC lymphocytes (TNC-L) possess reactivity against self-MHC molecules. The percentage of these autoreactive cells among TNC-L exceeds by far that of peripheral blood lymphocytes of the same donor. These results indicate that TNC-L constitute a T cell population enriched for self-MHC reactivity and these cells have undergone positive selection, but not yet been deleted or inactivated. Although the stage that maturing thymocytes interacts with TNCs is unknown, it has been found the intra-TNC lymophocytes are $CD4^{+}$ $CD8^{+}$ and express cell surface αβ type TCR, high levels of CD3 expression are detected (68). It was showed that TNC-Ls are Thy-1^{+}, $CD5^{+}$,MHC class I^{+} and cortical peanut agglutinin-positive (PNA^{+}) (69,70,71). Only $CD4^{+}$ $CD8^{+}$

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thymocytes are PNA^+. Thymic education is believed to occur at the double positive stage of T cell development which corresponds to the time when immature thymocytes interact with TNCs.

The molecules that mediate the interaction between the thymocytes and TNCs have not been defined. It has been showed that the molecules involved in the thymocyte-TNC interaction are not MHC molecules, because thymocyte binding to thymic nurse cells is trypsin-sensitive, while MHC molecules on the surface of cells are not trypsin sensitive. Previous studies have shown that the lymphocyte function-associated antigen (LFA-1) and ICAM-1 on thymic epithelial cells play an important role in thymocyte-thymic epithelial cell interaction. Thymocyte-thymic epithelial cell binding via the (LFA-1) and ICAM-1 can operate most effectively under physiologic conditions (50). The T cell specific CD2 antigen has also been shown to be the molecular target of LFA-3 in the formation of human red blood cell rosettes. Cortical peanut agglutinin-positive (PNA^+) thymocytes preferentially associate with TNCs and only $CD4^+$ $CD8^+$ thymocytes are PNA^+ (70,71,72). The actual function of these molecules in the binding process remains to be determined.

Very little is known about how the thymocytes are bound and internalized, and what kind of thymocytes interact with, and are taken up by thymic nurse cells. The reason for this lack of information is that the classical isolation procedures can not yield sufficient numbers of TNCs for thorough analyses, and freshly isolated TNCs have limited ability to grow in tissue culture. Also, freshly isolated TNCs do not internalize thymocytes in tissue culture. TNCs could only be identified and isolated using their multicellular phenotype at which time thymocytes have already been internalized. It was

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impossible to get pure TNCs without contamination of uninternalized lymphocytes. Fortunately we have been able to immortalize TNCs with SV40 and the temperature sensitive strain of SV40 (73), tsA58. These TNCs were found to bind and internalize freshly isolated thymocytes in culture.

We have shown that our TNC lines specifically bind and internalize $CD4^+$ $CD8^+$ thymocytes *in vitro* and the double positive cells that interact with TNCs were shown to express the cell surface $\alpha\beta$ TCR complex (74). These data are consistent with the theory that TNCs may have a role in thymic education.

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EXPERIMENTAL PROCEDURE

MICE.

Breeding pairs of C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). Three females were introduced into a cage with one male for 12 hours (overnight). The females were removed and examined 10 days later for pregnancy by abdominal palpation. Two weeks after birth, thymic nurse cells were isolated from newborns and used for infection with SV40.

CELLS.

Thymic nurse cells. TNCs were isolated using fetal calf serum density gradients (61). Briefly, thymi were minced and suspended in a 50 ml centrifuge tube containing phosphate-buffered saline (PBS). The PBS was removed and the large tissue fragments that settled at the bottom of the tube were subjected to two consecutive 5 minute digestions with 0.25% trypsin containing 1.5 mg/ml collagenase (Sigma, St. Louis, MO) at 37°C. The resulting cells were separated on a fetal calf serum gradient at 1 X g for 45 minutes. The gradient was repeated to increase enrichment of TNCs. The cells were suspended in growth media containing RPMI-1640 (Gibco, Grand Island, NY), 2% Nu Serum (Collaborative Research, Boston, MA), 0.01 % 2-mercaptoethanol (Sigma), 1% each of gentamicin (Gibco) and L-glutamine (Grand Island Biologicals, Grand Island, NY), and 10% fetal calf serum (Sigma). No particular lot of fetal calf serum was required. For immortalization, TNCs were allowed to release enclosed thymocytes and to form monolayers before exposure to SV40 (strain

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Rh911, originally obtained from Dr. V. Defendi) at a multiplicity of 10 PFU/cell at approximately 5 X 10^4 cells/ml.

Fetal Thymocytes. Cell suspensions were obtained from the thymus of two weeks to one month old C57BL/6 mice by mechanical dispersion in growth media. The cells were washed once with Gey's Complete Salts solution to remove erythrocytes. The remaining cells were suspended in growth media and seeded at 1 X 10^6 cells per dish containing a monolayer of TNCs.

GROWTH CURVE.

10^4 TNCs were plated in 35 mm tissue culture dishes, three dishes were counted for each time point, and viability was determined by Trypan blue exclusion.

BINDING ASSAY.

2 X10^6 thymocytes were added to each of four 35 mm plates containing monolayers of 1 X10^4 TNCs. After a 15 minute centrifugation at 3000 rpm, unattached lymphocytes were removed by three washes with PBS. The thymocytes collected in the wash were pooled and counted. The subset distribution of unbound cells was determined by counting the number of cells in each thymocyte subset after staining divided by the total number of cells counted times 100. 2 X10^6 live thymocytes from the pooled unbound fraction were then added to each of two 35 mm plates containing monolayers of 1 X10^4 TNCs.

ANTIBODIES AND IMMUNOFLUORESCENCE STAINING.

Fluoresceinated monoclonal antibodies against Mouse were purchased from Accurate Chemical (Westbury, NY). The hybridoma producing rat

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monoclonal antibody against CD4 (Clone GK1.5) was purchased from American Type Culture Collection (Rockville, MD). Anti-mouse CD3ε and CD45 were purchased from Boehringer Mannheim. (For thymic nurse cells) TNCs were stained with anti-A2B5 (American Type Culture Collection, A2B5 clone 105), a rabbit polyclonal anti-keratin antibody (Dr. T.-T. Sun, New York University Medical School), anti-Ia^b and anti-H2^b (Accurate Chemical). A hamster polyclonal anti- T antigen antibody was used to stain TNCs as an assay for transformation (provided by Dr. Robert Carrol, New York University Medical School). RGD peptides, polyclonal antibody against laminin, Fibronectin receptor and Vitronectin receptor was purchased from Telios (San Diego, CA). αβTCR was purchased from Pharmingen. All antibody preparations were airfuged prior to use, to remove aggregates. Affinity-purified second step reagents, mouse anti-rat immunoglobulin and goat anti-mouse immunoglobulin, were obtained from Bionetics Lab (Charleston, SC) as fluorescein conjugates. Fluoresceinated rabbit anti-hamster antibody was purchased from Cappel laboratory (Malvern, PA), and fluoresceinated goat anti-mouse IgM was obtained from Sigma. Goat anti-rat IgG TRITC (Tetramethylrhodamine Isothiocyanate) conjugate was obtained from Fisher Scientific.

Immunofluorescence staining was performed as follows: (For staining with antibodies against CD4 and CD8) Single cell suspensions were adjusted to 1 X 10^6 cells/ml in cold 5 mM HEPES-buffered Hank's balanced salt solution (HBSS) containing 1% bovine serum albumin (BSA) (Sigma), pH 7.4. The cells were fixed with a 1:1 methanol/acetone mixture for 3 minutes, washed 3 times with PBS, and one ml of the cell suspension was incubated for 15 minutes at 4^oC with a saturating concentration of monoclonal antibody. After three washes, the cells were incubated with the appropriate second step antibody for

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15 minutes. The same procedure was followed for the staining of thymocytes associated with TNCs grown on cover slips except the antibody preparations and washing solutions were applied to the top of the cover slip. In double label experiments, mouse anti-mouse IgM antibody made against CD8, and rat anti-mouse IgG made against CD4 were incubated in the first step. In the second step, goat anti-mouse IgM-FITC and goat anti-rat IgG TRITC antibodies were used to bind antibodies against CD8 and CD4, respectively. In control samples, cells were incubated with HBSS instead of the first step antibody prior to staining with the second step reagent. (For staining with antibodies to TCRαβ or CD3ε). Thymocytes internalized into TNCs were fixed and stained as described above. To obtain cell surface staining of thymocytes bound to TNCs, freshly isolated cells were stained without fixing in methanol:acetone. The staining and washing were done on ice. Stained thymocytes were centrifuged (15 minute centrifugation at 3000 rpm) onto monolayers of TNCs at $4^{o}C$, and washed 2 times before fixing with 2 % paraformaldehyde. The unbound fraction of thymocytes obtained in the wash was treated with 2 % paraformaldehyde and used as controls. All treated cells were viewed under phase and fluorescence optics on a Zeiss fluorescence microscope equipped with epi-illumination.

NORTHERN BLOT ANALYSIS.

Total cellular RNA were isolated from cells in 4M guanidine thiocyanate, 10µg of RNA will be separated overnight at 560 volt hours on a 1% agarose get containing formaldeghde in 0.2 Mmorpholinopropanesulfonic acid-acetate before blotting onto nitrocellulose. The blot was hybridized to a ^{32}P-labelled probe made with full length SV40 DNA. The nitrocellulose paper will be stripped and rehybridized to a ^{32}P-labelled actin probe.

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RELEASING ASSEY.

Thymocytes were isolated from 2 week to 1 month old C57Bl/6 mice and suspended in culture by mechanical dispersion. The cells were washed once with Gey's Complete Salts solution to remove erythrocytes and sorted (Coulter Elite fluorescence activated cell sorter (FACS) for $CD4^+CD8^+$ cells after double staining with an anti-CD4-phycoerythrin (PE) conjugate and an anti-CD8-fluorescein (FITC) conjugate (both fom Boehringer Mannheim). Sorted $CD4^+CD8^+$ cells were analyzed and shown to be > 99 % pure. $2X10^6$ double positive cells were plated in 35mm tissue culture dishes containing a monolayer of SVT-II2 cells (initial concentration $1X10^4$ cells/dish). TNCs were suspended in growth media containing RPMI-1640 (Gibco, Grand Island, NY), 2% Nu Serum (Collaborative Research, Boston, MA), 0.01 % 2-mercaptoethanol (Sigma), 1% each of gentamicin (Gibco) and L-glutamine (Grand Island Biologicals, Grand Island, NY), and 10% fetal calf serum (Sigma). After the appropriate incubation period, thymocytes were removed from tissue culture, washed and stained with anti- αβTCR-FITC (Pharmingen) or antibodies made against CD4 and CD8. The stained cells will be analyze using the FACS.

LONG TERM VIDEO MICROSCOPY

Thymocytes were co-incubated with SVT-II2 cells as described above will be viewed with a Nikon video microscope with Hoffman contrast optics.

CO-CULTURE EXPERIMENTS

Thymocytes were isolated from 2 week to 1 month old C57Bl/6 mice and suspended in culture by mechanical dispersion. The cells were washed once with Gey's Complete Salts solution to remove erythrocytes and then stained with both anti-CD4 PE and anti- CD8 FITC (Boehringer Mannheim) in

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preparation for FACS sorting. Thymocytes were resuspended at 5 $X10^6$ cells/ml and CD4 CD8 double positive thymocytes were sorted (> 97 % pure) using a Colter Epics Elite (model F09102). 5 $X10^6$ sorted thymocytes were incubated with 2 $X10^5$ tsTNC-1, or 3T3 cells for either 48 or 96 hrs at the appropriate temperature. After incubation the thymocytes were removed and washed three times with RPMI. The cells were stained again as described above and reanalyzed using the FACS. For the inhibition studies, antibody and thymocytes were added simultaneously. Antibody preparations were added daily in concentrations identical to those used for staining. Experiments designed to examine TNC interactive thymocytes exclusively were performed using thymocytes that remained bound to TNCs after vigorous washing.

DNA Fragmentation Assay

Cells (3 $X10^6$) were suspended in 0.5 ml of lysis buffer (10 mM EDTA, 50 mM Tris (pH 8.0), 0.5 % Triton X-100 with 0.5 mg of proteinase K) and incubated for 1.5 hr at 50^oC. The samples were extracted three times with phenol-chloroform and precipitated with ethanol. Each sample was resuspended in TE after drying and loaded onto 2 % agarose gels containing 0.05 mg ethidium bromide (75) . Photographs were taken under UV light using Polaroid film.

Modified TUNEL Assay

TNC-thymocytes complex or thymocytes are washed with 1xPBS and then fixed with freshly made 4% paraformaldehyde and .1% TritonX-100 on ice for 2 minutes. 5ul of terminal deoxynucleotidyl transferase (TdT) and 45ul fluorescein-labeled dUTP (Boehringer and Mannheim) to the slide and

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incubate at 37oC for 60 minutes. Wash samples with 1XPBS and analyze by FACS or fluorescence microscopy.

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RESULT

Isolation and Immortalization.

Thymic nurse cells (TNCs) were isolated using fetal calf serum gradients. All freshly isolated TNCs display the characteristic multicellular complex (Fig.1A). After 1-2 days in tissue culture,TNCs release enclosed thymocytes and form monolayers (Fig.1B), but have a limited proliferation patential (Fig. 2A). In order to get sufficent numbers of TNCs for our experiment, we infected freshly isolated TNCs with SV40 and temperature sensitive mutant tsA58. The size and morphology of the cells obtained post infection (Fig. 1C), flat with an expansive cytoplasm containing cable-like striations, are similar to TNCs found in fresh isolates (Fig. 1A). Cells taken in log phase of growth were stained with a polyclonal antibody made against the large T antigen, the SV40 transforming protein. Nuclear staining was readily visible in some cells (Fig. 1D), but other cells in the same culture displayed cytoplasmic staining with bright granules centered in the nucleus (Fig. 1E). This type of staining pattern may represent the plasma membrane-associated large T antigen fraction (76,77). Infected cells were cloned using limiting dilution, and a total of 18 clones was isolated from our original infection. Three of these clones have been maintained in culture for over a year. The other lines were stored in liquid nitrogen.

Increased growth of SV40-infected TNCs above normal was apparent from 10-14 days post infection, at which time they began to double every 18-20 hours (Fig. 2A). Rapid proliferation and the nuclear expression of large T antigen are characteristics of SV40-infected cells, but stable transformation requires the insertion of the SV40 genome into the genome of the host cell.

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IDENTIFICATION OF CELLS AS TNCS.

Because TNC-specific antibodies have not yet been developed, we stained each cell line with a panel of antibodies to verify their identity. The data presented in Figure 10 are the results of the analyses of SVT-Mp5. A2B5, a neuron-specific antibody that recognizes only TNCs in the thymus (65), was used as an initial screen for each clone isolated. Both normal TNCs and SVT-Mp5 were found to express the A2B5 specific antigen (Fig. 3A and B). The staining pattern obtained using anti-A2B5 suggests that the antigen recognized by this antibody may be associated with stress fibers in the cell. Also, extensive cytokeratin staining characteristic of normal TNCs (Fig. 3C) (73) was detected in SVT-Mp5 (Fig. 3D). SVT-Mp5 cells stained with antibodies made against the major histocompatibility complex class I (Fig. 3F) and class II antigens (Fig. 3H), but were Thy-1 and Mac-1 negative. No phenotypic changes have been detected in SVT-Mp5 cells after six months in culture. Together these data provide prelimenary evidence that the cells described above are thymic nurse cells transformed with SV40.

BINDING AND INTERNALIZATION OF THYMOCYTES BY TNCS

The TNC line SVT-II2, was developed by the infection of freshly isolated nurse cells from two week old C57BL/6 mice with SV40. In an attempt to visualize the dynamics of the process of internalization, thymocytes bound to a SVT-II2 cell were analyzed using long term video microscopy (Fig. 4). Photographs taken from the video monitor show the internalization of the thymocyte marked X. Each photograph, from left to right, represents an

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increase in time (the last digit in each frame number represents elapsed time in seconds). The thymocytes labeled 1, 2, and 3 are reference points for each photograph, and are bound to the external cell surface (unbound thymocytes were removed with vigorous washing). It is important to note that a channel (arrows, frame 778) is visible in the cytoplasm of the TNC before the initiation of internalization. As the thymocyte marked X moves into the membrane it becomes phase dark (frames 801, 804, 807, 809, 813, 816) and moves under the thymocytes bound to the cell surface without displacing them. The thymocytes marked 1, 2 and 3 (frames 822, 835, 853,873) would block the passage of thymocyte X if they were bound to the same surface. The only explanation is that thymocyte X has been internalized and the cytoplasmic membrane separates thymocyte X from the thymocytes bound to the external cell surface. Internalization is completed in frame 822. The internalized thymocyte (X) moves into the preformed channel mentioned above (frame 873, arrows). The internalized thymocyte continues to move in a directional manner within the channel (frames 889 and 897). Thymocytes are shown to move around the surface of the TNC as a function of their attachment to the TNC membrane (Fig.5, frames 3743-4108, cells labeled A and B).

TNCs EXCLUSIVELY BIND AND INTERNALIZE A SUBSET OF DOUBLE POSITIVE THYMOCYTES

SVT-II2 expresses the characteristics previously reported to define TNCs (Table 1), and are able to internalize freshly isolated thymocytes. To determine

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which T cell subset binds thymic nurse cells, thymocytes isolated from two weeks to one month old C57BL/6 mice were exposed to monolayers of SVT-II2 overnight. The cultures were washed vigorously to remove unattached cells and stained with antibodies to both CD4 and CD8 (Fig. 6). In every experiment, the only cells bound (Fig.6 ABC), or internalized (Fig.6DEF) by TNCs were double positive thymocytes. To confirm our results this experiment was repeated several times using fixed or unfixed preparations. Freshly isolated TNCs have the capacity to bind (Table 1), but are unable to internalize developing T cells in culture. We have performed this experiment with other TNC lines. Each line was shown to bind double positive thymocytes exclusively (data not shown). The relative number of double positive cells decreased after incubation with TNCs. The cells that remained unattached after the initial incubation with SVT-II2 were exposed to fresh preparations of TNCs to test their binding capacity (Table 2). Although background level binding was detected, the relative size of the double positive population remained unchanged. These results suggest that not all double positive cells have the ability to bind TNCs. Conversely, only a subset of cells within the double positive population display binding activity.

To further characterize the $CD4^{+}CD8^{+}$ lymphocytes bound to TNCs, we stained with antibodies to investigate T cell antigen receptor expression (Fig. 7.). The results of this experiment show internalized cells to express both αβ TCR (Fig. 7A.) and CD3ε. (Fig. 7C.). Again, the experiments shown in Fig. 7 do not distinguish between cytoplasmic and cell surface staining because the membranes must be permeabilized to permit antibody entry. To facilitate cell

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surface labeling, thymcoytes were stained and spun onto monolayers of SVT-II2 without fixation. The results of these experiments show the cell surface expression of TCRαβ (Fig. 8A) and CD3ε (Fig. 8C) on thymocytes bound to SVT-II2 cells.

SVT-II2 was shown to express both class I and class II MHC antigens (Table 1). Thymocytes that bind SVT-II2 were shown to express both CD4 and CD8, as well as CD3. As a result of these findings, the binding of thymocytes to SVT-II2 could be facilitated through the TCR/MHC complex. To test this possiblity, thymocytes were incubated with SVT-II2 in the presence of antibodies against CD3, CD4, CD8, Ia^b, $H2^b$ (Fig. 9A). All combinations of the antibodies were used but niether preparation interfered with binding. Because the assay time for binding is overnight, antibody activity could decrease with time. To ensure maximal antibody activity, the assay time was reduced using centrifugation to facilitate contact of thymocytes to TNCs (Fig. 9B). Although the overall percentage of bound thymocytes decreased using these assay conditions, there were no differences between the binding percentages obtained with or without antibody present.

Development and Characterization of Temperature Sensitive Thymic Nurse Cell Lines

To study the function of the interaction of TNC with double positive thymocytes, we created temperature sensitive clones.. A shift in the temperature from 32°C to 38°C prevents the interaction of thymocytes with TNCs and thus serves as an excellent system to study the role of TNCs in T cell development. Fresh thymic nurse cells were isolated from C57BL/6 mice and purified on fetal calf serum density gradients. The resulting cells were allowed

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to adhere to culture dishes and formed monolayers at 37°C. The monolayers were infected with tsA58 and then placed at 32°C, the permissive temperature for viral replication. Growing cells were passed several times and then cloned by limiting dilution. One of the resulting clones, tsTNC-1, was found to express temperature sensitive variations in morphology and growth. When tsTNC-1 cells were maintained at 32°C, the cells were smaller (Fig. 10 A) and had a higher growth rate (Fig. 10 C) than those analyzed after 48 hours at 38°C. At the higher temperature these cells became very large and flat, taking on a morphology that resembles freshly isolated TNCs (Fig. 10 B). Loss of the transformed phenotype upon the shift from 32°C to 38°C is concurrent with the loss of SV40 early region gene expression which contains the T antigen sequence (Fig.10 D). After about 48 hours at 38°C, mRNA for T antigen was undetectable. These data strongly suggest that the immortalization of tsTNC-1 cells is a function of tsA58 infection. To further characterize tsTNC-1 cells, samples were stained with a panel of monoclonal antibodies which are known to be expressed by TNCs. TsTNC-1 cells stained positively for class I ($H2K^b$) and class II MHC (Ia^b), cytokeratin, and A2B5, a neuroendocrine cell marker which has been shown to be expressed exclusively by TNCs in the cortex of the thymus (73). TsTNC-1 cells also expressed the cortical marker ER-TR-4, but were negative for ER-TR-5 (Table 3.), an antibody that recognizes a thymic medullary antigen (79).

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TEMPERATURE SENSITIVE BINDING AND INTERNALIZATION OF DOUBLE POSITIVE THYMOCYTES.

We have previously demonstrated that our SV40-transformed thymic nurse cell lines maintain the ability to both bind and internalize a subpopulation of the αβ TCR^{+}CD4^{+}CD8^{+} thymocytes. Binding assays were performed using tsTNC-1 cells at both 32°C and 38°C. Ts-TNC-1 cells, but not at 38°C (Fig. 11A). The wild type SV40-transformed TNC line MP5 showed equivalent binding efficiencies at both temperatures. The internalization of thymocytes at 32°C is shown in Figure 11B.

TSTNC-1 RESCUE OF DOUBLE POSITIVE THYMOCYTES FROM APOPTOSIS.

We have previously demonstrated that our SV40-transformed thymic nurse cell lines maintain the ability to both bind and internalize a subpopulation of the αβ TCR^{+}CD4^{+}CD8^{+} thymocytes. To determine the function of the thymocyte/TNC interaction *in vitro,* co-incubation experiments were performed using FACS-sorted double positive thymocytes. Double positive thymocytes were sorted to a purity of over 97% and placed on monolayers of tsTNC-1 cells at either 32°C or 38°C, and reanalyzed after 48 (Figure 12A) or 96 hours (Figure 12B) using forward versus side scatter. Survivors were sparse in control samples (1.9 % viability of thymocytes with 3T3; 0.5 % viability with tsTNC-1 at 38°C) but a relatively high frequency of viable cells were detectable in samples grown at 32°C in the presence of

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tsTNC-1 cells (55 % viability). Viable double positives in tsTNC-1 cultures at 32°C expressed relatively high levels of both CD4 and CD8 (Figure 12C, panels 1 and 2).

A recent study from our laboratory showed TNC binding to be restricted to viable thymocytes, but both live and preapoptotic double positives were detected within TNC vacuoles (74). The preapopototic thymocytes are small cells with irregular shapes and condensed chromatin in the nucleus. These data suggest that death of a selected double positive subset occurs within TNCs. Is thymocyte death a function of apoptosis? Enzymatic intranucleosomal DNA degradation produces detectable fragments during the process of programmed cell death (79). The DNA fragmentation assay was exploited for this investigation. Viable versus nonviable thymocytes were sorted using forward scatter after co-incubation with TNCs and 3T3 cells. It is important to note that in this experiment, the thymocytes analyzed were all TNC-adherent because nonadherent double positives were removed after a short incubation period to facilitate binding (a 2.5 hour incubation period). A much lower level of fragmentation was detected in thymocytes sorted in the rescued gates after 96 hours versus those that fall in the nonviable gates (Fig.13). Similarly, DNA fragmentation was greatly reduced in the TNC bound fraction at 32°C when compared to all unbound populations (Fig.13). The amount of DNA fragmentation increases within the bound thymocyte fraction with time (from 24-48 hours

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incubation periods), but remained much lower than that detected in unbound populations (Fig.13B). It is important to understand the advantages of studying each subset pair, live versus dead and bound versus unbound populations. Analysis of bound vs. unbound thymocytes allows one to monitor the apoptotic activity during the thymocyte/TNC interaction. In Figure 14, using the newly developed TUNEL assay, which results in terminal deoxynuceotidyl transferase-mediated labeling of DNA with numerous strand breaks, we verified that death of a subset of the internalized population is induced through the process of apoptosis. We then examined cocultured thymocytes separated into bound versus unbound fractions. Unlike the experiment reported in Figure14, which only examined the TNC-internalized population, this experiment was designed to analyze the effect of coculture on the entire double positive population. FACS - sorted double positives were exposed to cocultures as described above in Figure12, and the thymocytes resulting from 1 and 2 day cultures were analyzed using the TUNEL assay. Figure 15 shows apoptosis to be greatly reduced in the TNC-bound fraction at 32°C when compared to unbound populations at 32°C, in tsTNC-1 cultures grown alone. Collectively, these data suggest that viability results from an interaction with TNCs, however, both viable and apoptotic cells exist within the TNC-interactive population.

Several reports suggest an association between increased viability and Bcl-2 expression during thymocyte selection (80). The precise time of the activation and the function of Bcl-2 expression during the late stages of

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thymocyte development is currently the subject of debate. It has been shown that double positives express detectable levels of Bcl-2 but only in the $\alpha\beta TCR^{hi}$ subset. To determine the relationship between the thymocyte/TNC interaction and Bcl-2 expression, we analyzed thymocytes obtained from co-incubations after staining with anti- Bcl-2 antibody (Fig. 16). Bcl-2 expression is detectable in a subset of the TNC-bound population within 24 hours of culture. At 72 hours, most of the viable thymocytes co-cultured with tsTNC-1 cells express the Bcl-2 protein.

CELL SURFACE PHENOTYPE OF RESCUED THYMOCYTES.

TNC-interactive thymocytes were examined to determine the effect of this activity on thymocyte differentiation. Because antibody treatment of double positive thymocytes with CD4 results in an increased expression of cell surface TCR (81), TNC-interactive double positive thymocytes were selected as a function of their binding capacity. Bound thymocytes were recovered after an initial binding period of 2 hours and compared with those collected from 48 hour cultures. Analysis of the thymocytes bound to TNCs at 32°C showed the initial binding phenotype to be restricted to the $\alpha\beta TCR^{low}$ double positive population (Figure 17A). At 48 hours, a shift to the $\alpha\beta$ TCR^{int} phenotype was detected in the rescued population of tsTNC-1 cultures at 32°C (Figure 17A and C). Again, the percentage of surviving

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thymocytes was much higher in TNC cultures than that detected in control cultures (Figure 17A).

Recent reports suggest that the shift to the TCR^{int} phenotype is indicative of positive selection through an interaction between the TCR and the MHC complex antigens (82). We reported in an earlier study that antibodies against MHC class I and/or class II antigens do not interfere with the binding function between these two cell types (74). These framework antibody preparations were used to determine their effect on rescue. As seen in Figure 18 , the percent rescue was reduced in tsTNC-1 cultures at 32°C containing antibodies against either determinant , suggesting that any disturbance of or interference with the TCR/MHC interaction can reduce the rescue activity.

Using identical experimental conditions, the cell surface phenotype for the PNAr (83,84,85), HSA and CD69 of rescued thymocytes was characterized. TNC binding capacity was only found within the immature $PNAr^{high}$ phenotype, while the rescued thymocyte subset displayed the more mature $PNAr^{low}$ cell surface profile after a 48 hour incubation period (Figure 19A). No differences between the initial binding population and rescued thymocytes were detected in the cell surface expression of the early activator marker CD69 nor HSA (Figure 19B). A previous study reported TNCs to be deficient for complete antigen presentation alone, but in the presence of the cytokine IL-1β, the deficiency could be corrected (66,86).

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FOCUS

The capacity to discriminate between "self' and "nonself is acquired in the thymus where T cell progenitors develop to mature, antigen specific, self-tolerant T cells (6,7,8). This process is based on the direct contact of developing T cells with stroma cells and extracellular matrix constituents and the action of humoral factors secreted by nonlymphoid and lymphoid thymic components (28). The most widely accepted hypothesis postulates that thymocytes expressing T cell receptors with affinity to MHC class I and II molecules displayed on cortical epithelial cells are positively selected (MHC restriction) (87,88). In the medulla the interaction with (auto)antigen presenting bone marrow derived dendritic cells and/or macrophages entails clonal deletion of Thymocytes expressing potentially harmful TCR (negative selection) (89). The exact sites of positive and negative selection are not t yet known. However, thymic nurse cells seem to provide an optimal microenvironment for the former (69). If TNCs are also involved in negative selection is still an open question. Many attempts have been made to establish pure populations of thymic nurse cells to clarify both the identity of this critical cell type and to dissect the means by which it exerts its influence, either by direct cell-cell contact or perhaps by producing cytokine(s). Several groups have been reported methods for the establishment of short-term cultures of thymic stromal epithelial cells which play various roles in T cell development. But the studies have been difficult to interpret, because of the possibility that small numbers of macrophages, dendritic cells, or other cell types were present.

Thymic nurse cells are unique stromal epithelial cells found in the cortex of the thymus. They have been identified in a variety of organisms. Up to 200 proliferating lymphocytes can be found within specialized vacuoles in

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the TNC cytoplasm (22). TNCs express both class I and class II MHC antigens on their cell surface, as well as on the vacuolar membrane surrounding the internalized T cells (21, 33). They do not express lymphocyte specific markers such as Thy-1, Ly-1 (CD5), CD4 or CD8. Attempts at the characterization of TNC thymocytes have shown them to be a very heterogeneous population (69), and most of the cells have been found to be nonfunctional (90). It should be noted, however, that it is not possible to isolate a totally pure population of TNCs with internalized lymphocytes. There are always contaminating lymphocytes in the isolation procedure. Both immature and mature T cell phenotypes have been identified within TNCs. A low level of cytolytic activity was obtained in some TNC lymphocyte preparations (90) while others show a small population of cortisone-resistant cells expressing the phenotype of mature precursors of the helper T cell lineage. This fact may suggest that some degree of differentiation is occurring within the microenvironment of the thymic nurse cells, where immature non-functional cells are being stimulated to differentiate into mature functional cells.

In this study I have described the production of SV40 and ts-SV40 transformed thymic nurse cells. These cells appear to be of epithelial origin by morphologic and biochemical criteria. One of these ts-SV40 immortalized TNC lines SVT-II2, the temperature sensitive lines tsIV-2 and ts-TNC-1 have been shown to bind and internalize thymocytes *in vitro*. In order to understand the role of the TNCs in T cell development, my thesis was involved the following studies:

1) to characterize both the wild type and temperature sensitive TNCs lines. The growth and binding ability of these lines were studied and compared with freshly isolated TNCs by growth curve and modified binding assay. In

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addition, the growth and binding ability of the temperature sensitive line were studied at the permissive and nonpermissive temperature.

2) to identify the possible receptor(s) on TNCs which involve in thymocyte binding and internalization. Antibodies against all the possible ligands or receptors on either thymocytes or TNCs were used to block the binding and internalization process.

3) to expose fresh isolated thymocytes to TNC lines for uptake, and determine the phenotype of the subpopulation internalized. A panel of antibodies to T cell specific surface markers were used to identify the subsets of thymocytes bound internalized and released by TNCs.

4) to determine the fate of the thymocytes after internalized by TNCs. By using long term video microscopy and FACS, we were able follow the thymocytes which were bound and internalized to see if these cells were either killed released by TNCs cells, the phenotype of the thymcoytes after they interact with TNCs were checked by using antibody against CD4, CD8, αβTCR CD69, HSA, PNAr and other thymcoytes maturation markers.

5) to establish that thymocyte death in our system is the result of programmed cell death and not due to nutritional insufficiencies by using the DNA fragmentation assay and TUNEL assay.

This is the first report of the immortalization of TNCs with SV40 and formation of TNC/lymphocyte complexes *in vitro*. These cell lines along with

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freshly isolated lymphocytes represents a model system to study the binding and internalization process, as well as the role of TNCs in T cell development.

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DISCUSSION

A fundamental question in the study of Tcell biology is how T cells are made. Before the complex forces of negative and positive selection can imprint a pattern of self verse nonself discrimination on developing thymocytes, undifferentiated stem cells must commit themselves to become immature T cells first, rather than B cells, these maturing T cells interact with diverse components in thymus, then only the mature T cells can be released into the periphery.

In this study I developed a model system to study the function of thymic nurse cells in T cell differentiation in thymus. The study began with the isolation of thymic nurse cells followed by immortalization with SV40 and SV40 mutant tsA58. The immortalized TNCs maintained many of the characteristics of freshly isolated TNCs and retained the ability of internalizing thymocytes. This was the first time that internalization of thymocytes by TNCs was observed *in vitro* (73). The definitive identification of TNC binding and intra-TNC thymocyte population is crucial to the understanding of thymocyte development and the determination of thymic nurse cell function.

WHEN DO MATURING T CELLS INTERACT WITH TNCS ?

Intrathymic differentiation events are defined according to cell surface markers and the rearrangements of TCR genes which eventually lead to the generation of a complete CD3/αβTCR complex on mature thymocytes. Early

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$CD4^{lo}CD8^{-}CD3^{-}$ thymocytes, as defined by Shortman et al (91) are precursors of $\alpha\beta$ and $\gamma\delta$ T cells. $CD4^{lo}$cells are more active in repopulating the thymus than are triple-negative $CD3^{-}$ $CD4^{-}CD8^{-}$ cells. The sequential development of the $CD4^{lo}$ population is characterized by the expression of CD44 and /or CD25, and then by expression of CD4 and CD8. However, in order to follow these stages of development, it is necessary for $CD4^{lo}$ cells to downregulate the CD4 antigen and become $CD3^{-}CD4^{-}CD8^{-}$ (TN). Thus, the following transitions may be detected c-$kit^{++}CD44^{+}CD\ 25^{-}$ —> $CD44^{+}$ $CD25^{+}$ thymic precursors (TCR β and δ in germline configuration) —> $CD44^{-}$ $CD25^{+}$ (60% of the cells express the recombination-activating gene 1 (RAG-1) reflecting the start of TCRβ rearrangement (92). These steps are also characterized by rearrangement at the TCRγ locus and downregulation of c-kit protein expression. Interestingly thymic development is arrested at the $CD44^{-}CD25^{+}$ TN stage in RAG-1 knockout mice. Backcrosses of these mice with mice transgenic for TCR β lead to the generation of $CD4^{+}CD8^{+}$ thymocytes that are TCR $\beta^{+}\alpha^{-}$ and express surface CD3ε. A possible role for the incomplete CD3/TCRβ complex may be in the transition to the $CD4^{+}CD8^{+}$ double-positive (DP) stage, Indeed, earlier experiments indicated that CD3ε is expressed on the cell surface of double-negative (DN; $CD44^{-}CD25^{-}CD4^{lo}CD8^{lo}$) thymocytes at low concentrations, even before expression of the TCR β chain. Commitment to the $\alpha\beta$ or $\gamma\delta$ T cell DN lineages probably occurs at the TN $CD44^{-}CD25^{+}$ stage, since their immediate

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successors ($CD44^-CD25^-CD4^+CD8^+$) are the precursors of $\alpha\beta TCR^+CD4^+CD8^+$, which generate either CD4 or CD8 single positive (SP) mature T cells. In our study, we show that $CD3^+$ $\alpha\beta TCR^+CD4^+CD8^+$ thymocytes are the only cells bound by and internalized into thymic nurse cells (74). Also immortalized TNCs express both MHC classI and classII on their surface in early passages (73). Some SV-TNCs loss MHC class I expression in tissue culture, but upon treatment with γ-interferon these cells recover their MHC class I expression. The finding that MHC class I and class II expressing TNCs interact with $CD3^+\alpha\beta TCR^+CD4^+CD8^+$ thymocytes strongly suggest a role for thymic nurse cells in thymic education. The predominance of double positive cells is expected because of the cortical location of TNCs. Earlier studies by Kyewski et al also suggested that the majority of TNC associated thymocytes are $CD4^+CD8^+$(69).

HOW DO MATURING T CELLS INTERACT WITH TNCS ?

Recently many experiments have been directed at understanding the mechanism by which CD4 and CD8 contribute to antigen-specific recognition, activation and differentiation. It was suggested that CD4 and CD8 may be adhesion molecules that increase the overall avidity of a T cell and its partner by binding to their respective MHC class II and class I ligands thus facilitating specific TCR/antigen-MHC interactions. Expression of high levels of CD4 (93) or CD8 (94) in transfected cells or membrane vesicles increased the ability of

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those cells or vesicles to bind to cells or membranes expressing the appropriate MHC class II or class I ligand, demonstrating that CD4/class II or CD8/ class I interactions can influence the avidity of one cell for another. Expression of CD4 in a class-I-restricted hybridoma demonstrated that CD4 contributed to antigen-specific IL-2 production only when class II was present on the antigen-presenting cell (APC) (95). Similarly, expression of CD8 in a hybridoma in which the TCR was restricted to MHC class II influenced IL-2 production only when class I was present on the APC (96,97). These data are consistent with CD4 and CD8 acting as adhesion molecules. The relative adhesive effects of CD4 or CD8 proved to be minor, however compared with those of CD2 and LFA-3 (48).

Although it has been suggested that adhesion and entrance of thymocytes into TNCs can be partially reduced by anti-extracellular matrix ligands and anti-extracellular matrix receptor reagents, we showed that the binding and internalization of thymocytes by TNC cultures were not inhibited in the presence of antibodies against CD3, CD4, CD8, MHC classI class II, CD5, CD25, CD44, CD45, PNAr LFA-3, ICAM-1, CD2, LFA-1(74). The binding specificity of TNCs for this immature $CD3^{+}$double-positive subpopulation suggests that other molecules may be involved and the interaction is receptor mediated. Certainly a lot of work has to be done before the protein(s) involved in binding and internalizing this subset of thymocytes into TNCs can be found.

Identification of the cell surface molecules involved in thymocyte-TNC interactions has been approached by the production of mAbs reactive to thymic

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nurse cell surface Ags. A mAb against one such Ag, mouse thymic stroma (MTS) mAb MTS 23, stains a subset of thymic epithelium by immunohistology (98). In addition, it was found that, by flow cytometry, this antigen was constitutively expressed on peripheral B cells and macrophages as well as thymic and splenic dendritic cells. It was suggested that the Ag identified by MTS 23 represents a functional accessory molecule which is able to block up to 75% of T cell proliferation in soluble anti-CD3 and Ag-induced responses in a dose-dependent manner, but not under conditions in which no APCs were required. The molecule detected by this mAb has an apparent molecular mass of 120 kDa under reducing and nonreducing conditions. On the basis of these molecular properties and expression pattern, it is postulated that MTS 23 may detect an accessory molecule important for T cell activation. Its expression on thymic epithelium is consistent with the notion that T cell development is not solely a consequence of unique molecular interactions, but also of signals arising from combinations of interactions involving molecules expressed extrathymically as well.

A thymic epithelial adhesion complex that selectively binds double positive thymocytes and a small percentage of CD4 single positives was defined and characterized (99,100). However, this receptor appears to be restricted to E5 cells, a line of epithelial cells derived from the medulla of the thymus. It was proposed that this thymic medullary complex, gp 23/45/90, binds a subset of double positives that may be immediate precursors to single positive thymocytes. We propose that the thymocyte interaction with TNCs precedes

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thymocyte interaction with the gp 23/45/90 complex as defined by both the phenotype of the epithelial cells and thymocytes that interact with each epithelial cell type. TNCs are cortical cells, while cells of the E5 type were derived from the medulla. It is logical to propose that the thymocyte interaction with TNCs occurs before the encounter with medullary cells. Furthermore, double positive thymocytes that initiate the interaction with TNCs display a more immature $\alpha\beta$ $TCR^{lo}CD4^{lo}CD8^{lo}PNAr^{hi}CD69^{-}HSA^{hi}$ phenotype. The thymocytes become $\alpha\beta$ $TCR^{int}CD4^{hi}CD8^{hi}$ $PNAr^{lo}CD69^{-}HSA^{hi}$ after they interact with TNCs. Although the description of the double positive subset that interacts with E5 lacks information about cell surface expression levels for the TCR, HSA or CD69, it was reported that this thymocyte subset expressed high levels of both CD4 and CD8 which is similar to the rescued population released from TNCs (101,102).

Currently we are developing the monoclonal antibdy in an attempt to identify the receptor(s) involved in binding and internalization between thymocytes and our TNCs.

WHY DO MATURING T CELLS INTERACT WITH TNCS?

TNC-thymoyte complexes have been observed not only in the thymocyte TNC culture but also in the pre-B cell-bone marrow stromal cell culture (68). These interaction seem to be morphological reminiscences of the in vivo microenvironment for immature lymphocytes to proliferate and differentiate.

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Similar stuctures observed in the lymphocyte- high endothelial cell interaction in culture have been interpreted as a functional manifestation of high endothelial cells interacting with recirculating lymphocytes (64). Thus, it is presumed that thymocytes within the TNCs in culture may lie under similar circumstances, where immature thymocytes appear to contact TNCs directly and/or to be exposed to higher concentrations of thymic hormones and other soluble factors. MHC class I and class II expressing thymic nurse cells specifically interact with a subset of CD3 $\alpha\beta TCR^{+}CD4^{+}CD8^{+}$ thymocytes (25% of total double positive cells) indicate that the role of TNCs in thymic education and TNCs secifically rescue double positive thymocytes from programmed cell death, or apoptosis. It was shown that the MHC class I and class II present on the thymic epithelium of the host dictated the restriction specificity of the positively selected T cells. The issue of whether there is a strict requirement for stromal cells in positive selection has been addressed more recently using a number of approaches. Jacobs et al. (103) showed that the anti-HY receptor, which requires $H\text{-}2D^{b}$ for positive selection, was not selected in transgenic mice where the D^{b} allele was present only on bone marrow derived cells in chimeric mice. This was also shown with other TCR transgenic mice (104,105). A requirement for stromal cells in positive selection was also demonstrated in the experiments of Anderson et al. (106) who have worked out a very elegant but technically difficult reaggregate thymic organ culture system (107). In this system, differentiation occurred when stromal elements from fetal lobes were reaggregated with immature thymocytes. When this aggregate was prepared

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with stromal cells depleted of MHC class II^+ epithelial cells, the thymocytes were unable to differentiate. MHC class II^+ epithelial cells alone were able to induce the positive selection step from double positive to single positive cells. Additionally, two groups (108,109) were able to demonstrate positive selection on MHC molecules present on thymic epithelial cell lines injected directly into the thymus. In (A X B -> A) bone marrow chimaeras, T cells restricted to the MHC-A haplotype are positively selected, whereas MHC-B reactive thymocytes are not. Introduction of particular thymic stromal elements bearing MHC-B molecules could alter the fate of B-reactive T cells in these (A X B -> A) chimaeras. Thymic epithelial cell lines expressing H-2^b were introduced by intrathymic injection into (H-2b/s -> H2s) bone marrow chimaeras and one thymic epithelial cell line, 427.1, was able to positvely select CTLs specific for influenza and vesicular stomatitis virus antigens in association with MHC class I H-2^b molecules. In addition, line 427.1 can process cytoplasmic proteins for presentation to H-$2k^b$ and H-$2D^b$-restricted CTL. Recently, Bix et al (110) also provided direct evidence for the ability of BM-derived cells to dictate positive selection through the demonstration that β2-microglobulin (β2m)-deficient mice (lacking both class I MHC and $CD8^+$ T cells), when reconstituted with β2m positve bone marrow, do generate a $CD8^+$ T cell population restricted to the donor MHC haplotype. Subsequently, it was shown that the injection of fibroblasts into the thymus allowed positive selection of both class I (111) and class II (112) restricted T cells. Although several observations have shown that, under certain conditions, positive selection on MHC molecules present on cells

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other than epithelial cells occurs (17), a lof of evidence indicates that in the normal unmanipulated thymus, these MHC classI and class II^{+} thymic epithelial cells play a major role in mediating positive selection.

Negative selection of potentially autoreactive thymocytes occurs mainly in the thymus and is thought to be induced primarily by interaction with bone marrow-derived cells. However, recent studies have also reported a role for radioresistant thymic cells, which are probably epithelial in origin, in the deletion of thymocytes reacting to endogenous superantigens (102). It was previously demonstrated that thymic epithelial cell lines could induce thymocyte positive selection in vivo. In this work I showed TNCs bind 20% of the double positive population (74). My study also revealed that binding and internalization were restricted to live cells, but 11% of captured thymocytes die within TNC cytoplasmic vacuoles (113) within 12 hours . When double positive thymocytes were examined in cultures not containing TNCs, 80% of the cells died within 48 hours. These results suggest that thymocyte death is reduced as a function of the TNC interaction. In these studies, apoptosis was shown to be greatly reduced in the TNC-interactive thymocyte population. TNC rescue was consistently detected in all of the assays reported in this study (Figures 12,13and 15). A subset of the thymocytes remained viable throughout the incubation period in cultures containing tsTNC-1 at 32°C, the temperature at which thymocyte binding and internalization was observed.

Studies strongly suggest that many different cells including TNCs are capable of mediating both positive and negative selection of double positive

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thymocytes through multiple interactions between the TCRs and MHCs presented on several cell types of the thymic stroma while thymocytes migrate through the thymus. Apoptosis as well as rescue were observed within the TNC-interactive thymocytes (12 and 14). For these reasons we believe TNCs have the ability to select a subpopulation of thymocytes to continue the developmental process, while selecting another subset to die through the process of apoptosis. These data are consistent with studies that report positive and negative selection to occur within the same thymic stromal epithelial cell type (102). Our results also support the proposed idea that clonal deletion can occur at any time during the double positive stage, and need not follow positive selection (114). In our control cultures, we noticed that survival of double positive thymocytes co-incubated with 3T3 cells was consistently higher than that observed in co-cultures with TNCs at 38°C, or when thymocytes were cultured alone. The increased longevity of T cell subsets co-cultured with fibroblast has been described previously, but the mechanism of this activity has yet to be defined (115). Fibroblasts (3T3 cells) have also been shown to be required for the complete maturation of thymocytes in thymi reconstructed *in vitro* (106). A study of the effect of both fibroblast and TNCs on thymocyte development is currently being performed in our laboratory. Hugo et al (101,102) assessed the potential of thymic epithelial cells to delete thymocytes reacting to the staphyloccocal enterotoxin A or B superantigens in vitro. In the presence of staphyloccocal enterotoxin A or B they found that all thymic epithelial cell lines used in this study were capable of activating T cell hybrids

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or deleting $CD4^+CD8^+$ thymocytes expressing an appropriate TCR. The extent of superantigen-mediated thymocyte deletion mediated by thymic epithelial cell lines was comparable to that mediated by a thymic macrophage cell line. Similar results were obtained with three phenotypically distinct thymic cell lines, suggesting that the ability to induce thymocyte deletion might be a general feature of various subsets of thymic epithelium. The observations provided in this study, combined with our previous demonstration that the same thymic epithelial cell lines can participate in positive selection, suggest that a given stromal cell population might be capable of taking part both in positive and negative selection of thymocytes. Although experiments support that both epithelial and hematopoietic cells are able to mediate negative selection, the two cell types do not do so with equal efficiency. Negative selection is more efficient when the antigen is expressed by hematopoietic cells in systems where selection can be mediated by both stromal cell types. The reason for this difference in efficiency can be related to local concentrations of antigen, to the relative inability of epithelium to process and present antigen, to the requirement of costimulatory or adherence molecules between the maturing T cells and the antigen presenting cells. Also the quantity of MHC on cortical epithelium and TCR density on the majority of $CD4^+$ and $CD8^+$ T cells are lower, which may explain why epithelial cells can not induce negative selection as efficient as hematopoietic cells. While it is now clear that many different cell types are capable of mediating both positive and negative selection, this does not imply that they do so in an unmanipulated thymus.

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THE ROLE OF TNCS IN SELECTION MODELS

We have shown that temperature sensitive TNCs selectively interact with and rescue with αβ TCR^{lo} $CD4^{+}CD8^{+}$ thymocytes in vitro at permissive temperature. Survived thymocytes have a reduced level of programmed cell death and increased level of Bcl-2 expression (116). A subset of TNC rescued population matured from the αβ $TCR^{lo}/CD69^{-}$ phenotype to αβ $TCR^{in}/CD69^{-}$ expressing cells. In the presence of Interleukin-Il2β, the subpopulation of $\alpha\beta TCR^{lo}/CD69^{-}$ thymocytes can develop further within 48 hours incubation period to become a more mature αβ $TCR^{hi}/CD69^{+}$ phenotype.

Only a small number of the mature thymocytes are survived from positive and negative selection to molecules of the major histocompatibility complex (MHC) in the thymus. Immature thymocytes have a very rapid turnover, but there is only minimal evidence of cell death within the cortex of the thymus. In our system, we have shown that cortical epithelial TNCs bind and internalize $CD4^{+}$ and $CD8^{+}$ thymocytes and these internalized thymocytes go through apoptosis (Fig,14). The removal of apoptotic thymocytes by TNCs is extremely efficient and leads to rapid destruction of dying cells. The efficiency of TNCs in clearing apoptotic cells would explain the paradox that inspite of the presumed high rate of cell death in the normal thymus, it is not obvious in tissue sections. Thymocytes are dying from apoptosis but are cleared so rapidly by TNCs that cell death is inconspicuous. Recently, Surh and Sprent (117)

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presented direct evidence for apoptotic thymocytes scattered throughout the cortex in the thymus by using a modified TUNEL method for detecting apoptosis with DNA fragmentation,

As mentioned above, the early TCR^{lo} double positive cells encounter predominatly cortical stromal cells, most of them are epithelial cells. As thymocytes mature and upregulate TCR, they migrate into the medulla, where the hematopoietic, macrophages, and dendritic cells are more frequent. Early T cells require continuous signaling provided by TCR/MHC engagement to be rescued from programmed cell death and to promote maturation, the rescue signals have to come early in migration from the cortical cells. TNCs can be a very good candidate to play this role becuase of their location in the thymus and function in upregulating Bcl-2 which is believed to express in the cells rescued from apoptosis. If either or both MHC class I and class II are blocked, we observed more thymocytes dying from apoptosis (Fig. 18). Another cells that may occur frequently enough in the cortex to promote positive selection would be the thymcotyes themselves. If so, only class I-selected T cells could be generated in this manner because mouse thymoyctes do not express MHC class II while thymic epithelial cells including TNCs express both MHC class I and classII.

Althought there are still a lot of questions about the function of TNCs in T cell development, there are some evidence indicate that TNCs are involved in both positive and negective selection, we believe that using our

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tsTNCs lines developed in our lab will provide us a practical method to further study the role of TNCs in T cell development in thymus.

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FIGURE LEGENDS

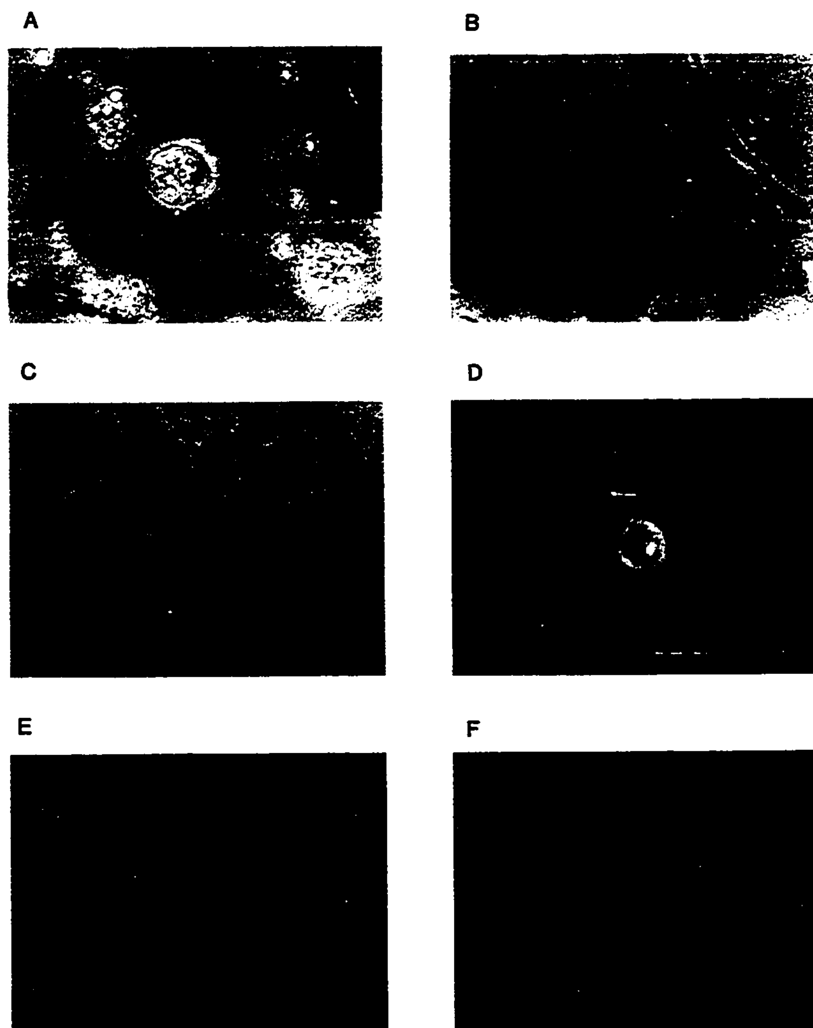

FIG. 1. Isolation and transformation of TNCs. (A) TNCs isolated from the thymus of 2-week-old C57BL/6 mice. Magnification 200x. (B) Isolated TNCs after 24-48 hours in tissue culture. Magnification 400x. (C) Phase contrast photograph of the SV40-transformed TNC line SVT-Mp5. Magnification 400x. (D and E) SVT-Mp5 stained with a hamster polyclonal anti-T antigen antibody. (F) SVT-Mp5 stained with no first step antibody.

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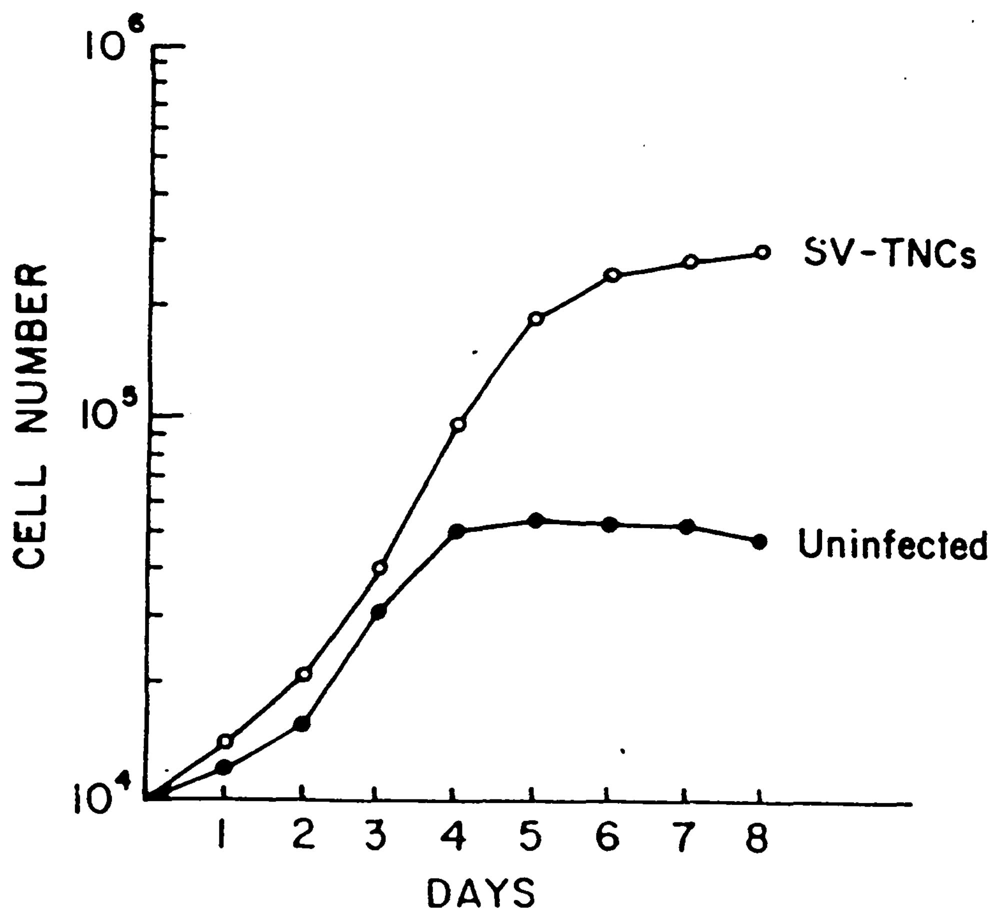

FIG. 2. Growth curve. Uninflected and SV40-infected TNCs were seeded at 10000 cells per 35-mm plate and fed with 3 ml of growth media. Cells were trypsinized and counted at 24-hr intervals. Each value is an average of three plates. Viability was determined by trypan blue exclusion.

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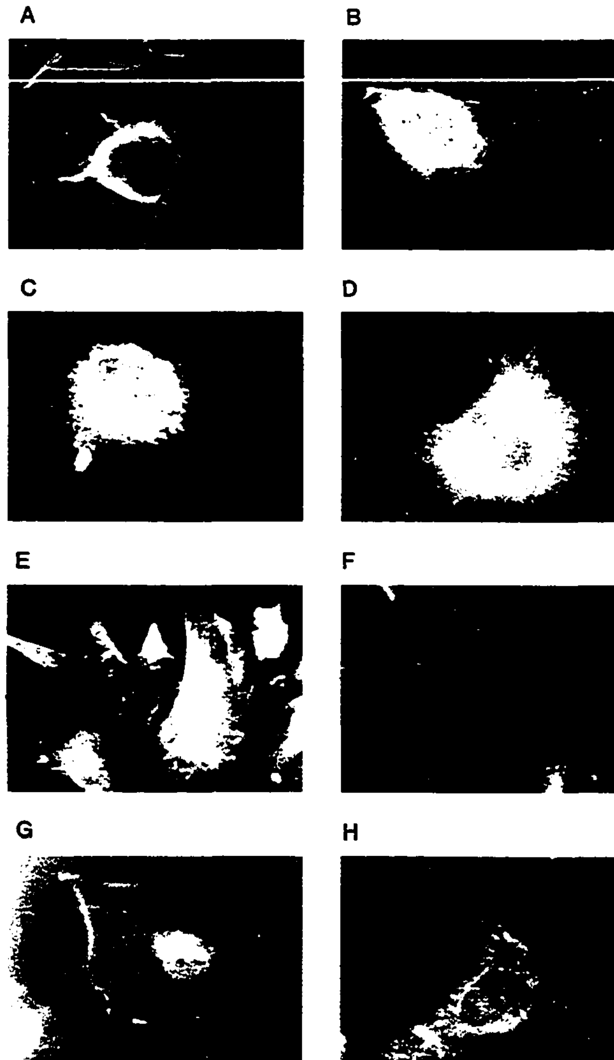

Fig. 3 Verification of SVT-Mp5 as trasformed TNC. (A) Freshly isolated TNCs stained with anti-A2B5 (ATCC. A2B5 clone 105) (B) SVT-MpS stained with anti-A2B5 (C) Freshly isolated TNCs stained with a rabbit polyclonal anti-keratin antibody (Dr. T-T Sun NYU Medical Center) (D) SVT-Mp5 stained with anti-keratin antibody (E) Freshly isolated TNCs stained with anti-lab (F) SVT-MpS stained with anti-lab antibody (G) Freshly isolated TNCs stained with anti-$H2^b$ (H) SVT-MpS stained with anti-$H2^b$ No staining was found in samples in which no first step antibody was used.

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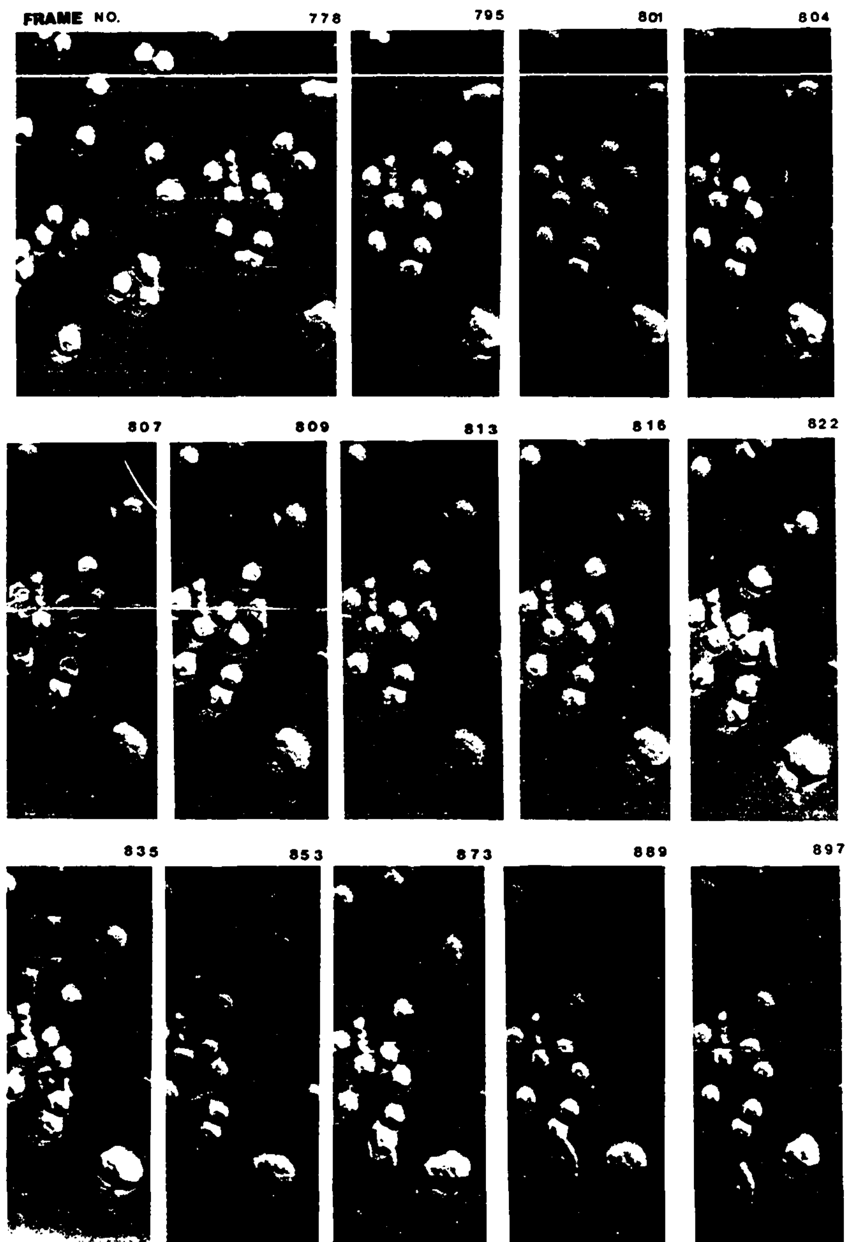

FIG. 4. The dynamics of thymocyte internalization. Freshly isolated thymocytes were added to monolayers of SVT-II2 cells and incubated overnight. Unattached thymocytes were removed with vigorous washing, and the remaining cells were observed using a Nikon Diaphat inverted microscope with Hoffman modulation 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 1-inch VCR. The same TNC is shown in each frame. The thvmocytes labeled 1. 2. and 3 are bound to the external surface of the TNC. The nucleus of the TNC is labeled N. The thymocyte labeled X is internalized in the series of micrographs. The arrows show a cntoplasmic channel that forms before the initiation of internalization.

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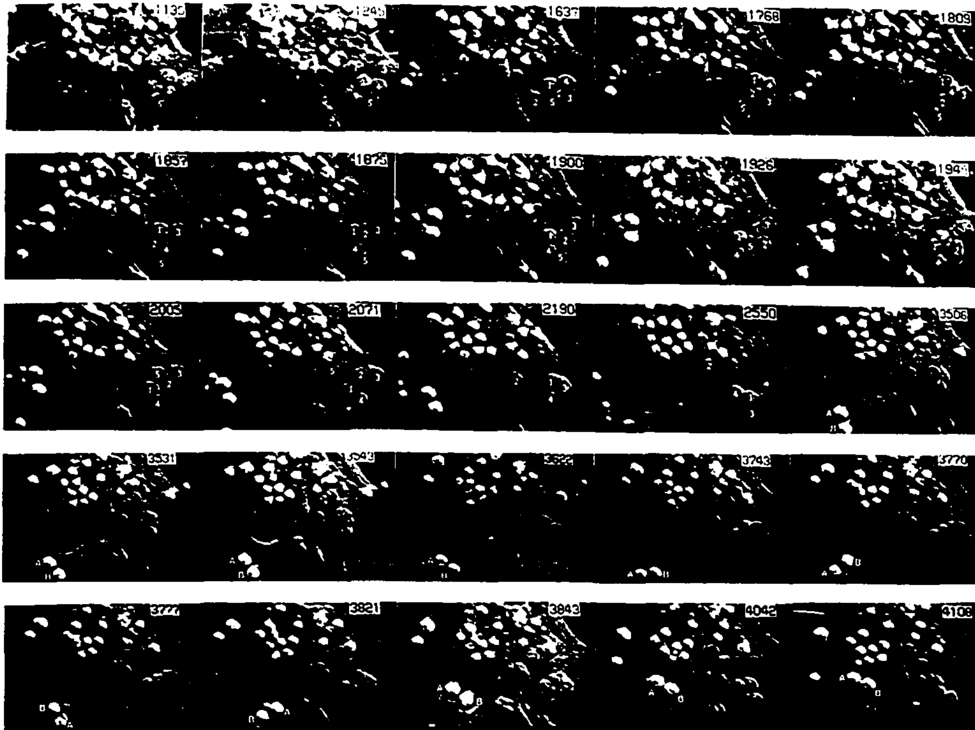

Fig. 5 Thymocyte movement within TNC vacuoles. Time-lapse video of a SVT-II2 TNC containing several bound and internilized thymocytes.. The thymocytes labeled 1-5 are internalized. Internalized cells can be distingtlished from bound cells because they are phase dark. Movement of these thymocyles is demonstrated by changes of the location of indivdual thymocytes relative to one another with increasing flame numbers (frames 1136-2550). The thymocytes labeled A and B are shown to bind to the TNC in frame 3743. These cells are then moved along the TNC cell surface toward the center of the cell TNC membrane movement can be seen immediately above thymocytes labeled A and B (3506-3543)

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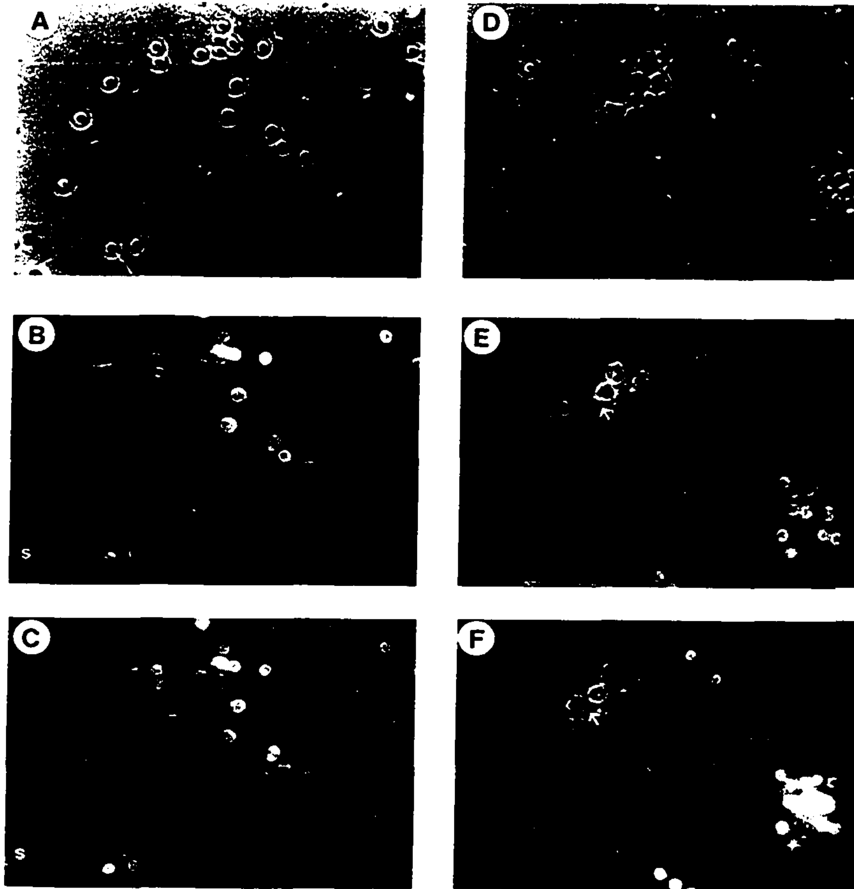

FIG. 6 Thymocyte subset binding and internalization Freshly isolated thymocytes from 2-week-old to 1 month-old mice were cocultivated with monolayers of SVT-112 The thymocytes were allowed to bind and unattached cells were removed by washing The remaining cells were fixed and stained with antibodies against CD4 and CD8. The same field is shown in (A-C) (A) Phase micrograph of thymocytes bound to SVT-II2, (B) fluorescence micrograph using mouse anti-mouse CD8 (IgM) and goat anti-mouse IgM FITC, and (C) fluorescence micrograph using rat anti-mouse CD4 (IgG) and goat anti-rat IgG TRITC The same field is shown in (D-F). (D) Phase micrograph of thymocytes,internalized by SVT-II2 (E) fluorescence micrograph using mouse anti-mouse CDX (IgM) and goat anti-mousc IgM FITC, and (F) fluorescence micrograph using rat anti-mouse CD4 (IgG) and goat anti-rat IgG TRITC Internalized cells undergo a visible phase shift and appear more evenly tinted (D). Initially internalized cells appear flattened between the TNC plasma membrane and appear to reside in a common cytoplasmic vacuole (E and F. arrows). S shows an unbound CD4 single positive thymocyte which represents an internal control for binding and the staining procedure.

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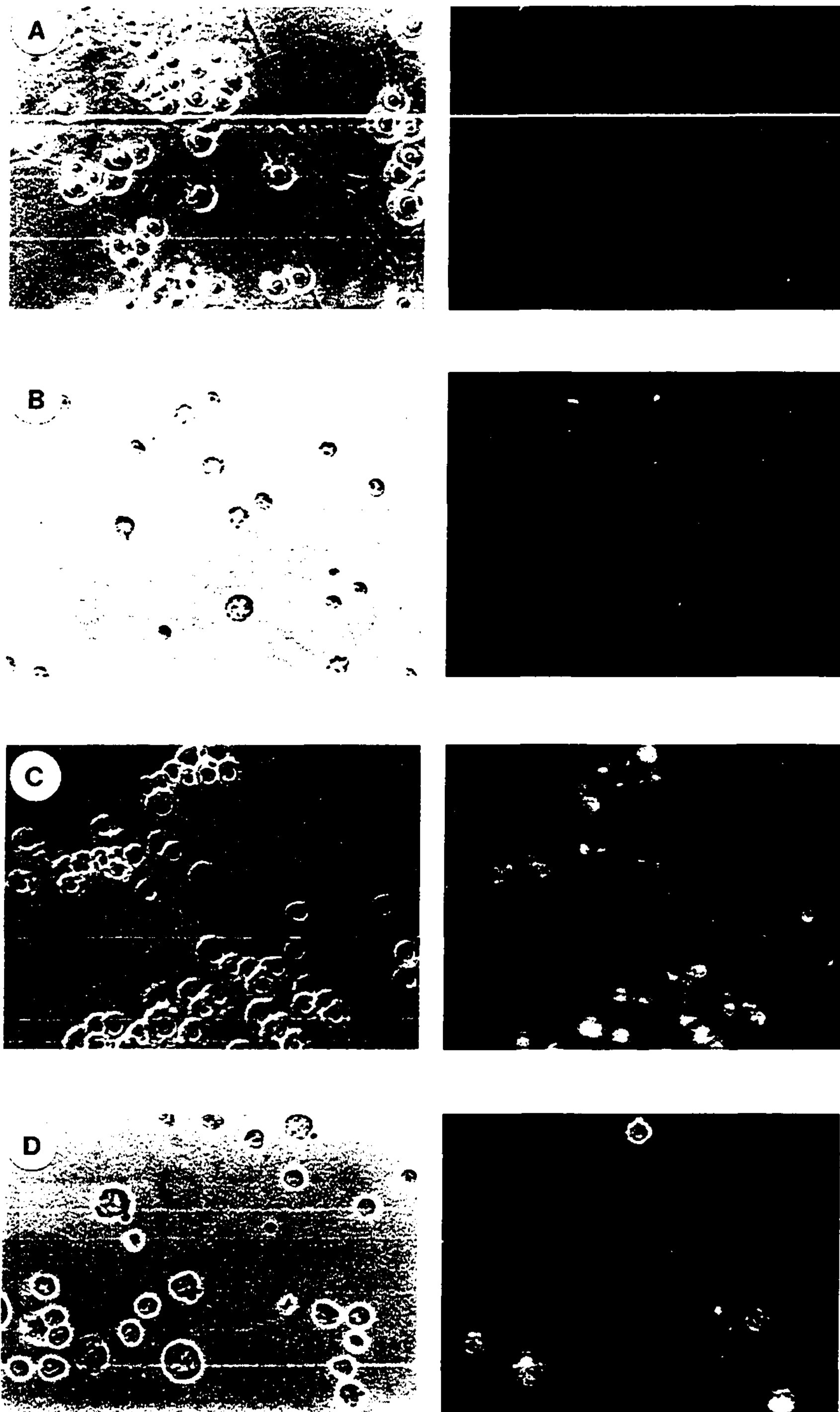

Fig. 7. Cell surface expression of αβTCR and CD3ε on bound thymocytes. Freshly isolated thymocytes were stained with antibodies against αβTCR (A) or CD3ε (C) before being centrifuged onto monolayers of SVT-II2 cells. Unattached cells were removed. and both bound and unbound preparations of thymocytes were exposed to 2%paraformaldehyde. Unattached thymocytes stained with antibodies against (αβTCR are shown in B. or CD3ε in D. The same field is shown in phase and fluorescence micrographs. Magnification, 599x.

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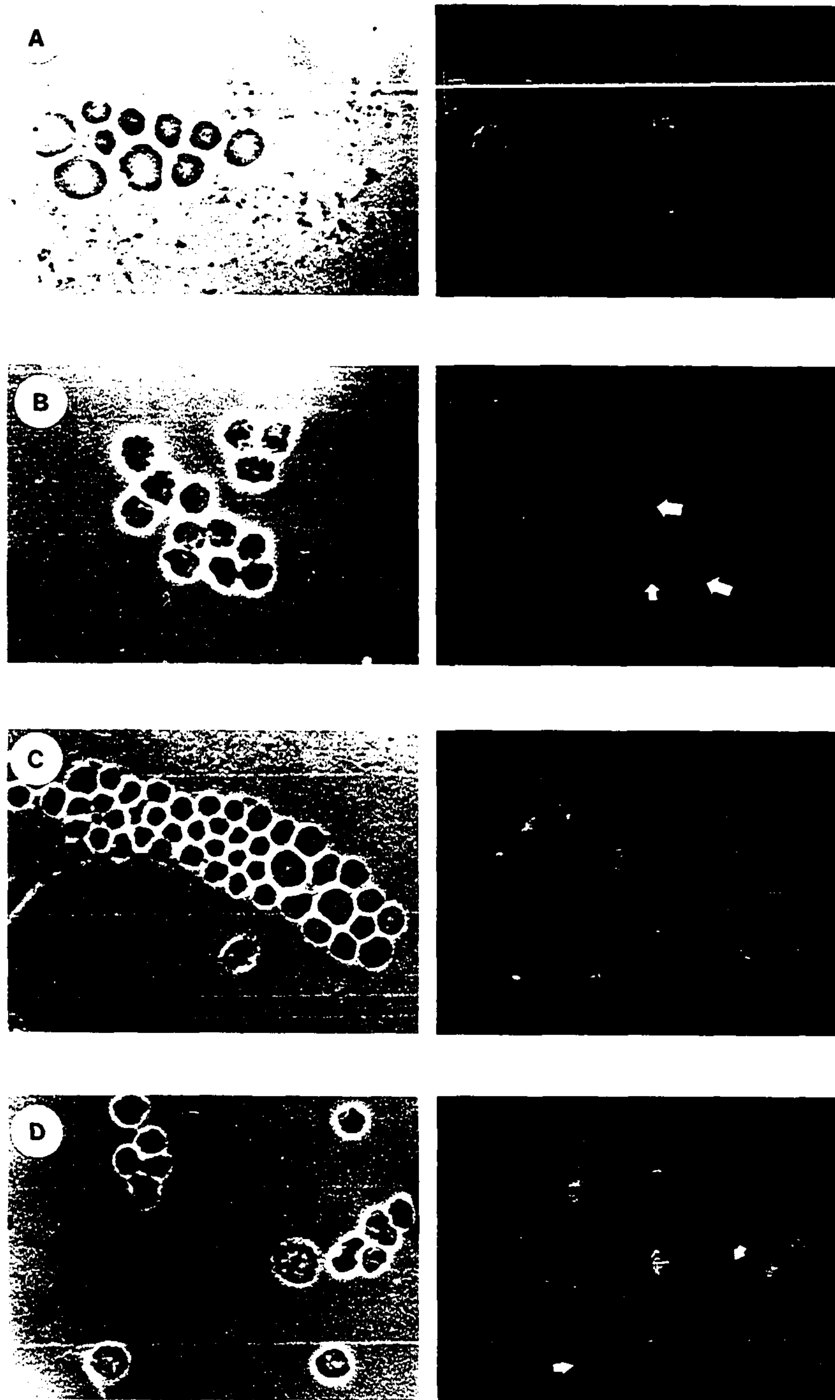

Fig. 8. αβTCR and CD3ε expression in internalized thymocytes. Freshly isolated thymocytes were allowed to bind SVT-II2 cells overnigh, fixed and stained with antibodies against αβTCR (A) or CD3ε(C). As a control for each experiment, the thymocytes were fixed and stained with antibodies against (B) αβ TCR or (D) CD3ε to show the selective staining procedures that were used. Arrows show the position of unstained cells. Magnification, 624x.

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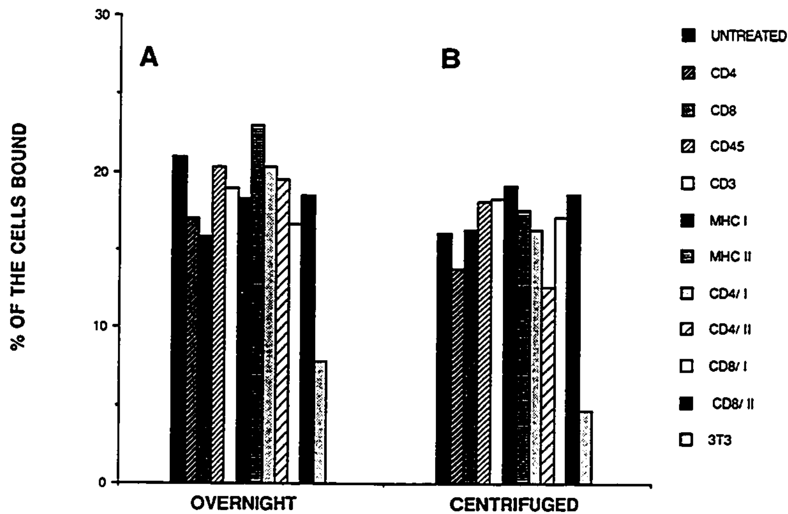

FIG.9. Antibody blocking. The binding assay was performed in the presence of monoclonal antibody against CD4,CD8,CD45,CD3, MHC class I, MHC class II. CD4 plus MHC class I (CD4/I),CD4plus MHC class II (CD4/II),CD8 plus MHC class I (CD8/1), and CD8 plus MHC class II (CD8/ll). Untreated cells were not exposed to antibody. Thymocytes and the appropriate antibody preparation were added simultaneously and (A) incubated overnight or (B) centnfuged at 3000 rpm for i5 min with a monolayer of SVT-II2 cells. Untreated thymocytes were added to a monolayer of 3T3 cells in each experiment as a control. Unattached cells were removed and the percentage of bound thymocytes was determined.

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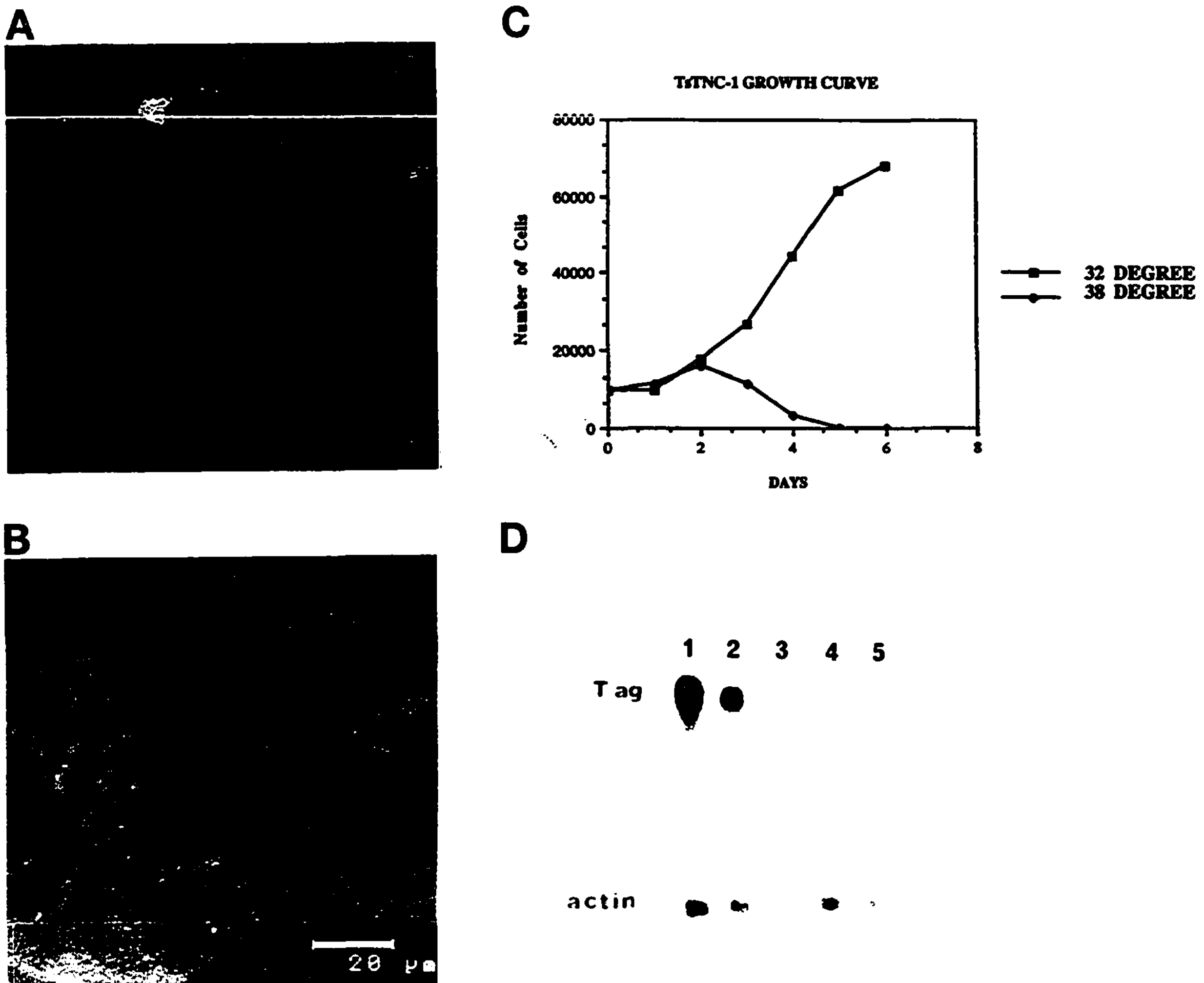

Fig. 10. The Characteristics of tsTNC-1 Cells

Scanning electron micrograph of tsTNC-1 cells at (A) 32°C, and (B) 38°C.

(C) Growth curve. TsTNC-1 cells were seeded at 1 X 10^4 cells per 35-mm plate and maintained at either 32°C or 38°C. Cells were trypsinized and counted at 24 hour intervals. Each point represents an average of three plates.

(D) Northern Blot Analysis of Large T Ag Expression. TsTNC-1 cells grown at 32°C, Lane 1, tsTNC-1 cells shifted to 38°C for 24 hours, Lane 2, 48 hours, Lane 3, 72 hours, Lane 4, and 96 hours, Lane 5. The blot was then stripped and rehybridized using an actin probe as a control for RNA concentration.

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Thymocyte Binding Assay

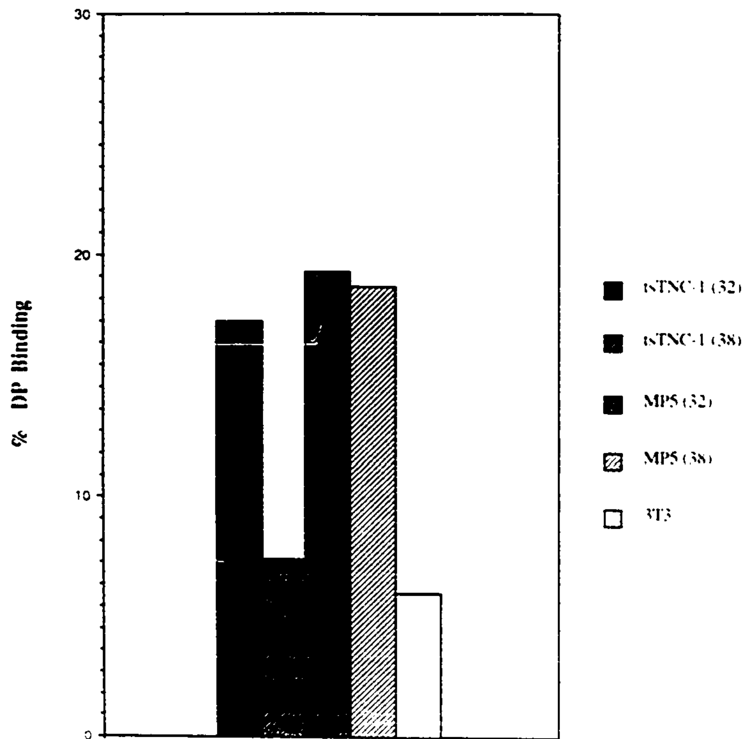

Fig. 11. Binding Assay

Monolayers of tsTNC-1 cells grown at 32°C and 38°C were incubated overnight with 2 X 10^6 freshly isolated thymocytes. Unbound thymocytes were recovered and counted. The percentage of bound cells was determined as a function of the remaining thymocyte population.

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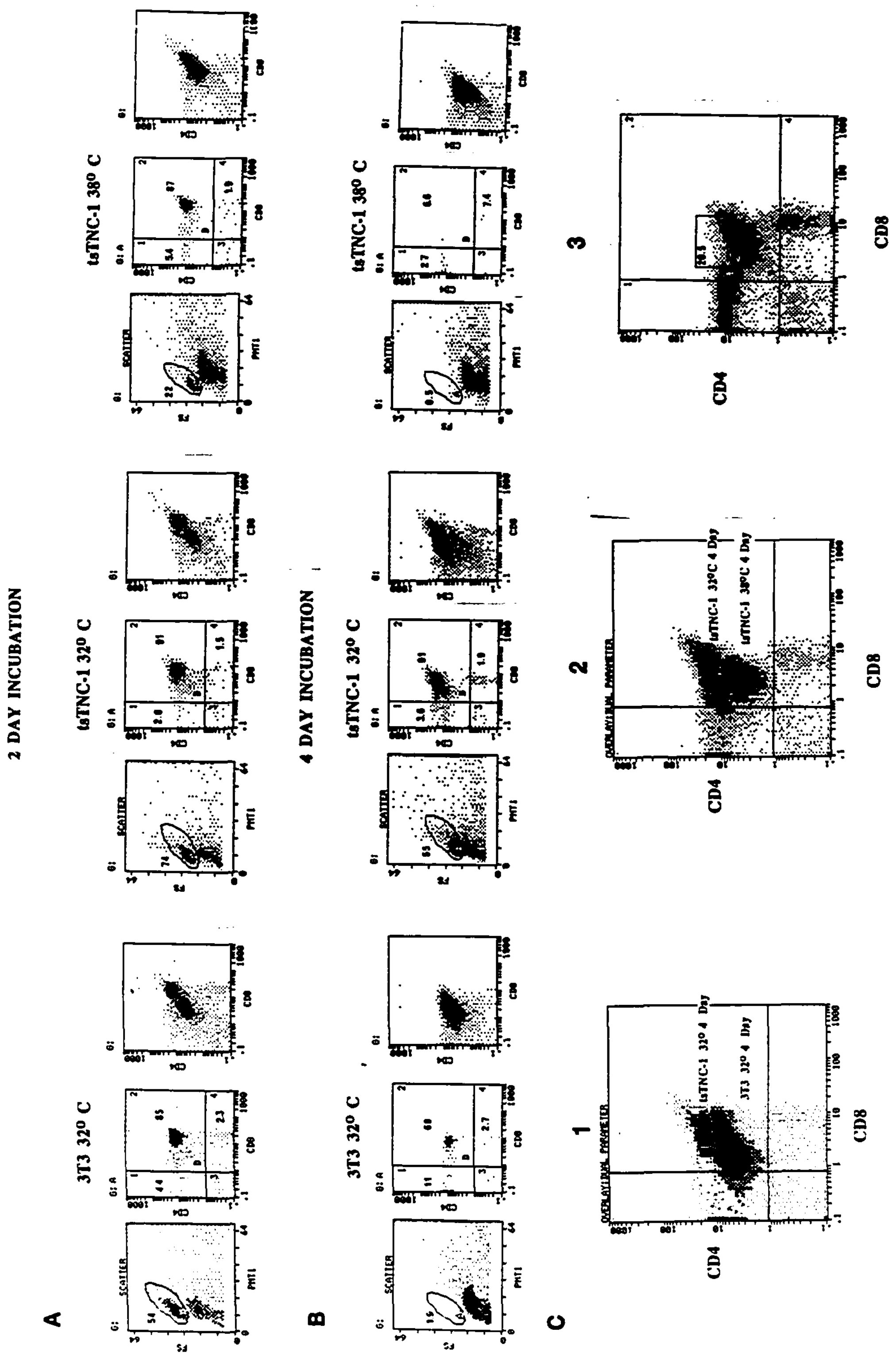

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Fig. 12. Co-incubation Experiments

Freshly isolated thymocytes were stained with antibodies against CD4 and CD8 and sorted to a purity of over 97%. 5×10^6 sorted thymocytes were incubated with 2×10^5 tsTNC-1 at 32°C or 38°C, or with 2×10^5 3T3 cells. Cocultures were maintained for either 48 hours (A), or 96 hours (B) at the appropriate temperature before FACS reanalyses of recovered thymocytes. The numbers within the histogram represent the percentage of the total number of cells in that quadrant or gate. The first histogram of each set displays the gates for viable thymocytes, the middle histogram shows the CD4/CD8 profile of gated viable thymocytes, and the third histogram shows CD4/CD8 staining of the total thymocyte population.

(C) Overlays of Co-incubation Data

The results obtained from four day co-incubation experiments presented in Figure 3B were used to compare the CD4/CD8 cell surface expression levels on thymocytes recovered from tsTNC-1 cultures at 32°C with those of thymocytes obtained from cocultures with 3T3 cells at 32°C (panel 1), tsTNC-1 cells at 38°C (panel 2) or freshly isolated thymocytes (panel 3). Panel 3 is a histogram of freshly isolated thymocytes stained with CD4 and CD8. The rectangular gate within quadrant 2 was set around rescued double positive thymocytes cocultured with tsTNC-1 cells at 32°C for 4 days (Fig. 3B). 20.5% of the freshly isolated thymocyte population show the equivalent high level expression of CD4 and CD8.

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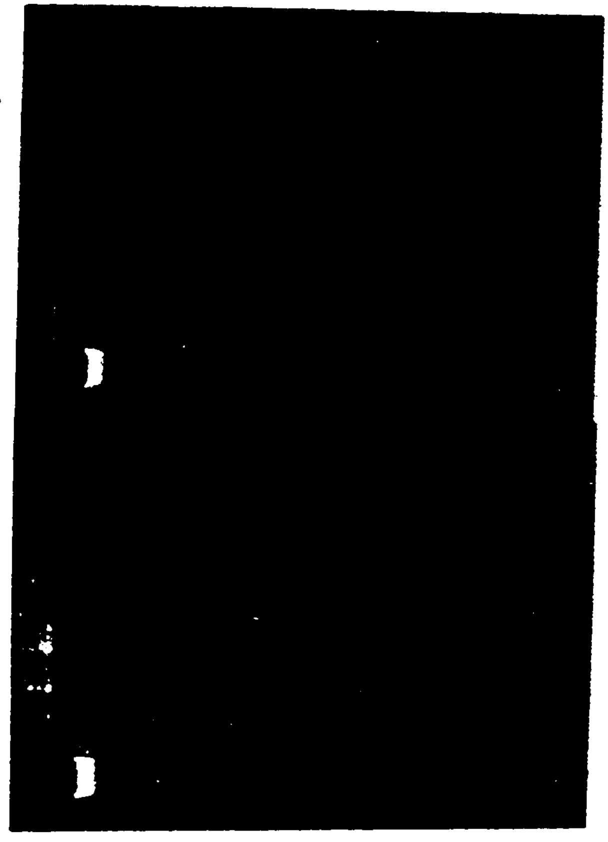

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Fig. 13. DNA Fragmentation Assay

(A) DNA recovered from TNC-adherent thymocytes after co-incubation for 4 days with tsTNC-1 cells at 32°C. Thymocytes were FACS-sorted into viable and nonviable populations before analysis using the DNA fragmentation assay. Lane 2, freshly isolated thymocytes treated for 2 hours with 10 mM hydrogen peroxide, Lane 3, freshly isolated thymocytes, Lane 4, viable double positive thymocytes obtained from 4 day cultures with tsTNC-1 cells at 32°C, Lane 5, nonviable double positive thymocytes obtained from 4 day cultures with tsTNC-1 cells at 32°C.

(B) Fragmentation analysis of DNA isolated from bound versus unbound fractions after 24 or 48 hours. FACS-sorted double positives were exposed to monolayers of TNCs or 3T3 cells as described in the coculture section of the Materials and Methods. The bound thymocyte subset remained attached to TNCs after three washes with PBS. The unbound fractions collected in the wash were pooled before analysis, and the bound fraction was obtained only after vigorous shaking. Lane 2, freshly isolated thymocytes treated for 2 hours with 10 mM hydrogen peroxide, Lane 3, freshly isolated thymocytes, Lane 4, bound thymocytes recovered from tsTNC-1 cells at 32°C, Lane 5, unbound thymocytes recovered from tsTNC-1 cells at 32°C, Lane 6, thymocytes collected from tsTNC-1 cells at 38°C, Lane 7, thymocytes collected from 3T3 cells at 32°C. Lane 1 contains the molecular weight marker for each gel.

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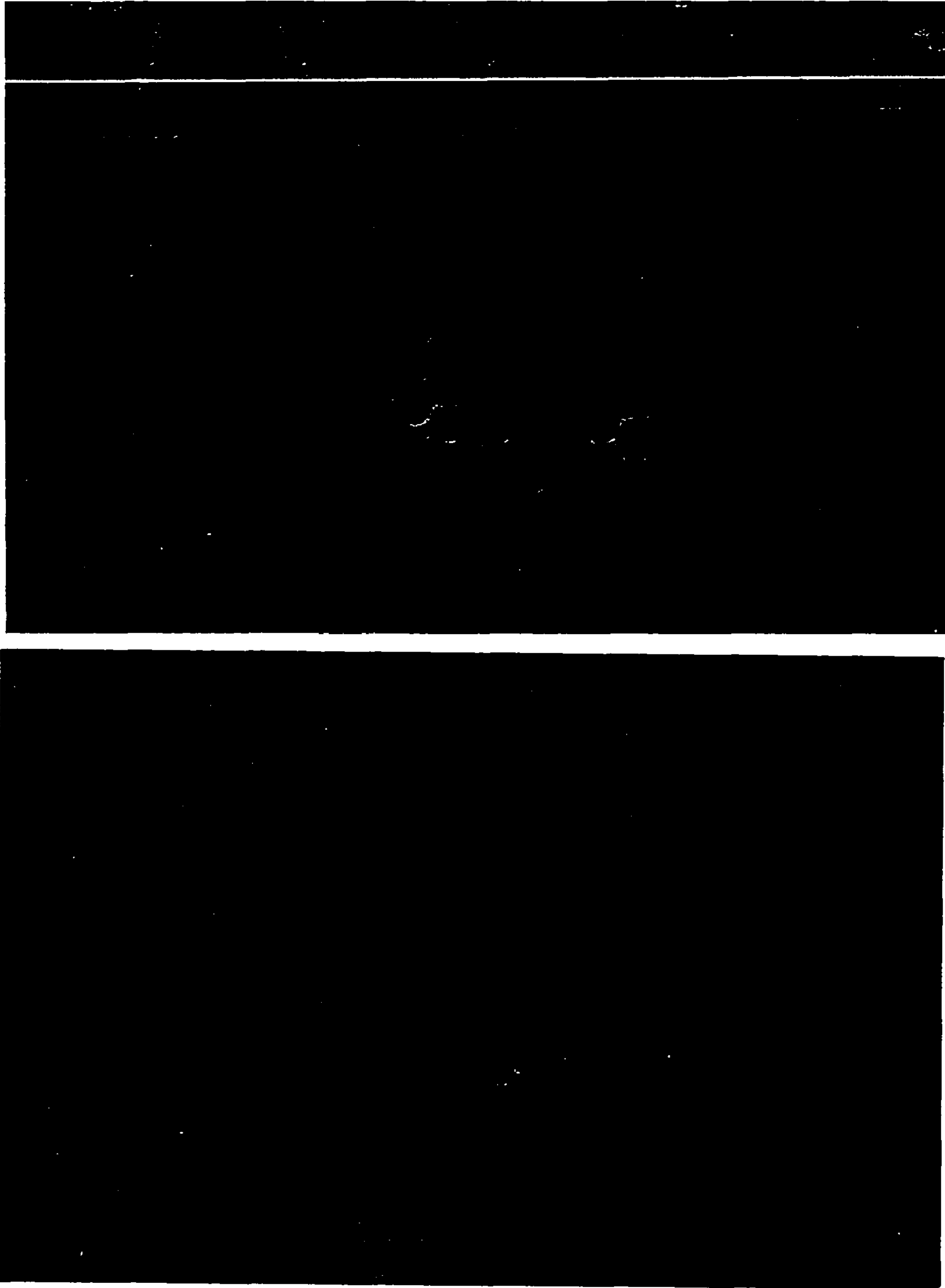

Fig. 14. Modified TUNEL assay.

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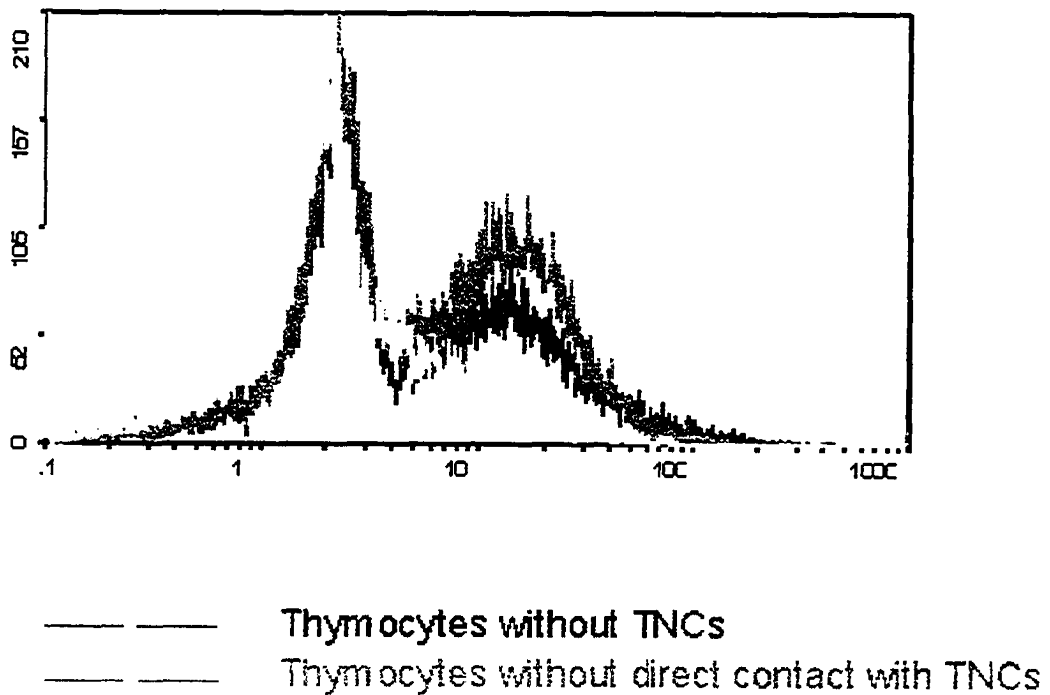

Fig. 15. Rescue assay by Modified TUNEL assay.

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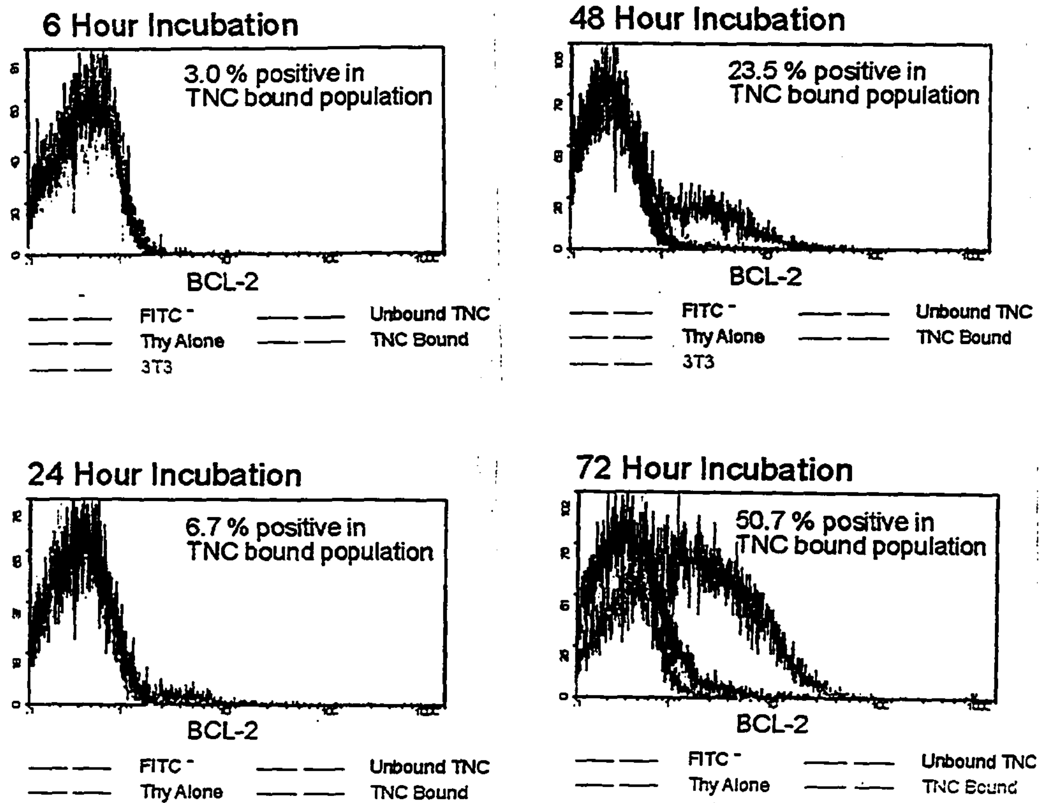

Fig. 16. Bcl-2 expression in rescued thymcoytes

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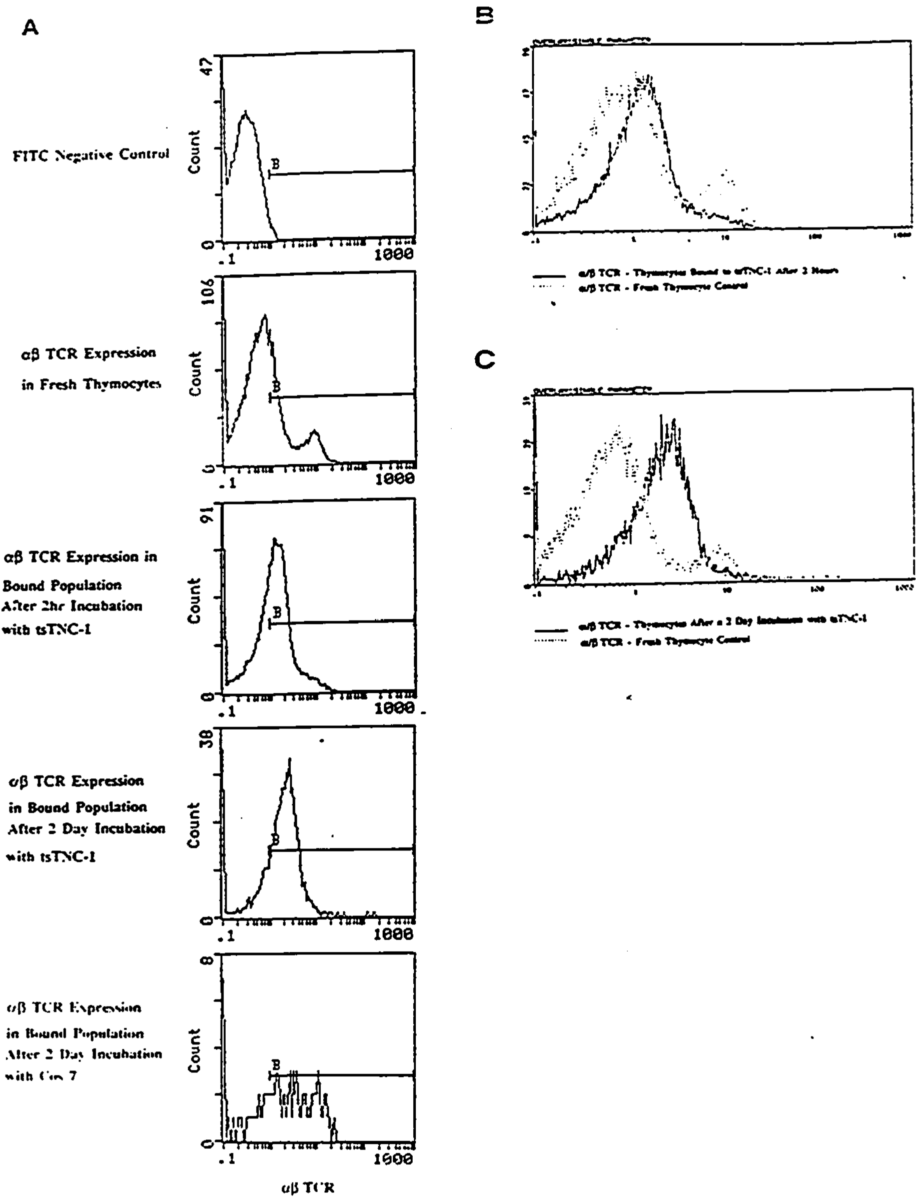

Fig. 17. αβ TCR Expression
(A) Freshly isolated thymocytes were exposed to monolayers of tsTNC-1 cells for 2 hours. Nonadherent thymocytes were removed after washing with PBS and the bound population was recovered and analyzed for αβ TCR expression after a 2 hour incubation period or after 2 days in culture. The TCR expression level obtained from these samples were compared to that detected on freshly isolated thymocytes and thymocytes recovered from cocultures with COS7 cells.
(B) An overlay of the TCR expression level on freshly isolated thymocytes and thymocytes bound to TNCs after 2 hours.
(C) An overlay of the TCR expression level on freshly isolated thymocytes and thymocytes from TNC cocultures after 2 days.

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Blocking of TNC Rescue with Antibodies to MHC

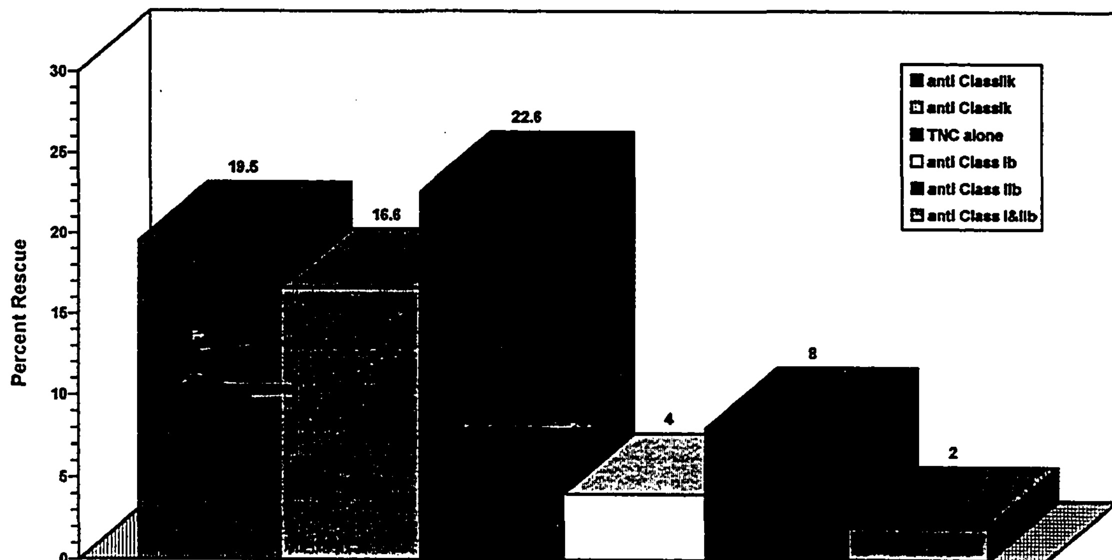

Fig. 18. The Effect of Class I or Class II MHC Antibodies on Thymocyte Rescue

Thymocytes were resuspended at 5 X 10^6 cells/ml and CD4 CD8 double positive thymocytes were sorted using a FACS Colter (model Elite) as described above. 5 X 10^6 sorted thymocytes were incubated with 2 X 10^5 tsTNC-1. Co-cultures were maintained for 48 hours at 32°C without antibody (A) , or with antibody to (B) rat IgG, (C) $H2K^b$ or (D) Ia^b before analysis for viability using FACS forward scatter. The numbers represent the percentage of cells under the viable gate.

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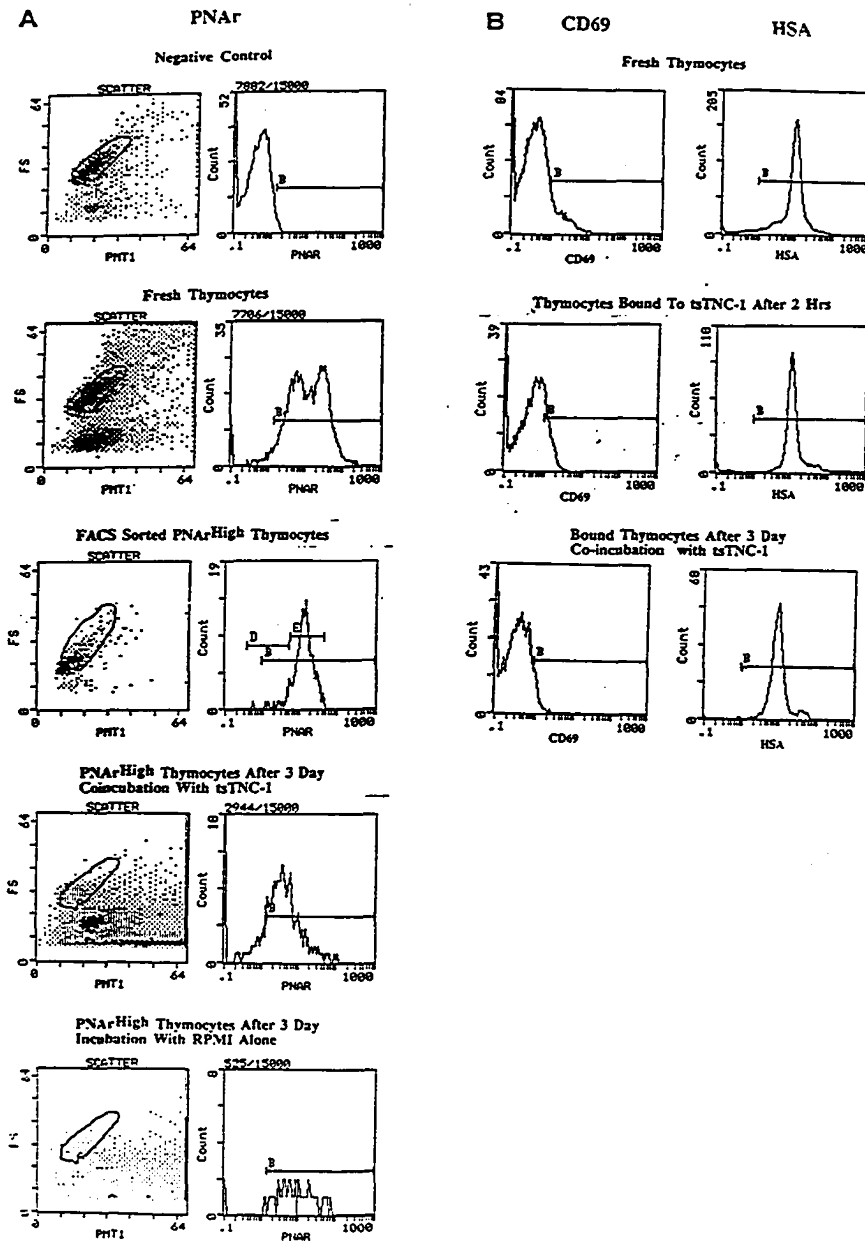

Fig. 19. Cell Surface Characteristics of Rescued Thymocytes
(A) PNAr^{high} thymocytes were sorted using a FACS. 5 X 10^6 sorted thymocytes were incubated with 2 X 10^5 tsTNC-1 and allowed to bind. Unbound cells were removed by washing. The remaining thymocytes were recovered and analyzed for initial binders (2 hours) or after 72 hours at 32°C. Control cultures contained no TNCs.
(B) CD69^{low} thymocytes were sorted using a FACS. 5 X 10^6 sorted thymocytes were incubated with 2 X 10^5 tsTNC-1. Thymocytes were recovered and analyzed after 2 hours or 72 hours at 32°C. TNC-adherent thymocytes were analyzed for HSA expression levels using a FACS after 2 hours or 72 hours at 32°C.

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Table I

Characteristics of the SV40-Transformed TNC line SVT-II2

	Antibodies					
	T ag[a]	A2B5	Keratin	Ia[b]	H2[b]	Internalization
TNCs (uninfected)	-	+	+	+	+	-
SVT-II2	+	+	+	+	+	+
TNC-depleted thymic stromal cell[b]	-	-	+	+/-	+	-

Note. Cells were stained with the appropriate primary antibody plus a fluorescent secondary antibody and visualized using fluorescence microscopy. + means that staining was obtained. - means that no staining was obtained.

a Anti- T antigen antibody.

b The procedure used to isolated TNCs was exploited here to obtain thymic stromal cells devoid of nurse cells. TNCs form pellets in fetal calf serum gradients, but the remaining cells settle at or above the interface between the fetal calf serum and culture media. This step was performed twice and all of the cells at or above the fetal a calf serum layer were collected and plated in tissue culture dishes. Those cells that formed monolayers were considered to be TNC-depleted thymic stromal cells.

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Table 2

Type and Percentage of Unbound Thymocytes

	$CD4^{-}CD8^{-}$	$CD4^{+}CD8^{+}$	$CD4^{+}CD8^{-}$	$CD4^{-}CD8^{+}$	Total added	Total recovered	Recovered from 3T3
A. Freshly isolated thymocytes[a]	25%	59%	7%	9%	-	-	-
B. After firstr spin onto SVT-II2	37%	34%	15%	14%	$2X10^6$	$1.43X10^6$	$1.89X10^6$
C. After second spin onto SVT-II2	34%	31%	17%	18%	$2X10^6$	$1.85X10^6$	$1.84X10^6$

Note. Thymocytes (2×10^6) were added to a of 1×10^4 TNCs. After a 15-min centrifugation at 3000 rpm, unattached lymphocytes were removed by washing with PBS. Unbound cells were then spun onto a fresh preparation of SVT-II2 cells, and unattached cells were collected. The total number of viable cells and the percentage of each unbound thymocyte subset were calculated after each centrifugation. The percentage of each thymocyte subset was determined after staining with antibodies to both CD4 and CD8. For thymocyte subset distribution, the total number of unbound cells counted in row A was 453, 382 for row B. and 295 for row C. The figures listed under the column labeled "Recovered from NIH 3T3" represent the number of cells recovered after 2.0×10^6 thymocytes were centrifuged onto a monolayer of 1×10^4 NIH 3T3 cells. The thymocyte profile after the first spin onto monolayers of 3T3 cells was 27% for CD4 CD8, 52% for $CD4^{+}CD8^{+}$, 10%, for $CD4^{+}$, and 11% for $CD8^{+}$. After the second spin onto monolayers of 3T3 cells. the thymocyte subset profile was 24% for $CD4^{-}$ $CD8^{-}$,56% for $CD4^{+}CD8^{+}$, 12% for $CD4^{+}$, and 8% for $CD8^{+}$.

[a] Freshly isolated thymocytes are the zero time values. The figures in this row represent the percentage of each subset in the original thymocyte population before exposure to TNCs.

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Table 3. TsTNC-1 Phenotype Summary

	32°C	38°C
Growth	+	-
T ag	+	-
Binding	+++	+/-
Internalization	+	-
A2B5	++	++
MHC class II*	+	+
MHC class I	+	+
Keratin	++	++
ER-TR-4	+	+
ER-TR-5	-	-

The characteristics of tsTNC-1 cells are presented above. The data obtained for the growth rate, Large T antigen expression (T ag), binding and internalization are shown in Figures 1C, 1D, 2A and 2B, respectively. TsTNC-1 cells stain with equal intensity at both temperatures for the neuroendocrine marker A2B5, class I MHC ($H2K^b$), class II MHC (Ia^b), ER-TR-4 and keratins.

*Class II expression is lost after long term growth but expression is recovered after a 48 hour treatment with 0.02 mg/ml INF-γ.

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